Regulation of Nucleolar Dominance within the Species *Drosophila melanogaster* and in *Drosophila* Interspecies Hybrids

by

Natalie Rose Warsinger-Pepe

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Molecular and Integrative Physiology)
in The University of Michigan
2020

Doctoral Committee:

Assistant Professor Jun Wu, Co-Chair
Professor Yukiko Yamashita, Co-Chair
Associate Professor Gyorgyi Csankovski
Associate Professor Sundeep Kalantry
Professor Scott Pletcher

Natalie Rose Warsinger-Pepe
nwarsing@umich.edu
ORCID ID: 0000-0002-9375-8990

© Natalie Rose Warsinger-Pepe 2020

Dedication

This dissertation is dedicated to all the untraditional graduate students who struggle with the toxic environment that academia can be. This dissertation is also dedicated to GEO, the Graduate Employees' Organization, and its members who work daily to ensure that graduate students are protected.

Acknowledgements

This work was supported through the mentorship of Dr. Yukiko Yamashita and advising by Dr. Jun Wu. The Yamashita lab members gave extensive feedback throughout the dissertation work; Dr. Madhav Jagannathan in particular provided extensive mentoring and training. Duojia (Dorcas) Li was instrumental in performing experiments to study female nucleolar dominance and copy number variation. Drs. Madhav Jagannathan and George J. Watase were instrumental in performing experiments to study satellite DNA divergence across the *Drosophila melanogaster* species complex. Jackie Fingerhut was instrumental in discussions exploring RNA Polymerase I factors in interspecies hybrids. Dr. Madhav Jagannathan designed and generated the rDNA BAC transgenic *D. melanogaster* stock. We thank Dr. Amanda Larracuente, Dr. Patricia Wittkopp, Bloomington *Drosophila* Stock Center, Kyoto *Drosophila* Stock Center, and the UC San Diego *Drosophila* Stock Center for reagents. We thank Dr. Sue Hammoud and the dissertation committee for discussion and/or comments throughout this dissertation work. This research was supported by Howard Hughes Medical Institute and in part by the NIH Career Training in Reproductive Biology (5T32HD079342-04) and the Rackham Predoctoral Fellowship.

Table of Contents

Dedication ii
Acknowledgements iii
List of Tables v
List of Figures vi
List of Appendices viii
List of Abbreviations ix
Abstract x
Introduction 1
Chapter I: Regulation of Nucleolar Dominance in *Drosophila melanogaster* 6
  Summary 7
  Introduction 8
  Materials and Methods 11
  Results 17
  Figures 27
  Discussion 40
Chapter II: Regulation of Nucleolar Dominance in *Drosophila* Interspecies Hybrids 45
  Summary 46
  Introduction 47
  Materials and Methods 50
  Results 53
  Figures 64
  Discussion 72
Chapter III: Satellite DNA Divergence Across Closely Related *Drosophila* Species 78
  Summary 79
  Introduction 80
  Materials and Methods 82
  Results 84
  Figures 88
  Discussion 95
Conclusions, Speculations, and Future Directions 98
Appendix A 103
Appendix B 110
Bibliography 120

List of Tables

A. 1 Oligonucleotide Probes Used to Study Nucleolar Dominance within *D. melanogaster* and *Drosophila* Interspecies Hybrids.....103
A. 2 Mating Success of Interspecies Hybrid Crosses.....104
A. 3 Rates of Bobbed Phenotype in Interspecies Hybrid Progeny.....105
A. 4 Combination of Probes Used for DNA FISH Mapping.....106
A. 5 Location of the Satellite DNAs on the Chromosomes of the *D. melanogaster* Species Complex.....107
A. 6 Satellite Composition of Each Chromosome in the *D. melanogaster* Species Complex.....108
A. 7 Estimating the Detection Limit of DNA FISH Method Used in this Study.....109

List of Figures

I.1 Nucleolar Dominance is not Established During Embryogenesis in Male Embryos.....27
I.2 Y rDNA Dominance is Established During Development in Males.....28
I.3 Heterochromatin Formation Aids in Y rDNA Dominance in Males.....29
I.4 Heterochromatin Formation Aids in Y rDNA Dominance.....30
I.5 The State of Nucleolar Dominance in Polyploid Tissues in *dcr-2* and *Su(var)3-9* Mutants.....31
I.6 X/X Females Express Both rDNA Loci Throughout Development.....32
I.7 ITS Probes that Differentially Identify Distinct X rDNA Variants.....33
I.8 Two X Chromosomes (ITS^{+} vs. $ITS^{\Delta 24}$ Variants) Exhibit Co-dominance Irrespective of Parental Origins.....34
I.9 The Y rDNA Can Dominate Over X rDNA in Female Cells.....35
I.10 Y rDNA Likely Has Information to Dictate Nucleolar Dominance.....37
I.11 Cytological Characterization of the $Xbb^{-}YS$ Chromosome.....39
II.1 Nucleolar Dominance is Established Across Developmental Time in Interspecies *Drosophila* Hybrid Males.....65
II.2 Nucleolar Dominance is Established Across Developmental Time in Interspecies *Drosophila* Hybrid Females.....67
II.3 Zhr^{1} Rescued *Drosophila* Interspecies Hybrids Exhibit Higher Rates of *D. simulans* rRNA Expression.....69
II.4 FM6 Balancer Can Rescue Female Embryonic Lethality and Disrupt Nucleolar Dominance.....71
III.1 DNA FISH (C1/C2) on Neuroblast Chromosome Spreads from *D. melanogaster*, *D. simulans*, *D. mauritiana*, and *D. sechellia*.....88
III.2 DNA FISH (C3/C4) on Neuroblast Chromosome Spreads from *D. melanogaster*, *D. simulans*, *D. mauritiana*, and *D. sechellia*.....89
III.3 DNA FISH (C5/C6) on Neuroblast Chromosome Spreads from *D. melanogaster*, *D. simulans*, *D. mauritiana*, and *D. sechellia*.....90
III.4 DNA FISH (C7/C8) on Neuroblast Chromosome Spreads from *D. melanogaster*, *D. simulans*, *D. mauritiana*, and *D. sechellia*.....91
III.5 DNA FISH (C11) on Neuroblast Chromosome Spreads from *D. melanogaster*, *D. simulans*, *D. mauritiana*, and *D. sechellia*.....92
III.6 DNA FISH on Neuroblast Chromosome Spreads from *D. simulans* T(Y:2) and T(Y:3) Translocation Strains.....93
III.7 DNA FISH (C9/C10) on Neuroblast Chromosome Spreads from *D. simulans*, *D. mauritiana*, and *D. sechellia*.....94
B.1 Guam X rDNA Can Dominate Over Zhr^{1} X rDNA in X/X Females.....110
B.2 Sequence Alignments of Various rDNA ITS.....112
B.3 Guam X rDNA Disrupts Female and Male Nucleolar Dominance.....113

B.4 IGS Transcription Occurs in Early Embryogenesis and is Higher in Male Embryos.115
B.5 rDNA on the Third Chromosome Disrupts Nucleolar Structure and Results in the Bobbed Phenotype.....116
B.6 ITS RNA *in situ* Probes are Species-Specific.....117
B.7 IGS is Transcribed from *D. melanogaster* rDNA in Interspecies Hybrid Embryos....118
B.8 Strains with rDNA Deletions and/or rDNA Additions Have Variable Effects on Female Hybrid Embryonic Rescue.....119

List of Appendices

A. Supplementary Tables.....103
B. Supplementary Figures.....110

List of Abbreviations

DNA, deoxyribonucleic acid
RNA, ribonucleic acid
rDNA, ribosomal DNA
rRNA, ribosomal RNA
D. melanogaster, *Drosophila melanogaster*
D. simulans, Drosophila simulans
SNP *in situ*, single nucleotide polymorphism *in situ* hybridization
RNA *in situ*, RNA *in situ* hybridization
DNA FISH, DNA fluorescent *in situ* hybridization
GSCs, germline stem cells
PBS, phosphate buffered saline
DAPI, 4',6-diamidino-2-phenylindole
SSC, saline-sodium citrate
BSA, bovine serum albumin
diH_2O, deionized water

Abstract

In eukaryotes, ribosomal RNA (rRNA) genes are tandemly repeated, forming ribosomal DNA (rDNA) loci that contain hundreds of rRNA genes to meet the demand of ribosome biogenesis. Individual rDNA loci can be entirely silenced or transcribed (termed nucleolar dominance) to control rRNA dosage. The mechanisms through which rDNA silencing is regulated has been extensively studied, however how or why particular loci are chosen to be silenced/transcribed remain elusive. This dissertation aims to understand how the large-scale silencing of nucleolar dominance is determined and how this "choice" might affect a cell or organism. This dissertation utilizes RNA *in situ* hybridization techniques and chromosome inversions/translocations to characterize nucleolar dominance within the species *D. melanogaster*. We show that nucleolar dominance is a developmentally-regulated program in *D. melanogaster* X/Y males, where the Y rDNA dominates in expression over the X rDNA locus over developmental time and in a tissue-specific manner. We expand on the observation that nucleolar dominance does not typically occur between two X rDNA loci in females across development, and that this difference between male and female nucleolar dominance is not due to cellular sex. We further show that the short arm of the Y chromosome, including its rDNA locus, likely contains elements that dictate its dominant expression over the X rDNA locus. This study on nucleolar dominance in *D. melanogaster* adds insights into how the "choice" of rDNA locus silencing/expression is made.

Nucleolar dominance was originally discovered to occur in interspecies hybrids where one species rDNA dominates over another. Whether or not nucleolar dominance is similar within a species and in interspecies hybrids has been extensively studied in *Arabidopsis*, but has been limited in studies of *Drosophila.* By utilizing RNA *in situ* hybridization probes that distinguish rRNA from the closely related species *D. melanogaster* and *D. simulans*, we expand on the characterization of nucleolar

dominance in *Drosophila* interspecies hybrids to different stages of development. We find that both rDNA loci in interspecies hybrid *Drosophila* are expressed in early embryogenesis and that nucleolar dominance (*D. melanogaster* rDNA dominance and *D. simulans* rDNA silencing) is established across development, similar to that seen in "pure species" *Drosophila* and *Arabidopsis.*

When *D. simulans* females and *D. melanogaster* males form hybrids, the female progeny die during early embryonic divisions as a result of chromosome bridge formation at the large X-chromosome specific heterochromatic block of 359-bp satellite DNA, a satellite DNA specific to *D. melanogaster*. The *D. melanogaster* mutant Zhr^1, which lacks 359-bp satellite DNA on the X chromosome, is known to rescue this female lethality. This dissertation reveals for the first time that Zhr^1-rescued interspecies hybrid females have reversed nucleolar dominance where *D. simulans* rDNA is expressed and *D. melanogaster* rDNA is silenced. This dissertation suggests that rDNA and *Zhr* (359-bp) have a relationship that may connect nucleolar dominance with female lethality seen in these *D. melanogaster – D. simulans* hybrids. This dissertation lays the groundwork to further understand how the organization and function of highly repetitive regions (rDNA) and proximal species-specific satellite DNAs (359-bp) may have relationships that act as barriers between species. In summary, this dissertation begins to uncover the mechanisms underlying nucleolar dominance within the species *D. melanogaster* and aims to understand how the regulation and divergence of nucleolar dominance and satellite DNAs may have consequences that manifest in barriers between species.

Introduction

Ribosomal RNA (rRNA), the catalytic component of ribosomes, is critically important for cellular metabolism. Levels of rRNA in a cell are modulated based on cellular energetic expenditure and can be decreased when nutrients are low, or increased when nutrients are high or growth rate is increased (e.g. in cancer) (Smetana and Busch 1964, Busch et al. 1979, Ghoshal et al. 2004, Grewal et al. 2005, Murayama et al. 2008, Aldrich and Maggert 2015). Ribosomal DNA (rDNA), the genes encoding rRNA, are highly repetitive (100s to 1000s of copies) and often exist as multiple loci on separate chromosomes (e.g. 2 loci in *Drosophila melanogaster*, 4 in *Arabidopsis*, 10-12 in *Mus musculus*, ~10 in *Homo sapiens* per diploid genome) (Long and Dawid 1980, Pontes et al. 2004). This expansive copy number is thought to meet the high demand of rRNA within the cell, given that rRNA accounts for ~60% of the total transcripts in a metabolically active cell (Moss and Stefanovsky 2002). Given the importance of maintaining proper rRNA levels within a cell, rDNA transcription has been found to be regulated at both the individual copy level and at entire chromosomal loci.

A phenomenon called nucleolar dominance is proposed to be a mechanism that regulates the dosage of rRNA at the chromosomal locus level, whereby individual rDNA loci are either entirely expressed or silenced (Preuss and Pikaard 2007). Nucleolar dominance has been noted to be one of the largest epigenetic mechanisms discovered thus far, second only to X-inactivation in eutherian mammals (Pikaard 2000). Nucleolar dominance was originally discovered in interspecies hybrids (i.e. *Xenopus* hybrids (Cassidy and Blackler 1974), *Arabidopsis* hybrids (Chen et al. 1998), *Drosophila* hybrids (Durica and Krider 1977, Goodrich-Young and Krider 1989, Oliveira et al. 2006), and mouse-human hybrid cell lines (Croce et al. 1977)), where rDNA loci inherited from one species are preferentially expressed and those from the other are silenced. Later,

nucleolar dominance was shown to occur within a species (i.e. non-hybrid context) (Lewis et al. 2004, Greil and Ahmad 2012, Zhou et al. 2012), indicating that nucleolar dominance is a mechanism to regulate rRNA expression/dosage and not purely as a result of incompatibilities between species.

Nucleolar dominance in *Arabidopsis* hybrids and *Arabidopsis thaliana* has been thoroughly studied and shown to be mechanistically similar, given that patterns of nucleolar dominance are consistent between hybrids and non-hybrids (Lewis et al. 2004). In both cases, nucleolar dominance is gradually established during development, where seedling cotyledons express rRNA from all rDNA loci (i.e. 'co-dominance'), transitioning to preferential expression of certain loci in mature tissues (Pontes et al. 2007, Earley et al. 2010). Studies in *Arabidopsis* have revealed the importance of small interfering RNAs (siRNAs) (Pontes et al. 2006, Preuss et al. 2008), histone deacetylation (Probst et al. 2004, Earley et al. 2006, Earley et al. 2010), DNA methylation (Chen et al. 1998, Lawrence et al. 2004, Pontes et al. 2006, Preuss et al. 2008, Costa-Nunes et al. 2010, Earley et al. 2010), histone methylation (Earley et al. 2010, Pontvianne et al. 2012), and chromosomal context/location (Chandrasekhara et al. 2016, Mohannath et al. 2016) in establishing nucleolar dominance. These studies indicate the mechanisms through which rDNA loci are silenced, but they do not necessarily indicate why nucleolar dominance occurs, why particular loci are "chosen" for silencing or expression, or if there are any consequences to nucleolar dominance.

Nucleolar dominance was found to occur in interspecies *Drosophila* hybrids where the *D. melanogaster* rDNA dominates in expression over *D. simulans* rDNA utilizing the presence of secondary constrictions at the active rDNA locus (Durica and Krider 1977). Nucleolar dominance in interspecies hybrids was further found to be influenced by specific regions of both the *D. melanogaster* X and Y heterochromatin (Durica and Krider 1978). Nucleolar dominance was later found to occur within the species *D. melanogaster*, where the rDNA on the Y chromosome ('Y rDNA') dominates over rDNA on the X chromosome ('X rDNA'), based on transcription-dependent deposition of GFP-tagged histone H3.3 onto the active rDNA locus (i.e. the Y rDNA

locus) (Greil and Ahmad 2012) and the presence of a secondary constriction of the active rDNA locus observed on condensed mitotic chromosomes of larval neuroblasts (Zhou et al. 2012). These methods used to study nucleolar dominance in *Drosophila* relied on readily available mitotic chromosomes, leaving the assessment of nucleolar dominance in other cell types (e.g. those not frequently undergoing mitosis) elusive. Given the previous technical limitations, many outstanding questions remain in the field. Little is known about what regulates nucleolar dominance in *Drosophila,* when nucleolar dominance is established, or if *Drosophila* interspecies nucleolar dominance is similar to that within the species *D. melanogaster.* Furthermore, it remains unknown why nucleolar dominance occurs, why particular loci are "chosen" for silencing or expression, or if there are consequences to this choice. To expand on previous studies of nucleolar dominance, in this dissertation I utilized species-specific sequence differences between rDNA loci as well as single nucleotide polymorphisms between *D. melanogaster* X and Y rDNA to visualize rRNA transcripts through multiple fluorescent *in situ* hybridization methodologies and characterized nucleolar dominance across development.

In the first two chapters of this dissertation, I expand on the knowledge of nucleolar dominance within the species *D. melanogaster* and separately within *Drosophila* hybrids generated from the sibling species *D. simulans* and *D. melanogaster*. I found that nucleolar dominance is established across developmental time in both *D. melanogaster* males and in *Drosophila* interspecies hybrids, reminiscent to what is known to occur in *Arabidopsis* (Pontes et al. 2007, Earley et al. 2010). The mechanisms regulating nucleolar dominance in *Drosophila* and *Arabidopsis* appear to be similar where heterochromatin formation and chromosome location are both important for establishing complete silencing of the non-dominant rDNA locus. However, these mechanisms reveal how the large-scale silencing of rDNA is implemented to achieve nucleolar dominance; in this dissertation, I further sought to understand the mechanisms that dictate the "choice" of nucleolar dominance in *Drosophila*, or rather, why the silencing/expression of particular rDNA loci is not random.

Nucleolar dominance appears to be a cellular or genetic "choice" given that patterns of nucleolar dominance are not random: a particular locus is always expressed or silenced. Factors that increase the rates of co-dominance are most likely involved in the mechanism of silencing an rDNA locus. Whereas these mechanisms are important for establishing rDNA silencing, we hypothesize that the silencing mechanisms are separate from the mechanism(s) of choice. Identifying factors that regulate the mechanism of choice in nucleolar dominance would require either 1) randomization of dominance, where 50% of all cells are dominant in expression of one rDNA locus and the other 50% of all cells are dominant in expression of the second rDNA locus, or 2) inducing a complete switch of which rDNA locus dominates in expression. This dissertation reveals that factor(s) present on the Y chromosome can induce nucleolar dominance within the species *D. melanogaster* (see Chapter I). Future studies are needed to dissect what element(s) on the Y chromosome and its rDNA locus dictate its ability to be dominantly expression and further to understand what element(s) on or lacking on the X chromosome that identify it to be silenced.

This dissertation also reveals that a *D. melanogaster* – specific satellite DNA may influence the choice of nucleolar dominance in *Drosophila* hybrids (see Chapter II). Satellite DNAs are short tandem repeats that are abundant and conserved features of eukaryotic genomes that have been typically considered "junk" DNA due to a lack of protein coding potential. However, many studies have implicated satellite DNA function in cellular processes such as kinetochore/centromere function, meiotic chromosome segregation, and X chromosome recognition (Dernburg et al. 1996, Karpen et al. 1996, Guenatri et al. 2004, Bouzinba-Segard et al. 2006, Usakin et al. 2007, Wong et al. 2007, Menon et al. 2014, Rosic et al. 2014). Studies have also revealed that closely related species display significant divergence in the abundance and sequence of individual satellite DNA repeats (Lohe and Brutlag 1987, Lohe and Roberts 2000, Bosco et al. 2007) leading to the hypothesis that rapid divergence of satellite DNA may play an important role in speciation (Yunis and Yasmineh 1971, Gatti et al. 1976). In support of this idea, it was shown that a satellite DNA on the *D. melanogaster* X chromosome (i.e., 359-bp satellite DNA) causes hybrid incompatibility and ultimate lethality when *D.*

melanogaster males are crossed with females of its closest sibling species, *D. simulans* (Sawamura et al. 1993, Ferree and Barbash 2009). When 359-bp is removed from the *D. melanogaster* X chromosome, not only do female hybrids survive the hybrid lethality (Sawamura et al. 1993, Ferree and Barbash 2009), in this dissertation I discovered that these female hybrids exhibit reversed patterns of hybrid nucleolar dominance. We speculate that there may be a relationship between rDNA and 359-bp which may shed light on how this hybrid incompatibility arises. This dissertation lays the groundwork to understand how the choice of nucleolar dominance is dictated in both *D. melanogaster* and *Drosophila* interspecies hybrids, and to understand how the developmental regime of nucleolar dominance may influence incompatibilities that arise between species.

Chapter I[1]: Regulation of Nucleolar Dominance in *Drosophila melanogaster*

[1] The majority of Chapter I is published in *Warsinger-Pepe et al, 2020*. Sections have been modified to reflect additions within this dissertation.

Summary

In eukaryotic genomes, ribosomal RNA (rRNA) genes exist as tandemly repeated clusters, forming ribosomal DNA (rDNA) loci. Each rDNA locus typically contains hundreds of rRNA genes to meet the high demand of ribosome biogenesis. Nucleolar dominance is a phenomenon whereby individual rDNA loci are entirely silenced or transcribed, and is believed to be a mechanism to control rRNA dosage. Nucleolar dominance was originally noted to occur in interspecies hybrids, and has been shown to occur within a species (i.e., nonhybrid context). However, studying nucleolar dominance within a species has been challenging due to the highly homogenous sequence across rDNA loci. By utilizing single nucleotide polymorphisms between X rDNA and Y rDNA loci in males, as well as sequence variations between two X rDNA loci in females, we conducted a thorough characterization of nucleolar dominance throughout development of *Drosophila melanogaster*. We demonstrate that nucleolar dominance is a developmentally regulated program that occurs in nonhybrid, wild-type *D. melanogaster*, where Y rDNA dominance is established during male embryogenesis, whereas females normally do not exhibit dominance between two X rDNA loci. By utilizing various chromosomal complements (e.g., X/Y, X/X, X/X/Y) and a chromosome rearrangement, we show that the short arm of the Y chromosome including the Y rDNA likely contains information that instructs the state of nucleolar dominance. Our study begins to reveal the mechanisms underlying the selection of rDNA loci for activation/silencing in nucleolar dominance in the context of nonhybrid *D. melanogaster*.

Introduction

Ribosomal DNA (rDNA), genes encoding the catalytic RNA components of ribosomes, is highly repetitive (100s to 1000s of copies) and often exists as multiple loci on separate chromosomes (e.g. 2 loci in *Drosophila melanogaster*, 4 in *Arabidopsis*, 10-12 in *Mus musculus*, ~10 in *Homo sapiens* per diploid genome) (Long and Dawid 1980, Pontes et al. 2004). This expansive copy number may come to no surprise, considering that the transcription of rDNA accounts for ~60% of the total transcription of a metabolically active cell (Moss and Stefanovsky 2002). The regulation of ribosomal RNA (rRNA) expression is critically important for adjusting cellular energetic expenditure: when nutrients are low rRNA synthesis is downregulated, whereas the opposite occurs when nutrients are high or growth rate is increased (e.g. in cancer) (Smetana and Busch 1964, Busch et al. 1979, Ghoshal et al. 2004, Grewal et al. 2005, Murayama et al. 2008, Aldrich and Maggert 2015). Accordingly, transcription of rRNA is expected to require precise regulation.

A phenomenon called nucleolar dominance, whereby individual rDNA loci are either entirely expressed or silenced, is proposed to be a mechanism that regulates the dosage of rRNA (Preuss and Pikaard 2007). Nucleolar dominance has been noted to be one of the largest epigenetic mechanisms, second only to X-inactivation in eutherian mammals (Pikaard 2000). Nucleolar dominance was originally discovered in interspecies hybrids (i.e. *Xenopus* hybrids (Cassidy and Blackler 1974), *Arabidopsis* hybrids (Chen et al. 1998), *Drosophila* hybrids (Durica and Krider 1977, Goodrich-Young and Krider 1989, Oliveira et al. 2006), and mouse-human hybrid cell lines (Croce et al. 1977)), where rDNA loci inherited from one species are preferentially expressed and those from the other are silenced. Later, nucleolar dominance was shown to occur within a species (i.e. non-hybrid context) (Lewis et al. 2004, Greil and Ahmad 2012,

Zhou et al. 2012), indicating that nucleolar dominance is a mechanism to regulate rRNA expression/dosage instead of a result of interspecies incompatibility.

Nucleolar dominance has been thoroughly studied in *Arabidopsis*, both in *A. suecica* (the interspecies hybrid between *A. thaliana* crossed to *A. arenosa*) as well as non-hybrid *A. thaliana* (Pontes et al. 2007, Earley et al. 2010). In both cases, nucleolar dominance is gradually established during development, where seedling cotyledons express rRNA from all rDNA loci (i.e. 'co-dominance'), transitioning to preferential expression of certain loci in mature tissues (Pontes et al. 2007, Earley et al. 2010). Several mechanisms have been shown to mediate the silencing of chosen rDNA loci, including small interfering RNAs (siRNAs) (Pontes et al. 2006, Preuss et al. 2008), DNA methylation (Chen et al. 1998, Lawrence et al. 2004, Pontes et al. 2006, Preuss et al. 2008, Costa-Nunes et al. 2010, Earley et al. 2010), histone methylation (Earley et al. 2010, Pontvianne et al. 2012), and histone deacetylation (Probst et al. 2004, Earley et al. 2006, Earley et al. 2010). These mechanisms reveal how the large-scale silencing of rDNA is implemented to achieve nucleolar dominance, however, what factor(s) influence the choice of which rDNA loci are silenced or activated remain elusive.

Nucleolar dominance is likely a wide-spread phenomenon across many species. For example, only a subset of rDNA loci are transcribed in human cell lines (Roussel et al. 1996) and human lymphocytes (Roussel et al. 1996, Heliot et al. 2000), implying that these cells also may undergo nucleolar dominance, although which loci are silenced and/or expressed remains unknown. Nucleolar dominance was found to occur in 'pure species' or 'non-hybrid' *D. melanogaster* larval neuroblasts, where the rDNA on the Y chromosome ('Y rDNA') dominates over rDNA on the X chromosome ('X rDNA'), based on transcription-dependent deposition of GFP-tagged histone H3.3 onto the active rDNA locus (i.e. the Y rDNA locus) (Greil and Ahmad 2012) and the presence of a secondary constriction of the active rDNA locus observed on the condensed mitotic chromosomes (Zhou et al. 2012). These methods relied on readily available mitotic chromosomes, leaving the assessment of nucleolar dominance in other cell types (e.g. those not frequently undergoing mitosis) elusive. Recently, we adapted a single nucleotide

polymorphism RNA fluorescent *in situ* hybridization (SNP *in situ*) protocol and showed that nucleolar dominance (Y rDNA dominance) also occurs in male germline stem cells (Levesque et al. 2013, Lu et al. 2018). This method utilizes SNPs between the X rDNA and the Y rDNA to differentially label their products (X- vs. Y-derived rRNA), allowing assessment of nucleolar dominance without requiring mitotic chromosomes.

In this study, we utilized SNP *in situ* to comprehensively examine the state of nucleolar dominance in *D. melanogaster* (i.e. 'non-hybrid' context) during development and across different tissues. We show that nucleolar dominance in *D. melanogaster* is gradually established during development, similar to the observations in *A. thaliana*, supporting the notion that nucleolar dominance is a regulatory mechanism that occurs in non-hybrid organisms. We have further examined the state of nucleolar dominance between two X rDNA loci in females by isolating X rDNA with distinct sequences that enables RNA *in situ* hybridization to distinguish transcripts from two X rDNA loci. Our results show that the two X rDNA loci in females exhibit co-dominance in essentially all tissues, expanding the previous finding of co-dominance in female larval neuroblasts (Greil and Ahmad 2012). Moreover, by utilizing the various karyotypes (e.g. X/X females, X/Y males, vs. X/X/Y females) and a chromosome rearrangement strain, we show that Y chromosome element(s) (within Y rDNA as well as non-rDNA element(s) of the Y chromosome) may aid in the 'choice' mechanism that underlies nucleolar dominance: i.e. the preferential expression of the Y rDNA locus and the preferential silencing of the X rDNA locus. These results provide insights into how specific rDNA loci may be preferentially transcribed/silenced, and will provide the foundation for future studies aimed at understanding the regulation and biological meaning of nucleolar dominance.

Materials and Methods

Fly Husbandry and Strains

Unless otherwise stated, all fly stocks were raised on standard Bloomington medium at room temperature (RT). Unless otherwise stated, all flies used for wild-type experiments were the standard lab wild-type strain y^1w^1, referred to as *yw*, that contains the X and Y chromosomes with mapped rDNA SNPs (Lu et al. 2018). Stocks used to study female nucleolar dominance were obtained from the UC San Diego *Drosophila* Stock Center and the culture was established by using single pair parents to minimize heterogeneity of rDNA within each stock.

The X and Y chromosomes from wild type (*yw*) were introduced into genotypes of interest analyzed in this study to keep the rDNA loci consistent across experiments. When it was not feasible to introduce the wild type (*yw*) X and/or Y chromosomes into a genetic background of interest, their rDNA was sequenced to find SNPs between the X and Y rDNA and the appropriate SNP *in situ* probes were used.

Assessing Bobbed Phenotype with Addition of Third rDNA Locus

All bobbed assays were performed at room temperature (RT). For each vial, one virgin female and one young male (all less than or equal to 5 days old) were paired. Single flies were paired to more accurately assess any defects in chromosome segregation. After one week post pairing, P0 flies were flipped to new vials, and continued to flip for four weeks. After the fourth week, P0 flies were tossed. Vials were only scored if P0 survived all four weeks and if F1 were present in the vials. All F1 were collected and scored up until 20 days after pairing in each vial. Progeny were scored for sex, bobbed phenotype which here is visualized by cuticular etching and/or peeling, the presence of a marked Y chromosome, and the presence of the rDNA BAC on the third chromosome.

RNA in situ Hybridization

Third instar larval or adult tissues were dissected in RNase-free 1X PBS, fixed in RNase-free 4% formaldehyde, and incubated overnight in 70% EtOH at 4ºC to permeabilize the tissues. Embryos were collected according to a modified protocol from (Wilk et al. 2010) by allowing parents to lay eggs on an apple-agar plate at room temperature for a range of collection time (3 – 17hrs). Embryos were transferred to glass scintillation vials with glass Pasteur pipettes and were washed of any yeast in 1X PBS then dechorionated in 50% bleach for 30 sec and washed again in PBS. The embryos were then devitellinized and fixed in 50:50 heptane:4% RNase-free formaldehyde during vigorous, manual shaking for 20 min, then again in 50:50 heptane:methanol twice for 30 sec, washed in methanol then stored in methanol at -20ºC for at least one night before proceeding to *in situ* hybridization.

In situ hybridization was performed as previously described with slight modifications (Lu et al. 2018). In short, X/Y and X/X/Y samples were washed with wash buffer (10% formamide in 1X SSC and 0.1% Tween-20) for 5 min, then incubated with the hybridization mix (10% formamide, 1X SSC, 10% Dextran sulfate (w/v) (Sigma, D8906), 100nM each *in situ* fluorescent probe (X and Y rDNA SNP probes), and 300nM each mask oligo (for SNP *in situ*) overnight in a 37ºC water bath. For assessment of intergenic spacer (IGS) transcripts, 100nM of anti-sense IGS fluorescent oligonucleotide probe was added to the hybridization mix. Samples were then washed twice in wash buffer for 30 min each at 37ºC and stored in Vectashield H-1200 (Vector Laboratories) with DAPI. Hybridization and washes for X/X females were performed at 42ºC. Images were taken using a Leica TCS SP8 confocal microscope with 63X oil-immersion objectives and processed using Adobe Photoshop software.

See Table A.1 for fluorescent *in situ* oligonucleotide probes. Unless otherwise stated, all four X rDNA SNP *in situ* probes and all four Y rDNA SNP *in situ* probes were used for each experiment visualizing X and Y rRNA. SNP *in situ* oligonucleotide probes were custom ordered from Biosearch Technologies (Lu et al. 2018). Fluorescent *in situ*

oligonucleotide probes used to study female nucleolar dominance were designed using Integrated DNA Technologies Oligo Analyzer.

Identification of SNPs in rDNA

To sequence X rDNA, genomic DNA was extracted from 10-15 female flies of a genotype of interest. To sequence Y rDNA, male flies of the genotype of interest were crossed to C(1)DX/Y female flies, which lack X rDNA, and 10-15 female progeny (which have the Y chromosome of interest and C(1)DX) was subjected to genomic DNA extraction. PCR was performed on the extracted genomic DNA to amplify three regions of the rDNA with the primers listed in Table A.1.

PCR products were verified by agarose gel electrophoresis and purified using a PCR Purification Kit (Qiagen). Sanger sequencing was performed on the purified PCR products using the same PCR primers (University of Michigan Biomedical Research DNA Sequencing Core Facility). Sequencing data was analyzed using the free downloadable software ApE: A plasmid Editor, by M. Wayne Davis.

Larval Brain Squash and DNA FISH on Mitotic Chromosomes

We utilized a modified DNA fluorescent *in situ* hybridization (FISH) protocol described previously (Larracuente and Ferree 2015, Jagannathan et al. 2017). In short, third instar larvae were collected and brains were dissected in 1X PBS. Larval brains were fixed in 45:55 acetic acid:4% formaldehyde in 1X PBS on Superfrost Plus Microscope Slides (Fisherbrand 22-037-246). The sample was then covered with a coverslip, manually squashed, and submerged in liquid nitrogen until frozen. The coverslips were quickly removed and the slides were treated with 100% ethanol at room temperature (RT) for 5 min. If the Stellaris 18S probe was included in the hybridization, RNase treatment was performed before hybridization (see Lu and Nelson et al. 2018 for 18S Stellaris probe details). RNase treatment was carried out with 2mg/mL RNase A in 1X PBS for 1 hour at RT in a humid chamber in the dark. Slides were then dehydrated in 100% ethanol at RT for five min and dried. 20µL of hybridization buffer (50% formamide, 10% dextran sulfate, 2X SSC buffer, 0.5µM of each probe) was added to

the sample, covered with a cover slip, and the sample was heat-denatured at 95°C for 5 min, followed by incubation in a humid chamber in the dark overnight at room temperature. Samples were washed three times for 15 min in 0.1X SSC and then mounted in Vectashield H-1200 (Vector Laboratories) with DAPI. Probe sequences are provided in Table A.1. Images were taken using a Leica TCS SP8 confocal microscope with 63X oil-immersion objectives and processed using Adobe Photoshop software.

Hybrid Dysgenesis Assays

All hybrid dysgenesis assays were performed at 25ºC as described in (Engels and Preston 1979). For each vial, two virgin females and one young male (all less than or equal to 5 days old) were paired. After one week post pairing, P0 flies were tossed. Female F1 were collected, aged for 2-5 days, and their ovaries were dissected in 1X PBS and assessed for hybrid dysgenesis. Ovaries were deemed dysgenic if at least 1 ovary was atrophied, deformed, or absent.

Immunofluorescence on Mitotic Chromosome Spreads

A protocol described in (Blum et al. 2017) was used to conduct immunofluorescence on mitotic chromosome spreads. Briefly, larval brains from third instar larvae were dissected and incubated in 30µL of 0.5% sodium citrate on Superfrost Plus Microscope Slides (Fisherbrand 22-037-246) for 10-20 min. Sodium citrate was gently removed using a micropipette. 25µL of 4% formaldehyde was gently added to the slide over the sample, removed with a micropipette and replaced with another fresh 25µL of 4% formaldehyde and fixed for 4 min. During fixation, the larval brains were dissected into smaller pieces. Any imaginal discs and/or the ventral nerve cord were removed during this process. After fixation, the sample was covered with a coverslip, squashed and submerged in liquid nitrogen until frozen. After removal of the coverslips, slides were washed in PBS for 30 - 60 min and incubated overnight with primary antibodies (Chicken anti-Cid, 1:200) in 3% BSA in 1X PBST at 4°C in a humid chamber. The slides were washed in 1X PBST, three times for 20 min each, then incubated with secondary antibodies (Goat anti-Chicken Alexa Fluor 488 Invitrogen, A-11039, 1:200) in 3% BSA in 1X PBST for 45 min at room temperature in a humid chamber in the dark.

Slides were washed in 1X PBST, three times for 20 min and mounted in Vectashield H-1200 (Vector Laboratories) with DAPI. Images were taken using a Leica TCS SP8 confocal microscope with 63X oil-immersion objectives and processed using Adobe Photoshop software.

Immunofluorescence on Whole Mount Tissues and Microscopy

Immunofluorescence staining was performed as described (Cheng et al. 2008). Briefly, tissues were dissected in 1X PBS, transferred to 4% formaldehyde in PBS and fixed for 30 min. Tissues were then rinsed twice in 1X PBST and then washed for at least 60 min at RT for permeabilization. This was followed by incubation with primary antibody in 3% BSA in 1X PBST at 4°C overnight. Samples were washed three times for 20 min per wash in 1X PBST, incubated with secondary antibody in 3% BSA in 1X PBST at 4°C overnight, washed again as above, and mounted in Vectashield H-1200 (Vector Laboratories) with DAPI. The following primary antibodies were used: rat anti-vasa (1:20; DSHB; developed by A. Spradling), guinea pig anti-D1 (Covance) (Jagannathan et al. 2019), rabbit anti-Fibrillarin (1:200; Abcam ab5821). Images were taken using a Leica TCS SP8 confocal microscope with 63X oil-immersion objectives and processed using Adobe Photoshop software.

Quantification and Statistical Analysis of Nucleolar Dominance

Nucleolar dominance was quantified manually from images generated using a Leica TCS SP8 confocal microscope. For each embryo, larval brain, imaginal disc, larval anterior midgut, and adult anterior midgut sample, 1-3 representative images of each tissue were captured for scoring purposes. Imaginal discs were randomly scored without intentionally excluding any imaginal disc type, therefore all imaginal discs were included in the category of ‘imaginal discs’ for scoring purposes. Z-stacks were generated with maximum projections for pre-gastrulation embryos, larval anterior midgut and adult anterior midgut images for scoring. Whole tissues were scored for salivary glands and larval fat bodies. All cells were identified and scored based on nuclear DAPI staining and morphology. Note that the call of dominance vs. co-dominance was straightforward, owing to consistent signal intensity across samples based on the RNA

in situ procedure described above. The number of cells and the number of tissues scored per genotype are listed in each corresponding figure legend. p-values were calculated using an unpaired Student's *t*-test with Welch's correction (assuming unequal variances) with n representing number of tissues scored.

Quantification and Statistical Analysis of Relative rDNA Locus Size

Quantification of relative rDNA fluorescence was performed using a protocol modified from (Lu et al. 2018). Images of rDNA from chromosome spreads were taken using a Leica TCS SP8 confocal microscope with 63X oil-immersion objectives. Images settings were modified to detect saturation to ensure that acquired signals were not saturated. ImageJ was used to quantify fluorescence from the acquired z-stacks. Slices in the stack for each channel to be quantified were summed together in a z-projection. Signals measured from each chromosome were manually encircled and signal was measured as Raw Integrated Density and compared between the two chromosomes. p-values were calculated using an unpaired Student's *t*-test with Welch's correction (assuming unequal variances) with n representing number of cells scored.

Results

Y rDNA Dominance is Gradually Established During Male Development

Thorough characterization of nucleolar dominance within a species (i.e. in the context of non-hybrids) has been limited to *A. thaliana* (Tucker et al. 2010). Prior analysis of nucleolar dominance in non-hybrid *D. melanogaster* has been limited to larval neuroblasts and adult male germline cells (Greil and Ahmad 2012, Zhou et al. 2012, Lu et al. 2018). To extend the analysis of nucleolar dominance in non-hybrid *D. melanogaster*, we applied the SNP *in situ* hybridization method that differentiates X rDNA-derived rRNA vs. Y rDNA-derived rRNA (Lu et al. 2018). Using this technique, we comprehensively analyzed the state of nucleolar dominance during development of *D. melanogaster* (Figure I.1). In all experiments reported in this study, X and Y chromosomes with defined rDNA SNPs from a wild type strain (*yw*) were introduced into the genetic background of interest. Alternatively, distinct SNPs were identified by sequencing X and Y rDNA loci, if introduction of the *yw* strain sex chromosomes was complicated/impossible (see Materials and Methods).

We first focused on nucleolar dominance in male embryos: 48.6% of the total embryos scored (n = 368) contained both the Y rDNA and X rDNA SNP signals, which we deemed as male embryos. Note that not all nuclei within an embryo necessarily contained both X rRNA and Y rRNA signals, but the presence of any Y rRNA-containing nuclei within an embryo indicates that they are male embryos. On the contrary, 51.4% of embryos contained only X rDNA SNP signal in all nuclei within an entire embryo, which were deemed as female embryos. Since our SNP *in situ* probes cannot discriminate rRNA signals from two X rDNA loci in females, their state of nucleolar dominance cannot be determined by these experiments (Figure I.1G-J) (see below for nucleolar dominance in females). We found that in early male embryos (pre-gastrulating, around syncytial cycle 13-14), the majority of nuclei expressed both the X

and Y rDNA (i.e. co-dominant) (94.8 ± 13.2%) (Figure I.1B-D). It has been reported that larval neuroblasts (Greil and Ahmad 2012), male germline stem cells (GSCs) and spermatogonia (Lu et al. 2018) exhibit Y rDNA dominance, suggesting that nucleolar dominance may be established during the course of development. To address this possibility, we examined the state of nucleolar dominance along the course of development through embryonic stages, larval development and into adulthood. Although the pre-gastrulating embryos exhibited a high frequency of co-dominance (~95%), we observed a decrease in the percentage of co-dominant nuclei, with a concomitant increase in Y rDNA-dominant cells as male embryos progressed through development (Figure I.1B-F). Male embryos during early gastrula or germband extension stages show 55.3 ± 27.5% co-dominant nuclei and 42.9 ± 27.0% Y rDNA dominant nuclei (Figure I.1B-F). Later during segmentation, co-dominant nuclei further decreased to 33.7 ± 7.1%, as Y rDNA dominant nuclei increased to 64.6 ± 7.9% (Figure I.1B).

As development proceeds to the larval stage, we observed much higher rates of Y rDNA dominance in most tissues: larval brains (83.5 ± 4.6%) similar to what has been previously reported (Greil and Ahmad 2012), imaginal discs (93.6 ± 3.1%), larval fat bodies (95.8 ± 5.1%), and larval anterior midgut enterocytes (82.9 ± 12.1%) (Figure I.2A, 2C-I). Salivary glands, which undergo a high degree of polyploidization, showed only a moderate degree of Y rDNA dominance (51 ± 19.4%) (Figure I.2A-B). Y rDNA dominance in the anterior midgut further increased in the adult (from 82.9% ± 12.1% in third instar larvae to 99.7 ± 0.7% in adult) (Figure I.2A, 2J-K). These data suggest that nucleolar dominance in *D. melanogaster* males is gradually established over the course of development. This is similar to what was reported in *Brassica* (Chen and Pikaard 1997*)* and separately in *Arabidopsis* hybrids (Pontes et al. 2007, Earley et al. 2010), where seedling cotyledons exhibit co-dominance and nucleolar dominance is established in later stages of development depending on the tissue, suggesting conservation of this phenomenon whether it be within a species or in hybrids.

Histone Methyltransferase Su(var)3-9 Aids in the Establishment of Y rDNA Dominance in Males Across Tissues

Small interfering RNAs (siRNAs) in *Arabidopsis* (Preuss et al. 2008) and long non-coding, promoter-associated RNAs in mammalian cell lines (Mayer et al. 2006) were shown to regulate rDNA silencing. These non-coding RNAs recruit factors that induce heterochromatinization of rDNA through DNA methylation (Lawrence et al. 2004, Preuss et al. 2008, Schmitz et al. 2010), histone methylation (Lawrence et al. 2004, Santoro and Grummt 2005, Pontvianne et al. 2012) and histone deacetylation (Santoro and Grummt 2005, Earley et al. 2006). The siRNA pathway and heterochromatin factors have also been shown to influence nucleolar morphology in *D. melanogaster* larval tissues, which may reflect disrupted rDNA expression (Peng and Karpen 2007). Based on these previous studies, we wondered whether the siRNA machinery and/or heterochromatin formation play a role in nucleolar dominance in *D. melanogaster*. To test this, we assessed nucleolar dominance in the mutants of *dicer-2* (*dcr-2*), an endonuclease critical for the siRNA pathway (Kim et al. 2006), and *Su(var)3-9*, a histone methyltransferase critical for depositing heterochromatin-associated histone methylation (Elgin and Reuter 2013). We found that $dcr\text{-}2^{L811fsx}$/ $dcr\text{-}2^{p[f06544]}$ mutants showed only a slight (although statistically significant) change in Y rDNA dominance in larval brains (71.4 ± 7.5% compared to control 83.5 ± 4.6%) and imaginal discs (81.8 ± 10.9% compared to control 93.6 ± 3.1%) (Figure I.3A-B and Figure I.4A-B, D-E), and no change in adult germline stem cells (GSCs) (57.3 ± 25.0% compared to control 70.1 ± 14.9%) (Figure I.3C) suggesting that the siRNA mechanism might not play an important role in nucleolar dominance, as opposed to what is reported in *Arabidopsis* (Pontes et al. 2006, Preuss et al. 2008). However, the potential redundancy between *dcr-2* and *dcr-1* could not be excluded as the *dcr-1* mutant was too sick to generate compound double mutants with *dcr-2* (*dcr-1/+; dcr-2/+*).

$Su(var)3\text{-}9^{1}/Su(var)3\text{-}9^{2}$ mutants showed a marked decrease in Y rDNA dominance in larval brain (46.5 ± 16.5%, compared to control 83.5 ± 4.6%), imaginal discs (61.4 ± 21.0%, compared to control 93.6 ± 3.1%), and adult GSCs (53.5 ± 17.7%, compared to control 70.1 ± 14.9%) (Figure I.3A-C and Figure I.4A, C, D, F), consistent

with the previous finding that *Su(var)3-9* is involved in silencing of X rDNA in male neuroblasts (Greil and Ahmad 2012). *dicer-2* and *Su(var)3-9* mutants had minimal effects on nucleolar dominance in polyploid tissues (salivary glands, larval fat bodies, larval anterior midgut, and adult anterior midgut enterocytes) (Figure I.5). These results suggest that the siRNA pathway does not play a major role in silencing of X rDNA for establishment of nucleolar dominance, whereas heterochromatin formation mediated by *Su(var)3-9* is important for nucleolar dominance of diploid tissues in *D. melanogaster* males.

We further examined piRNA pathway components in the germline, as the piRNA pathway is an important silencing mechanism in the germline (Yamashiro and Siomi 2018). However, neither *piwi* (p-element induced wimpy testes) or *ago3* (*argonaute 3*) mutants compromised nucleolar dominance in male GSCs (Figure I.3C). Piwi and Ago3 are involved in the nuclear and cytoplasmic arms of the piRNA pathway, respectively (Yamashiro and Siomi 2018). The GSCs of $piwi^{1}/piwi^{06843}$ males showed no change in Y rDNA dominance (73.4 ± 11.8% compared to wild-type control 70.1 ± 14.9%) (Figure I.3C). Likewise, $ago3^{t2}/ago3^{t3}$ mutants did not change rates of Y rDNA dominance (65.7 ± 19.7% compared to wild-type control 70.1 ± 14.9%) (Figure I.3C). These results suggest that the piRNA pathway does not play a major role in silencing of X rDNA in nucleolar dominance in male GSCs.

Co-dominance is Commonly Observed in X/X Female Tissues

It was previously shown that nucleolar dominance does not occur in female larval neuroblasts (Durica and Krider 1977, Greil and Ahmad 2012). We sought to determine the state of nucleolar dominance in females (between two X rDNA loci) across tissues and developmental stages. Doing so requires two distinct X rDNA loci with detectable differences, similar to SNP *in situ* hybridization described above for X vs. Y rDNA. Our initial searches for SNPs between X rDNA loci from multiple laboratory strains revealed no SNPs. However, sequencing of X rDNA from geographically separated *D. melanogaster* strains led us to the identification of a 24-bp deletion in the internal transcribed spacer (ITS1) of the X rDNA in a strain originating from Guam, compared to

most other strains sequenced (i.e. *yw,* Oregon-R, Canton-S, Beijing, Pohnpei, Samoa, Port Moresby, Le Réduit) (Figure I.6A). We designed oligonucleotide probes to distinguish the rRNA from the Guam strain ($ITS^{\Delta 24}$) vs. other strains (ITS^{+}) (see Materials and Methods, Figure I.6A). The Guam strain exhibited signals only from $ITS^{\Delta 24}$ rDNA (Figure I.7). Among other strains that have the ITS^{+} variant, the Le Réduit strain had the least background signal with the $ITS^{\Delta 24}$ probe (Figure I.7), whereas females from other strains revealed weak $ITS^{\Delta 24}$ signal in addition to predominant ITS^{+} signal (data not shown), possibly because these strains may contain a small fraction of rDNA copies with the $ITS^{\Delta 24}$ variant. Based on these results, we decided to utilize the Guam and Le Réduit strains to determine the state of nucleolar dominance between two X rDNA loci in females.

We crossed Guam strain males with Le Réduit strain females and tissues from the resulting F1 females were assessed for the state of nucleolar dominance by RNA *in situ* using the $ITS^{\Delta 24}$ and ITS^{+} probes (Figure I.6B). We found that X/X female cells predominantly show expression from both rDNA loci (i.e. co-dominant) in larval brains (67.1 ± 15.6% co-dominant) (Figure I.6C). We found that X/X female larval imaginal discs also exhibit primarily co-dominance (77.3 ± 8.1%) (Figure I.6C). Adult tissues revealed even higher rates of co-dominance compared to larval tissues: anterior midgut enterocytes (97.6 ± 2.1%) and GSCs (96.8 ± 10.7%) (Figure I.6C-E). It should be noted that we did not assess nucleolar dominance in female embryos because the Guam strain Y rDNA shares the same ITS sequence as Le Réduit X rDNA (ITS^{+}), making the accurate sexing of embryos impossible. However, female embryos in the experiments described in Figure I.1H-J mostly exhibited two nucleoli per nucleus, suggesting that female embryos also exhibit co-dominance.

The co-dominant state of X rDNA loci in the progeny of Guam and Le Réduit parents did not change even when parental origin of $ITS^{\Delta 24}$ vs. ITS^{+} rDNA loci was switched (i.e. Guam females crossed to Le Réduit males) (Figure I.8B). It should be noted that in this direction of the cross, females exhibited hybrid dysgenesis, leading to a high frequency of degenerated ovaries (Figure I.8A). Despite high rates of hybrid

dysgenesis, all cells scored exhibited co-dominance (Figure I.8B). These results establish that females exhibit co-dominance in a broad range of tissues and developmental stages, extending the previous findings in female neuroblasts (Durica and Krider 1977, Durica and Krider 1978, Greil and Ahmad 2012).

Y rDNA Dominates in Female Cells

The above results reveal a striking difference in the state of nucleolar dominance between males and females: Y rDNA dominates over X rDNA in males, whereas two X chromosomes are co-dominant in females. What accounts for this striking difference in the state of nucleolar dominance between males and females? A previous study showed that nucleolar dominance in *D. melanogaster* is not likely due to imprinting during the parents' gametogenesis, as reversing inheritance of X vs. Y chromosomes (i.e. X from father, Y from mother) did not influence the state of Y rDNA dominance (Greil and Ahmad 2012). Others have speculated that distinct sequence differences between the loci, in this case the X rDNA vs. Y rDNA loci, allow selective expression/silencing of particular rDNA loci (Kidd and Glover 1981, Macleod and Bird 1982, Labhart and Reeder 1984, Grimaldi et al. 1990, Heix and Grummt 1995, Neves et al. 1995, Houchins et al. 1997, Caudy and Pikaard 2002, Felle et al. 2010). Yet another possibility is that chromosomal context, or location within a particular chromosome (Chandrasekhara et al. 2016, Mohannath et al. 2016) may determine whether or not a particular rDNA locus may be expressed/silenced. In addition, cellular sex might determine whether or not nucleolar dominance occurs.

Because parental imprinting unlikely contributes to the regulation of nucleolar dominance (Greil and Ahmad 2012), we sought to test the possibility that X and/or Y rDNA contain specific elements that determine the state of nucleolar dominance. To this end, we examined the state of nucleolar dominance in females that carry a Y chromosome. C(1)RM is a compound X chromosome (two X chromosomes are fused and it contains X rDNA) and C(1)RM/Y flies develop as females (Bridges 1916, Bridges 1921). The rDNA on C(1)RM was found to share all SNPs with the *yw* X rDNA (see Materials and Methods) and the Y rDNA SNP *in situ* probes did not cross-hybridize

(Figure I.9E). Utilizing these SNPs, we determined the state of nucleolar dominance between C(1)RM rDNA and Y rDNA in female tissues (e.g. diploid larval tissues, the adult anterior midgut and adult ovary). Surprisingly, C(1)RM/Y females exhibited a strikingly high frequency of Y rDNA dominance in many cell types (Figure I.9A-C, F). This is in stark contrast to X/X females, where two X chromosomes exhibit co-dominance across tissues (Figure I.9A-D). These results indicate that nucleolar dominance is determined by the presence of a Y chromosome (e.g. sequence information within Y rDNA or other elements on the Y chromosome), and disfavors the possibility that cellular sex determines whether or not nuclear dominance occurs.

Interestingly, adult GSCs and cystoblasts showed a high degree of co-dominance in C(1)RM/Y females (Figure I.9D, G) (GSCs: 97.2 ± 8.9% co-dominance), whereas some nurse cells showed Y rDNA dominance (Figure I.9H). The somatic follicle cells of the egg chambers showed mostly co-dominance (Figure I.9I). These data together suggest that, whereas the Y rDNA dominates irrespective of cellular sex, it is not the sole factor to determine nucleolar dominance, and that cell-type specific information may modulate the state of nucleolar dominance.

Element(s) within the Y Chromosome Contribute to Nucleolar Dominance

The above data that the Y rDNA dominates over the X rDNA irrespective of cellular sex in most cell types indicate that the Y chromosome may contain element(s) that regulate nucleolar dominance. Such information may be embedded in the Y rDNA locus itself, such as variable sequences in the coding and/or spacer sequences (Tautz et al. 1987, Tautz et al. 1988, Schlotterer et al. 1994, Caudy and Pikaard 2002). Additionally, the entire chromosome in which the rDNA is located may dictate the state of silencing/activation (Chandrasekhara et al. 2016, Mohannath et al. 2016). To address whether the Y rDNA contains information that influences its dominance, we utilized an X chromosome that contains Y rDNA due to chromosomal rearrangements. This chromosome, In(1)$sc^{4L}sc^{8R}$+Tp(1;YS)bb^{AM7}(referred to as Xbb^{-}YS) lacks X rDNA (Xbb^{-}) but instead contains Y rDNA together with other element(s) of the Y chromosome short arm (YS) (Rasooly and Robbins 1991) (Figure I.11A and Figure I.12). We first

sequenced the rDNA from the Xbb^{-}YS chromosome and found that its rDNA exhibited 3 SNPs compared to *yw* X rDNA (see Materials and Methods). Using these three sets of SNP *in situ* probes, we found that Xbb^{-}YS/X females exhibit intermediate patterns of nucleolar dominance: in larval brain, imaginal discs, and adult anterior midgut enterocytes, Xbb^{-}YS rDNA mostly dominates over X rDNA, as opposed to co-dominance in typical X/X females (Figure I.11B-D, F, G, I-J). However, the degree of Xbb^{-}YS rDNA dominance was lower than Y rDNA dominance in X/Y males (Figure I.11B-C, J) and in C(1)RM/Y females (Figure I.11D-E, G-H). GSCs from Xbb^{-}YS/X females exhibited high rates of co-dominance, similar to X/X females (Figure I.11K) as well as C(1)RM/Y females (compare to Figure I.9D). These results suggest that Y rDNA and/or its proximal region within YS carries critical information that allows for establishment of nucleolar dominance, where Y rDNA is transcribed and X rDNA is silenced. This is similar to what has been seen in *Arabidopsis* where nucleolar dominance is "allelic" and possibly influenced by rDNA sequence differences (Rabanal et al. 2017). Additionally, the observation that the degree of Y rDNA dominance in Xbb^{-}YS/X females is much less than that in X/Y males or C(1)RM/Y females indicates that the chromosomal context (Chandrasekhara et al. 2016) (e.g. being embedded in the entire Y chromosome) and/or with other factor(s) on the long arm of the Y chromosome also play a role in the determination of Y rDNA dominance (see Discussion).

An X rDNA Locus Can Exhibit Nucleolar Dominance

Through our search for Y chromosome elements that may influence nucleolar dominance, we observed the presence of nucleolar dominance between two X chromosomes, *Zhr^{1}* and the X chromosome from the *D. melanogaster* strain from Guam (Guam X). *Zhr^{1}* is a compound X:Y chromosome that contains the central most portion of the Y chromosome (majority of YL and the Y-centromere) and the euchromatic arm of the X chromosome (Figure B.1A). *Zhr^{1}* acts as an X chromosome in sex determination so we functionally define it as an X chromosome. *Zhr^{1}* ITS is intact (ITS^{+}) (Figure B.1B), allowing the analysis of nucleolar dominance with Guam X rDNA (ITS$^{\Delta 24}$). RNA *in situ* of female adult tissues containing both Guam X and *Zhr^{1}* revealed strikingly high rates of Guam X rDNA dominance (Figure B.1D-H). We do not believe this is due to the inability

of *Zhr*^{1} to express its rDNA since *Zhr*^{1}/ *Zhr*^{1} female control tissues still revealed rRNA signal, albeit we cannot discriminate *Zhr*^{1}/ *Zhr*^{1} female tissues from *Zhr*^{1}/ *Zhr*^{1}/Y female tissues with these probes (Figure B.1D-E). Despite this, Guam X is able to dominate over *Zhr*^{1}, revealing that the majority of YL+Y-centromere are not necessary for nucleolar dominance and simultaneously that nucleolar dominance can in fact occur between two X chromosomes in females.

In our attempt to understand why Guam X rDNA can dominate over *Zhr*^{1} rDNA, we observed that Guam X rDNA appeared much larger than *Zhr*^{1} rDNA (Figure B.3A). We therefore wondered if rDNA locus size could influence this dominance. Previous reports showed that small differences in rDNA copy number did not correlate with the ability of the Y rDNA locus to dominate in expression over X rDNA (Greil and Ahmad 2012), but we wondered if larger differences in rDNA size could induce dominance in X/X females. We compared relative rDNA size of various X and Y chromosomes utilizing DNA FISH of mitotic chromosomes and comparison of raw integrated fluorescence density (Figure B.2A-B and Materials and Methods). We confirmed that Guam X 18S is much larger than *Zhr*^{1} 18S (Figure B.3A, 3C). We also found that Guam X 18S is much larger than all other rDNA loci assessed (Figure B.3C). Guam X IGS is larger than all rDNA loci assessed, albeit to a lesser extent than 18S, but IGS of the Y chromosome from *yw* stock (Y^{yw}) is much larger than Guam X IGS (Figure B.3B, 3D).

To further understand if rDNA locus size corresponds to ability to dominate in expression, we assessed nucleolar dominance in adult tissues between Guam X rDNA and *yw*, Y^{yw}, and Y from Guam stock (Y^{Guam}) (Figure B.3E-N) utilizing our ITS^{+} and ITS$^{\Delta 24}$ *in situ* hybridization probes (Figure B.2). We found that Guam X rDNA can dominate over *yw*, Y^{yw}, and Y^{Guam} in adult gut enterocytes (Figure B.3E, 3G, 3I, 3K). We also found that Guam X rDNA is co-dominant in GSCs in X^{Guam}/X^{yw} females and co-dominant in GSCs in X^{Guam}/Y^{Guam} and X^{Guam}/Y^{yw} males (Figure B.3F, 3H, 3J, 3L). Shockingly, these data reveal that an X rDNA locus can dominate over other X rDNA loci in females but can also dominate over Y rDNA loci in male tissues, revealing exceptions to patterns previously described. Despite Guam X rDNA being larger, Guam

X rDNA and Le Réduit rDNA are able to co-dominate. Given the additional finding that Guam X rDNA is able to dominate over *yw* rDNA despite *yw* relative rDNA size equaling that of Le Réduit X rDNA, we do not believe that relative rDNA size is a strong indicator of dominance, similar to what has previously been shown (Greil and Ahmad 2012). We were unable to test nucleolar dominance between Le Réduit X rDNA and *yw* rDNA due to lack of sequence differences between the two rDNA loci. Given the presumed majority of shared identity between the Guam X chromosome, Le Réduit X chromosome, and *yw* chromosome, these patterns of nucleolar dominance suggest that Guam X rDNA specifically contains elements that can dictate its expression. What these element(s) are and what element(s) Le Réduit X rDNA has to evade silencing will be key to understand the regulation of nucleolar dominance in future investigations.

Figures

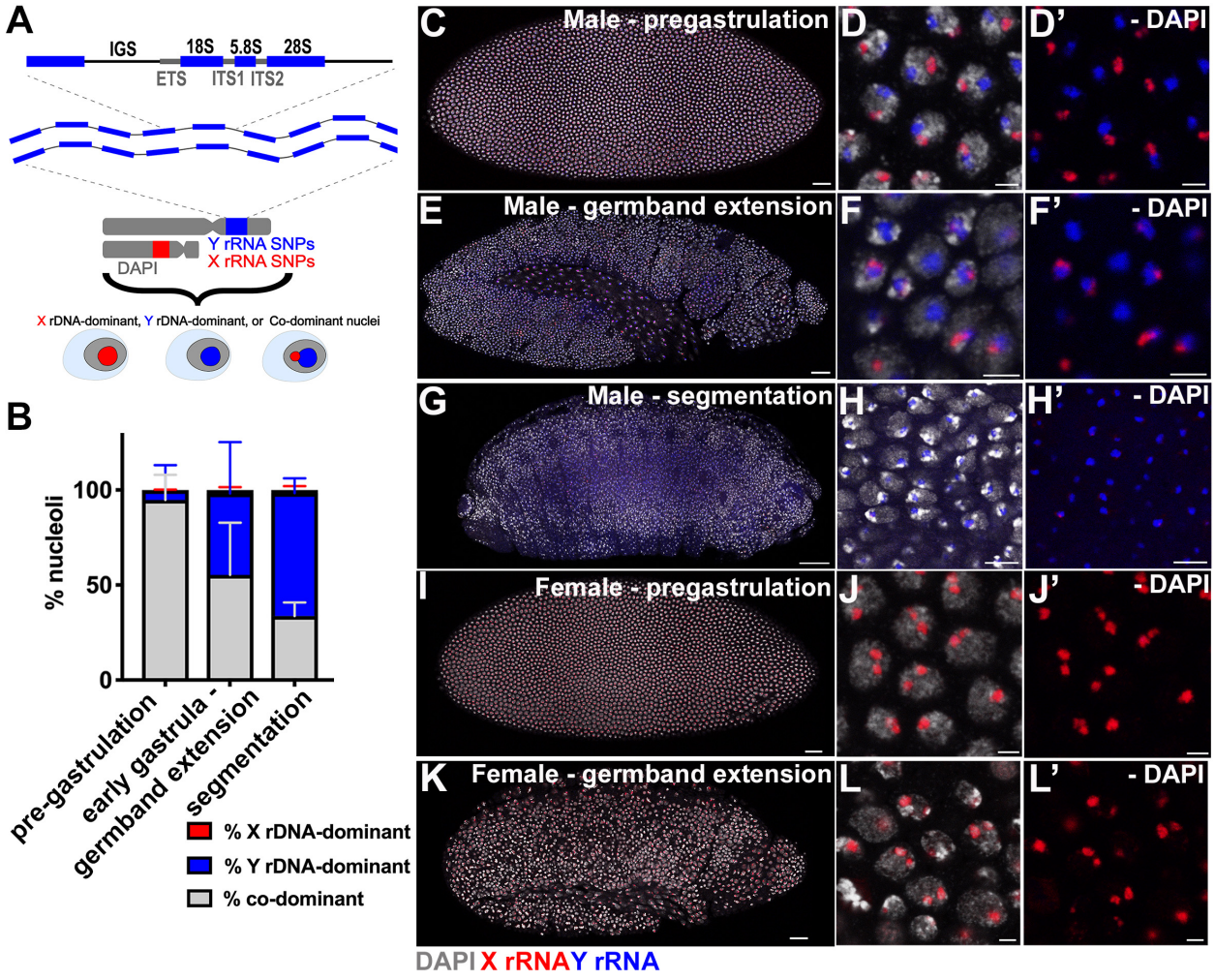


Figure I.1: Nucleolar Dominance is not Established During Embryogenesis in Male Embryos. A) Schematic of rDNA repeats (IGS = intergenic spacer, ETS = external transcribed spacer, ITS = internal transcribed spacer, 18S, 5.8S and 28S = rRNA coding region). X and Y rDNA can be distinguished by SNP *in situ* hybridization. Definition of X rDNA-dominant, Y rDNA-dominant, or co-dominant is shown. B) Quantification of nucleolar dominance across embryogenesis in males: pre-gastrulation (n = 748 cells from 7 embryos), early gastrula through germband extension (n = 1086 cells from 12 embryos), and segmentation (n = 1242 cells from 10 embryos). Red = % X rDNA-dominant, blue = % Y rDNA-dominant, grey = % co-dominant. C) Male pre-gastrulation embryo, scale = 25μm, D) zoomed image of nuclei from male pre-gastrulation embryo, scale = 3μm, D') no DAPI. E) Male embryo at germband extension stage, scale = 25μm, F) zoomed image of male embryo at germband extension stage, scale = 3μm, F') no DAPI. G) Male embryo at segmentation stage, scale = 50μm, H) zoomed image of male embryo at segmentation state, scale = 8μm, H') no DAPI. I) Female pre-gastrulation embryo, scale = 25μm, J) zoomed image of female pre-gastrulation embryo, scale = 3μm, J') no DAPI. K) Female embryo at germband extension stage, scale = 25μm, L) zoomed image of female embryo at germband extension stage, scale = 3μm, L') no DAPI. Red = X rRNA, blue Y rRNA, white = DAPI.

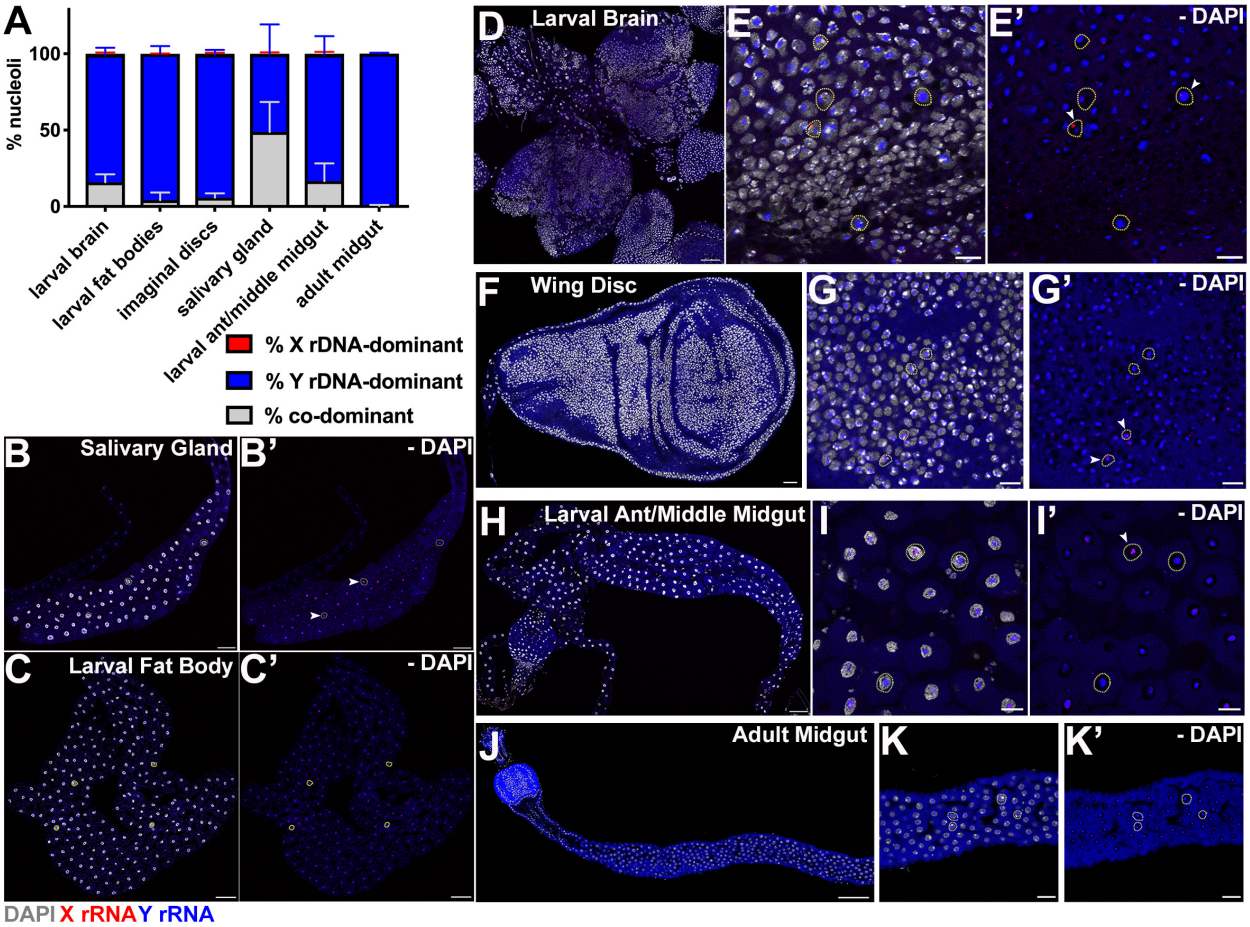


Figure I.2: Y rDNA Dominance is Established During Development in Males.
A) Quantification of nucleolar dominance in larval and adult tissue(s) in males: larval brain (n = 1594 cells from 6 brains), larval fat bodies (n = 1575 cells from 17 fat bodies), imaginal discs (n = 1251 cells from 5 imaginal discs), salivary gland (n = 878 cells from 15 salivary glands), larval anterior/middle midgut (n = 81 cells from 6 guts), adult anterior midgut (n = 922 cells from 7 guts). Red = % X rDNA-dominant, blue = % Y rDNA-dominant, grey = % co-dominant. B) Representative images of whole mount salivary gland, scale = 100μm, B') no DAPI. C) larval fat body, scale = 100μm, C') no DAPI. D) larval brain, scale = 50μm, E) zoomed image of larval brain, scale = 10μm, E') no DAPI. F) wing disc, scale = 25μm, G) zoomed image of wing disc, scale = 8μm, G') no DAPI. H) larval anterior midgut, scale = 100μm, I) zoomed image of larval anterior midgut, scale = 25μm, I') no DAPI. J) adult anterior midgut, scale = 100μm, K) zoomed image of adult anterior midgut, scale = 25μm, K') no DAPI. Red = X rRNA, blue Y rRNA, white = DAPI. Yellow circles accent examples of SNP *in situ* signal in nuclei. Arrowheads = examples of co-dominant nuclei.

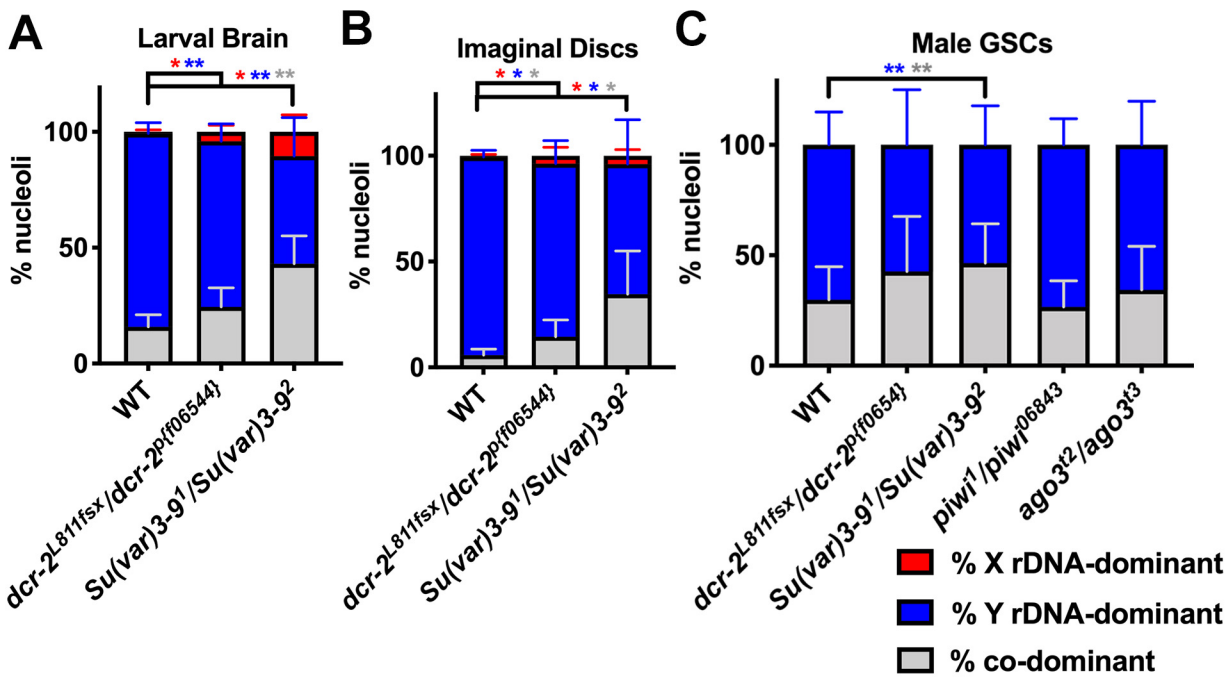


Figure I.3: Heterochromatin Formation Aids in Y rDNA Dominance in Males.
Quantification of male nucleolar dominance in A) larval brains of wild type (*yw*) (n = 1594 cells from 6 brains), *dcr-2*^{L811fsx}*/dcr dcr-2*$^{p[f06544]}$ mutants (n = 504 cells from 6 brains) and *Su(var)3-9*^{1}*/Su(var)3-9*^{2} mutants (n = 461 cells from 6 brains) (the same wild type data from Figure I.2 for comparison). B) Quantification of nucleolar dominance in male imaginal discs of wild type (n = 1251 cells from 5 imaginal discs), *dcr-2*^{L811fsx}*/ dcr-2*$^{p[f06544]}$ mutants (n = 579 cells from 9 imaginal discs), and *Su(var)3-9*^{1}*/Su(var)3-9*^{2} mutants (n = 432 cells from 6 imaginal discs) (the same wild type data from Figure I.2 for comparison). C) Quantification of male nucleolar dominance in adult germline stem cells of wild type (*yw*) (n = 87 cells from 11 testes), *dcr-2*^{L811fsx}*/dcr dcr-2*$^{p[f06544]}$ mutants (n = 122 cells from 15 testes), *Su(var)3-9*^{1}*/Su(var)3-9*^{2} mutants (n = 298 cells from 37 testes), *piwi*^{1}*/piwi*^{06843} mutants (n = 191 cells from 20 testes), and *ago3*^{t2}*/ago3*^{t3} mutants (n = 220 cells from 28 testes). Red = % X rDNA-dominant, blue = % Y rDNA-dominant, grey = % co-dominant nuclei. p-values calculated using Welch's unpaired, unequal variances *t*-test using n = number of tissues. (no star) = not significant, * = < 0.05, ** = < 0.01. Colors of asterisks correspond to colors of bars for which p-values were calculated (e.g. blue asterisk for Y rDNA-dominant p-values).

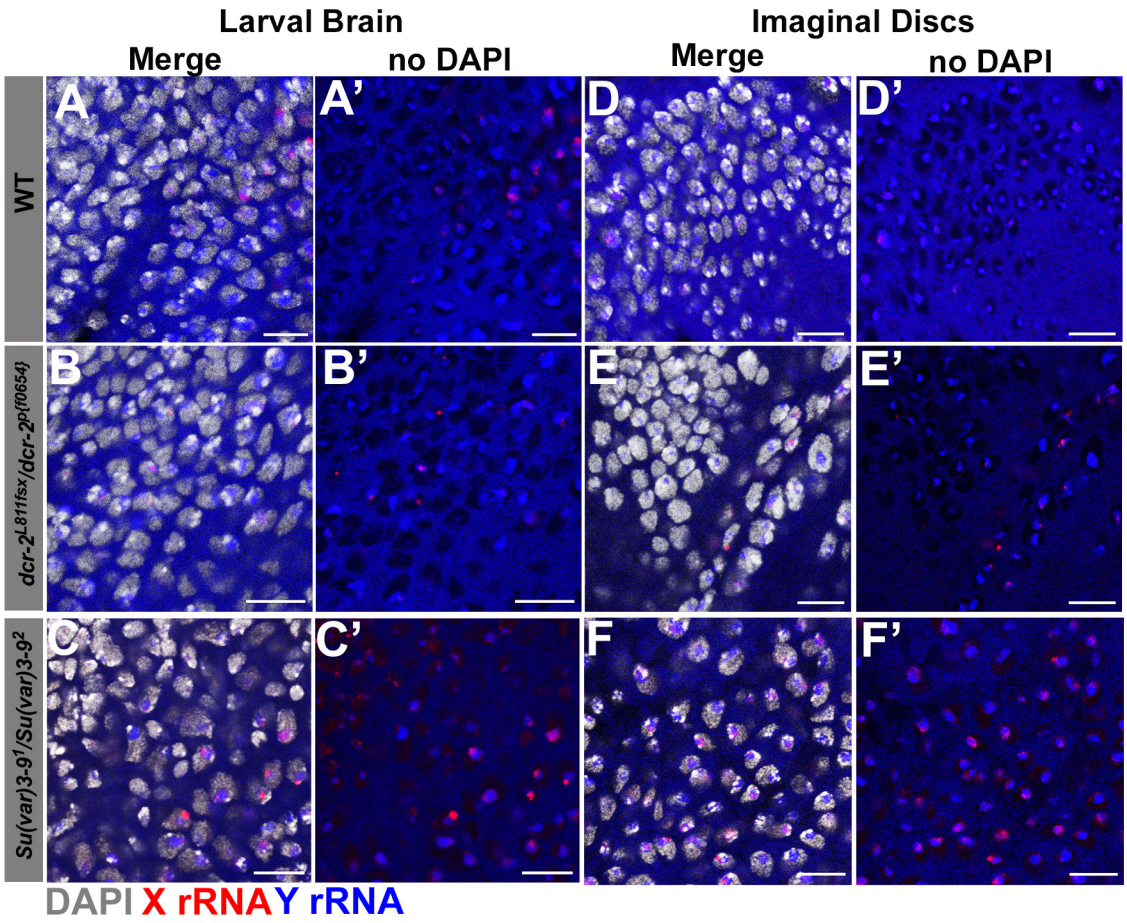


Figure I.4: Heterochromatin Formation Aids in Y rDNA Dominance.
Representative images of larval brains from A) wild type (*yw*), B) *dcr-2*^{L811fsx}*/ dcr-2*$^{p[f06544]}$ mutants, and C) *Su(var)3-9*^{1}*/Su(var)3-9*^{2} mutants. Representative images of imaginal discs from D) wild type, E) *dcr-2*^{L811fsx}*/ dcr-2*$^{p[f06544]}$ mutants, and F) *Su(var)3-9*^{1}*/Su(var)3-9*^{2} mutants. Red = X rRNA, blue Y rRNA, white = DAPI. All scale bars = 10μm.

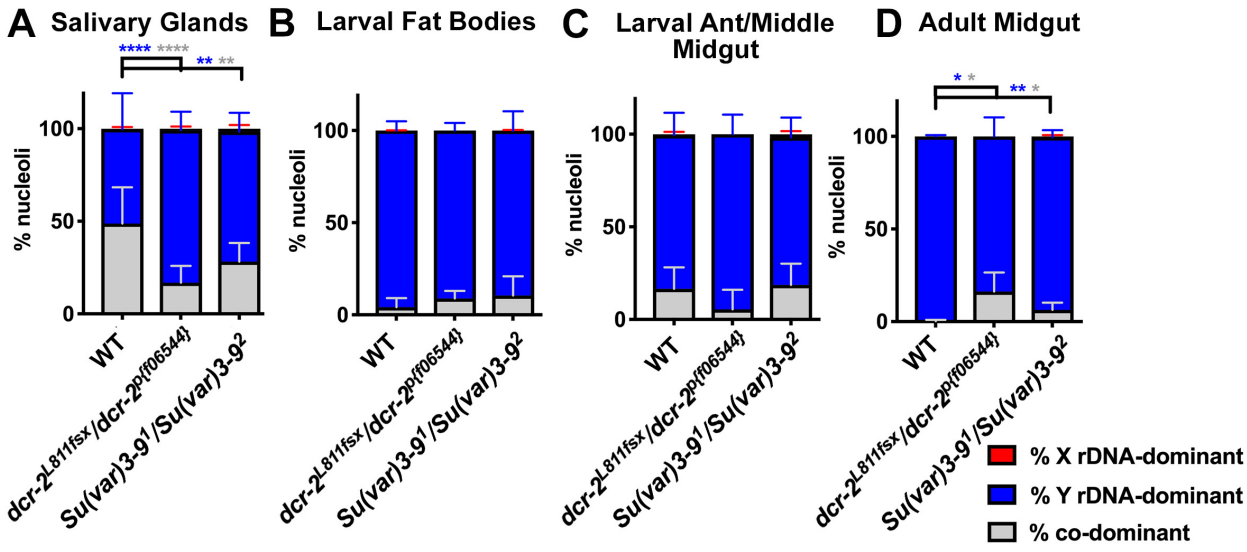


Figure I.5: The State of Nucleolar Dominance in Polyploid Tissues in *dcr-2* and *Su(var)3-9* Mutants. Quantification of nucleolar dominance in males in A) salivary glands of wild type (*yw*) (n = 878 cells from 15 salivary glands), *dcr-2*^{L811fsx}/ *dcr-2*$^{p[f06544]}$mutants (n = 373 cells from 8 salivary glands), and *Su(var)3-9*^{1}*/Su(var)3-9*^{2} mutants (n = 423 cells from 11 salivary glands), B) larval fat bodies of wild type (n = 1575 cells from 17 fat bodies), *dcr-2*^{L811fsx}/ *dcr-2*$^{p[f06544]}$ mutants (n = 320 cells from 4 fat bodies), and *Su(var)3-9*^{1}*/Su(var)3-9*^{2} mutants (n = 351 cells from 7 fat bodies), C) larval anterior/middle midgut enterocytes of wild type (n = 181 cells from 6 guts), *dcr-2*^{L811fsx}/ *dcr-2*$^{p[f06544]}$mutants (n = 150 cells from 5 guts), and *Su(var)3-9*^{1}*/Su(var)3-9*^{2} mutants (n = 172 cells from 5 guts), and D) adult anterior midgut enterocytes of wild type (n = 922 cells from 7 guts), *dcr-2*^{L811fsx}/ *dcr-2*$^{p[f06544]}$ mutants (n = 614 cells from 6 guts), and *Su(var)3-9*^{1}*/Su(var)3-9*^{2} mutants (n = 476 cells from 6 guts). Red = % X rDNA-dominant, blue = % Y rDNA-dominant, grey = % co-dominant nuclei. p-values calculated using Welch's unpaired, unequal variances *t*-test using n = number of tissues. (no star) = not significant, * = < 0.05, ** = < 0.01, **** = < 0.0001. Colors of asterisks correspond to colors of bars for which p-values were calculated (e.g. blue asterisk for Y rDNA-dominant p-values).

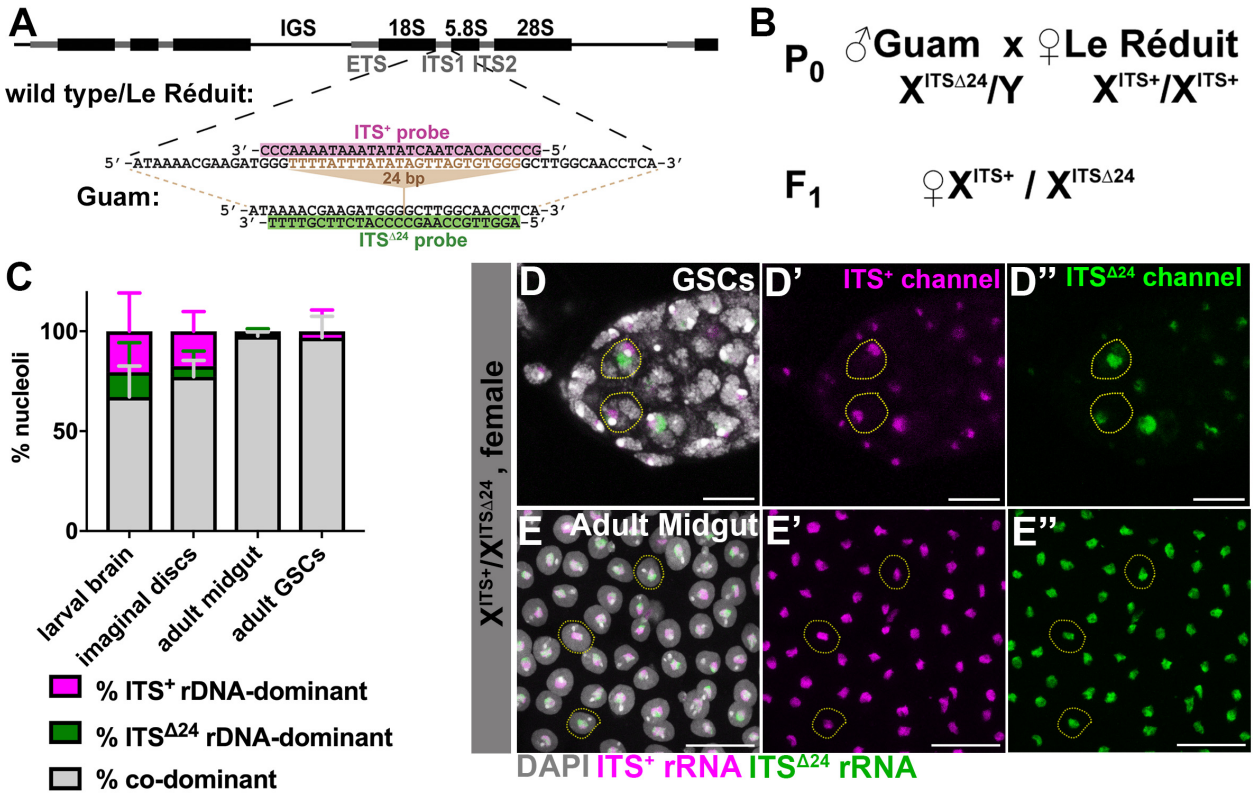


Figure I.6: X/X Females Express Both rDNA Loci Throughout Development.
A) Oligonucleotide probe design to differentially visualize two distinct X-chromosome rDNA internal transcribed spacer (ITS) transcripts, utilizing a 24-bp deletion in the rDNA ITS between wild type/ Le Réduit and Guam *D. melanogaster* strains. B) The cross to generate female F1 with one X chromosome with wild type ITS (ITS^{+}) and the other X chromosome with the ITS with 24bp deletion ($ITS^{\Delta 24}$). C) Quantification of nucleolar dominance between two X rDNA in female larval and adult tissue(s): larval brain (n = 2616 cells from 9 brains), imaginal discs (n = 2575 cells from 9 imaginal discs), adult anterior midgut (n = 904 cells from 9 guts), adult GSCs (n = 150 cells from 57 germarium). Magenta = % ITS^{+} rDNA-dominant, green = % $ITS^{\Delta 24}$ rDNA-dominant, grey = % co-dominant nuclei. Representative images of D-D'') GSCs, scale = 8μm, and E-E'') adult anterior midgut enterocytes, scale = 25μm. Magenta = ITS^{+} rRNA, green = $ITS^{\Delta 24}$ rRNA, white = DAPI.

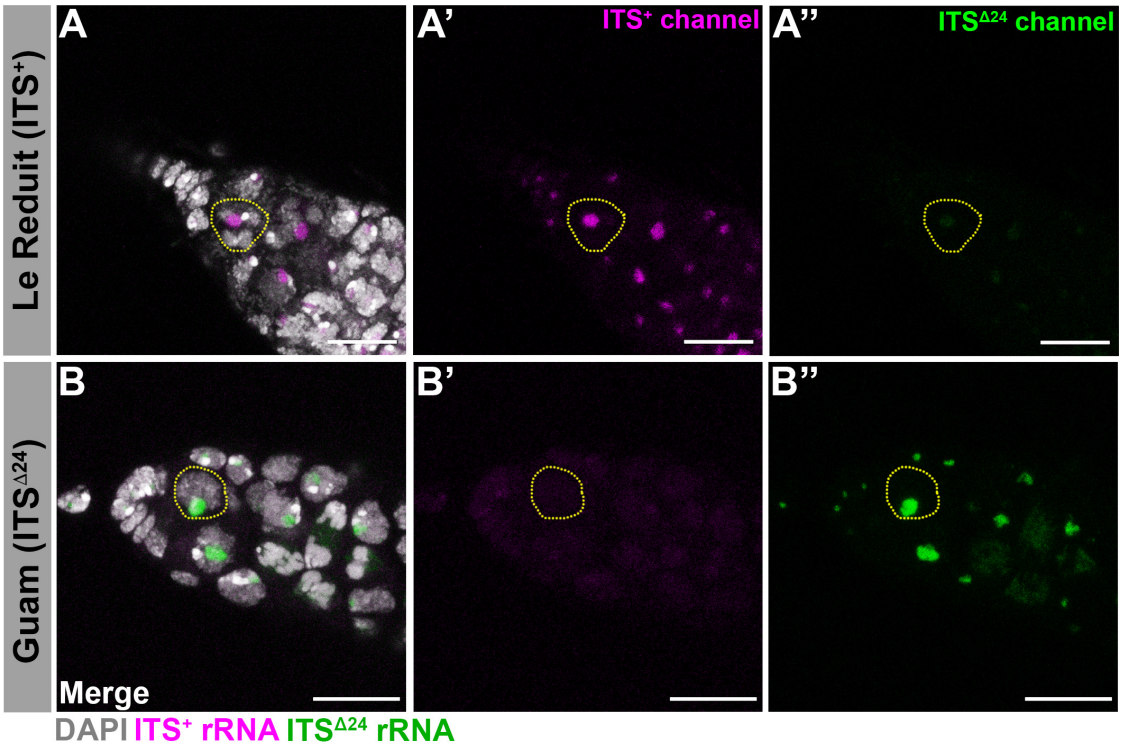


Figure I.7: ITS Probes that Differentially Identify Distinct X rDNA Variants.
A) Representative images of germarium from Le Réduit, A') ITS^{+} channel only, A'') ITS$^{\Delta 24}$ channel only, and B) Guam *D. melanogaster* strains, B') ITS^{+} channel only, B'') ITS$^{\Delta 24}$ channel only. All scale bars = 10μm. Magenta = ITS^{+} rDNA transcript, green = ITS$^{\Delta 24}$ rDNA transcript, white = DAPI. Yellow circles to denote an example of a germline stem cell (GSC).

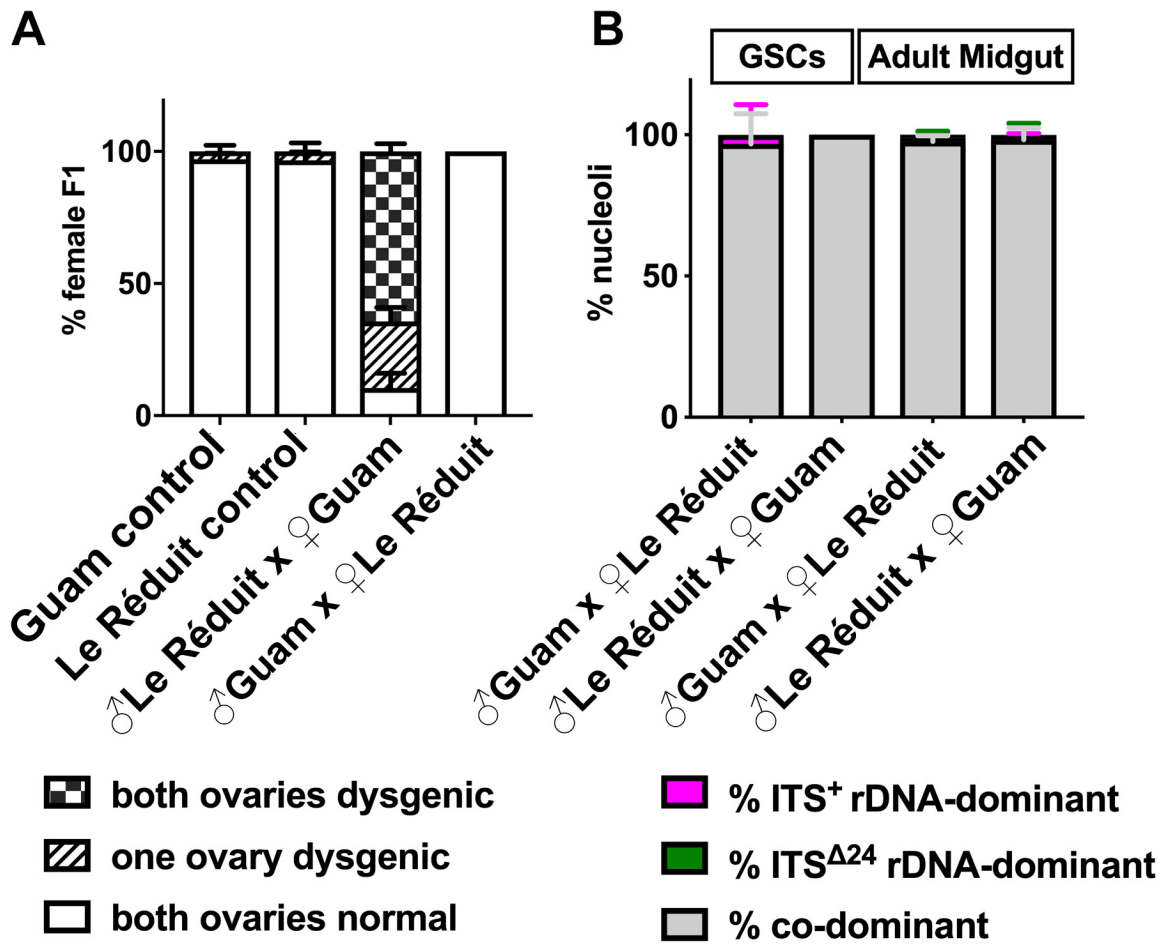


Figure I.8: Two X Chromosomes (ITS^{+} vs. ITS$^{\Delta 24}$ Variants) Exhibit Co-dominance Irrespective of Parental Origins.
A) Quantification of hybrid dysgenesis in the crosses of ♂Guam x ♀Guam controls (n = 104 females from 4 vials), ♂Le Réduit x ♀Le Réduit controls (n = 185 females from 4 vials), and ♂Le Réduit x ♀Guam (n = 145 females from 3 vials), and ♂Guam x ♀Le Réduit (n = 202 females from 4 vials). B) Quantification of female nucleolar dominance between two X rDNA comparing both cross directions (data from Figure I.6 is reproduced to show that the parental origin minimally influence the state of nucleolar dominance). GSCs scored: ♂Guam x ♀Le Réduit (n = 150 cells from 57 germarium),♂Le Réduit x ♀Guam (n = 107 cells from 51 germarium). Adult anterior midgut scored: ♂Guam x ♀Le Réduit (n = 904 cells from 9 guts),♂Le Réduit x ♀Guam (n = 962 cells from 9 guts).

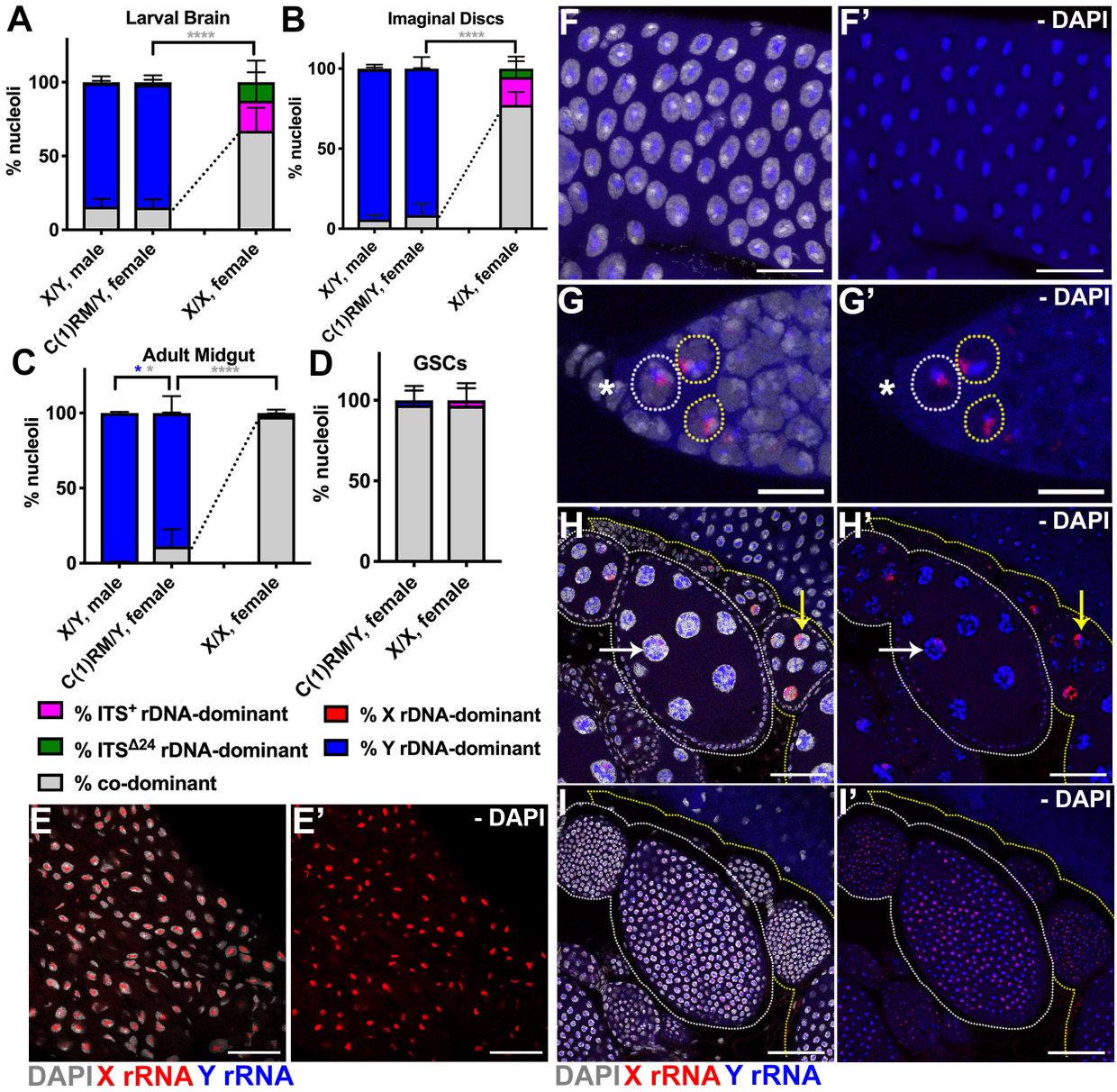


Figure I.9: The Y rDNA Can Dominate Over X rDNA in Female Cells.

Figure I.9: The Y rDNA Can Dominate Over X rDNA in Female Cells.
A-D) Quantification of nucleolar dominance in C(1)RM/Y females compared to males (data from Figure I.2 for comparison) and typical X/X females (data from Figure I.6 for comparison) in larval brain (A, n = 914 cells from 9 brains), imaginal discs (B, n = 1068 cells from 12 imaginal discs), adult anterior midgut enterocytes (C, n = 870 cells from 7 guts), and female GSCs (D, n = 54 cells from 21 germarium). Dotted lines denote differences in rates of co-dominance between C(1)RM/Y and typical X/X females. p-values calculated using Welch's unpaired, unequal variances *t*-test using number of tissues scored. p-values between C(1)RM/Y and XX females were only calculated for % co-dominant. (no star) = not significant, * = < 0.05, **** = < 0.0001. Colors of asterisks correspond to colors of bars for which P-values were calculated (e.g. blue asterisk for Y rDNA-dominant p-values). E) Representative control image of C(1)RM female adult midgut enterocytes, scale = 50μm, E') no DAPI, F) Representative image of C(1)RM/Y female adult anterior midgut enterocytes, scale = 25μm, F') no DAPI. G) C(1)RM/Y female GSCs (white circle) and cystoblasts (yellow circles), * = terminal filament, scale = 10μm, G') no DAPI. H) two C(1)RM/Y ovarioles (separately circled in white or yellow), scale = 50μm, H') no DAPI. Arrows indicating nurse cells with low X rDNA expression (white) and high X rDNA expression (yellow). I) Follicle cells from C(1)RM/Y ovarioles corresponding to H (different Z-depth), scale = 50μm, I') no DAPI. Red = X rRNA, blue Y rRNA, white = DAPI.

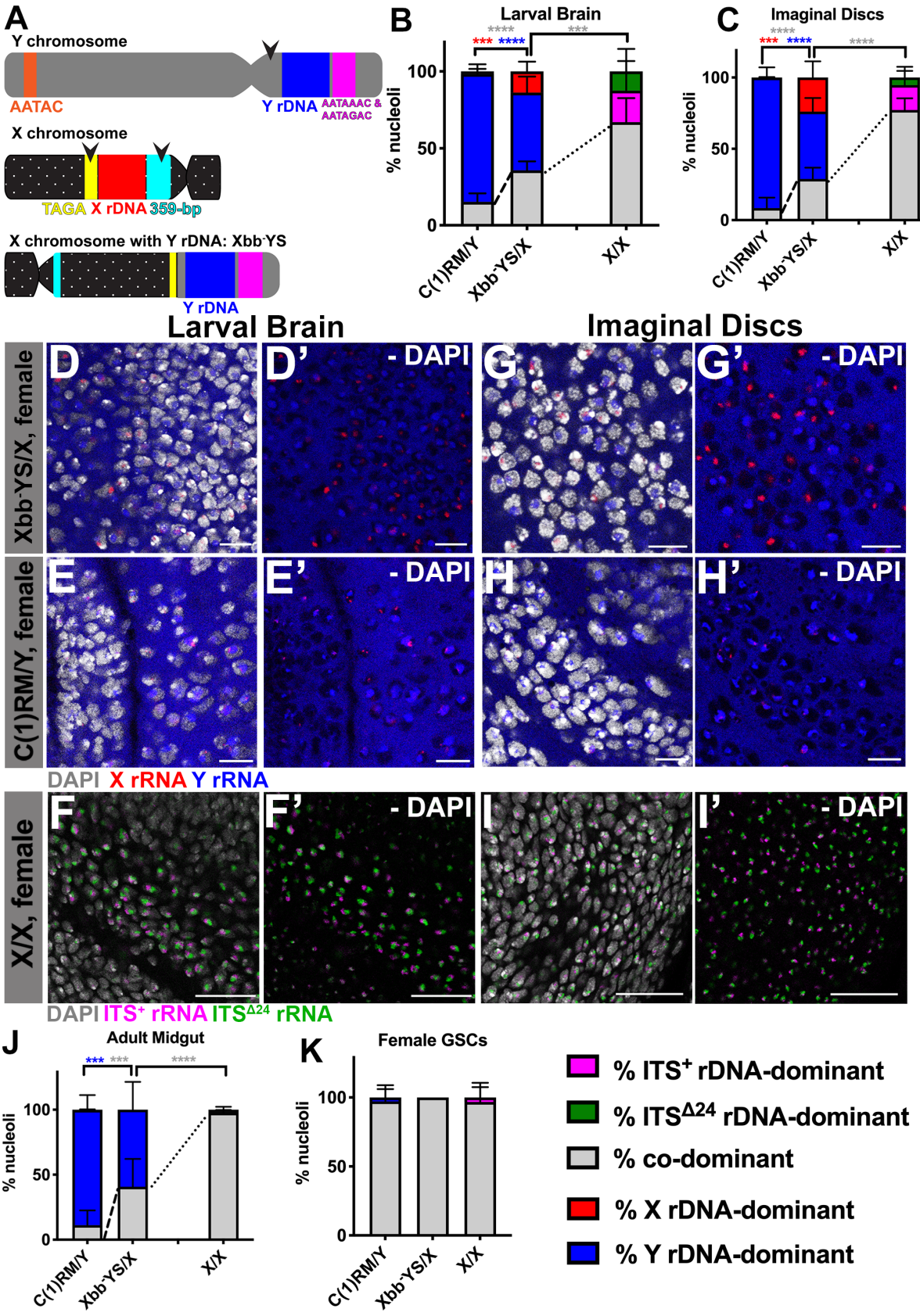


Figure I.10: Y rDNA Likely Has Information to Dictate Nucleolar Dominance.

Figure I.10: Y rDNA Likely Has Information to Dictate Nucleolar Dominance.
A) Structure of a wild type Y chromosome, a wild type X chromosome, and the Xbb^{-}YS chromosome based on Figure I.11. Constriction represents centromere location. Black arrows mark location of chromosome break. B-C) Quantification of nucleolar dominance in Xbb^{-}YS/X females compared to C(1)RM/Y females (data from Figure I.9 for comparison) and X/X females (data from Figure I.6 for comparison) in larval brain (B, n = 1400 cells from 8 brains) and imaginal discs (C, n = 1362 cells from 9 imaginal discs). Red = % X rDNA-dominant, blue = % Y rDNA-dominant, grey = % co-dominant, magenta = % ITS^{+} rDNA-dominant, green = % ITS$^{\Delta 24}$ rDNA-dominant nuclei. p-values calculated using Welch's unpaired, unequal variances *t*-test using n = number of tissues. p-values between Xbb^{-}YS/X and XX females were only calculated for % co-dominance. Dotted lines denote differences in rates of co-dominance between Xbb^{-}YS/X and X/X females. Dashed lines denote differences in rates of co-dominance between Xbb^{-}YS/X females and C(1)RM/Y females. *** = < 0.001, **** = < 0.0001. Colors of asterisks correspond to colors of bars for which p-values were calculated (e.g. blue asterisk for Y rDNA-dominant p-values). Representative images of larval brain from D) Xbb^{-}YS/X females, scale = 10μm, D') no DAPI, E) C(1)RM/Y females, scale = 10μm, E') no DAPI, and F) X/X females, scale = 25μm, F') no DAPI. Representative images of imaginal discs from G) Xbb^{-}YS/X females, scale = 8μm, G') no DAPI, H) C(1)RM/Y females, scale = 10μm, H') no DAPI, I) X/X females, scale = 25μm, I') no DAPI. Red = X rRNA, blue Y rRNA, white = DAPI, magenta = ITS^{+} rDNA transcript, green = ITS$^{\Delta 24}$ rDNA transcript. J) Quantification of nucleolar dominance in Xbb^{-}YS/X females compared to both C(1)RM/Y females (data from Figure I.9 for comparison) and X/X females (data from Figure I.6 for comparison) in adult anterior midgut enterocytes (n = 1213 cells from 13 guts), and K) female GSCs (n = 122 cells from 51 germarium). Red = % X rDNA-dominant, blue = % Y rDNA-dominant, grey = % co-dominant, magenta = % ITS^{+} rDNA-dominant, green = % ITS$^{\Delta 24}$ rDNA-dominant nuclei. p-values calculated using Welch's unpaired, unequal variances *t*-test. p-values between Xbb^{-}YS/X and XX females were only calculated for % co-dominance. Dotted lines denote differences in rates of co-dominance between Xbb^{-}YS/X and X/X females. Dashed lines denote differences in rates of co-dominance between Xbb^{-}YS/X females and C(1)RM/Y females. (no star) = not significant, *** = < 0.001, **** = < 0.0001.

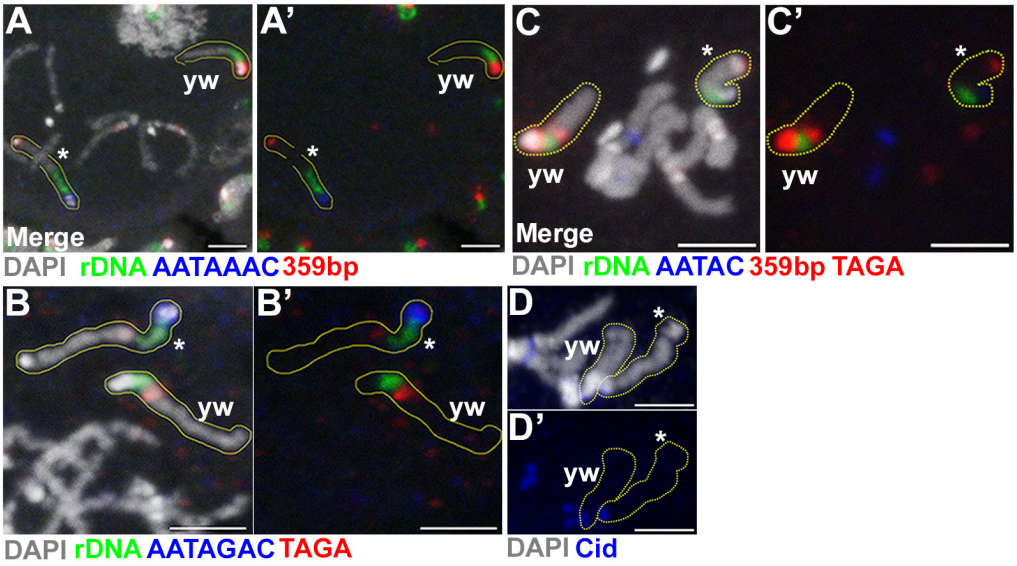


Figure I.11: Cytological Characterization of the Xbb^{-}YS Chromosome.
DNA fluorescent *in situ* hybridization (FISH) on mitotic chromosome spreads from larval brains of Xbb^{-}YS/X females with A) AATAAAC (blue) and 359-bp (red), A') no DAPI. B) AATAGAC (blue) and TAGA (red), B') no DAPI. C) AATAC (blue), TAGA, and 359-bp, C') no DAPI. D) Immunofluorescence (IF) of centromeric protein Cid (blue) on mitotic chromosome spreads, D') no DAPI. Xbb^{-}YS (*) and wild type X (wt), both outlined in yellow. White = DAPI. All scale bars = 3μm.

Discussion

In this chapter, we conducted a thorough characterization of nucleolar dominance in the context of non-hybrid *D. melanogaster*. Our work extends the previous discovery in *D. melanogaster* male larval neuroblasts that nucleolar dominance occurs within a species (Greil and Ahmad 2012, Zhou et al. 2012) to a broader range of tissues and developmental stages. Our work shows that nucleolar dominance is a developmentally regulated process, being established gradually during the course of development. This is reminiscent of what was seen in *Brassica* and *Arabidopsis* (Chen and Pikaard 1997, Pontes et al. 2007, Earley et al. 2010), and supports the notion that nucleolar dominance is not limited to interspecies hybrids.

Earlier studies (Lawrence et al. 2004, Santoro and Grummt 2005, Earley et al. 2006, Earley et al. 2010, Greil and Ahmad 2012, Pontvianne et al. 2012), confirmed here, revealed heterochromatin formation as a critical aspect of nucleolar dominance. The mechanism through which heterochromatin factors are assembled to the silenced rDNA locus appear to vary across species: siRNAs direct silencing marks in *Arabidopsis* (Preuss et al. 2008), long promoter associated RNAs silence rDNA in mammalian cell lines (Santoro et al. 2010), and rDNA silencing in *Drosophila* does not appear to involve siRNAs or piRNAs. Although these studies reveal the need of heterochromatinization to silence rDNA loci that were chosen to be silenced, they do not provide the mechanism of ‘choice’ that dictates which particular rDNA loci are to be silenced or activated.

In this regard, elements within rDNA have been suggested to influence the ‘choice’ of nucleolar dominance, including the rDNA-specific retrotransposons R1 and R2 (Eickbush et al. 2008) and the intergenic spacer sequence (IGS), which contains rDNA promoters (Coen and Dover 1982) and enhancer function (Labhart and Reeder 1984). Different species’ IGS was shown to compete for rDNA transcription factors

where longer spacers outcompete short spacers in *Xenopus laevis* oocytes (Reeder et al. 1983, Labhart and Reeder 1984). This difference in transcription factor binding based on spacer length was speculated to influence nucleolar dominance in interspecies *Xenopus* hybrids since larger *Xenopus laevis* spacers are able to dominate over the short *Xenopus borealis* spacers when injected into either species' oocyte (Reeder and Roan 1984). Because the IGS sequences are known to be highly diverged compared to the coding region of rDNA (Tautz et al. 1987, Tautz et al. 1988), it is tempting to speculate that differences in IGS sequence between X and Y rDNA loci can differentiate two loci to instruct nucleolar dominance. However, differences in IGS sequences in *Brassica rapa* and *Brassica oleracea* did not dictate nucleolar dominance in their hybrid, *Brassica napus* (Frieman et al. 1999), suggesting that there may be a considerable variation in the mechanisms that instruct nucleolar dominance across species. Previous reports have noted the expression of IGS in mammalian cells *in vitro* (Mayer et al. 2006) and *in vivo* in *Xenopus laevis* (Moss 1983) given the presence of promoters embedded in the IGS (Coen and Dover 1982, Kohorn and Rae 1982, Labhart and Reeder 1984, De Winter and Moss 1986, Kuhn and Grummt 1987, Grimaldi and Di Nocera 1988). In support of IGS transcription, we visualized IGS transcripts in an array of tissues, starting as early as pre-gastrulation embryos, appearing before rRNA expression and continuing through development (Figure B.4, larval data not shown). Interestingly, 92.16% (47/51) of male embryos have more IGS associated with the Y rRNA signal than with the X rRNA signal. 70.18% (40/57) female embryos have low or no IGS signal and 29.82% (17/57) have bright IGS signal. Given these differences in IGS expression between sexes and chromosome complement (X/Y vs. X/X), and given the ability of IGS RNA to silence rDNA expression in mammalian cells in *trans* (Santoro et al. 2010), it is intriguing to speculate whether or not IGS RNA regulates nucleolar dominance in *D. melanogaster*. However, modifying the large and highly repetitive IGS (~200-250 copies/chromosome) and/or knocking down its transcripts to test these possibilities remain a technical challenge. The role of other rDNA *cis* sequence elements in nucleolar dominance, like the rDNA-specific retrotransposons R1 and/or R2 whose insertion into rDNA can effectively silence that repeat, remain questions for future studies (Eickbush et al. 2008).

Elements within heterochromatin adjacent to the rDNA locus have been shown to influence nucleolar dominance in interspecies hybrids between *D. melanogaster* and *D. simulans*. Durica and Krider found that the long arm of the Y chromosome (YL) with rDNA was not able to induce nucleolar dominance over *D. simulans* rDNA (Durica and Krider 1978). This appears to be similar in "pure species" *D. melanogaster* since Zhr^1 that contains a majority of YL cannot dominate over Guam X rDNA in females (Figure B.1). The short arm of the Y chromosome (YS) with rDNA was able to induce nucleolar dominance in interspecies hybrids, but to a lesser extent than a complete Y chromosome (Durica and Krider 1978). This suggests that YS has a stronger influence on nucleolar dominance than YL, but YL may contain some element(s) that aids in complete establishment of nucleolar dominance in interspecies hybrids. These findings in *Drosophila* hybrids are similar to what we report in non-hybrid female *D. melanogaster*, where YS with rDNA exhibits a strong influence on its dominance over the X rDNA, albeit incomplete (Figure I.11), suggesting that the underlying mechanism of nucleolar dominance in non-hybrids vs. interspecies hybrids may be similar in *Drosophila*, as suggested by studies of *Arabidopsis*. In experiments described in this chapter, where the entire or portion of the Y chromosome was introduced into the context of females (C(1)RM/Y and Xbb^-YS/X), the Y rDNA exhibited a high degree of dominance even in female cells, suggesting that the Y chromosome harbors certain information that dictates Y rDNA transcription and/or X rDNA silencing. It remains elusive if the element(s) of the Y chromosome are selectively activating the Y rDNA (acts in *cis*) and/or selectively suppressing X rDNA transcription (acts in *trans*). However, considering the involvement of Su(var)3-9 in nucleolar dominance (Figure I.3), it is likely that the Y chromosome contains the information to dictate the silencing of X rDNA. Whether the Y chromosome also has information to simultaneously maintain its own transcription remains unknown.

Most importantly, why a locus-wide mechanism, i.e. nucleolar dominance, has evolved to regulate rDNA expression is a fundamental question. Our previous study revealed preferential copy number loss from the Y rDNA in male germline stem cells

during aging (Lu et al. 2018), potentially as a result of intrachromatid recombination occurring during active transcription (Takeuchi et al. 2003). Once Y rDNA copy number is reduced, X rDNA became active, shifting toward co-dominance (Lu et al. 2018). These observations may point to a possibility that nucleolar dominance may protect X rDNA locus from degeneration, and may serve as a mechanism to monitor and regulate rDNA copy number. Additionally, the release of silencing from the X rDNA locus in aged male GSCs suggest that a cell can sense/count the number of rDNA loci and/or levels of rRNA present and adjust the activation/silencing of whole rDNA loci depending on cellular need (Planta 1997, Nomura 2001, Goetze et al. 2010, Lu et al. 2018, Iida and Kobayashi 2019). Modulation of rRNA levels has been shown to be important for proper cell proliferation (Zhang et al. 2014) and development (Grewal et al. 2005); improper regulation of rRNA levels has been associated with cancer and other disease states (Drygin et al. 2011, Calo et al. 2018).

Given the ability of GSCs to “sense” the decrease in rDNA copy number on the Y chromosome, it appears that a cell can count rDNA abundance and that nucleolar dominance is influenced by rDNA locus size. However, nucleolar dominance appears to be multifaceted. Using a *D. melanogaster* strain with a bacterial artificial chromosome (BAC) containing a portion of an rDNA locus (~51kb) inserted in the euchromatic arm of the third chromosome via PhiC31 integrase-mediated transgenesis of the P[acman] system (through BestGene Inc), referred to as rDNA on III, we found that a third rDNA locus can disrupt rDNA expression, as shown by high rates of the bobbed phenotype and nucleolar disruption (Figure B.5) (Ritossa et al. 1966, Mohan and Ritossa 1970, Durica and Krider 1978, Lu et al. 2018). Future studies are needed to fully understand how this third rDNA locus disrupts typical rDNA expression and whether or not nucleolar dominance results from an rDNA counting mechanism. We additionally found that a very large rDNA locus (Guam X rDNA) exhibits differential patterns of nucleolar dominance when in the presence of two different X chromosomes with similar amounts of rDNA (Figure B.3). These data together suggest that there is not a clear relationship between rDNA locus size and nucleolar dominance. Together, these data further suggest that some locus-counting mechanism may be present and that other factors,

possibly tissue specific information or other unknown information within the rDNA locus may be drivers of nucleolar dominance.

In summary, our work in this chapter expands on previous studies in *Arabidopsis* and *Drosophila* and supports the notion that nucleolar dominance is not constrained to interspecies hybrids, and represents a mechanism of rRNA regulation within a species. Our work suggests that the Y rDNA and/or the short arm of the Y chromosome may have elements that dictate Y rDNA's dominance (either by X rDNA silencing and/or Y rDNA activation) in *D. melanogaster*. The precise identity of the element(s) of the Y rDNA/Y chromosome short arm, and how they mediate nucleolar dominance (whether via preferential transcription of Y rDNA and/or silencing of X rDNA) await future investigation. This work lays the foundation to identify element(s) within and outside of the X and Y rDNA that regulate nucleolar dominance and to understand the underlying mechanisms needed to achieve nucleolar dominance within the species *D. melanogaster*.

Chapter II: Regulation of Nucleolar Dominance in *Drosophila* Interspecies Hybrids

Summary

Nucleolar dominance is a large-scale silencing phenomenon whereby entire ribosomal DNA loci, the highly repeated genes that encode ribosomal RNA (rRNA) the catalytic components of ribosomes, are preferentially silenced/expressed. Nucleolar dominance was first discovered to occur in interspecies hybrids where one species rDNA loci are preferentially silenced, suggesting that nucleolar dominance occurred as a result of incompatibilities arising within the interspecies hybrid. However, with the discovery that nucleolar dominance is a developmental phenomenon that occurs within a species to potentially regulate rRNA dosage, nucleolar dominance in interspecies hybrids has been suggested to be reminiscent of typical development. In this study, we expand previous knowledge by further characterizing nucleolar dominance in interspecies hybrids between the closely related species *Drosophila melanogaster* and *Drosophila simulans* across stages of hybrid development. We find that nucleolar dominance in interspecies hybrids is established across developmental time, similar to that seen to occur during development of *D. melanogaster* males. This dissertation work implies that the embryonic lethality that occurs to female progeny of *D. simulans* females and *D. melanogaster* males may be intertwined with the regulation of nucleolar dominance. This study lays the groundwork to understand if and how nucleolar dominance may influence incompatibilities that arise within these hybrids.

Introduction

Nucleolar dominance was originally discovered to occur in interspecies hybrids where one species' rDNA is expressed while the other species' rDNA is silenced. Nucleolar dominance was discovered to occur in *Xenopus* hybrids (Cassidy and Blackler 1974), *Arabidopsis* hybrids (Chen et al. 1998), *Drosophila* hybrids (Durica and Krider 1977, Goodrich-Young and Krider 1989, Oliveira et al. 2006), and mouse-human hybrid cell lines (Croce et al. 1977) and then subsequently found to occur within a species (see Chapter I).

Nucleolar dominance in interspecies hybrids has been found to be influenced by a range of factors that vary across species: intergenic spacer size and abundance has been shown to influence nucleolar dominance in *Xenopus* hybrids (Busby and Reeder 1983, Reeder et al. 1983) and regions of both X and Y heterochromatin have been shown to be important in establishing nucleolar dominance in *Drosophila* hybrids (Durica and Krider 1977, Durica and Krider 1978). Nucleolar dominance in *Arabidopsis* hybrids and *Arabidopsis thaliana* has been thoroughly studied and shown to be mechanistically similar, given that patterns of nucleolar dominance are consistent between hybrids and non-hybrids (Lewis et al. 2004). Studies in *Arabidopsis* have revealed the importance of small interfering RNAs (siRNAs) (Pontes et al. 2006, Preuss et al. 2008), histone deacetylation (Probst et al. 2004, Earley et al. 2006, Earley et al. 2010), DNA methylation (Chen et al. 1998, Lawrence et al. 2004, Pontes et al. 2006, Preuss et al. 2008, Costa-Nunes et al. 2010, Earley et al. 2010), histone methylation (Earley et al. 2010, Pontvianne et al. 2012), and chromosomal context/location (Chandrasekhara et al. 2016, Mohannath et al. 2016) in establishing nucleolar dominance. These comparative studies within the species *Arabidopsis thaliana* and in interspecies *Arabidopsis* provide us with the understanding that nucleolar dominance is

not limited to interspecies hybrids and that the mechanisms underlying these two phenomena are similar.

Nucleolar dominance in *Drosophila* was first discovered in interspecies hybrids and later within the species *D. melanogaster*. These initial studies of nucleolar dominance required the use of condensed mitotic chromosomes, limiting our knowledge of nucleolar dominance to highly mitotic cell types. In this dissertation, I expand our understanding of nucleolar dominance in *Drosophila* interspecies hybrids to aid in understanding if mechanisms of nucleolar dominance between "pure" species and interspecies hybrids are the same. Utilizing sequence differences between the closely related species *Drosophila simulans* and *Drosophila melanogaster,* we use RNA *in situ* hybridization to characterize nucleolar dominance across the development of *Drosophila* interspecies hybrids. Our data show that nucleolar dominance is established over developmental time in *Drosophila* interspecies hybrids, similar to that known to occur in *Drosophila melanogaster* and *Arabidopsis* (see Chapter I) (Pontes et al. 2007, Earley et al. 2010, Warsinger-Pepe et al. 2020), further supporting the conservation of this developmental phenomenon.

Drosophila interspecies hybrids often exhibit defects common to progeny that arise when two species interbreed (Sturtevant 1920, Barbash 2010). When male *D. melanogaster* mate with females of their closely related sibling species *D. simulans*, they produce mostly sterile male progeny (Sturtevant 1920, Sawamura et al. 1993). Female progeny from this cross are inviable, but on rare occasion some can survive to adulthood (Sturtevant 1920, Sawamura et al. 1993). More often, female hybrid progeny from this cross were found to die during early embryogenesis as a result of incomplete chromosome segregation of the *D. melanogaster* X chromosome (Sawamura and Yamamoto 1993, Sawamura et al. 1993, Ferree and Barbash 2009). Sawamura et al found that the lethality of these female embryos was due to a heterochromatic locus, termed zygotic hybrid rescue (*Zhr*), removal of which results in rescue of the lethality (Sawamura and Yamamoto 1993, Sawamura et al. 1993). The *Zhr* locus corresponds to the *D. melanogaster* specific 359-bp heterochromatic block (Lohe et al. 1993,

Sawamura and Yamamoto 1993); removal of 359-bp from the *D. melanogaster* X chromosome within hybrids produced from *Zhr*^{1}/Y P0 males rescues this hybrid female embryonic lethality (Sawamura et al. 1993, Ferree and Barbash 2009). Given that *Zhr* presence does not cause 100% lethality in hybrids, Sawamura and Yamamoto speculated that the efficacy of *Zhr*-induced lethality may be influenced by proximal elements, potentially heterochromatin spreading via position-effect variegation or some relationship with nucleolar dominance (Sawamura and Yamamoto 1993). Here we report for the first time that *Zhr*^{1} rescued hybrid females reverse patterns of nucleolar dominance, where *D. simulans* rDNA dominates in expression in adult tissues. We speculate that there may be a relationship between rDNA and 359-bp which may shed light on how this hybrid incompatibility arises. This study lays the groundwork to understand how nucleolar dominance is regulated in *Drosophila* interspecies hybrids and to further understand how this regulation may influence the genomic instability of these *Drosophila* hybrid female embryos.

Materials and Methods

Fly Husbandry and Strains

Unless otherwise stated, all fly stocks were raised on standard Bloomington medium at room temperature (RT). Unless otherwise stated, all *D. melanogaster* flies used for interspecies hybrid experiments were the standard lab wild-type strain Oregon-R and all *D. simulans* flies used for interspecies hybrid experiments were strain tsimbazaza.

Interspecies Hybrid Female Embryonic Lethality Rescue Assay

Unless otherwise stated, all rescue assays were performed at room temperature (RT). For each rescue assay, many vials of animals were set up to account for pre-zygotic mating barriers between *D. melanogaster* and *D. simulans*. Data for the rescue assays were compiled from many assays performed at different times due to low mating success rates for particular crosses. Data in Figure II.3, II.4, and Figure B.8 include vials (n) that resulted in progeny. See Table A.2 for complete number of crosses, including number resulting in zero progeny, sample size of successful crosses, and the mating success rate of each cross.

For each vial, five virgin females and 5 young males (all less than or equal to 5 days old) were paired. Every week for four weeks, these flies were flipped to new vials, regardless if any P0 flies died. P0 were discarded at the end of four weeks. Each vial was scored for progeny based on sex, genotype (if necessary), and bobbed (bb) phenotype. The bb phenotype was scored as cuticular etching and/or peeling. Rates of bb can be found in Table A.3. Progeny was scored from each vial until 20 days post P0 pairing in each vial.

Tissue Collection and *RNA in situ Hybridization*

Third instar larval or adult tissues were dissected in RNase-free 1X PBS, fixed in RNase-free 4% formaldehyde, and incubated overnight in 70% EtOH at 4º to permeabilize the tissues. Embryos were collected according to a modified protocol from (Wilk et al. 2010) by allowing parents to lay eggs on an apple-agar plate at room temperature for a range of collection time (3 – 17hrs). Embryos were transferred to glass scintillation vials with glass Pasteur pipettes and were washed of any yeast in 1X PBS then dechorionated in 50% bleach for 30 sec and washed again in PBS. The embryos were then devitellinized and fixed in 50:50 heptane:4% RNase-free formaldehyde during vigorous, manual shaking for 20 min, then again in 50:50 heptane:methanol twice for 30 sec, washed in methanol then stored in methanol at -20º for at least one night before proceeding to *in situ* hybridization.

In situ hybridization was performed as previously described with slight modifications (Lu et al. 2018). In short, samples were washed with wash buffer (10% formamide in 1X SSC and 0.1% Tween-20) for 5 min, then incubated with the hybridization mix (10% formamide, 1X SSC, 10% Dextran sulfate (w/v) (Sigma, D8906), 100nM each *in situ* fluorescent probe overnight in a 37º water bath. Samples were then washed twice in wash buffer for 30 min each at 37º and stored in Vectashield H-1200 (Vector Laboratories) with DAPI. Images were taken using a Leica TCS SP8 confocal microscope with 63X oil-immersion objectives and processed using Adobe Photoshop software. See Table A.1 for fluorescent *in situ* oligonucleotide probes. Fluorescent *in situ* oligonucleotide probes were designed using Integrated DNA Technologies Oligo Analyzer.

Identification of Sequence Differences in rDNA

To sequence rDNA from separate species, rDNA from the X chromosome was sequenced. Genomic DNA was extracted from 10-15 female flies of a genotype of interest. PCR was performed on the extracted genomic DNA to amplify three regions of the rDNA with the primers listed in Table A.1.

PCR products were verified by agarose gel electrophoresis and purified using a PCR Purification Kit (Qiagen). Sanger sequencing was performed on the purified PCR products using the same PCR primers (University of Michigan Biomedical Research DNA Sequencing Core Facility). Sequencing data was analyzed using the free downloadable software ApE: A plasmid Editor, by M. Wayne Davis.

Quantification and Statistical Analysis of Nucleolar Dominance

Nucleolar dominance was quantified manually from images generated using a Leica TCS SP8 confocal microscope. For each embryo, larval brain, imaginal disc, larval anterior midgut, and adult anterior midgut sample, 1-3 representative images of each tissue were captured for scoring purposes. Imaginal discs were randomly scored without intentionally excluding any imaginal disc type, therefore all imaginal discs were included in the category of 'imaginal discs' for scoring purposes. Z-stacks were generated with maximum projections for pre-gastrulation embryos, larval anterior midgut and adult anterior midgut images for scoring. Whole tissues were scored for salivary glands and larval fat bodies. All cells were identified and scored based on nuclear DAPI staining and morphology. Note that the call of dominance vs. co-dominance was straightforward, owing to consistent signal intensity across samples based on the RNA *in situ* procedure described above. The number of cells and the number of tissues scored per genotype are listed in each corresponding figure legend. p-values were calculated using an unpaired Student's *t*-test with Welch's correction (assuming unequal variances) with n representing number of tissues scored.

Results

Nucleolar Dominance in Interspecies Hybrids is Established Over Developmental Time.

Thorough characterization of nucleolar dominance in interspecies hybrids has been limited to *Arabidopsis* (Tucker et al. 2010) and to *Drosophila* larval neuroblasts (Durica and Krider 1977, Durica and Krider 1978). These earlier studies in *Drosophila* hybrids required the use of highly condensed, mitotic chromosomes, thereby limiting the studies to larval neuroblasts. To extend the analysis of nucleolar dominance in *Drosophila* interspecies hybrids to other stages of development, we utilized sequence differences between the rDNA of the closely related species *D. melanogaster* and *D. simulans* (see Table A.1 for sequencing primers) and performed RNA *in situ* hybridization on hybrid tissues throughout development with species-specific oligonucleotide probes (Figure B.1 and Table A.1). Using RNA *in situ* hybridization, we comprehensively analyzed the state of nucleolar dominance during development of *Drosophila* interspecies hybrids (Figure II.1).

We first focused on nucleolar dominance in embryos generated from female *D. simulans* and male *D. melanogaster* (Figure II.1A). Since our RNA *in situ* hybridization probes cannot aid us in determining the sex of the embryos, all embryos were scored as unknown sex. However, when female *D. simulans* and male *D. melanogaster* mate, the female progeny typically exhibit nuclear morphology defects as seen in Figure II.1B-C and mostly die during early embryogenesis (Sawamura et al. 1993, Sawamura et al. 1993), suggesting that most F1 embryos and larvae assessed from this cross are male. We found that in early embryos (pre-gastrulating, around syncytial cycle 13-14), the majority of nuclei expressed rDNA from both *D. simulans* and *D. melanogaster* loci (i.e. co-dominant) (89.7 ± 00.0%) (Figure II.1D-E, 1R). Given that nucleolar dominance in male *D. melanogaster* is established during the course of development (Warsinger-

Pepe et al. 2020) and that interspecies *Drosophila* larval neuroblasts reveal dominance of *D. melanogaster* rDNA (Durica and Krider 1977), we wondered if interspecies hybrid nucleolar dominance is also established over developmental time. To address this, we examined the state of nucleolar dominance along the course of development through embryonic stages and larval development. Whereas the pre-gastrulating embryos exhibited a high frequency of co-dominance, we observed a decrease in the percentage of co-dominant nuclei, with a concomitant increase in *D. melanogaster* rDNA-dominant nuclei in later stages of embryogenesis (Figure II.1D-K, 1R). Embryos during early gastrula stage show 34.1 ± 29.8% co-dominant nuclei and 41.4 ± 51.4% *D. melanogaster* rDNA dominant nuclei (Figure II.1F-G, 1R). Embryos during germband extension stage show 56.6 ± 6.5% co-dominant nuclei and 18.4 ± 13.5% *D. melanogaster* rDNA dominant nuclei (Figure II.1H-I, 1R). Later during segmentation, co-dominant nuclei further decreased to 54.8 ± 14.9%, as *D. melanogaster* rDNA dominant nuclei increased to 31.8 ± 22.3% (Figure II.1J-K, 1R). This progression towards *D. melanogaster* rDNA dominance does not appear to be linear; this may be due to variability of nucleolar dominance establishment in hybrids or due to low sample size.

As development proceeds to the larval stage, we observed much higher rates of *D. melanogaster* rDNA dominance in all hybrid tissues: larval brains (92.5 ± 6.1%) similar to what has been previously reported (Durica and Krider 1977) (Figure II.1L-M, 1R), imaginal discs (96.4 ± 5.2%) (Figure II.1N-O, 1R), salivary glands (96.7 ± 9.2%) (Figure II.1R), and larval anterior midgut enterocytes (100.0 ± 0.0%) (Figure II.1R). Hybrid larval tissues were also scored without knowing the sex of the animal since hybrid larvae do not develop testes, which is key for distinguishing male vs. female larvae. However, ~80% of larvae from this cross at room temperature can be presumed to be male (Figure II.3O) (Durica and Krider 1977, Sawamura et al. 1993, Sawamura et al. 1993). Adult hybrids (male and female) showed almost complete establishment of *D. melanogaster* rDNA dominance in gut enterocytes (98.5 ± 3.1%) (Figure II.1P-Q, 1R). These data suggest that nucleolar dominance in *D. melanogaster – D. simulans* interspecies hybrids is regulated across their development. This is similar to what was reported in *Brassica* (Chen and Pikaard 1997*)* and separately in *Arabidopsis* hybrids

(Pontes et al. 2007, Earley et al. 2010), where seedling cotyledons exhibit co-dominance and nucleolar dominance is established in later stages of development. These similarities suggest conservation of this phenomenon whether it be within a species or in hybrids. However, nucleolar dominance in interspecies *Drosophila* hybrids appears to be established sooner than that seen within the species *D. melanogaster*. This developmental time difference in establishment of nucleolar dominance suggests that the mechanisms that regulate nucleolar dominance within a species may be dysregulated in interspecies hybrids, reminiscent of previous reports on gene dysregulation in hybrids (Wittkopp et al. 2004).

To identify whether or not this similar developmental pattern of nucleolar dominance occurs in female interspecies hybrids, we utilized the same RNA *in situ* hybridization with F1 embryos generated from the reverse cross direction (*D. melanogaster* females crossed to *D. simulans* males) (Figure II.2A). When *D. melanogaster* females generate progeny with *D. simulans* males, the female F1 survive to adulthood (but are sterile) and the male F1 hybrids do not develop fully into large third instar larvae and die during larval development (Sturtevant 1920). We analyzed female embryos from this cross given the understanding that *D. melanogaster* rDNA always dominates in expression in both hybrid female and male larval brains regardless of the direction of the P0 cross (Durica and Krider 1977); we therefore assume that nucleolar dominance in hybrid females from *D. melanogaster* females and *D. simulans* males will be similar to that in hybrid females from *D. simulans* females and *D. melanogaster* males, similar to what we report between two *D. melanogaster* strains in females (Warsinger-Pepe et al. 2020). Since *D. simulans* males do not have rDNA on the Y chromosome (Lohe and Roberts 1990), and therefore cannot undergo nucleolar dominance with one rDNA locus, embryos with 100% of their nuclei expressing *D. melanogaster* rDNA (n = 14/29) were deemed male (Figure II.2B, bottom). All other embryos showing any amount of *D. melanogaster* rDNA expression in addition to *D. simulans* rDNA expression (n = 15/29) were deemed female (Figure II.2B, top). We were thus able to quantify nucleolar dominance specifically in female hybrids. Nucleolar dominance scoring of F1 from this cross was only reported for female interspecies

embryos. Female embryos generated from P0 *D. melanogaster* females crossed to *D. simulans* males reveal high rates of co-dominance during the early gastrula stage (75.1 ± 5.4% co-dominant nuclei and 9.5 ± 4.2% *D. melanogaster* rDNA dominant nuclei) (Figure II.2C-D, 2M). Embryos during germband extension stage show 59.5 ± 12.5% co-dominant nuclei and 22.5 ± 13.4% *D. melanogaster* rDNA dominant nuclei (Figure II.2E-F, 2M). Later during segmentation, co-dominant nuclei further decreased to 44.7 ± 20.3%, as *D. melanogaster* rDNA dominant nuclei increased to 49.9 ± 23.0% (Figure II.2G-H, 2M).

As these hybrids proceed to the larval stage, given that male F1 hybrids generated from female *D. melanogaster* and male *D. simulans* do not develop fully into large third instar larvae and die during larval development (Sturtevant 1920), we assume that all third instar larval tissues scored are from female animals. In the larval stage, we observed much higher rates of *D. melanogaster* rDNA dominance in all female hybrid tissues: larval brains (90.9 ± 0.0%) similar to what has been previously reported (Durica and Krider 1977) (Figure II.2I-J, 2M), imaginal discs (81.8 ± 7.4%) (Figure II.2K-L, 2M), and salivary glands (100.0 ± 0.0%) (Figure II.2M). Despite low sample size, these data suggest that nucleolar dominance in *Drosophila* interspecies hybrids is established across development, occurs independent of embryonic sex or parental sex/genotype, and occurs at an earlier time frame in development than nucleolar dominance within “pure species” *D. melanogaster.*

D. simulans rDNA Dominates in Expression in Female Hybrids Rescued by Zhr^{1}

Progeny between closely related species are oftentimes inviable or are sterile/have impaired fertility if viable (Sturtevant 1920, Lachaise et al. 1986). These phenotypes are thought to arise between organisms within a species that evolve away from each other enough to form interspecies boundaries by way of pre- or post-zygotic barriers (hybrid incompatibilities) (Orr 1995). *Drosophila* interspecies hybrids follow these patterns. Progeny from female *D. melanogaster* and the closely related male *D. simulans* only survive as sterile females; male progeny die during larval development (Sturtevant 1920). The underlying incompatibilities involved in this hybrid male lethality

have not been fully elucidated, but reports have found that various factors originating from either parental species (*Hmr*, *Lhr, gfzf*) influence this hybrid-specific lethality (Hutter and Ashburner 1987, Barbash et al. 2000, Brideau et al. 2006, Phadnis et al. 2015) (reviewed in (Barbash 2010)). Progeny from male *D. melanogaster* and female *D. simulans* typically only survive as sterile males; females die during early embryogenesis, although some exceptional ("escaper" or "escapee") females survive to adulthood (Sturtevant 1920, Sawamura et al. 1993). The underlying incompatibility involved in this hybrid female embryonic lethality was mapped to the *Zhr* locus on the *D. melanogaster* X chromosome that is composed of the 359-bp satellite block (Sawamura et al. 1993, Ferree and Barbash 2009) and is not influenced by other known hybrid incompatibility genes that induce lethality in male hybrids (e.g. *Hmr*, *Lhr*) (Sawamura et al. 1993, Sawamura et al. 1993).

Previous reports found that *D. melanogaster* males with the Zhr^1 mutant X chromosome that lacks the majority of the 359-bp satellite block were able to rescue interspecies hybrid female embryonic lethality when mated to *D. simulans* females (Sawamura et al. 1993, Ferree and Barbash 2009) (Figure II.3A). We were able to confirm this rescue by Zhr^1 *D. melanogaster* males at room temperature (F1 hybrid females: 46.2 ± 10.9%) whereas typical *D. melanogaster* (Oregon R) males do not rescue hybrid female embryonic lethality (F1 hybrid females: 17.5 ± 5.7%) (Figure II.3O) (Sawamura et al. 1993, Ferree and Barbash 2009). Sawamura and Yamamoto noted that *Zhr* was located in close proximity to the heterochromatic region on *D. melanogaster* X chromosome previously determined to influence nucleolar dominance in *Drosophila* interspecies hybrids (Durica and Krider 1978, Sawamura and Yamamoto 1993). Sawamura and Yamamoto questioned whether nucleolar dominance was related to *Zhr*-induced hybrid incompatibility (Sawamura and Yamamoto 1993). To attempt to understand if nucleolar dominance and *Zhr*-induced hybrid incompatibility are in fact related, we first sought to characterize nucleolar dominance in hybrid embryos rescued by the *D. melanogaster* Zhr^1 X chromosome. Using our species-specific rRNA *in situ* hybridization probes (Figure B.1), we found that Zhr^1 rescued embryos exhibited high rates of *D. simulans* rDNA dominance throughout: early gastrula stage (11.2 ± 3.4%)

(Figure II.3B-C, 3M), germband extension stage (34.8 ± 28.0%) (Figure II.3D-G, 3M), and segmentation (41.5 ± 27.3%) (Figure II.3H-K, 3M). Individual embryos were often highly *D. simulans* rDNA dominant (Figure II.3D-E, 3H-I) or co-dominant (Figure II.3B-C, 3F-G, 3J-K) (from germband extension and segmentation stages combined, 6/19 embryos are mostly co-dominant, 10/19 are mostly *D. simulans* rDNA dominant, and 3/19 are mostly *D. melanogaster* rDNA dominant), suggesting potential differences between sexes, however we were not able to determine sex of these embryos with the RNA *in situ* hybridization probes used.

As hybrids generated from *D. simulans* females and Zhr^1/Y *D. melanogaster* males proceed to the larval stage, we observed moderate rates of *D. simulans* rDNA dominance: larval brains (12.6 ± 23.3%) (Figure II.3L, 3M), imaginal discs (19.7 ± 27.2%), salivary glands (13.6 ± 31.7%) and larval anterior midgut enterocytes (16.2 ± 28.9%) (Figure II.3M). These rates of *D. simulans* rDNA dominance are lower than that seen during embryogenesis, which may be due to high variability caused by the presence of two distinct populations of larval tissues, appearing either highly *D. melanogaster* rDNA dominant (13/20 larval tissues) or highly *D. simulans* rDNA dominant (4/20 larval tissues) (Figure II.3L). Strikingly, as hybrids proceed to adulthood, adult hybrid female gut enterocytes exhibited an almost complete dominance of *D. simulans* rDNA (71.6 ± 13.3%) (Figure II.3N). This suggests that an intact *Zhr* locus may be required for the establishment of *D. melanogaster* rDNA dominance in Zhr^1 rescued hybrid females.

Interestingly, $Zhr^1/X^{D.\ sim}$ hybrid female adult gut enterocytes exhibit distinct patterns of nucleolar dominance that appears to correspond to the origin of maternal oocyte cytoplasm. For $Zhr^1/X^{D.\ sim}$ hybrid females from *D. melanogaster* maternal cytoplasm (P0: ♀*D. melanogaster* x ♂*D. simulans*), high rates of co-dominance were observed (79.2 ± 10.7%) (Figure II.3N). This is in contrast to the dominance of *D. simulans* rDNA in $Zhr^1/X^{D.\ sim}$ hybrid females from *D. simulans* maternal cytoplasm (P0: ♀*D. simulans* x ♂*D. melanogaster*) (Figure II.3N). This suggests that lack of 359-bp in $Zhr^1/X^{D.\ sim}$ hybrid females is not sufficient to completely suppress *D. melanogaster*

rDNA expression. These data also suggest that other factor(s) may aid in the complete suppression of *D. melanogaster* rDNA in $Zhr^1/X^{D.\ sim}$ hybrid females. What these factor(s) are and how they interact with rDNA and *Zhr* remain questions for future investigation.

Hybrid Females that Escape Embryonic Lethality are Frequently Bobbed

The above results suggest an intriguing possibility that rDNA expression, or the developmental establishment of nucleolar dominance, might influence female hybrid lethality. In line with this possibility, we noticed when we crossed *D. simulans* females with *D. melanogaster* Oregon R males, the hybrid female progeny that "escaped" the embryonic lethality were highly bobbed (71.0 ± 15.1% bobbed escaper females). The bobbed phenotype, which often manifests in shorter bristles and/or cuticular etching, is known to indicate rDNA insufficiency (Ritossa et al. 1966), defects in rRNA metabolism (Durica and Krider 1978), and/or slowed rates of rDNA transcription (Mohan and Ritossa 1970) (Figure II.3P, 3Q). The bobbed phenotype has been noted in F1 hybrids that escape lethality as well as in introgressed hybrids, but what this phenotype means for hybrids remains unknown (Sturtevant 1920, Barbash et al. 2000, Sawamura et al. 2000, Barbash and Ashburner 2003). The high rate of the bobbed phenotype that we observed in escapee females does not appear to be a general feature of all *simulans* - *melanogaster* interspecies hybrids since the hybrid male progeny are not bobbed (5.8 ± 2.3%) (Figure II.3P). Additionally, the female hybrid progeny from the cross with *D. melanogaster* (Oregon R) females and *D. simulans* males are not bobbed (Figure II.3R), further suggesting that defects that result in the bobbed phenotype are only occurring in female hybrid progeny from *D. simulans* females and *D. melanogaster* males. We suspect that component(s) within or lacking in the maternal cytoplasm of *D. simulans* oocytes influence this bobbed phenotype.

To see if the observed bobbed phenotype can inform us of the nature of the female embryonic lethality relationship with rDNA, we scored for cuticular etching in the hybrid progeny from *D. simulans* females and Zhr^1/Y *D. melanogaster* males. We found that interspecies hybrids from the cross with *D. simulans* females and Zhr^1/Y *D.*

melanogaster males were significantly less bobbed, including both the hybrid males (F1 hybrid males: 1.5 ± 1.6%) and the rescued hybrid females (F1 hybrid females: 1.3 ± 1.6%) (Figure II.3P). These data suggest that the presence of the *Zhr* locus may influence the bobbed phenotype seen in the escaper female hybrid progeny, suggesting that the *Zhr* locus may somehow regulate rRNA metabolism in these hybrid animals. The exact means of interaction between *Zhr* and rDNA remain unknown but preliminary data hinting at the nature of their relationship are discussed below.

Proximity of Zhr with rDNA May Influence Female Hybrid Embryonic Lethality and Nucleolar Dominance

Hybrid female embryos generated from *D. simulans* females and *D. melanogaster* males were found to die as a result of incomplete chromosome segregation and the formation of chromosome bridges in early syncytial embryonic divisions (Ferree and Barbash 2009), revealing genomic instability in these hybrids. Chromosome bridges formed at the 359-bp satellite block which were relieved by the Zhr^{1} X chromosome (Ferree and Barbash 2009). These chromosome bridges indicate possible recombination (Sofueva et al. 2011), DNA replication stress (Jacome and Fernandez-Capetillo 2011), replication failure (Ferree and Barbash 2009, Aguilera and Garcia-Muse 2013), and/or conflicts between adjacent heterochromatin regions (Ferree et al. 2014). Given that rDNA is adjacent to the 359-bp satellite block on the X chromosome in *D. melanogaster* (Figure II.4A) (Lohe et al. 1993, Sawamura and Yamamoto 1993, Jagannathan et al. 2017) and is present in the chromosome bridges of dying female hybrid embryos (Ferree and Barbash 2009), it is intriguing to speculate if conflicts occur between these two loci or potentially at their juncture. We noted that rRNA expression appears at a similar embryonic stage as chromosome bridge formation in hybrids (Figure II.1B-C) making us wonder if transcription of rDNA may be influencing this conflict.

To test if the physical proximity of rDNA and *Zhr* influence this conflict and ultimately hybrid lethality, we utilized the FM6 balancer chromosome which we found has a portion of *Zhr* and rDNA separated from each other (Figure II.4B). We performed

female hybrid rescue assays with FM6/Y males and found that FM6 was able to rescue hybrid female embryonic lethality (FM6/Yy^{+} males, F1 females: 52.9 ± 4.0%) with the caveat that this rescue may be influenced by the background of the stock (*yw*/Yy^{+} control males, F1 females: 43.7 ± 1.9%) (Figure II.4C). However, when FM6 was paired with the Y chromosome from Oregon R (Y^{OrR}) after one outcross, female hybrids were also rescued (FM6/Y$^{OrR\text{-}1}$ males, F1 females: 50.0 ± 0.0%) (Figure II.4C). These results are similar to that found in previous work that suggested that the *Bar* mutants (an eye phenotype marker found on FM6) can rescue embryonic lethality and may be functionally linked to *Zhr* (Sawamura et al. 1993). Given that *Bar* and *Zhr* are tightly physically linked, we wondered if embryonic survival from *Bar* mutants was in fact due to physical separation of *Zhr* away from rDNA, as we see with the *Bar* marked FM6, instead of a relationship with *Bar* and *Zhr* (Sawamura et al. 1993). Our preliminary hybrid rescue data suggests that *Zhr* and rDNA may have physical conflicts that result in hybrid female embryonic lethality.

To understand the relationship of *Zhr* and rDNA proximity in hybrid females further, and whether or not proximity of these elements influences nucleolar dominance, we assessed nucleolar dominance in adult female hybrids rescued by FM6. Strikingly, embryos generated from female *D. simulans* and male FM6/Yy+ *D. melanogaster* revealed high rates of *D. simulans* rDNA dominant cells (Figure II.4E). Interestingly, this dominance was overcome by high rates of co-dominance in adulthood: female adult hybrid gut enterocytes rescued by FM6/Yy^{+} showed high rates of co-dominance compared to female hybrids that escape embryonic lethality (58.3 ± 22.2% compared to 16.7 ± 16.4% (Oregon R), duplicated from Figure II.R) (Figure II.4F). This is similar to rates of co-dominance in the reverse cross with *D. melanogaster* FM6 females and *D. simulans* males (66.3 ± 19.7%) (Figure II.4F). Thus, regardless of maternal cytoplasm, in adult gut enterocytes of FM6$^{D.\ mel}$/X$^{D.\ sim}$, we see high rates of co-dominance (Figure II.4F). Whether or not the high rates of co-dominance seen here are due to proximity of *Zhr* (359-bp) and rDNA or some other factor(s) remain unclear. However, this preliminary data suggests that FM6 is able to disrupt patterns of nucleolar dominance in interspecies hybrids. This is in opposition to previous findings that suggested that an

FM6 (In(1) sc8) was unable to disrupt nucleolar dominance in female hybrids (Durica and Krider 1978). However, this discrepancy may be due to differences in chromosomes used: Durica and Krider described FM6 as In(1)sc^{8} (Durica and Krider 1978) whereas our currently used FM6 (FlyBase ID FBba0000003) was created from either the precursor In(1)sc^{4} (FlyBase ID FBab0004187) or In(1)FM6 (FlyBase ID FBab0003928) both of which have different reported chromosome breakpoints than In(1)sc^{8}.

The hybrid female progeny of *D. simulans* females and male FM6/Yy+ *D. melanogaster* with the genotype FM6$^{D.\ mel}$/X$^{D.\ sim}$ had very low rates of the bobbed phenotype compared to control vials with either Oregon R or *yw*/Yy^{+} *D. melanogaster* males (4.6 ± 8.8% compared to 71.0 ± 15.1% (Oregon R) or 64.1 ± 10.9% (*yw*/Yy^{+})) (Figure II.4D). Given that FM6 has *Zhr* (359-bp), the lack of the bobbed phenotype implies that some factor(s) on FM6 not only allows for rescue of female embryonic lethality, but also allows for proper rRNA metabolism. Whether these factor(s) are the same and whether the bobbed phenotype is related to this hybrid rescue remains unknown. From these data, we hypothesize that the physical proximity of 359-bp and rDNA may influence both of these phenomena. Future studies are needed to test if there are indeed consequences to the physical proximity of the highly repetitive (359-bp and rDNA) and highly transcribed (rDNA) regions. Male hybrids of *D. simulans* females and male FM6/Yy+ *D. melanogaster* had higher rates of the bobbed phenotype with the addition of Yy^{+}, a marked Y chromosome containing *Zhr* (16.6 ± 7.6%) (Figure II.4D) (Ferree and Barbash 2009). This is consistent with the observation that Yy^{+} can induce male hybrid embryonic lethality through chromosome bridge formation (Sawamura and Yamamoto 1993, Ferree and Barbash 2009). In male hybrids, this conflict (and bobbed phenotype) is not determined by proximity of *Zhr* and rDNA as they are far apart on Yy^{+} (Sawamura and Yamamoto 1993), however, heterochromatin elements adjacent to *Zhr* have been found to correspond to chromosome bridge formation in ring chromosomes (Ferree et al. 2014), suggesting that possibly heterochromatin organization of the highly heterochromatic Y chromosome may influence this lethality.

In summary, these preliminary data suggest that the *Zhr*^{1} and the FM6 X chromosome balancer disrupt patterning of interspecies hybrid nucleolar dominance and relieve hybrid female embryonic lethality. Further studies are needed to fully understand how *Zhr* and rDNA influence each other in female interspecies hybrids and if their physical proximity impacts chromosome bridge formation and eventual lethality.

Figures

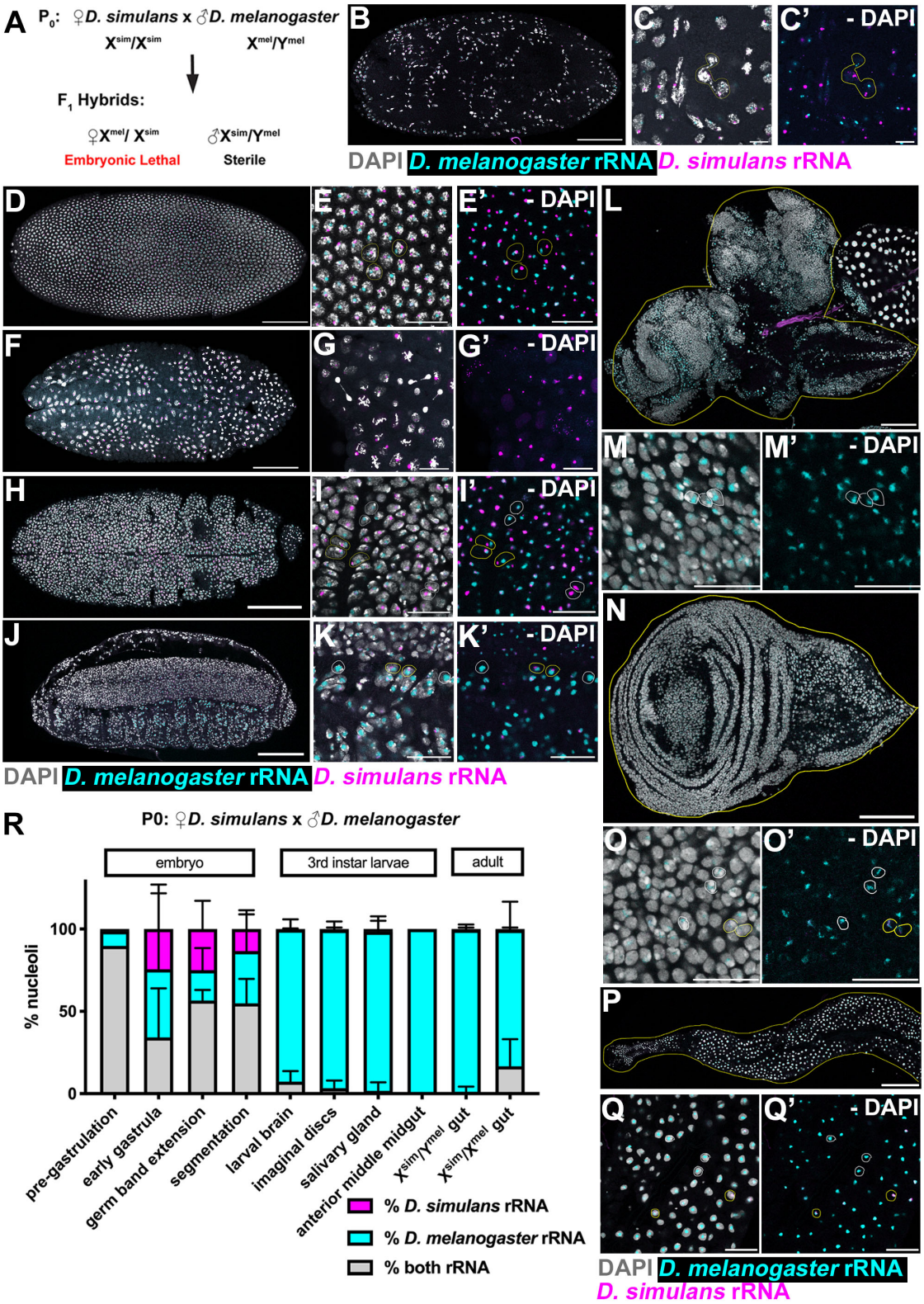


Figure II.1: Nucleolar Dominance is Established Across Developmental Time in Interspecies *Drosophila* Hybrid Males.

Figure II.1: Nucleolar Dominance is Established Across Developmental Time in Interspecies *Drosophila* Hybrid Males.
A) Schematic of interspecies hybrid crosses with P0 ♀*D. simulans* and ♂*D. melanogaster* and the resulting F1 hybrid progeny. B) Representative image of deformed early stage hybrid embryo (most likely female) from P0 *D. simulans* females and *D. melanogaster* males revealing expression of both rDNA loci, scale = 100μm. C) Zoomed image from embryo in (B) showing mitotic errors (encircled), scale = 10μm, C') no DAPI, scale = 10μm. D) Representative image of a pre-gastrulation stage embryo, scale = 100μm. E) Zoomed image of embryo in (D), E') no DAPI, scale = 20μm. F) Representative image of an early gastrula stage embryo, scale = 100μm. G) Zoomed image of embryo in (F), G') no DAPI, scale = 20μm. H) Representative image of a germband extension stage embryo, scale = 100μm. I) Zoomed image of embryo in (H), I') no DAPI, scale = 20μm. J) Representative image of a segmentation stage embryo, scale = 100μm. K) Zoomed image of embryo in (J), K') no DAPI, scale = 20μm. L) Larval brain, scale = 100μm. M) Zoomed image of larval brain from (L), M') no DAPI, scale = 20μm. N) Wing disc, scale = 100μm. O) Zoomed image of wing disc from (N), O') no DAPI, scale = 20μm. P) Representative image of a female adult anterior/middle midgut, scale = 200μm. Q) Zoomed image of adult anterior/middle midgut from (P), Q') no DAPI, scale = 50μm. Magenta = *D. simulans* rRNA, turquoise = *D. melanogaster* rRNA, grey = DAPI. Cells encircled with yellow are co-dominant. Cells encircled with white are dominant in expression. R) Quantification of nucleolar dominance across development in interspecies hybrids from P0 ♀*D. simulans* females and ♂*D. melanogaster* males: pre-gastrulation stage embryos (n = 156 cells from 1 embryo), early gastrula stage embryos (n = 196 cells from 3 embryos), germband extension stage embryos (n = 884 cells from 5 embryos), segmentation stage embryos (n = 4779 cells from 24 embryos), larval brain (n = 738 cells from 6 brains), imaginal discs (n = 1066 cells from 10 discs), salivary gland (n = 661 cells from 17 salivary glands), larval anterior midgut (n = 48 cells from 1 gut), X^{sim}/Y^{mel} hybrid male adult gut enterocytes (n = 966 cells from 7 guts), X^{sim}/X^{mel} hybrid female adult gut enterocytes (n = 385 cells from 3 guts). X^{sim} = X chromosome originating from *D. simulans,* X^{mel} = X chromosome originating from *D. melanogaster*, Y^{mel} = Y chromosome originating from *D. melanogaster*. Magenta = % *D. simulans* rDNA-dominant, turquoise = % *D. melanogaster* rDNA-dominant, grey = % co-dominant.

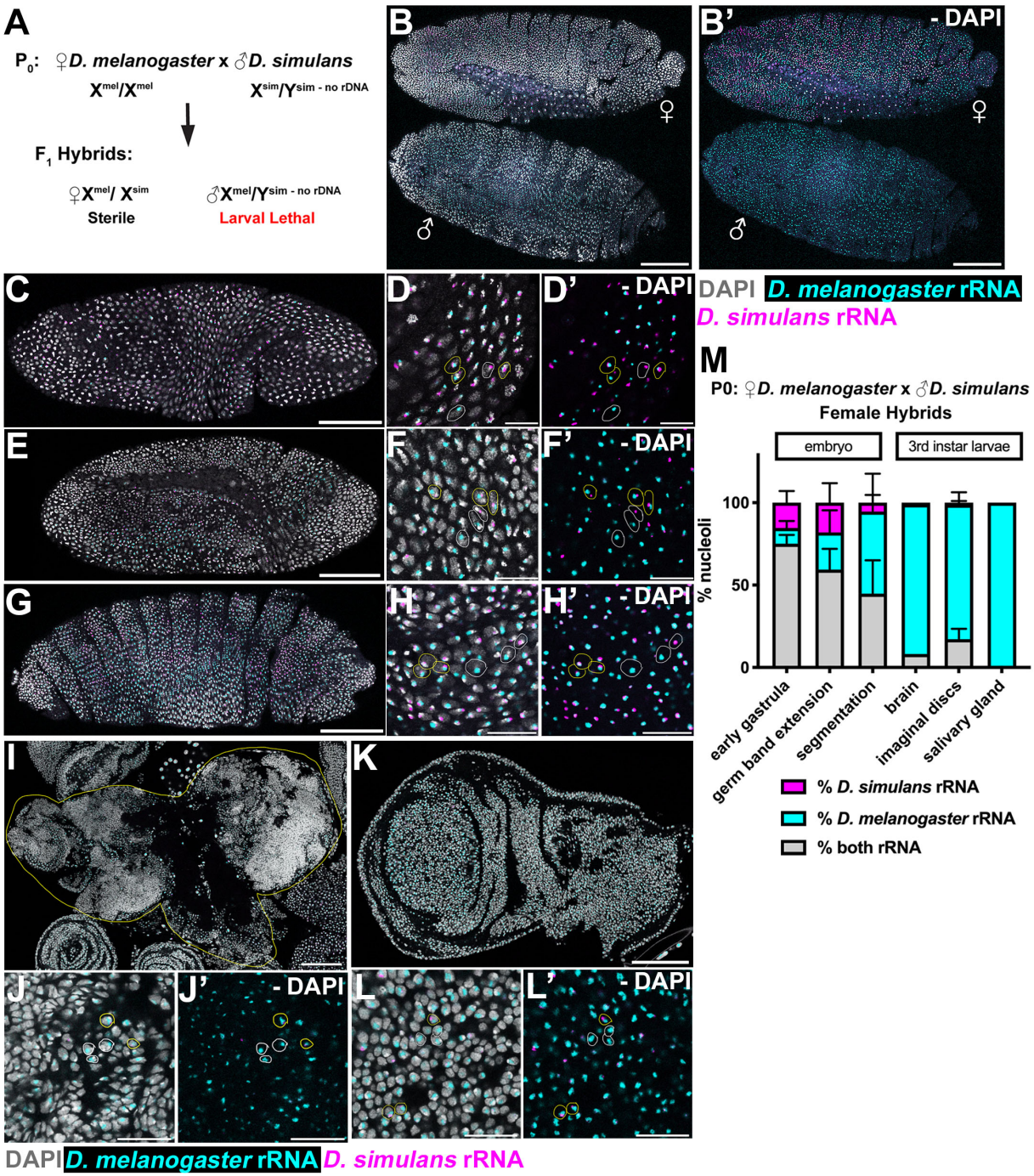


Figure II.2: Nucleolar Dominance is Established Across Developmental Time in Interspecies *Drosophila* Hybrid Females.

Figure II.2: Nucleolar Dominance is Established Across Developmental Time in Interspecies *Drosophila* Hybrid Females.
A) Schematic of interspecies hybrid crosses with P0 ♀*D. melanogaster* and ♂*D. simulans* and the resulting F1 hybrid progeny. B) Representative image of embryos predicted to be female (top) and male (bottom) from P0 ♀*D. melanogaster* and ♂*D. simulans*, given that *D. simulans* Y chromosome does not have rDNA, B') no DAPI, scale = 100μm. C) Representative image of an early gastrula stage embryo, scale = 100μm. D) Zoomed image of embryo in (C), D') no DAPI, scale = 20μm. E) Representative image of a germband extension stage embryo, scale = 100μm. F) Zoomed image of embryo in (E), F') no DAPI, scale = 20μm. G) Representative image of a segmentation stage embryo, scale = 100μm. H) Zoomed image of embryo in (G), H') no DAPI, scale = 20μm. I) Larval brain, scale = 100μm. J) Zoomed image of larval brain from (I), J') no DAPI, scale = 20μm. K) Wing disc, scale = 100μm. L) Zoomed image of wing disc from (K), L') no DAPI, scale = 20μm. Magenta = *D. simulans* rRNA, turquoise = *D. melanogaster* rRNA, grey = DAPI. Cells encircled with yellow are co-dominant. Cells encircled with white are dominant in expression. M) Quantification of nucleolar dominance across development in interspecies hybrid females from P0 ♀*D. melanogaster* and ♂*D. simulans*: early gastrula stage embryos (n = 370 cells 4 embryos), germband extension stage embryos (n = 1024 cells from 10 embryos), segmentation stage embryos (n = 591 cells from 4 embryos), larval brain (n = 208 cells from 1 brain), imaginal discs (n = 488 cells from 3 discs), and salivary gland (n = 189 cells from 3 salivary glands). Magenta = % *D. simulans* rDNA-dominant, turquoise = % *D. melanogaster* rDNA-dominant, grey = % co-dominant.

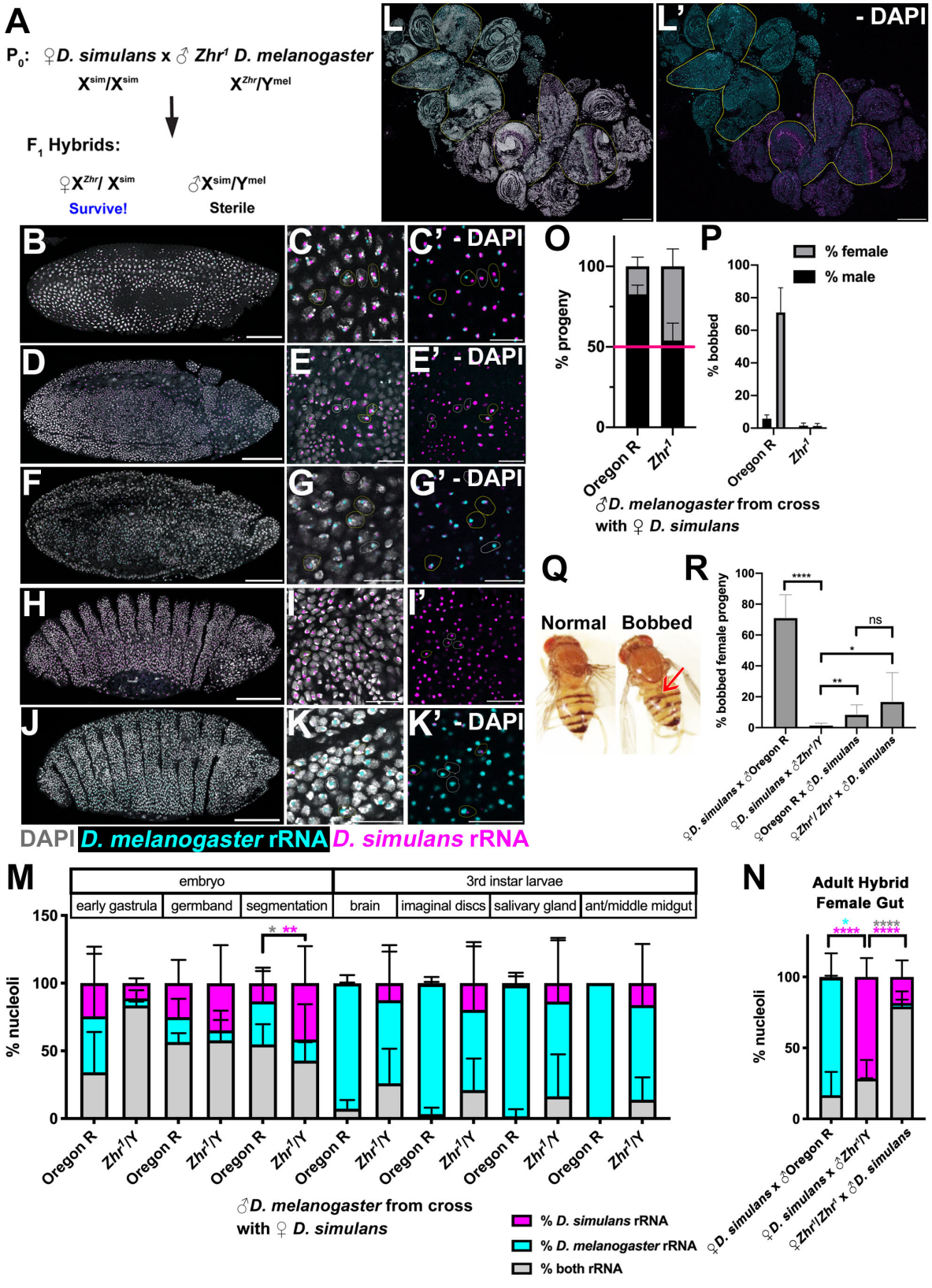


Figure II.3: *Zhr*^{1} Rescued *Drosophila* Interspecies Hybrids Exhibit Higher Rates of *D. simulans* rRNA Expression.

Figure II.3: *Zhr[1]* Rescued *Drosophila* Interspecies Hybrids Exhibit Higher Rates of *D. simulans* rRNA Expression.
A) Schematic of interspecies hybrid crosses with P0 ♀*D. simulans* and ♂*D. melanogaster* (*Zhr[1]*/Y) and the resulting F1 hybrid progeny. B) Representative image of an early gastrula stage embryo, scale = 100μm. C) Zoomed image of embryo in (B), C') no DAPI, scale = 20μm. D) Representative image of a germband extension stage embryo showing high rates of *D. simulans* rDNA-dominance, scale = 100μm. E) Zoomed image of embryo in (D), E') no DAPI, scale = 20μm. F) Representative image of a germband extension stage embryo showing high rates of co-dominance, scale = 100μm. G) Zoomed image of embryo in (F), G') no DAPI, scale = 20μm. H) Representative image of a segmentation stage embryo showing high rates of *D. simulans* rDNA-dominance, scale = 100μm. I) Zoomed image of embryo in (H), I') no DAPI, scale = 20μm. J) Representative image of a segmentation stage embryo showing high rates of *D. melanogaster* rDNA-dominance, scale = 100μm. K) Zoomed image of embryo in (J), K') no DAPI, scale = 20μm. L) Larval brains showing either high rates of *D. melanogaster* rDNA expression (top left) or high rates of *D. simulans* rDNA expression (bottom right), L') no DAPI, scale = 200μm. Magenta = *D. simulans* rRNA, turquoise = *D. melanogaster* rRNA, grey = DAPI. Cells encircled with yellow are co-dominant. Cells encircled with white are dominant in expression. M) Quantification of nucleolar dominance across development in interspecies hybrids from P0 ♀*D. simulans* and ♂*D. melanogaster* Oregon R compared to *Zhr[1]*/Y males (F1 data from ♀*D. simulans* and Oregon R males from Figure II.1 for comparison). Sample sizes for hybrids from P0 ♀*D. simulans* crossed to *Zhr[1]*/Y males: early gastrula stage embryos (n = 259 cells from 3 embryos), germband extension stage embryos (n = 431 cells from 4 embryos), segmentation stage embryos (n = 2295 cells from 15 embryos), larval brain (n = 915 cells from 5 brains), imaginal discs (n = 443 cells from 5 discs), salivary gland (n = 298 cells from 6 salivary glands), and larval anterior midgut (n = 309 cells from 4 guts). N) Quantification of nucleolar dominance in female adult enterocytes in interspecies hybrids (P0: ♀ x ♂): *D. simulans* x Oregon R (n = 385 cells from 3 guts, from Figure II.1 for comparison), *D. simulans* x *Zhr[1]*/Y (n = 1168 cells from 9 guts), *Zhr[1]*/ *Zhr[1]* x *D. simulans* (n = 622 cells from 5 guts). Magenta = % *D. simulans* rDNA-dominant, turquoise = % *D. melanogaster* rDNA-dominant, grey = % co-dominant. p-values calculated using Welch's unpaired, unequal variances *t*-test. (no star) = not significant, * = < 0.05, ** = < 0.01, **** = < 0.0001. Colors of asterisks correspond to colors of bars for which p-values were calculated (e.g. magenta asterisk for *D. simulans* rDNA-dominant p-values). O) Female embryonic hybrid rescue assay with *Zhr[1]*/Y males (n = 12 vials) and control Oregon R males (n = 15 vials). Pink line denotes 50% of progeny for visual ease. P) Rates of bobbed, a cuticular etching/peeling phenotype indicative of rDNA insufficiency, in the hybrid F1 from the corresponding crosses in (O). Grey = % female, Black = % male. Q) Representative images of female *Drosophila* with normal cuticular etching (left) and bobbed cuticular etching (right). Red arrow denotes cuticular etching. R) Percentage of female progeny from hybrid crosses with the bobbed phenotype (P0: ♀ x ♂): *D. simulans* x Oregon R (n = 15 vials), *D. simulans* x *Zhr[1]*/Y (n = 12 vials), Oregon R x *D. simulans* (n = 10 vials), *Zhr[1]*/ *Zhr[1]* x *D. simulans* (n = 9 vials). Values from P0 ♀*D. simulans* x Oregon R males and from P0 ♀*D. simulans* x *Zhr[1]*/Y males are duplicated from (P) for comparison and statistical analysis. Rescue assays for the crosses with ♀*D. melanogaster* and ♂*D. simulans* were not reported since no male progeny survive to adulthood. p-values calculated using Welch's unpaired, unequal variances *t*-test. (no star) = not significant, * = < 0.05, ** = < 0.01, **** < 0.0001. Grey = % female.

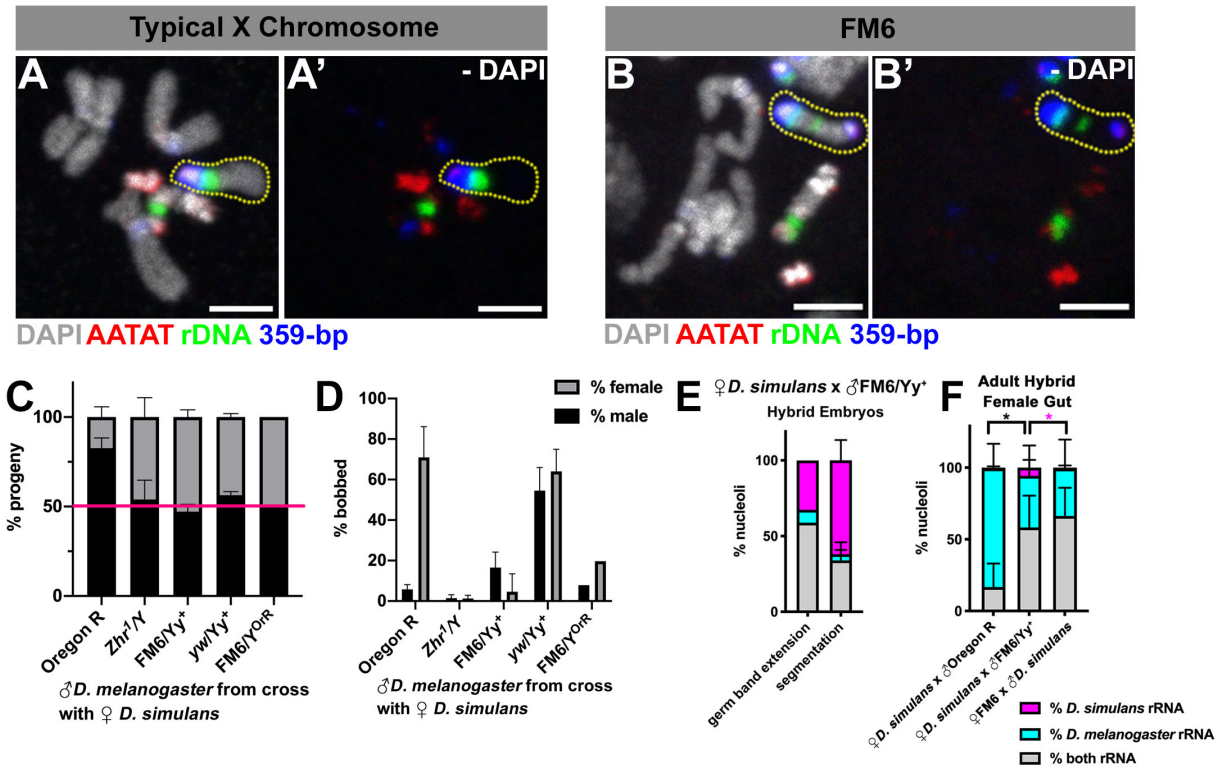


Figure II.4: FM6 Balancer Can Rescue Female Embryonic Lethality and Disrupt Nucleolar Dominance.
DNA FISH of mitotic chromosome spreads from larval neuroblasts of A) *D. melanogaster* X/Y male with a typical X chromosome, A') no DAPI, and from B) *D. melanogaster* FM6/Y male, B') no DAPI. All scale bars = 3µm. Grey = DAPI, red = AATAT, green = rDNA IGS, blue = 359-bp. C) Female embryonic hybrid rescue assay. Pink line denotes 50% of progeny for visual ease. Sample size are as follows (♂*D. melanogaster* genotype from P0 cross with ♀*D. simulans*): Oregon R (duplicated from Figure II.3) = 15, *Zhr*^{1}/Y (duplicated from Figure II.3) = 12, FM6/Yy^{+} = 4, *yw*/Yy^{+} = 7, FM6/Y^{OrR-1} = 1. D) Percentage of progeny from crosses corresponding to (C) with the bobbed phenotype. Bobbed is a cuticular etching/peeling phenotype indicative of rDNA insufficiency. Grey = % female, Black = % male. P-values were not calculated. E) Quantification of nucleolar dominance during embryogenesis in interspecies hybrids from P0 ♀*D. simulans* and ♂*D. melanogaster* FM6/Yy^{+}: germband extension stage embryos (n = 236 cells from 1 embryo), segmentation stage embryos (n = 1962 cells from 10 embryos). F) Quantification of nucleolar dominance in interspecies hybrid female adult gut enterocytes (P0: ♀ x ♂): *D. simulans* x Oregon R (n = 385 cells from 3 guts, from Figure II.1 for comparison), *D. simulans* x FM6/Yy^{+} (n = 988 cells from 10 guts), and FM6 x *D. simulans* (n = 1440 cells from 13 guts). Magenta = % *D. simulans* rDNA-dominant, turquoise = % *D. melanogaster* rDNA-dominant, grey = % co-dominant. p-values calculated using Welch's unpaired, unequal variances *t*-test. (no star) = not significant, * = < 0.05. Black asterisk to indicate that all categories compared were similar p-values.

Discussion

This study on nucleolar dominance in interspecies *Drosophila* hybrids expands previous knowledge to other tissues and developmental stages (Durica and Krider 1977). In interspecies hybrids of *Drosophila simulans* and *Drosophila melanogaster*, the rDNA locus from *D. melanogaster* dominates in expression over *D. simulans* rDNA in both X/X females, X/Y males, and X/X/Y females (Durica and Krider 1977). By utilizing sequence differences between *D. simulans* and *D. melanogaster* rDNA internal transcribed spacer (ITS), we expanded on previous studies and characterized nucleolar dominance using RNA *in situ* hybridization by visualizing nucleolar dominance across development. We show that nucleolar dominance in these *Drosophila* interspecies hybrids is not established until larval development. This temporal regulation of nucleolar dominance is similar to what has been found to occur in "pure species" *Arabidopsis thaliana, Arabidopsis* hybrids (Pontes et al. 2007, Earley et al. 2010) and in X/Y "pure species" *D. melanogaster* males (Warsinger-Pepe et al. 2020), although nucleolar dominance in *Drosophila* interspecies hybrids appears to be established earlier. This difference in establishment of nucleolar dominance may in part be due to dysregulation of developmental mechanisms in interspecies hybrids (Wittkopp et al. 2004).

Other studies of nucleolar dominance have implicated that both sequence *cis* elements within rDNA (Kidd and Glover 1981, Macleod and Bird 1982, Labhart and Reeder 1984, Grimaldi et al. 1990, Heix and Grummt 1995, Neves et al. 1995, Houchins et al. 1997, Caudy and Pikaard 2002, Eickbush et al. 2008, Felle et al. 2010) and chromosome elements/environment (Chandrasekhara et al. 2016, Mohannath et al. 2016) influence nucleolar dominance. Sequence differences within the intergenic spacer sequences (IGS) of rDNA were previously suggested to regulate nucleolar dominance. Different species' IGS can compete for rDNA transcription factors where longer spacers outcompete short spacers in *Xenopus laevis* oocytes (Reeder et al. 1983, Labhart and

Reeder 1984) and was thought to influence nucleolar dominance in *Xenopus* interspecies hybrids (Reeder and Roan 1984). Because the IGS sequences in *Drosophila* are known to be highly diverged compared to the coding region of rDNA (Tautz et al. 1987, Tautz et al. 1988), it is tempting to speculate that differences in IGS between *D. simulans* and *D. melanogaster* can instruct nucleolar dominance. Previous studies (Tautz and Dover 1986) and our preliminary data in Chapter I reveal that IGS is transcribed in "pure species" *Drosophila* (Figure B.4), and additional preliminary data of ours suggests that IGS is still transcribed in interspecies *Drosophila* hybrids (Figure B.7). Preliminary observations reveal higher IGS RNA signal co-localizing with the *D. melanogaster* rRNA signal in *Drosophila* interspecies hybrid embryos (Figure B.7). This may be due to the inability of *D. simulans* rRNA to be expressed since we do not see high IGS signal from *D. simulans* female embryos (Figure B.7B). We see IGS expression in male *D. simulans* (one rRNA signal) where IGS appears not to co-localize with rRNA signal (Figure B.7). The *D. simulans* Y rDNA locus has IGS but does not have rDNA coding repeats, so we believe this IGS signal is from the Y chromosome (Figure B.7). Female *D. simulans* (two rRNA signals) show faint IGS expression (Figure B.7). This is similar to the observation noted in "pure species" *D. melanogaster* embryos where most females have relatively low IGS signal (Figure B.4). It is intriguing to speculate whether this difference in IGS RNA levels impacts establishment of nucleolar dominance. Whether or not IGS RNA influences nucleolar dominance in either "pure species" or interspecies *Drosophila* hybrids remain difficult to elucidate given the size and repetitive nature of IGS and awaits future investigation.

The aforementioned sequence divergence of the rDNA spacer sequence despite generally high conservation of rDNA sequence (Tautz et al. 1987, Ganley and Kobayashi 2007), has been speculated to arise from molecular co-evolution of the promoter regions embedded in IGS and RNA Polymerase I (RNA Pol I) components (Dover and Flavell 1984) that utilize those promoters. Early studies on rDNA expression in human and mouse cells revealed that different species rDNA promoters and RNA Pol I components are not fully compatible, suggesting species-specificity and divergence (Grummt et al. 1982). Many have speculated that molecular co-evolution of rDNA

promoters and the RNA Pol I components occurs over time to result in this species-specificity (Dover and Flavell 1984, Reeder 1984, Moss et al. 1985), however RNA Pol I component replacement studies have not been performed to test this. Interestingly, *D. virilis and D. melanogaster* which are speculated to have diverged ~40 million years ago (Morales-Hojas et al. 2011) are unable to utilize the others cytoplasmic components (e.g. RNA Pol I) to transcribe its rDNA, revealing support for the molecular co-evolution hypothesis (Kohorn and Rae 1982), albeit it remains unknown whether this conflict arises between DNA-protein interactions, protein-protein interactions, or both (Heix and Grummt 1995).

Chromosome context, or the location of rDNA and its proximal heterochromatic elements, has been shown to influence nucleolar dominance in *Arabidopsis* (Chandrasekhara et al. 2016). Similarly, chromosome components that influence nucleolar dominance were thoroughly characterized in *Drosophila* interspecies hybrids by Durica and Krider (Durica and Krider 1978). Durica and Krider found that the short arm of the Y chromosome (YS) influences nucleolar dominance in interspecies *Drosophila* hybrids. This is similar to what we report where YS is able to induce nucleolar dominance in “pure species” X/X *D. melanogaster* females. What aspects of YS and its rDNA influence nucleolar dominance remain unknown, but the influence of chromosome context of the rDNA locus in these cases is reminiscent of what has been reported in *Arabidopsis* (Chandrasekhara et al. 2016).

Durica and Krider also explored which element(s) of the *D. melanogaster* X chromosome are important for hybrid nucleolar dominance. They found that two X chromosome deficiencies ($Df(1)y^{x2}$ and $Df(1)mal^{12}$) completely switched nucleolar dominance, where *D. simulans* rRNA is expressed and *D. melanogaster* rDNA is silenced (Durica and Krider 1978). Both X chromosomes however did not have secondary constrictions in their original “pure species” stocks, suggesting smaller or potential complete lack of rDNA, perhaps indicating reason for the switch in nucleolar dominance. Durica and Krider used other inverted X chromosomes and found that heterochromatin proximal to the rDNA on the *D. melanogaster* X chromosome is

important for establishing nucleolar dominance (Durica and Krider 1978). This is supported by our data that *Zhr*^{1}, which lacks heterochromatin proximal to the rDNA (359-bp satellite block) disrupts nucleolar dominance in adult female hybrids (Figure II.3N). Two inverted X chromosomes used in their study, In(1)sc^{S1}+In49 and In(1)sc^{S1}+S, both exhibited relatively high rates of co-dominance (Durica and Krider 1978). The original In(1)sc^{S1} chromosome from their mapping reveal that the majority of the 359-bp satellite block is intact and adjacent to the rDNA locus, albeit on the most distal portion of the long arm of the chromosome. Additionally, a portion of 359-bp satellite block (the smallest portion mapped by Sawamura and Yamamoto 1993 to induce female embryonic lethality) remains intact on In(1)sc^{S1} and in its original location. Whether In(1)sc^{S1} induces hybrid lethality remains unknown. In combination with our data that shows the ability of FM6 to disrupt nucleolar dominance, the ability of In(1)sc^{S1} to disrupt nucleolar dominance suggests that inverting and/or disrupting the X heterochromatin can allow for de-repression of *D. simulans* rRNA in interspecies hybrids. Exactly how these inversions/disruptions function to de-repress *D. simulans* rRNA (whether through position effect or separation of conflicting elements), remains unclear.

To further explore whether proximity of rDNA and *Zhr* can influence female hybrid nucleolar dominance and viability, our lab generated a *D. melanogaster* strain containing an rDNA locus in the euchromatin on the third chromosome (see Chapter I for details), referred to as rDNA on III. We used this rDNA locus in combination with a complete rDNA deletion on X (*bb*^{158}) to test whether complete separation of rDNA from *Zhr* (via a different chromosome) could rescue hybrid female embryonic lethality. Males lacking rDNA on their X chromosome while containing rDNA on III were able to rescue hybrid female embryonic lethality (*bb*^{158}/Y;; rDNA/TM6B P0 males, F1 females: 62.4 ± 0.0%) (Figure B.8). *bb*^{158} X chromosome on its own may have some ability to rescue (Figure B.8), however this rescue may be due to partial deletion of the *Zhr* locus on *bb*^{158} (Sawamura and Yamamoto 1993). This preliminary data suggests that an rDNA locus separate from the X chromosome may be able to rescue female embryonic

lethality. Whether or not this rDNA on III disrupts hybrid nucleolar dominance remains unknown.

The physical nature of chromosome arrangements not only has implications in nucleolar dominance but may also influence genomic stability. A previous study revealed that chromosome bridges (a hallmark of genome instability) (Aguilera and Garcia-Muse 2013) formed from circular chromosomes that contained specific organization of satellite DNA (Ferree et al. 2014). Ferree et al suggested that chromosome bridge formation of the circular chromosomes and ultimate lethality of the corresponding *Drosophila* embryo is due to 359-bp satellite DNA and its proximity to additional heterochromatic repeats (Ferree et al. 2014). The connection between nucleolar dominance and hybrid female lethality therefore may lie within the arrangement of rDNA and heterochromatic elements. 359-bp satellite DNA in proximity to rDNA appears to result in female hybrid death; separating these two elements on X or even inverting these two away from the centromere can often either disrupt nucleolar dominance and/or rescue hybrid female lethality (Figure II.4) (Durica and Krider 1978, Sawamura et al. 1993). 359-bp additions on the Y chromosome can cause chromosome bridges and male hybrid embryonic lethality, despite 359-bp and rDNA being separate from each other (Sawamura and Yamamoto 1993, Ferree and Barbash 2009); since the Y chromosome is almost entirely heterochromatic, this reveals potential other conflicts between satellite DNAs in a hybrid environment.

But through what mechanism(s) might physical proximity, or position effects, result in these genomic conflicts/instabilities? Studies in yeast have shown that transcription-replication conflicts can occur on the rDNA and result in increased rates of recombination and rDNA copy loss (Kobayashi et al. 1998, Defossez et al. 1999, Kaeberlein et al. 1999, Johzuka and Horiuchi 2002, Merker and Klein 2002, Takeuchi et al. 2003). Replication conflicts do appear to occur in lethal hybrid embryos, given the enrichment of Topoisomerase I along the chromosome bridge (Ferree and Barbash 2009). This suggests that the 359-bp element is not being fully replicated and that supercoiling may be abundant. It is intriguing to speculate whether torsional stress from

high transcription of rDNA (Liu and Wang 1987) cannot be relieved in these hybrids and/or if replication fork progression is deterred (Olavarrieta et al. 2002, Takeuchi et al. 2003), resulting in collisions with the transcriptional machinery. Whether or not supercoiling, torsional stress and incomplete DNA replication are involved in the relationship between the repetitive 359-bp satellite DNA block and high transcription of the rDNA repeats remain for future studies.

External factor(s) also appear to play a role in interspecies hybrid nucleolar dominance and female hybrid lethality. Our data reveals that lack of 359-bp is not sufficient to suppress *D. melanogaster* rDNA expression in female hybrids (Figure II.3N). It is intriguing to speculate that a maternal factor (or lack thereof) may aid in the complete reversal of nucleolar dominance that we see in $Zhr^{1}/X^{D.\ sim}$ females, since $Zhr^{1}/X^{D.\ sim}$ females generated from P0 female *D. melanogaster* exhibit co-dominance instead of *D. simulans* rDNA dominance. Additionally, we see bobbed hybrids when 359-bp is present in *D. simulans* maternal cytoplasm (P0: ♀*D. simulans* x ♂*D. melanogaster*) for both male and female hybrids (Figure II.4D), further suggesting that a *D. simulans* maternal cytoplasmic factor may influence rDNA expression in hybrids. Previous reports found that a *D. simulans* maternal factor, *maternal hybrid rescue* (*mhr*), causes lethality in both male and female hybrids that developed in *D. simulans* maternal cytoplasm (P0: ♀*D. simulans* x ♂*D. melanogaster*) (Sawamura et al. 1993). It is intriguing to speculate if the female hybrid rescue seen by removal of 359-bp and the disruptions in nucleolar dominance/rDNA metabolism described above, are all influenced by *mhr* or another *D. simulans* maternal factor. Future studies are required to understand the exact relationship between these maternal factor(s), rDNA expression, and 359-bp satellite DNA and to understand if and how these relationships influence hybrid nucleolar dominance and hybrid lethality.

Chapter III[2]: Satellite DNA Divergence Across Closely Related *Drosophila* Species

[2] The majority of Chapter III is published in *Jagannathan, Warsinger-Pepe, Watase and Yamashita 2017*. Text has been slightly modified to fit the formatting of this dissertation.

Summary

Satellite DNAs are highly repetitive sequences that account for the majority of constitutive heterochromatin in many eukaryotic genomes. It is widely recognized that sequences and locations of satellite DNAs are highly divergent even in closely related species, contributing to the hypothesis that satellite DNA differences may underlie speciation. However, due to its repetitive nature, the mapping of satellite DNAs has been mostly left out of recent genomics analyses, hampering the use of molecular genetics techniques to better understand their role in speciation and evolution. Satellite DNAs are most extensively and comprehensively mapped in *Drosophila melanogaster*, a species that is also an excellent model system with which to study speciation. Yet the lack of comprehensive knowledge regarding satellite DNA identity and location in its sibling species (*D. simulans*, *D. mauritiana*, and *D. sechellia*) has prevented the full utilization of *D. melanogaster* in studying speciation. To overcome this problem, we initiated the mapping of satellite DNAs on the genomes of the *D. melanogaster* species complex (*D. melanogaster*, *D. simulans*, *D. mauritiana*, and *D. sechellia*) using multi-color fluorescent in situ hybridization (FISH) probes. Our study confirms a striking divergence of satellite DNAs in the *D. melanogaster* species complex, even among the closely related species of the *D. simulans* clade (*D. simulans*, *D. mauritiana*, and *D. sechellia*), and suggests the presence of unidentified satellite sequences in these species.

Introduction

Short tandem repetitive or “satellite” DNAs are abundant and conserved features of eukaryotic genomes. Although typically considered “junk” DNA due to a lack of protein coding potential, decades of study have implicated satellite DNA function in cellular processes such as kinetochore/centromere function, meiotic chromosome segregation, and X chromosome recognition (Dernburg et al. 1996, Karpen et al. 1996, Guenatri et al. 2004, Bouzinba-Segard et al. 2006, Usakin et al. 2007, Wong et al. 2007, Menon et al. 2014, Rosic et al. 2014). Moreover, aberrant transcription of satellite DNAs has been associated with human diseases such as cardiomyopathy and cancer, suggesting critical importance of the regulation of this underappreciated component of eukaryotic genomes (Gaubatz and Cutler 1990, Feber et al. 2011, Ting et al. 2011, Haider et al. 2012). Yet, other than these examples, the functions of the majority of satellite DNAs remain obscure.

The common fruit fly, *Drosophila melanogaster*, is an excellent model system with which to study satellite DNAs. Approximately 21% of the *D. melanogaster* genome is comprised of satellite DNA (Lohe and Brutlag 1986) and extensive efforts have mapped the location of 15 unique repeats on *D. melanogaster* chromosomes (Waring and Pollack 1987, Bonaccorsi and Lohe 1991, Abad et al. 1992, Lohe et al. 1993, Dernburg et al. 1996). Efforts to identify satellite repeats and map them onto chromosomes have been made in many species including *Abracris flavolineata* (Bueno et al. 2013), *Aegilops geniculate* and wheat (Koo et al. 2016), various *Arabidopsis* species (Kamm et al. 1995, Ito et al. 2007, Kawabe and Charlesworth 2007), maize (Lamb et al. 2007), *Turritis glabra* (Kawabe and Nasuda 2006), rodent species including *Phodopus sungorus* (Paco et al. 2014), *Cricetus cricetus* and *Microtus arvalis* (Louzada et al. 2015), *Xenopus* (Schmid and Steinlein 2015), and human (Altemose et al. 2014), revealing general patterns of centromeric, pericentromeric, and telomeric satellite

distribution. The rich history of *D. melanogaster* genetics has resulted in the comprehensive identification and mapping of satellite DNA to individual chromosomes; *D. melanogaster* remains the only species with this resolution (Waring and Pollack 1987, Bonaccorsi and Lohe 1991, Abad et al. 1992, Lohe et al. 1993, Dernburg et al. 1996). Even in *Drosophila* sibling species such as *D. simulans*, *D. mauritiana*, and *D. sechellia* (together called the *D. simulans* clade), satellite composition and chromosome location has only been partially examined (Lohe and Brutlag 1987, Larracuente 2014). Interestingly, it has been shown that even closely related species display significant divergence in the abundance and sequence of individual satellite DNA repeats (Lohe and Brutlag 1987, Lohe and Roberts 2000, Bosco et al. 2007). These observations led to the hypothesis that rapid divergence of satellite DNA may play an important role in speciation by causing reproductive isolation between closely related species (Yunis and Yasmineh 1971, Gatti et al. 1976). In support of this idea, it was shown that a satellite DNA on the *D. melanogaster* X chromosome (i.e., 359-bp repeats) causes hybrid incompatibility when crossed with its closest sibling species, *D. simulans* (Sawamura et al. 1993, Ferree and Barbash 2009). However, a lack of information regarding satellite DNAs in other species hinders efforts to identify whether there are more instances of hybrid incompatibility caused by satellite DNA among closely related species. In this chapter, we have used DNA FISH to map known *Drosophila* satellite DNA repeats on the mitotic chromosomes of the sibling species *D. melanogaster*, *D. simulans*, *D. mauritiana*, and *D. sechellia* (collectively categorized as “the *D. melanogaster* species complex”). We reveal a remarkable divergence in the abundance and location of specific satellite DNA repeats in these closely related sibling species, which can be used as a resource for future work on *Drosophila* chromosome biology and speciation.

Materials and Methods

Drosophila Strains and Fly Husbandry

All fly stocks were raised on standard Bloomington medium at 25ºC, and male third instar wandering larvae were used. For better chromosome squashes, larvae cultured at 18ºC were used. The following fly stocks were used: *D. melanogaster yw*, *D. simulans* w^{501} (DSSC#14021-0251.195), *D. sechellia* w^{1} (DSSC#14021-0248.30), and *D. mauritiana* w^{1} (DSSC#14021-0241.60).

Larval Brain Squash, Chromosome DNA FISH, and Microscopy

We adapted a simple DNA FISH protocol against squashed chromosomes published by Larracuente and Ferree (2015) with small modifications (Larracuente and Ferree 2015). Briefly, male third instar wandering larvae were collected and brains were dissected in 1X PBS. Larval brains were fixed in 25 mL of acetic acid: 4% formaldehyde in 1X PBS (45%:55%) for 4 min on Sigmacote-coated coverslips (Sigma: SL2 SIGMA). The whole sample was quickly applied to a clean Superfrost plus slide and the sample was manually squashed via thumb/stamp over coverslip, over sample, on top of the slide. The slide/coverslip/sample was immediately submerged in liquid nitrogen until it stopped boiling. Slides were removed from liquid nitrogen and coverslips were quickly flicked off the slide with a razor blade. Slides were then washed in 100% ethanol at room temperature for 5 min then dried in a dark, dust-free location. Hybridization was performed in 50% formamide, 10% dextran sulfate, 2X SSC buffer, 0.5 mM of each probe, and up to 20 mL of diH_2O. Hybridization buffer was added to the samples and covered with a coverslip. Slides were heated at 95ºC for 5 min, cooled briefly, wrapped in parafilm, and incubated in a humid chamber in the dark overnight at room temperature. Coverslips were removed and slides were washed three times for 15 min in 0.1X SSC, removed of excess buffer, and mounted in Vectashield mounting medium containing DAPI. Images were taken using an upright Leica TCS SP8 confocal

microscope with a 63X oil immersion objective (NA = 1.4) and processed using Adobe Photoshop software. Images were modified solely for the purpose of clarity. Modified images were not quantified. Detailed sequences of probes used are provided in Table A.4.

Results

Based on previous studies (Simeone et al. 1985, Lohe and Brutlag 1987, Waring and Pollack 1987, Lohe and Roberts 1990, Abad et al. 1992, Carmena et al. 1993, Lohe et al. 1993, Abad et al. 2000, Hoskins et al. 2015), we designed 17 probes that are known to exist in *D. melanogaster* (some of which are also known to exist in *D. simulans*), and two probes that are known to exist in *D. simulans* but are supposedly absent in *D. melanogaster* (Table A.4). We used these 19 probes in 11 different combinations (C1–11, Table A.4), with each combination consisting of Alexa488-, Cy3-, and Cy5-conjugated probes. We then performed DNA FISH on the mitotic chromosomes of squashed larval brains from *D. melanogaster*, *D. simulans*, *D. mauritiana*, and *D. sechellia* using a method described previously (Larracuente and Ferree 2015). We selected male third instar larvae based on their gonad morphology such that the mitotic chromosome spread would always contain a Y chromosome.

The results are shown in Figure III.1, Figure III.2, Figure III.3, Figure III.4, and Figure III.5, and the chromosomal location(s) of each satellite DNA repeat in all four species are summarized in Table A.5 and Table A.6. We observed a dramatic divergence of satellite repeat abundance and distribution that clearly demarcates the chromosomes of these closely related species. It should be noted that the probe used for 372-bp repeats perfectly colocalized with the 359-bp probe on the mitotic chromosomes in all four species, suggesting that these two probes do not differentiate between the 359-bp satellite and the 372-bp repeat sequences, as well as other derivatives such as 353- and 356-bp repeats in *D. melanogaster* (Losada and Villasante 1996, Abad et al. 2000), and 360 family sequences in the *D. simulans* clade (Strachan et al. 1985). Indeed, our 70-bp-long probe designed for 372-bp repeats shows 74% identity to the 359-bp satellite consensus sequence. One puzzling observation is that the 372-bp repeats were originally identified as middle repetitive elements dispersed

along the X chromosome euchromatin in *D. melanogaster* (Waring and Pollack 1987). Later, it was found that the 372-bp repeats are a part of the 1.688 g/cm^3 class of satellite DNAs, which is present at numerous locations throughout the X chromosome euchromatin (DiBartolomeis et al. 1992). Our mitotic chromosome DNA FISH did not detect hybridization of 372- or 359-bp probes to these euchromatic regions. This might be due to the fact that euchromatic loci of the 1.688 g/cm^3 class of satellite DNAs (including 372-bp repeats) are below the detection limit of our DNA FISH technique (DiBartolomeis et al. 1992, Kuhn et al. 2012). In addition, the detection of such low abundance sequences in euchromatic regions might require polytenization, where the euchromatic 372-bp repeat was originally identified (Waring and Pollack 1987). We additionally designed a 260-bp probe, as neither of our 359- or 372-bp probes was expected to hybridize to 260-bp repeats. This probe still cross-hybridized to 359/372-bp repeats, but successfully detected an additional locus (Figure III.5), likely representing bona fide 260-bp repeats on the *D. melanogaster* second chromosome as reported previously (albeit at a low signal intensity) (Abad et al. 2000). In *D. mauritiana*, the 260-bp probe hybridized to the same chromosomal location as 359/ 372-bp repeats, suggesting this might also represent cross-hybridization. However, in *D. simulans* and *D. sechellia*, the 260-bp repeat probe did not show any signal, suggesting either that (1) *D. simulans* and *D. sechellia* 359/372-bp repeats are more diverged in the region that is covered by our 260-bp repeat probe or that (2) cross-hybridization of the 260-bp probe to 359/372-bp repeats is very weak and slight differences in hybridization conditions yield different results.

While two *D. simulans*-specific satellites (AACAAAC and GAACAGAACATGTTC) had been previously identified by cesium chloride centrifugation (Lohe and Brutlag 1987), their chromosomal locations were unknown. Here, we map their locations on the *D. simulans* chromosomes (Figure III.3, Table A.5, and Table A.6) and extend this mapping to both *D. mauritiana* and *D. sechellia*. The GAACAGAACATGTTC repeat is completely absent in *D. melanogaster* and is predominantly present on the autosomes of *D. simulans*, *D. mauritiana*, and *D. sechellia*. On the other hand, the AACAAAC satellite repeat is specifically present on the *D. simulans* Y chromosome and is absent

from *D. mauritiana* and *D. sechellia*. Although AACAAAC was reported to be absent in *D. melanogaster*, we observed probe hybridization on the *D. melanogaster* second chromosome, albeit at a low level. It is possible that a slightly diverged sequence on the *D. melanogaster* chromosome is still able to hybridize to the AACAAAC probe. Alternatively, the *D. melanogaster* genome could have acquired/amplified this satellite repeat in the time between the previous report and this study (~30 years).

An important aspect of the satellite DNA maps in *D. melanogaster* is the knowledge of chromosome-specific satellite sequences, a feature that has facilitated the study of chromosome-specific behaviors (Dernburg et al. 1996, Joyce et al. 2012, Christophorou et al. 2013). Although our DNA FISH experiments revealed differences in the contents of satellite DNA repeats on the autosomes of the *D. simulans* clade, it is nevertheless difficult to determine which chromosome (second or third) corresponds to which satellite composition, as these two autosomes are morphologically indistinguishable in all species. We have attempted to overcome this issue in *D. simulans* using two stocks, Dsim\T(Y;2) and Dsim\T(Y;3), where a translocation event between the Y and second or third chromosomes allows us to unambiguously distinguish the second and third chromosomes. By using a probe combination that focuses on *D. simulans* autosomes (C10, Table A.4), we observed these translocation stocks of *D. simulans*. Our data show that the majority of the dodeca satellite repeats are present on the *D. simulans* second chromosome (Figure III.6). Considering the fact that the dodeca satellite repeat is present on the third chromosome in *D. melanogaster*, it is clear that each autosome does not have “signature” sequences (i.e., chromosome-specific sequences) that are conserved among the four species examined in this study. Due to the lack of translocation stocks in *D. mauritiana* and *D. sechellia*, the identity of the second and third chromosomes with respect to satellite DNA composition remains unclear in these species.

Finally, we observed that the GAACAGAACATGTTC and 359/372-bp satellite repeats were asymmetrically distributed between the *D. mauritiana* second and third chromosomes (Figure III.2, C4 and Figure III.3, C6); that is, one chromosome (second

or third) contained more GAACAGAACATGTTC satellites than the other, and similarly, one chromosome contained more 359/372-bp repeats than the other chromosome. Also, we observed a similar asymmetric distribution of the GAACAGAACATGTTC and the dodeca satellite repeats on the *D. sechellia* second and third chromosomes (Figure III.3, C5 and C6). To determine the relationship between these satellite repeats on the autosomes of *D. mauritiana* and *D. sechellia*, we used two probe combinations (C9 and C10) (Figure III.7 and Table A.4). This revealed that the abundances of 359-bp and GAACAGAACATGTTC satellite repeats are inversely correlated in *D. mauritiana*; the autosome that contains more 359-bp repeats contains less GAACAGAACATGTTC compared to the other autosome. Also, in *D. mauritiana*, the abundances of GAACAGAACATGTTC and dodeca satellite repeats are positively correlated. In *D. sechellia*, the abundance of GAACAGAACATGTTC and dodeca are positively correlated; the autosome that contained more GAACAGAACATGTTC also contained more dodeca compared to the other autosome.

Figures

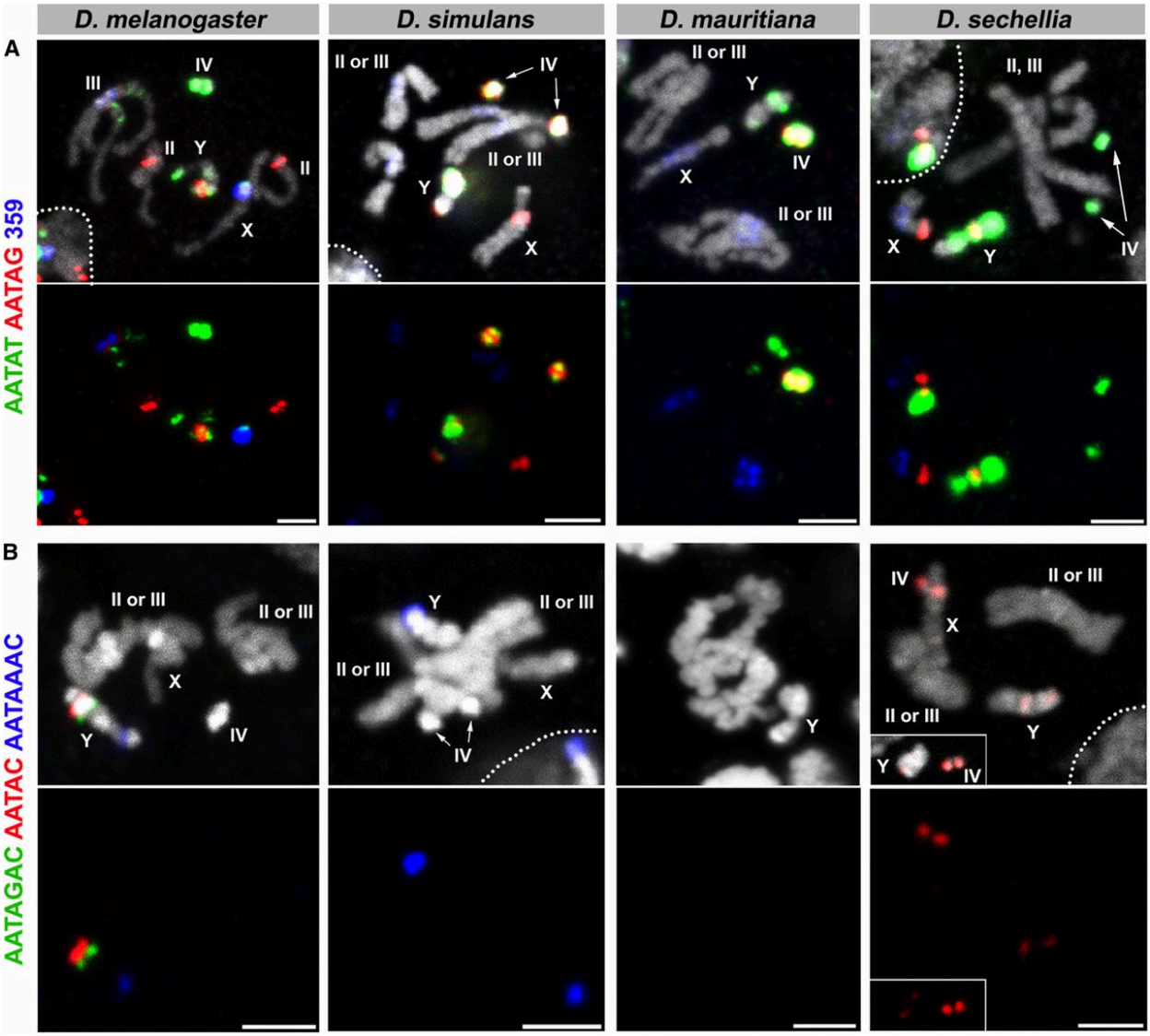


Figure III.1: DNA FISH (C1/C2) on Neuroblast Chromosome Spreads from *D. melanogaster*, *D. simulans*, *D. mauritiana*, and *D. sechellia*.
A) C1 probes and B) C2 probes. Probe sequences are indicated by the colored text. The top panels show three probes combined with DAPI, and the bottom panels show only probe hybridization signals. Bar, 2.5 µm. C = combination.

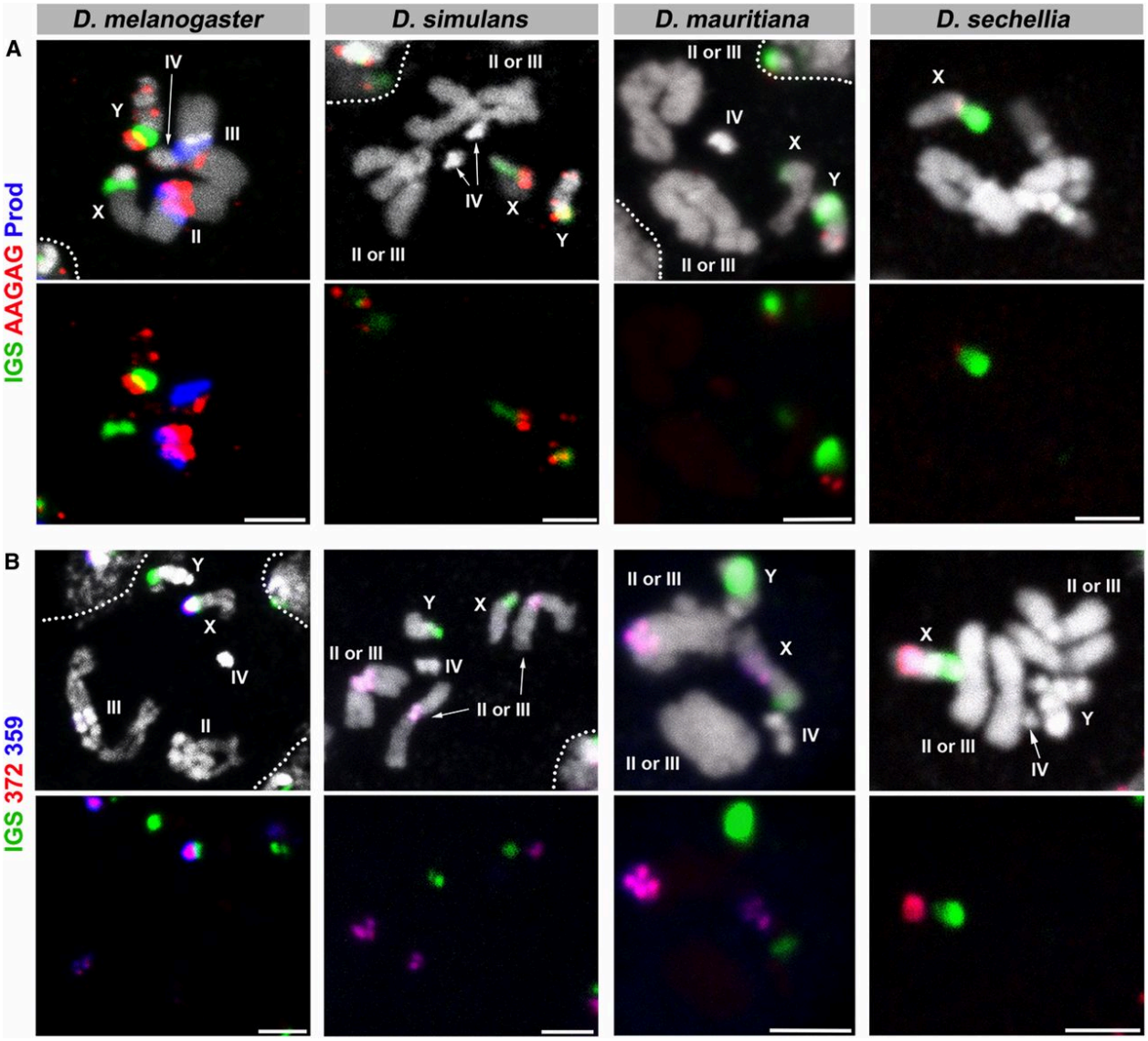


Figure III.2: DNA FISH (C3/C4) on Neuroblast Chromosome Spreads from *D. melanogaster*, *D. simulans*, *D. mauritiana*, and *D. sechellia*.
A) C3 probes and B) C4 probes. Probe sequences are indicated by the colored text. The top panels show three probes combined with DAPI, and the bottom panels show only probe hybridization signals. Bar, 2.5 µm. C = combination.

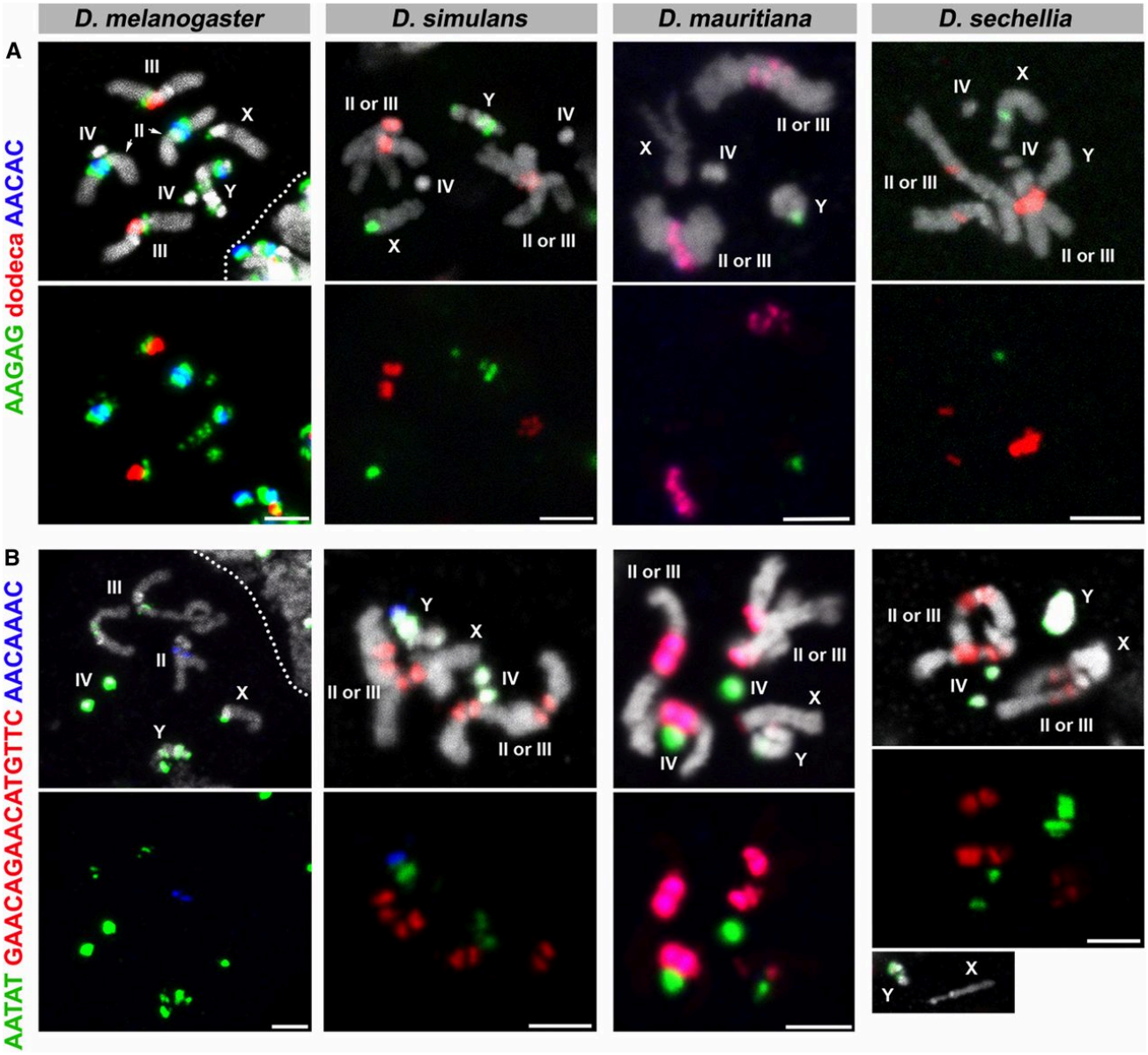


Figure III.3: DNA FISH (C5/C6) on Neuroblast Chromosome Spreads from *D. melanogaster*, *D. simulans*, *D. mauritiana*, and *D. sechellia*.
A) C5 probes and B) C6 probes. Probe sequences are indicated by the colored text. The top panels show three probes combined with DAPI, and the bottom panels show only probe hybridization signals. Bar, 2.5 µm. C = combination.

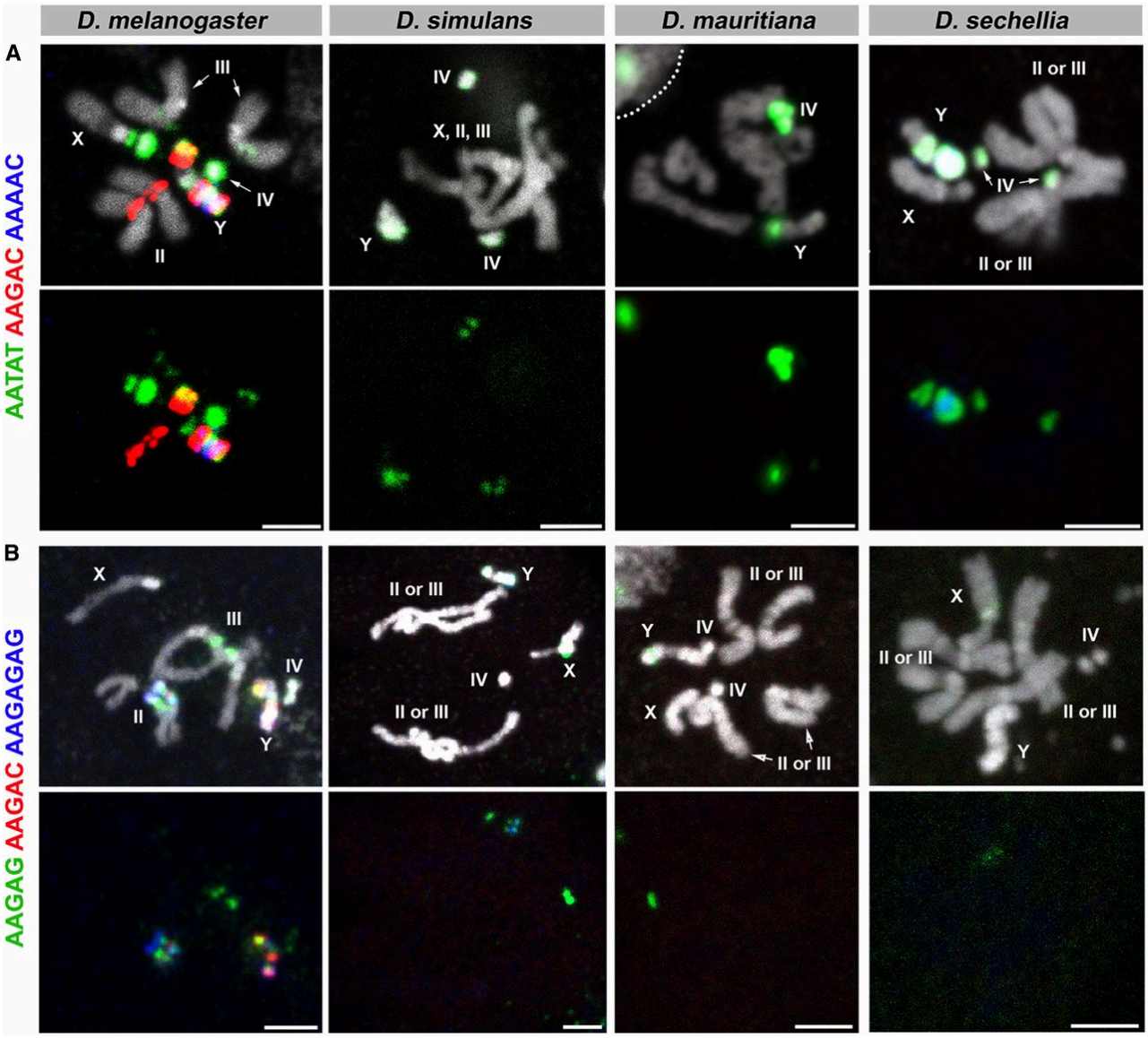


Figure III.4: DNA FISH (C7/C8) on Neuroblast Chromosome Spreads from *D. melanogaster*, *D. simulans*, *D. mauritiana*, and *D. sechellia*.
A) C7 probes and B) C8 probes. Probe sequences are indicated by the colored text. The top panels show three probes combined with DAPI, and the bottom panels show only probe hybridization signals. Bar, 2.5 µm. C = combination.

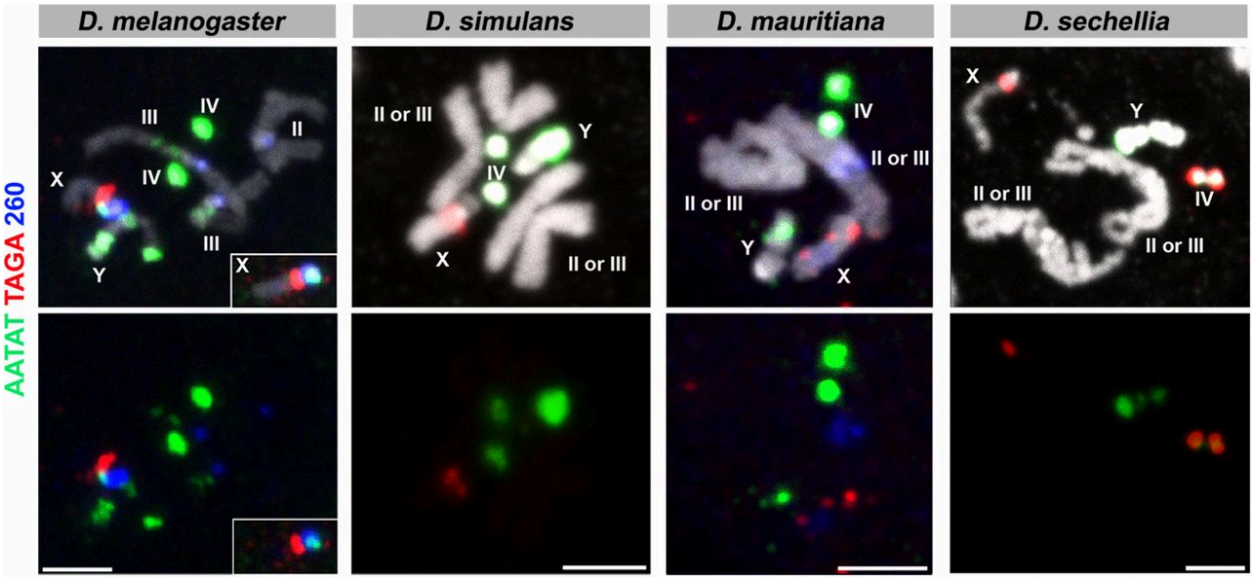


Figure III.5: DNA FISH (C11) on Neuroblast Chromosome Spreads from *D. melanogaster*, *D. simulans*, *D. mauritiana*, and *D. sechellia*.
C11 probes were used. Probe sequences are indicated by the colored text. The top panels show three probes combined with DAPI, and the bottom panels show only probe hybridization signals. Bar, 2.5 μm. C = combination.

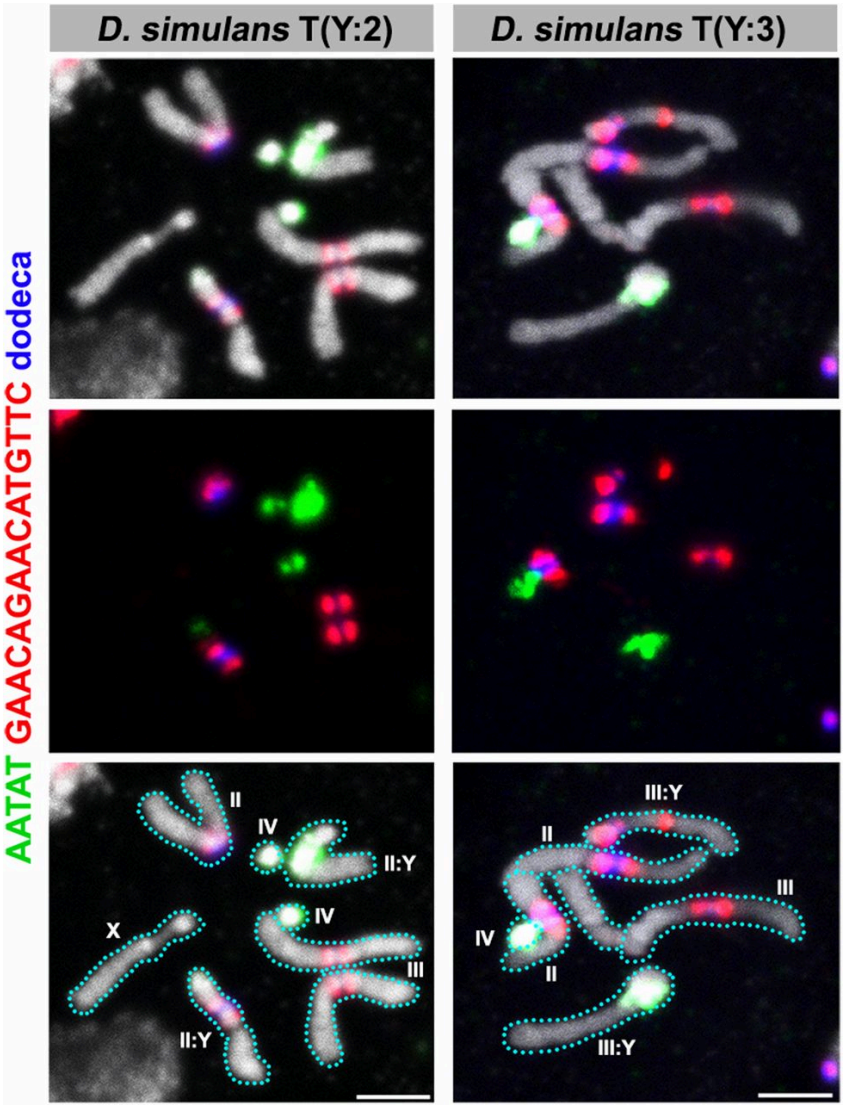


Figure III.6: DNA FISH on Neuroblast Chromosome Spreads from *D. simulans* T(Y:2) and T(Y:3) Translocation Strains.
C10 probes were used. Probe sequences are indicated by the colored text. The top panels show three probes combined with DAPI, the middle panels show only probe hybridization signals and the bottom panels indicate the interpretation. Bar, 2.5 µm. C = combination.

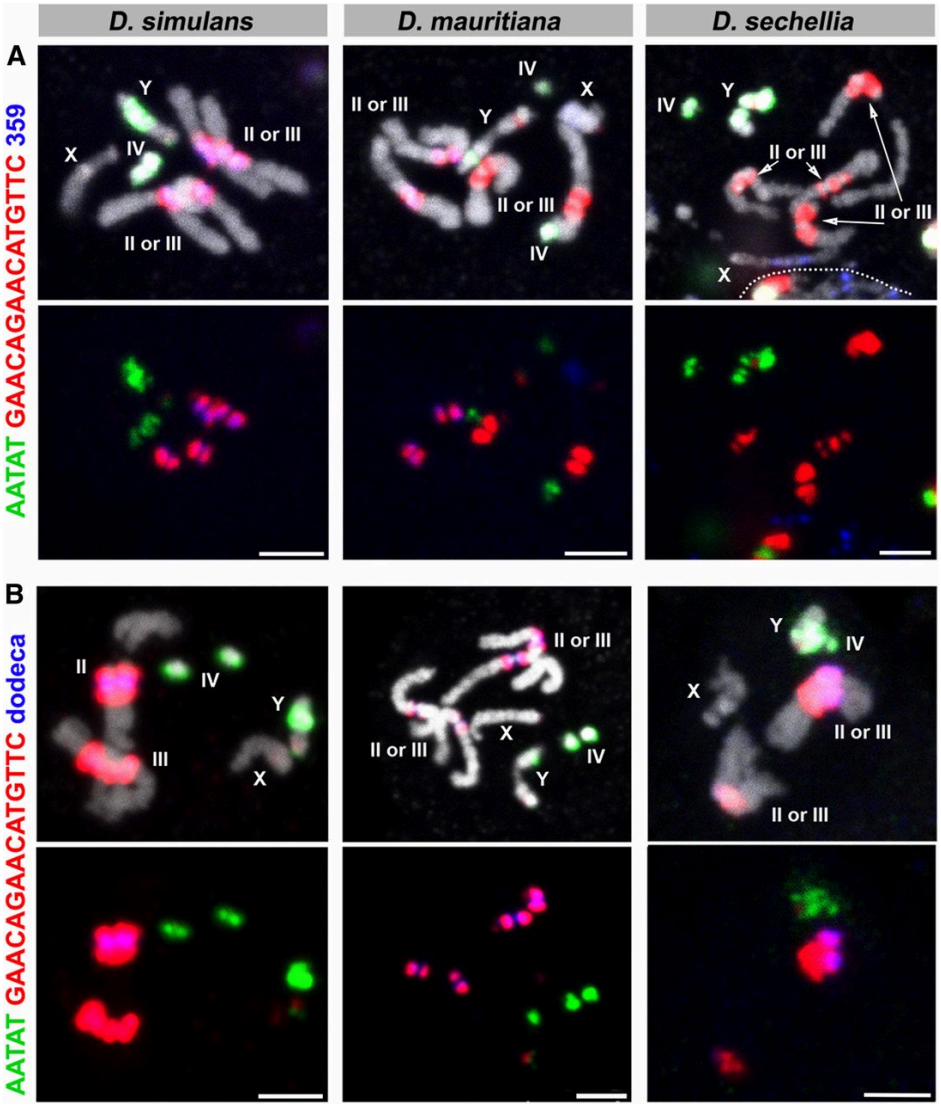


Figure III.7: DNA FISH (C9/C10) on Neuroblast Chromosome Spreads from *D. simulans*, *D. mauritiana*, and *D. sechellia*.
A) C9 probes and B) C10 probes. Probe sequences are indicated by the colored text. The top panels show three probes combined with DAPI, and the bottom panels show only probe hybridization signals. Bar, 2.5 µm. C = combination.

Discussion

D. melanogaster is one of the few species where satellite DNA has most comprehensively been mapped (Lohe et al. 1993). In previous studies, the mapping effort was carried out by the use of radioactive probes, allowing precise quantitation of satellite DNA repeats. In addition, the extensive and unique set of chromosomal aberrations in this historical model organism facilitated the mapping of these repeats onto specific chromosomal loci. However, these mapping experiments cannot be extended to the sibling species of *D. melanogaster* due to the lack of a similar set of chromosomal aberrations. Therefore, other approaches are required to resolve the relationship of multiple satellite repeats on the chromosomes of sibling species. Here, by using multi-color DNA FISH, we have mapped previously identified satellite DNAs onto the genomes of the *D. melanogaster* species complex.

We estimate that the detection limit of our method is between 110 and 140 kb. By comparing our DNA FISH results with the previous mapping effort by Lohe et al. (1993), which used 3 H-labeled radioactive probes and is more accurate in quantification, the detection limit of our method can be inferred (Table A.7) (Lohe et al. 1993). Except for (AATAG)n on the third chromosome, which was estimated to be 30 kb and detected with our method, all satellite repeats that were estimated below 110 kb by Lohe et al. (1993) were not detected with our method, whereas all satellite repeats that were estimated above 140 kb by Lohe et al. (1993) were detectable with our method (Table A.7) (Lohe et al. 1993). Therefore, it is possible that the satellite repeats that exist below 140 kb in the *D. simulans* clade species were missed. Our work provides a few critical insights into the arrangement and evolution of satellite DNA and reveals the need for future mapping efforts in these species.

Satellite DNA Divergence in the D. melanogaster Species Complex

Lohe and Brutlag (1987) suggested that after *D. melanogaster* and *D. simulans* diverged from a common ancestor, the satellite DNA sequences between both of these species remained similar in sequence, although different in abundance (Lohe and Brutlag 1987). Although quantification is not possible with our method of analysis, our study confirms that some satellite DNAs are visibly different in abundance between these species (e.g., 359-bp, AATAAAC, AATAC, AAGAG, AACAC, AAGAC, AAGAGAG, GAACAGAACATGTTC, and prod satellite). Furthermore, our mapping in this study reveals that *D. melanogaster* and *D. simulans* differ from each other in the patterning and location of these satellite DNA repeats. Among four species of the *D. melanogaster* species complex, a few satellite DNA repeats are found to be absent in one or more species, suggesting species specificity of certain satellites. Overall, it is clear that satellite DNA distribution/composition in *D. melanogaster* is most distant within the *D. melanogaster* species complex, whereas those of three species in the *D. simulans* clade (*D. simulans*, *D. mauritiana*, and *D. sechellia*) are much closer to each other, just like their evolutionary relationship. However, it is apparent that there are drastic differences in satellite DNA contents even among species of the *D. simulans* clade. This suggests that changes in satellite DNA sequence/patterning occurs rapidly over the short evolutionary timespan that separates sibling species, as suggested by Lohe and Brutlag (1987) and Hey and Kliman (1993) (Lohe and Brutlag 1987, Hey and Kliman 1993).

In addition to distinct composition of satellite DNA repeats in these species, multi-color DNA FISH revealed that satellites that are present in distinct locations in one species are overlapping in another species. For instance, the dodeca satellite and AACAC are present at distinct loci on separate autosomes in *D. melanogaster*, but completely overlap in *D. mauritiana* (Figure III.3, C5). In another example, GAACAGAACATGTTC and AACAAAC overlap in *D. mauritiana* but are separate in *D. simulans*, where GAACAGAACATGTTC remains on the autosomes and AACAAAC is only present on the Y chromosome (Figure III.3, C6). Overlapping signals likely reflect that those satellite sequences are interspersed among each other without forming large blocks of repeats composed of a single sequence. The fact that the repeats are

interspersed in one species, but completely separate in another species, indicates that satellite evolution cannot be explained by a single simple translocation event that cuts/copies and pastes a large segment of a chromosome to another. Instead, multiple events of one or many mechanisms must occur to account for the diverse patterning and abundance of satellite DNA repeats in closely related species. Potential underlying mechanisms include transposition and nonhomologous recombination among satellites (Ugarkovic and Plohl 2002, Plohl et al. 2012).

Possible Presence of Unidentified Satellite DNA Sequences in the D. simulans Clade Species

In this chapter, we mainly utilized satellite DNA probes that were previously identified in *D. melanogaster* and performed comparative mapping in the entire *D. melanogaster* species complex. This leaves the possibility that there are unidentified satellite repeats in *D. simulans* clade species that were left out of our mapping effort. Although we were able to cover the majority of the Y chromosomes of *D. melanogaster*, *D. simulans*, and *D. sechellia* using our satellite DNA probes, only a minor portion of the *D. mauritiana* Y chromosome was visualized by our probe set. As it was shown that the *D. mauritiana* Y chromosome contains a large amount of transposons (HeT-A and TART) (Berloco et al. 2005), it is possible that these transposons are the major constituent of Y chromosome heterochromatin in *D. mauritiana*. It will be of future interest to identify unknown satellite repeats in *D. simulans* clade species. It is possible that those unidentified satellites belong to the classes of “complex repeats,” such as 180- and 500-bp repeats, as indicated previously (Strachan et al. 1982).

In summary, this comparative mapping of satellite DNA using multicolor DNA FISH probes has confirmed the highly divergent nature of satellite DNAs among the *D. melanogaster* species complex, and identified species-specific and chromosome-specific satellites to serve as a resource for future work on chromosome biology and speciation in these model organisms

Conclusions, Speculations, and Future Directions

In the majority of this dissertation, I described aspects of nucleolar dominance in *Drosophila melanogaster* to understand how the identification/selection of entire ribosomal DNA (rDNA) loci occurs. I found that nucleolar dominance is developmentally regulated in X/Y *D. melanogaster* males whereby the Y rDNA locus dominates in expression and the X rDNA locus is silenced across development. The ability of the Y rDNA locus to dominate does not depend on the sex of the animal, as it can dominate in X/X/Y females. I further found that elements on the short arm of the Y chromosome including the Y rDNA locus aids in allowing the Y rDNA locus to dominate in expression. Furthermore, this dissertation lays the groundwork to understand if and how the developmental progression of nucleolar dominance within the species *D. melanogaster* has detrimental consequences in *Drosophila* interspecies hybrids.

rDNA is one of the most highly transcribed regions of the genome, given the necessity of ribosomal RNAs (rRNA) for ribosome function (Moss and Stefanovsky 2002). The combined repetitive nature of rDNA and the fact that it is the most highly transcribed region of the genome in all eukaryotes make rDNA one of the most unstable regions of the genome (Salim et al. 2017, Nelson et al. 2019, Salim and Gerton 2019): breaks and errors are frequent events in the transcribed loci occurring from high rates of transcription-replication conflicts (Datta and Jinks-Robertson 1995, Helmrich et al. 2013, Garcia-Muse and Aguilera 2016), transcription induced errors (Voelkel-Meiman et al. 1987, Datta and Jinks-Robertson 1995, Jinks-Robertson and Bhagwat 2014), and structural burdens like supercoiling (Liu and Wang 1987) and R-loops (Aguilera and García-Muse 2012, Hamperl et al. 2017). The high rates of replication and repair errors (Liao and Weiner 1995), gene slippage (Lower et al. 2019), gene conversion (Strachan et al. 1985, Dover 1987, Tautz et al. 1987, Liao and Weiner 1995), and unequal exchange/crossing over (Strachan et al. 1985, Dover 1987, Tautz et al. 1987, Lower et al. 2019) can frequently occur in rDNA and drive rapid change through the locus (Salim

and Gerton 2019) and within a population (Dover and Tautz 1986, Tautz et al. 1986, Dover 1989, Dover 1994, Ganley and Kobayashi 2007, Dion-Cote and Barbash 2017). Similar to the wide range of satellite DNAs and their patterning across diverse species, as this dissertation explored in Chapter III (Lohe and Brutlag 1987, Lohe et al. 1993, Lohe and Roberts 2000, Jagannathan et al. 2017), rDNA also diverges rapidly. Sequence analysis of rDNA repeats between and within strains/species reveal that the coding region of rDNA, essential for rRNA production and ribosome function, are highly similar whereas the intergenic spacer sequences (IGS) are more diverged (Tautz et al. 1987, Ganley and Kobayashi 2007). The high sequence homology within the rDNA repeats within strains and species reveal that rDNA undergoes concerted evolution (Coen et al. 1982, Tautz et al. 1987, Dover et al. 1993, Elder and Turner 1995, Averbeck and Eickbush 2005, Ganley and Kobayashi 2007, Kuhn et al. 2012). Concerted evolution occurs via the homogenization of particular sequences within regions of repetitive DNA. Changes in the DNA sequence is then thought to drive change in the corresponding DNA binding proteins and their networks through the process of molecular co-evolution, further aiding in divergence between species (Dover and Flavell 1984, Ohta and Dover 1984, Dover et al. 1993).

The IGS of rDNA, which contains functional promoters and transcriptional enhancers (Coen and Dover 1982, Labhart and Reeder 1984), has been hypothesized to undergo molecular co-evolution with RNA Polymerase I (RNA Pol I) and/or its transcription factors given the high sequence diversity of IGS (Dover and Flavell 1984, Tautz et al. 1987, Carter and Drouin 2009). This model of molecular co-evolution postulates that rapid sequence changes in IGS, that occur via mechanisms discussed previously, promote rapid changes within IGS binding proteins, particularly that of RNA Pol I complex proteins and/or its transcription factors (Carter and Drouin 2009). The molecular co-evolution model of IGS and RNA Pol I is further supported by the observations that 1) rDNA is often transcribed by species-specific RNA Pol I components (Croce et al. 1977, Grummt et al. 1982, Kohorn and Rae 1982, Dover and Flavell 1984, Heix and Grummt 1995), 2) different species RNA Pol I components and transcription factors can be incompatible with each other (Heix and Grummt 1995), 3)

species-specific rDNA is preferentially transcribed in interspecies hybrids (Cassidy and Blackler 1974, Durica and Krider 1977, Chen and Pikaard 1997), and 4) RNA Pol I components evolve faster than both RNA Pol II and RNA Pol III components, reflecting similar high rates of evolution of their transcribed genes (Carter and Drouin 2009, Viktorovskaya et al. 2013, Viktorovskaya and Schneider 2015). However, any functional studies to thoroughly test which RNA Pol I components are rapidly evolving and how this is influenced by IGS sequence changes remain an ambitious goal for future studies.

Through the studies of molecular co-evolution and concerted evolution, it has been thought that the divergence in repetitive regions across species, like that seen in IGS and in satellite DNAs, suggests selection or drive for particular sequences/repeats may occur (Dover et al. 1993, Charlesworth et al. 1994, Csink and Henikoff 1998, Henikoff et al. 2001, Satyaki et al. 2014). It is indeed intriguing to speculate whether there are any forms of selective forces driving change within the rDNA locus, although no evidence currently exists showing that this occurs. Other repetitive regions within heterochromatin, particularly the centromere and telomeres, have been shown to exhibit drive-like tendencies, where DNA binding proteins and their corresponding DNA sequences undergo an “arms-race” with other DNA binding proteins/DNA sequences to be selected and utilized by the cell (Henikoff et al. 2001, Henikoff and Malik 2002, Lee et al. 2017). Satellite DNAs and their binding proteins have also been speculated to undergo similar forms of selection given the diversity of satellite DNAs across species, the flexibility of satellite DNA binding proteins, and their importance in chromocenter formation (Dover et al. 1993, Charlesworth et al. 1994, Elder and Turner 1995, Csink and Henikoff 1998, Abad et al. 2000, Lohe and Roberts 2000, Bayes and Malik 2009, Jagannathan et al. 2017, Jagannathan and Yamashita 2017, Jagannathan et al. 2018, Jagannathan et al. 2019). Finding more satellite-specific binding proteins, particularly those with specificity for 359-bp (e.g. Bub1 (Abad et al. 2000) or other unknown proteins (Hsieh and Brutlag 1979)), and assessing their function may aid in future studies to understand both the divergence of repetitive DNAs and their binding proteins and whether an “arms race” occurs between satellite DNA binding proteins within the chromocenter.

No studies have currently identified the presence of an arms race occurring between IGS/rDNA repeats and their associated proteins, however multiple characteristics of rDNA suggest that some mechanism of drive or adaptation may be at play. The dynamic nature of the rDNA locus as described above along with its ability to rapidly magnify or contract at specific loci (Hawley and Tartof 1985) allow for rapid changes in sequence elements within the rDNA. In addition to the rapid evolution of rDNA, the utilization of particular loci through nucleolar dominance suggests preferential functional selection which has been shown to at least be partially influenced by sequence elements within the rDNA locus (Lu et al. 2018, Warsinger-Pepe et al. 2020). This preferential selection for expression has functional consequences that can result specifically in rDNA copy number loss of the transcribed locus in male germline stem cells (Lu et al. 2018) suggesting a potential detriment to the locus selected for expression. Similar to the centromere paradox, too severe of changes or copy loss could result in the inability of that rDNA locus to be transmitted to future progeny, given that rDNA is important for sex chromosome pairing in *Drosophila* male meiosis (Merrill et al. 1992, Ren et al. 1997, McKee et al. 1998) as well as its necessity in cellular metabolism (Moss and Stefanovsky 2002). Together these studies suggest that molecular co-evolution that occurs with rDNA and its binding proteins may be influenced by a similar “arms race” and/or drive-like mechanism. Future studies are needed to elucidate if or how this occurs.

Furthermore, it is intriguing to speculate if the unstable nature of the highly transcribed rDNA locus may have detrimental relationships with neighboring satellite DNAs. Proximity of elements has been shown to impact the function/stability of the affected genes/regions, particularly through position-effect variegation (Henikoff 1981, Elgin and Reuter 2013), chromosome pairing and transvection (Henikoff 1994, Henikoff et al. 1995, Fukaya and Levine 2017, King et al. 2019), topologically associated domains (TADs) (Bompadre and Andrey 2019), and still unknown mechanisms (Ferree et al. 2014). In this dissertation, I reveal that the previously reported rescue of embryonic lethality by *Zhr*^{1} seen in *D. simulans – D. melanogaster* hybrids corresponds

to high rates of silenced *D. melanogaster* rDNA expression. We believe that the expression of *D. melanogaster* rDNA in these hybrid females may be detrimental to their survival. Preliminary data in this dissertation further implies that rDNA and the adjacent 359-bp satellite DNA block may have detrimental relationships that arise in hybrid *Drosophila*. Future studies are needed to understand if *D. melanogaster* rDNA expression in these *Drosophila* hybrids creates instabilities/conflicts with the adjacent 359-bp satellite DNA block. Preliminary data reveals that separating these two loci may relieve the conflicts that underlie the hybrid incompatibility that can cause embryonic lethality. Future high-resolution studies are needed to elucidate how rDNA expression in *Drosophila* hybrids is dysregulated, if this dysregulation results in physical conflict with the 359-bp heterochromatic block, and what factor(s) in the *D. simulans* oocyte cytoplasm contribute to this conflict or what is lacking in the *D. simulans* oocyte needed to resolve this conflict. Whether or not the relationship between rDNA and 359-bp involves physical conflicts between these loci, and whether these conflicts are driven by differences in DNA sequence and/or binding proteins remain unknown. Future studies are needed to further explore how rDNA and satellite DNAs and their binding proteins change over time and how these changes may influence incompatibilities with each other and between species.

Appendix A

Oligo name	Sequence (5' -> 3')	Use
SNP1 for X (18S)	AAAAAATACAAGTATTTAATCACATA-/Quasar 570/	SNP *in situ*
SNP2 for X (ITS1)	GTTTCTTCGATTTTCATGTTCGAAAC-/Quasar 570/	SNP *in situ*
SNP3 for X (ITS1)	AAATATTTATTAACGGTAAGGATATT-/Quasar 570/	SNP *in situ*
SNP4 for X (28S)	TTAGGCATTTTTGTTTTACTTGAAAA-/Quasar 570/	SNP *in situ*
SNP1 for Y (18S)	AAAAGATACAAGTATTTAATCACATA-/Quasar 670/	SNP *in situ*
SNP2 for Y (ITS1)	GTTTTTTCGATTTTCATGTTCGAAAC-/Quasar 670/	SNP *in situ*
SNP3 for Y (ITS1)	AAATGTTTATTAACGGTAAGGATATT-/Quasar 670/	SNP *in situ*
SNP4 for Y (28S)	TTAGCCATTTTTGTTTTACTTGAAAA-/Quasar 670/	SNP *in situ*
SNP1 mask	TATGTGATTAAATACT	SNP *in situ*
SNP2 mask	GTTTCGAACATGAAAA	SNP *in situ*
SNP3 mask	AATATCCTTACCGTTA	SNP *in situ*
SNP4 mask	TTTTCAAGTAAAACAA	SNP *in situ*
$ITS^{\Delta 24}$	/Cy3/-AGGTTGCCAAGCCCCATCTTCGTTTT	RNA *in situ*
ITS^{+}	/Cy5/-GCCCCACACTAACTATATAAATAAAACCC	RNA *in situ*
18S Forward Primer	GAAACGGCTACCACATCTAAGG	PCR
18S Reverse Primer	GGACCTCTCGGTCTAGGAAATA	PCR
ITS Forward Primer	CTTGCGTGTTACGGTTGTTTC	PCR
ITS Reverse Primer	ACAGCATGGACTGCGATATG	PCR
28S Forward Primer	AGCCCGATGAACCTGAATATC	PCR
28S Reverse Primer	CATGCTCTTCTAGCCCATCTAC	PCR
rDNA (IGS)	/Alexa488/-AGTGAAAAATGTTGAAATATTCCCATATTCTCTAAG TATTATAGAGAAAAGCCATTTTAGTGAATGGA	DNA FISH
359-bp	CCACATTTTGCAAATTTTGATGACCCCCCTCCTTACAAAAAATGCG	DNA FISH
TAGA	(TAGA)8	DNA FISH
AATAAAC	(AATAAAC)6	DNA FISH
AATAC	(AATAC)6	DNA FISH
AATAGAC	(AATAGAC)6	DNA FISH
IGS, antisense	/Alexa488/-TCCATTCACTAAAATGGCTTTTCTCTATAATACTTA GAGAATATGGGAATATTTCAACATTTTTCACT	RNA *in situ*
D. melanogaster ITS	/Cy5/-TCTACCAATTATATTAACACTTAT	RNA *in situ*
D. simulans ITS	/Cy3/-TTATGGTCCATATACACACAAACTTA	RNA *in situ*

Table A.1: Oligonucleotide Probes Used to Study Nucleolar Dominance within *D. melanogaster* and *Drosophila* Interspecies Hybrids.
Probes listed for DNA FISH have 5-prime fluorescent dyes, but probes were used with one or two different dyes, so dyes are not listed here.

Cross (female x male)	# vials with no progeny	#vials with progeny	% mating success*
tsimbazaza x Oregon R	2	15	88.24
tsimbazaza x Zhr^{1}/Y	4	12	75.00
tsimbazaza x OrR;; rDNA/III	2	3	60.00
tsimbazaza x OrR;; rDNA/TM6B	0	5	100.00
tsimbazaza x bb^{158}/Yy^{+}	10	12	54.55
tsimbazaza x FM6/Yy^{+}	17	4	19.05
tsimbazaza x yw/Yy^{+}	1	7	87.50
tsimbazaza x bb^{158}/Y^{OrR}	1	4	80.00
tsimbazaza x FM6/Y^{OrR}	4	1	20.00
tsimbazaza x bb^{1}/Y	6	1	14.29
tsimbazaza x bb^{l}/BsY	6	1	14.29
tsimbazaza x bb^{158}/Y;; rDNA/III	14	0	0.00
tsimbazaza x bb^{158}/Y;;rDNA/TM6B	3	1	25.00
Zhr^{1}/Zhr^{1} x tsimbazaza	1	11	91.67
Oregon R x tsimbazaza	0	10	100.00

Table A.2: Mating Success of Interspecies Hybrid Crosses.
*percentage of vials with progeny.

Cross (female x male)	Average ± S.D.% bobbed, females	Average ± S.D.% bobbed, males
tsimbazaza x Oregon R	71.0 ± 15.1	5.8 ± 2.3
tsimbazaza x *Zhr*^{1}/Y	1.3 ± 1.6	1.5 ± 1.6
tsimbazaza x OrR;; rDNA/III	0.0 ± 0.0	27.8 ± 25.5
tsimbazaza x OrR;; rDNA/TM6B	90.0 ± 6.2	24.4 ± 7.9
tsimbazaza x *bb*^{158}/*Yy*$^{+}$	10.0 ± 7.7	17.5 ± 20.0
tsimbazaza x FM6/*Yy*$^{+}$	4.6 ± 8.8	16.6 ± 7.6
tsimbazaza x *yw*/*Yy*$^{+}$	64.1 ± 10.9	54.5 ± 11.4
tsimbazaza x *bb*^{158}/Y^{OrR-1}	50.0 ± 35.3	13.6 ± 9.6
tsimbazaza x FM6/Y^{OrR-1}	19.7 ± 0.0	7.9 ± 0.0
tsimbazaza x *bb*^{1}/Y	25.0 ± 0.0	0.0 ± 0.0
tsimbazaza x *bb*^{l}/BsY	67.6 ± 0.0	23.6 ± 0.0
tsimbazaza x *bb*^{158}/Y;;rDNA/TM6B	41.4 ± 0.0	57.1 ± 0.0
Zhr^{1}/*Zhr*^{1} x tsimbazaza	6.4 ± 4.8	0
Oregon R x tsimbazaza	8.6 ± 4.8	0

Table A.3: Rates of Bobbed Phenotype in Interspecies Hybrid Progeny.
S.D. = standard deviation
tsimbazaza = *D. simulans* strain
OrR = Oregon R, *D. melanogaster* strain

Combination	488 (Green)	Red (Cy3)	Blue (Cy5)
C1	(AATAT)6	(AATAG)6	359: AGGATTTAGGGAAATTAATTTTTGGA TCAATTTTCGCATTTTTTGTAAG
C2	(AATAGAC)6	(AATAC)6	(AATAAAC)6
C3	IGS: AGTGAAAAATGTTGAAATATTCCCATATTCTCTAAGTATT ATAGAGAAAAGCCATTTTAGTGAATGGA	(AATAG)6	PROD: (AATAACATAG)3
C4	IGS: AGTGAAAAATGTTGAAATATTCCCATATTCTCTAAGTATT ATAGAGAAAAGCCATTTTAGTGAATGGA	372: TATTTTGATCAAAACATTGAAAATAATGGCCCAAATA TGGAATGTCATACCTCGTTGAGTTTGTTTTTTA	359: AGGATTTAGGGAAATTAATTTTTGGA TCAATTTTCGCATTTTTTGTAAG
C5	(AAGAG)6	DODECA: ACCGAGTACGGGACCGAGTACGGGACCAGTACGG GACCAGTACGGG	(AACAC)6
C6	(AATAT)6	(GAACAGAACATGTTC)2	(AACAAAC)5
C7	(AATAT)6	(AAGAC)6	(AAAAC)6
C8	(AAGAG)6	(AAGAC)6	(AAGAGAG)5
C9	(AATAT)6	(GAACAGAACATGTTC)2	359: AGGATTTAGGGAAATTAATTTTTGGA TCAATTTTCGCATTTTTTGTAAG
C10	(AATAT)6	(GAACAGAACATGTTC)2	DODECA: ACCGAGTACGGGACCGAGTACGGG ACCAGTACGGGACCAGTACGGG
C11	(AATAT)6	(TAGA)8	260: CATATTTGCAAATTTTGATGAATGCG AAAATTAACC

Table A.4: Combination of Probes Used for DNA FISH Mapping.

	D. melanogaster	*D. simulans*	*D. mauritiana*	*D. sechellia*
(AATAT)n	Y, X, III, IV	Y, IV	Y, IV	Y, IV
(AATAG)n	Y, II, III[a]	Y, X, IV	IV	Y, X
359/372	X[b], III[b]	II[b], III[b]	X[b], II or III[b]	X[b]
260	X[b], III[b], II	None	X[b], II or III[b]	None
(AATAGAC)n	Y	None	None	None
(AATAC)n	Y	None	None	Y, IV
(AATAAAC)n	Y	Y	None	None
IGS	X, Y	X, Y[c]	X, Y	X (not on Y[c,d])
(AAGAG)n	X, Y, II, III, IV	X, Y	Y	X
Prod	II, III	None	None	None
dodeca	Y[e], III	II, III	II, III	II, III
(AACAC)n	Y, II, III[a]	None	II[a], III[a]	None
(GAACAGAACATGTTC)n	None	II, III	II, III, X[a], Y[a]	II, III
(AACAAAC)n	II[a]	Y	II[a], III[a]	None
(AAGAC)n	Y, II	None	None	None
(AAAAC)n	Y	None	None	Y
(AAGAGAG)n	Y, II	Y	None	None
(TAGA)n	X	X	X	X, IV

Table A.5: Location of the Satellite DNAs on the Chromosomes of the *D. melanogaster* Species Complex.

a) Sequences that are obviously low in abundance.

b) Likely cross-hybridization among 359-bp, 372-bp, 260-bp, and other related repeats.

c) Without rDNA.

d) Roy et al (2005) reports the presence of rDNA (using Py12 and 240-bp probes) on *D. sechellia* Y, although much weaker than X rDNA. This may be because our IGS-240 probe does not cover the entire length of the 240-bp sequence, and our probe sequence does not hybridize well to *D. sechellia* IGS (Roy et al. 2005).

e) Yadlapalli and Yamashita (2013) described its presence on Y but this chromosome spread did not find dodeca on Y, possibly due to difference in hybridization conditions or strains (Yadlapalli and Yamashita 2013).

	D. melanogaster	*D. simulans*	*D. mauritiana*	*D. sechellia*
X	AATAT 359/372/260 IGS AAGAG TAGA	 IGS AAGAG AATAG TAGA	 359/372/260 IGS GAACAGAACATGTTC TAGA	 359/372/260 IGS AAGAG AATAG TAGA
Y	AATAT AAGAC AATAC AATAG AATAGAC AATAAAC IGS AAGAG AACAC AAAAC AAGAGAG	AATAT AATAG AATAAAC IGS AAGAG AAGAGAG AACAAAC	AATAT IGS AAGAG GAACAGAACATGTTC[a]	AATAT AATAC AATAG AAAAC
II	AATAG AAGAG Prod AACAC AAGAC AACAAAC AAGAGAG 260	 359/372 dodeca GAACAGAACATGTTC	 AACAC AACAAAC 359/372/260 (only II or III) dodeca GAACAGAACATGTTC	 dodeca GAACAGAACATGTTC
III	AATAT AATAG AAGG 359/372/260 Prod dodeca	 359/372 dodeca GAACAGAACATGTTC	 359/372/260 (only II or III) dodeca AACAAAC GAACAGAACATGTTC	 dodeca GAACAGAACATGTTC
IV	AATAT AAGAG	AATAT AATAG	AATAT AATAG	AATAT AATAC TAGA

Table A.6: Satellite Composition of Each Chromosome in the *D. melanogaster* Species Complex
a) Sequence that is obviously low in abundance.

Satellite	X	Y	II	III	IV
(AATAT)n	**600**	**5800**	10	**630**	**2700**
(AATAG)n	8.1	**310**	**200**	**30**[a]	78
(AATAC)n	0	**3500**	0	0	0
(AAAAC)n	0	**400**	0	0	0
(AAGAC)n	81	**8500**	**1800**	110	0
(AAGAG)n	**1200**	**7200**	**5500**	**1100**	**170**
(AATAAAC)n	0	**1600**	0	0	0
(AATAGAC)n	0	**1600**	0	0	0
(AAGAGAG)n	**270**	**1800**	**1700**	**140**	100
(AATAACATAG)n	0	0	**1900**	**1600**	0
359-bp	**11000**	0	0	0	0

Table A.7: Estimating the Detection Limit of DNA FISH Method Used in this Study
The numbers indicate the amounts (kilobases) of satellite repeats in the *D. melanogaster* genome estimated by Lohe *et al.* (1993) (Lohe et al. 1993). Bold numbers indicate the satellite repeats that were successfully detected with our DNA FISH method.
a) Except for 30 kb of $(AATAG)_n$, which was detected on the third chromosome, the detection limit with our method is likely between 110 and 140 kb [110 kb of $(AAGAC)_n$ being undetectable and 140 kb of $(AAGAGAG)_n$ being detectable].

Appendix B

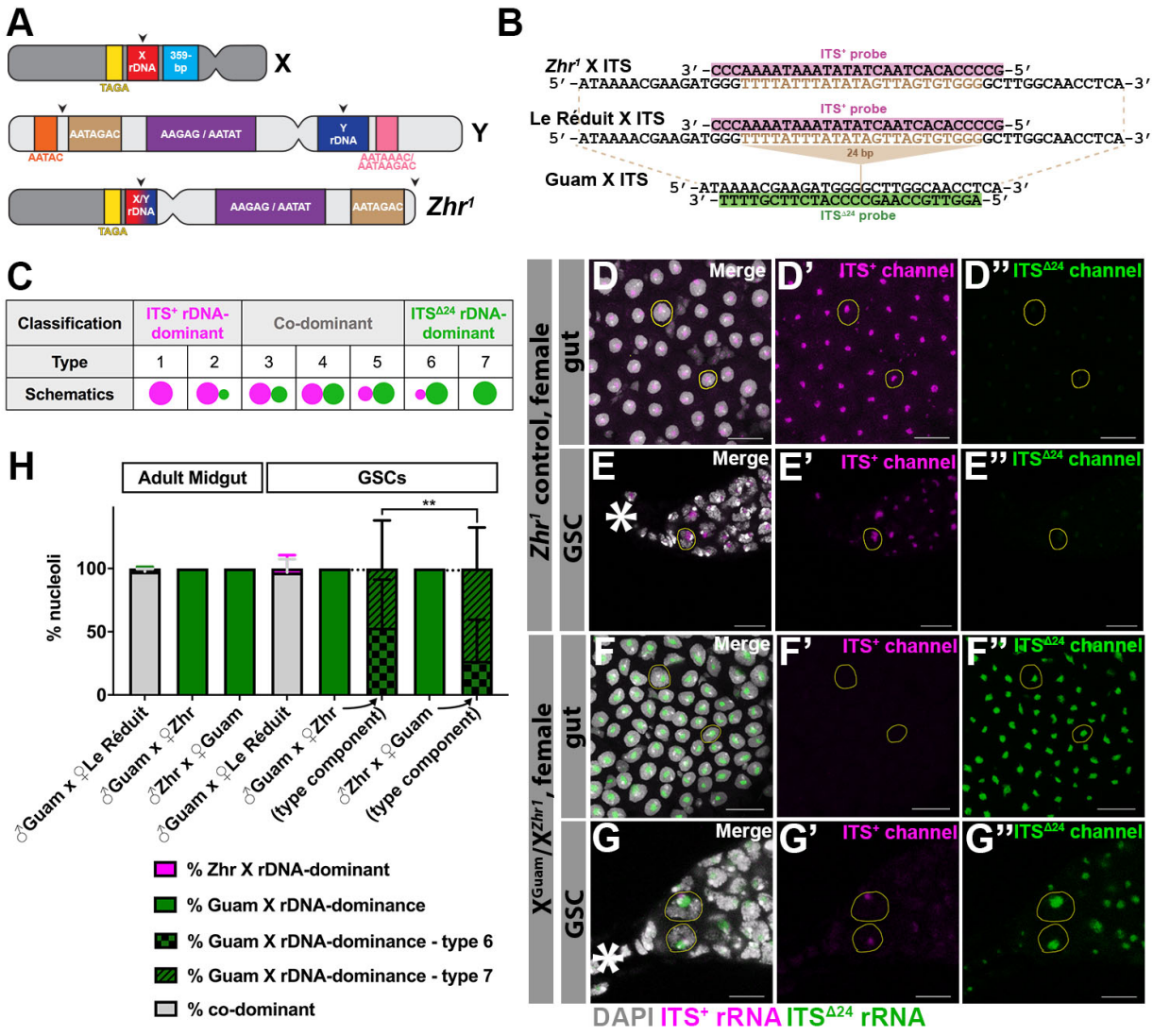


Figure B.1: Guam X rDNA Can Dominate Over *Zhr¹* X rDNA in X/X Females.

Figure B.1: Guam X rDNA Can Dominate Over *Zhr[1]* X rDNA in X/X Females.
A) Structure of a wild-type X chromosome, a wild-type Y chromosome, and the *Zhr[1]* chromosome based on cytological characterization data (not shown) and (Ferree and Barbash 2009). Constriction represents centromere location. Black arrows mark location of chromosome break/recombination. We believe that a recombination/break point occurred in the rDNA of *Zhr[1]* since sequencing of the rDNA revealed the presence of SNPs that align with both our X and Y rDNA (from *yw*) (SNP data not shown). B) *Zhr[1]* X ITS sequence can be specifically visualized by the ITS^{+} probe in comparison to Le Réduit X and Guam X ITS sequences. C) Nucleolar dominance scoring classification and type schematics for female GSCs. Representative images of D) *Zhr[1]* control female adult anterior midgut, D') ITS^{+} channel only, D'') $ITS^{\Delta 24}$ channel only, scale bar = 25μm, E) *Zhr[1]* control female GSCs, E') ITS^{+} channel only, E'') $ITS^{\Delta 24}$ channel only, scale bar = 10μm, F) F1 female from P0 ♂ *Zhr[1]* x ♀Guam adult anterior midgut, F') ITS^{+} channel only, F'') $ITS^{\Delta 24}$ channel only, scale bar = 25μm, and G) F1 female from P0 ♂ *Zhr[1]* x ♀Guam GSCs, G') ITS^{+} channel only, G'') $ITS^{\Delta 24}$ channel only, scale bar = 8μm. Asterisks label germarium terminal filament. Magenta = ITS^{+} rRNA, green = $ITS^{\Delta 24}$ rRNA, white = DAPI. H) Quantification of nucleolar dominance between Guam X rDNA and *Zhr[1]* rDNA in adult female tissues comparing both cross directions (♂Guam x ♀Le Réduit data from Figure I.6 is reproduced for comparison): F1 adult anterior midgut enterocytes from P0 ♂Guam x ♀Le Réduit (n = 904 cells from 9 guts), F1 adult anterior midgut enterocytes from P0 ♂Guam x ♀*Zhr[1]* (n = 1016 cells from 9 guts), F1 adult anterior midgut enterocytes from P0 ♂*Zhr[1]* x ♀Guam (n = 765 cells from 6 guts); F1 adult GSCs from P0 ♂Guam x ♀Le Réduit (n = 150 cells from 57 germarium), F1 adult GSCs from P0 ♂Guam x ♀*Zhr[1]* (n = 86 cells from 40 germarium), F1 adult GSCs from P0 ♂Guam x ♀*Zhr[1]* $ITS^{\Delta 24}$ rDNA (Guam X) dominant type component (n = 86 cells from 40 germarium), F1 adult GSCs from P0 ♂*Zhr[1]* x ♀Guam (n = 90 cells from 40 germarium), F1 adult GSCs from P0 ♂*Zhr[1]* x ♀Guam ITS^{+} rDNA (Guam X) dominant type component (n = 90 cells from 40 germarium). "Type component" bars represent the detailed type scored dominance within the $ITS^{\Delta 24}$ rDNA dominant classification described of its respective bar (see arrow for relationship) in C. p-values calculated only to compare type components using Welch's unpaired, unequal variance *t*-test using n = number of tissues. ** = < 0.01. Magenta = % ITS^{+} rDNA-dominant, green without pattern = % $ITS^{\Delta 24}$ rDNA-dominant, green with checker pattern = % $ITS^{\Delta 24}$ rDNA-dominant – type 6, green with slash pattern = % $ITS^{\Delta 24}$ rDNA-dominant – type 7, grey = % codominant nuclei.

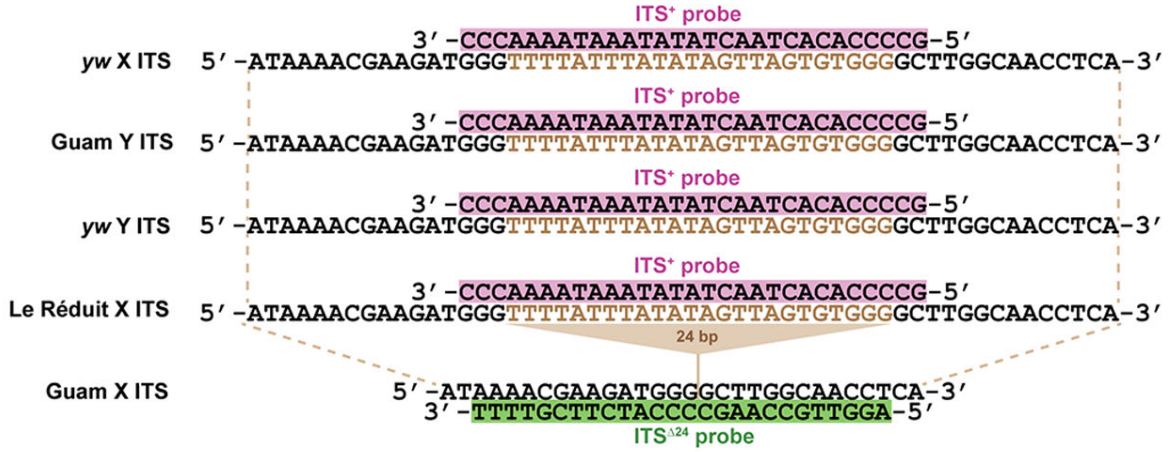


Figure B.2: Sequence Alignments of Various rDNA ITS.
yw (X), Guam Y, and *yw* stock Y ITS sequences that can be specifically visualized by the ITS^{+} probe in comparison to Le Réduit X (ITS^{+}) and Guam X ITS (ITS$^{\Delta 24}$) sequences.

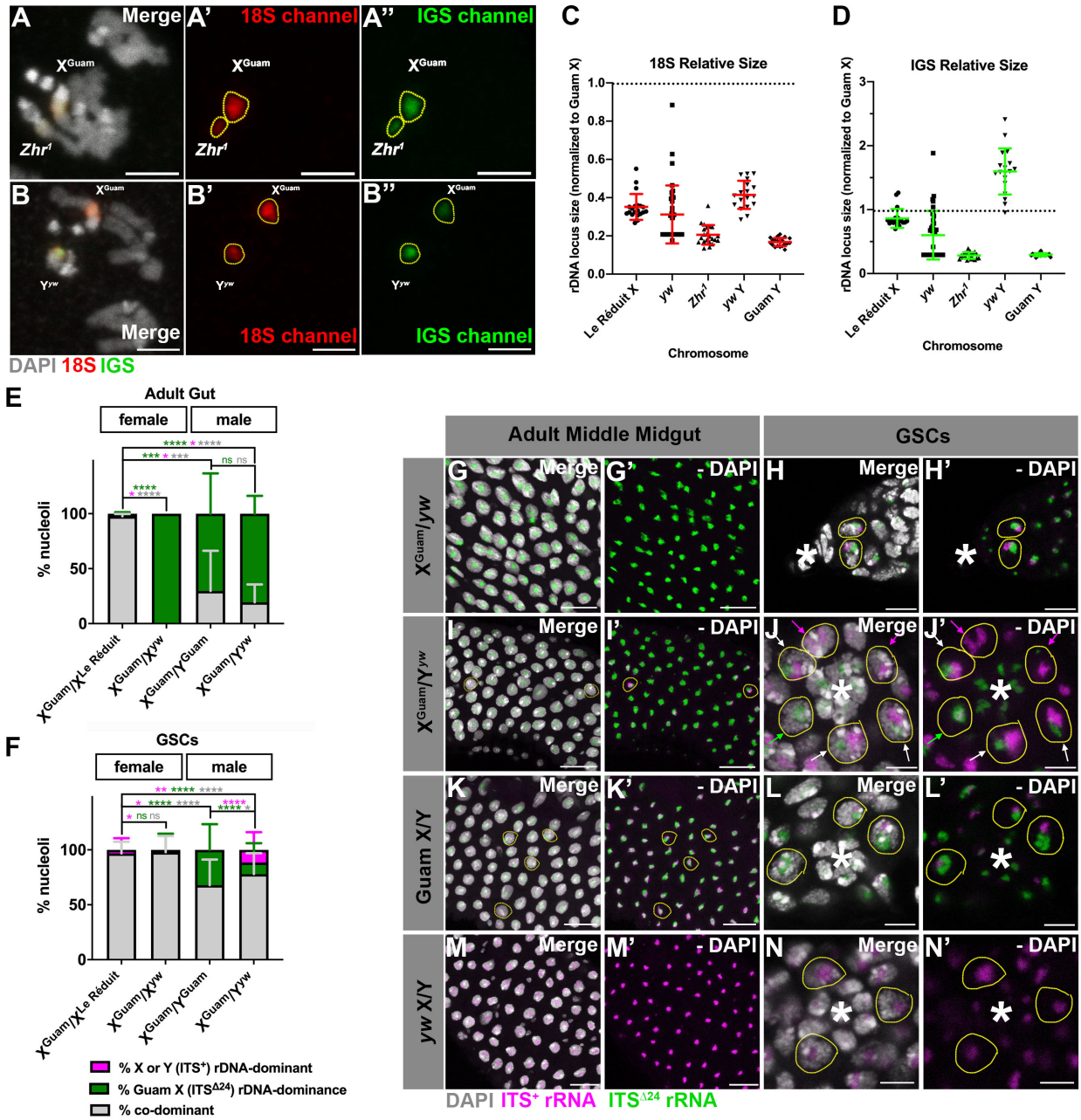


Figure B.3: Guam X rDNA Disrupts Female and Male Nucleolar Dominance.

Figure B.3: Guam X rDNA Disrupts Female and Male Nucleolar Dominance.
Representative images of mitotic chromosome spreads from third instar larval brains for relative rDNA size quantification from A) Zhr^{1}/X^{Guam} female larval neuroblast, A') 18S channel alone, A'') IGS channel alone, and B) X^{Guam}/Y^{yw} male larval neuroblast, B') 18S channel alone, B'') IGS channel alone. Yellow circles to accent each rDNA locus signal. All scale bars = 3μm. Grey = DAPI, red = 18S rDNA, green = IGS rDNA. C) Quantification of 18S relative size by raw integrated fluorescence intensity, all normalized to internal Guam X 18S control raw integrated fluorescence intensity. D) Quantification of IGS relative size by raw integrated fluorescence intensity, all normalized to internal Guam X IGS control raw integrated fluorescence intensity. E) Quantification of nucleolar dominance in adult gut enterocytes with varying chromosome complements ($X^{Guam}/X^{Le\ Réduit}$ data reproduced from Figure I.6 for comparison): X^{Guam}/X^{yw} (n = 756 cells from 6 guts), X^{Guam}/Y^{Guam} (n = 1037 from 10 guts), X^{Guam}/Y^{yw} (n = 1129 from 7 guts). F) Quantification of nucleolar dominance in adult GSCs with varying chromosome complements ($X^{Guam}/X^{Le\ Réduit}$ data reproduced from Figure I.6 for comparison): X^{Guam}/X^{yw} (n = 100 GSCs from 46 germarium), X^{Guam}/Y^{Guam} (n = 442 GSCs from 48 testes), X^{Guam}/Y^{yw} (n = 465 GSCs from 55 testes). Magenta = % ITS^{+} rDNA-dominant, green = % $ITS^{\Delta 24}$ rDNA-dominant, grey = % codominant nuclei. Representative images of G) X^{Guam}/X^{yw} adult middle midgut, G') no DAPI, scale = 25μm, H) X^{Guam}/X^{yw} GSCs, H') no DAPI, scale = 8μm, I) X^{Guam}/Y^{yw} adult middle midgut, I') no DAPI, scale = 25μm, J) X^{Guam}/Y^{yw} GSCs, J') no DAPI, scale = 5μm, K) X^{Guam}/Y^{Guam} adult middle midgut, K') no DAPI, scale = 25μm, L) X^{Guam}/Y^{Guam} GSCs, L') no DAPI, scale = 5μm, M) X^{yw}/Y^{yw} adult middle midgut, M') no DAPI, scale = 25μm, N) X^{yw}/Y^{yw} GSCs, N') no DAPI, scale = 5μm. Asterisks label germarium terminal filament (females) or hub (males). Yellow circles in adult middle midgut images denote examples of co-dominant cells. Yellow circles in apical germarium/testes tip denote GSCs. Magenta arrow = ITS^{+} rDNA-dominant, green arrow = $ITS^{\Delta 24}$ rDNA-dominant, white arrow = co-dominant. Magenta = ITS^{+} rRNA, green = $ITS^{\Delta 24}$ rRNA, white = DAPI.

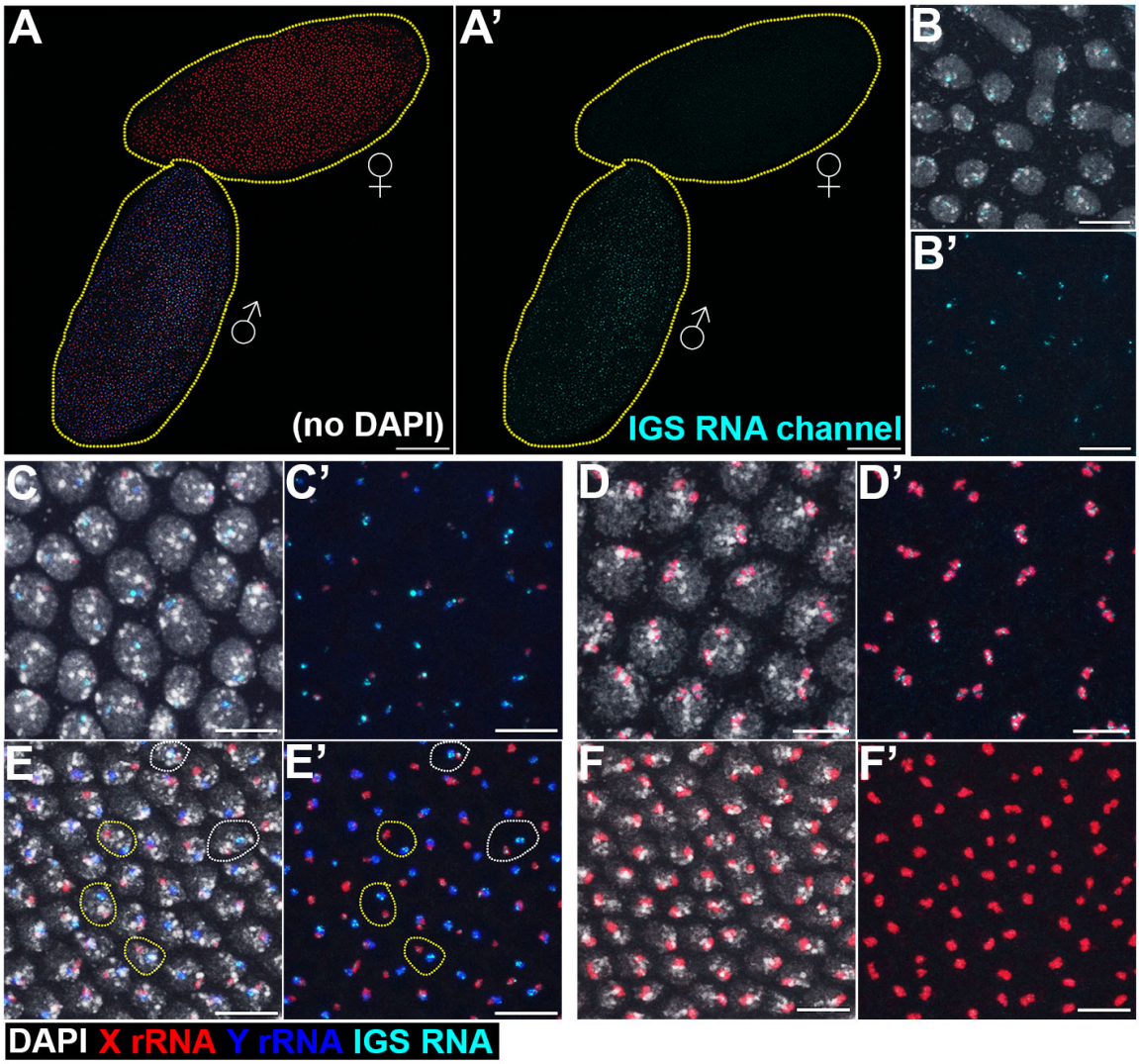


Figure B.4: IGS Transcription Occurs Early in Embryogenesis and is Higher in Male Embryos. A) Representative image of a male and female embryo, no DAPI, scale = 100μm. A') showing drastic differences in IGS signal, scale = 100μm. B) Syncytial division ~11 embryo revealing early transcription of IGS RNA and no rRNA, scale = 10μm, B') no DAPI, scale = 10μm. C) Syncytial division ~12 male embryo, scale = 8μm, C') no DAPI, scale = 8μm. D) Syncytial division ~12 female embryo, scale = 8μm, D') no DAPI, scale = 8μm. E) Syncytial division 13-14 male embryo, scale = 10μm, E') no DAPI, scale = 10μm, yellow circles denote nuclei with higher IGS associated with Y rRNA and white circles denote nuclei with high IGS associated with both X and Y rRNA. F) Syncytial division 13-14 female embryo, scale = 10μm, F') no DAPI, scale = 10μm. Red = X rRNA, blue = Y rRNA, grey = DAPI, turquoise = IGS RNA.

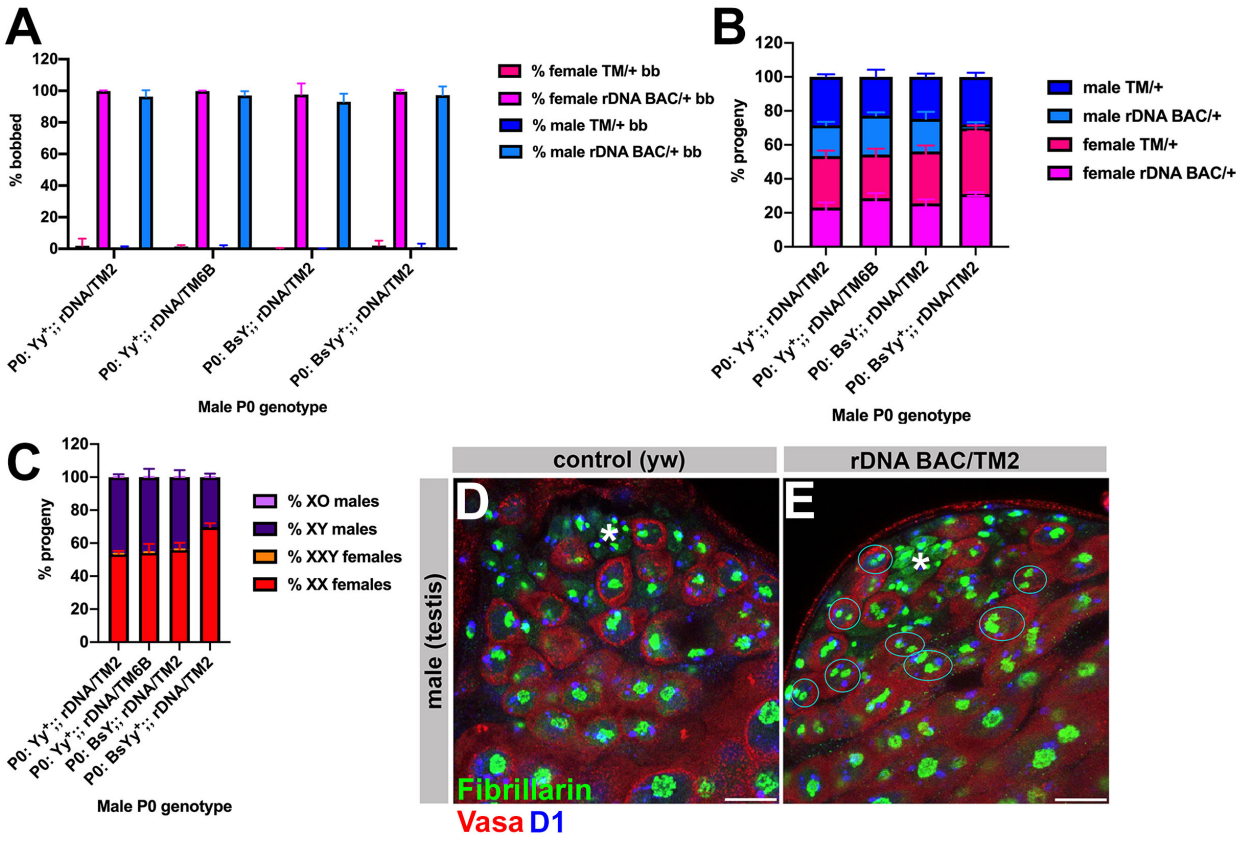


Figure B.5: rDNA on the Third Chromosome Disrupts Nucleolar Structure and Results in the Bobbed Phenotype.

A) Percentage of progeny exhibiting the bobbed phenotype (cuticular etching and/or peeling) from crosses with *yw* females and males of the listed genotype. B) Percentage of progeny from the crosses in A depicted by sex and genotype. C) Percentage of progeny from the crosses in A based on chromosome content. D) Representative images of immunofluorescence on whole mount young testes of control (*yw*), scale = 10μm, and E) young testes of rDNA BAC/TM2 males, scale = 10μm. Red = vasa, green = fibrillarin, blue = D1.

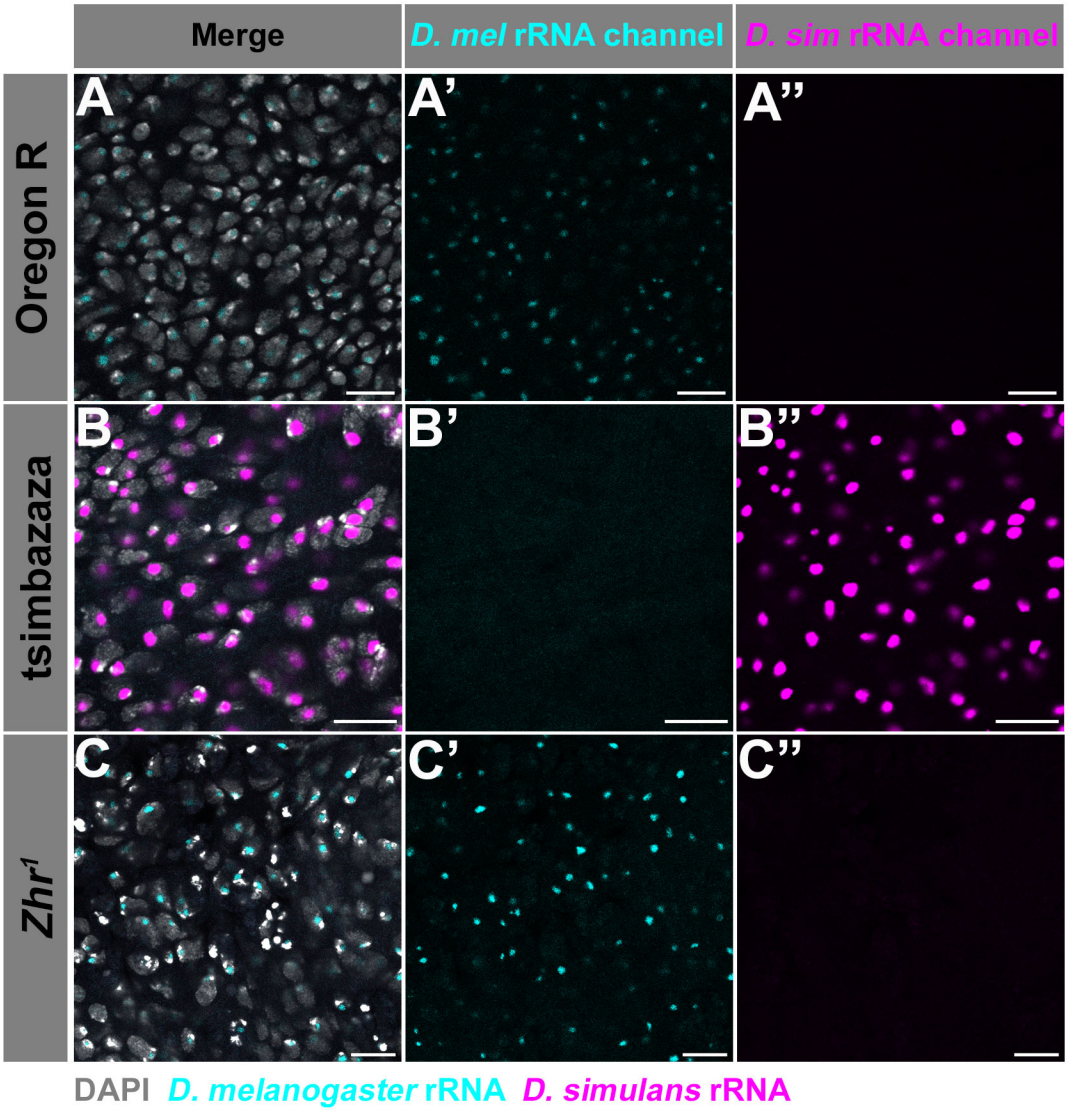


Figure B.6: ITS RNA *in situ* Probes are Species-specific.
A) Zoomed image of a *D. melanogaster* stock Oregon R embryo, A') *D. melanogaster* rRNA channel only, A'') *D. simulans* rRNA channel only. B) Zoomed image of an *D. simulans* tsimbazaza strain embryo, B') *D. melanogaster* rRNA channel only, B'') *D. simulans* rRNA channel only. C) Zoomed image of a *D. melanogaster Zhr*^{1} stock embryo, C') *D. melanogaster* rRNA channel only, C'') *D. simulans* rRNA channel only. All embryos are of unknown sex. All scale bars = 10μm. Magenta = *D. simulans* rRNA, turquoise = *D. melanogaster* rRNA, grey = DAPI.

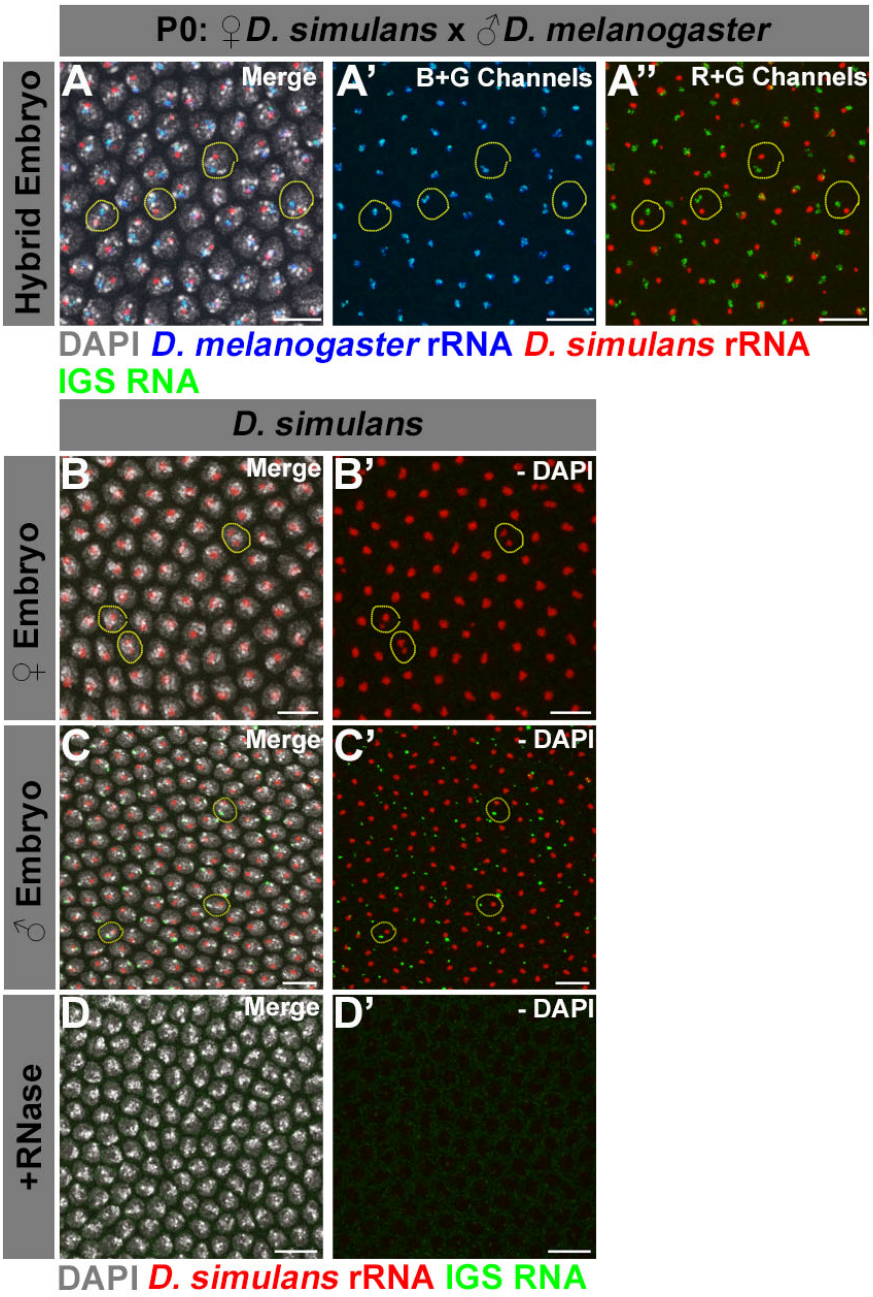


Figure B.7: IGS is Transcribed from *D. melanogaster* rDNA in Interspecies Hybrid Embryos.
A) Zoomed image of a syncytial interspecies hybrid embryos from P0 ♀*D. simulans* and ♂*D. melanogaster*, A') *D. melanogaster* rRNA and IGS RNA channels only, A'') *D. simulans* rRNA and IGS RNA channels only. B) Zoomed image of a syncytial *D. simulans* female embryo (sex determined by presence of two distinct rRNA puncta), B') no DAPI. C) Zoomed image of a syncytial *D. simulans* male embryo (sex determined by presence of one distinct rRNA puncta), C') no DAPI. D) Zoomed image of a syncytial *D. simulans* embryo of unknown sex after RNase treatment, D') no DAPI. All scale bars = 10μm. Red = *D. simulans* rRNA, Blue = *D. melanogaster* rRNA, Green = IGS RNA, Grey = DAPI

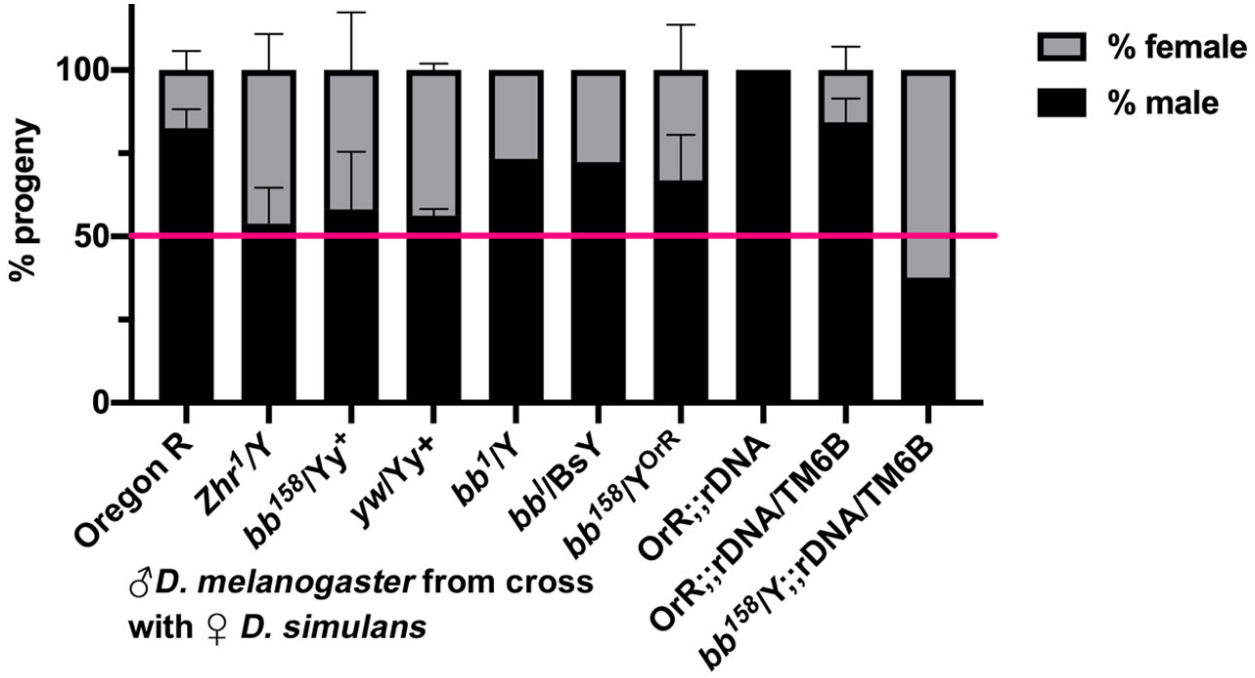


Figure B.8: Strains with rDNA Deletions and/or rDNA Additions Have Variable Effects on Female Hybrid Embryonic Rescue.
Female embryonic hybrid rescue assay with *D. melanogaster* males from a variety of strains with rDNA deficiencies and/or rDNA additions. Pink line denotes 50% of progeny for visual ease. Sample sizes are as follows (♂*D. melanogaster* from P0 cross with ♀*D. simulans*): Oregon R = 15 (data replicated from Figure II.3 for comparison), *Zhr*^{1} = 12 (data replicated from Figure II.3 for comparison), *bb*^{158}/Yy^{+} = 12, *yw*/Yy^{+} = 7, *bb*^{1}/Y = 1, *bb*^{l}/BsY = 1, *bb*^{158}/Y^{OrR-1} = 4, OrR-1;;rDNA = 3, OrR-1;;rDNA/TM6B = 5, *bb*^{158}/Y;;rDNA/TM6B = 1. OrR = sex chromosomes from Oregon R stock. Grey = % female, black = % male.

Bibliography

Abad, J. P., M. Agudo, I. Molina, A. Losada, P. Ripoll, et al. (2000). "Pericentromeric regions containing 1.688 satellite DNA sequences show anti-kinetochore antibody staining in prometaphase chromosomes of Drosophila melanogaster." Mol Gen Genet **264**(4): 371-377.

Abad, J. P., M. Carmena, S. Baars, R. D. Saunders, D. M. Glover, et al. (1992). "Dodeca satellite: a conserved G+C-rich satellite from the centromeric heterochromatin of Drosophila melanogaster." Proc Natl Acad Sci U S A **89**(10): 4663-4667.

Aguilera, A. and T. Garcia-Muse (2013). "Causes of genome instability." Annu Rev Genet **47**: 1-32.

Aguilera, A. and T. García-Muse (2012). "R loops: from transcription byproducts to threats to genome stability." Mol Cell **46**(2): 115-124.

Aldrich, J. C. and K. A. Maggert (2015). "Transgenerational inheritance of diet-induced genome rearrangements in Drosophila." PLoS Genet **11**(4): e1005148.

Altemose, N., K. H. Miga, M. Maggioni and H. F. Willard (2014). "Genomic characterization of large heterochromatic gaps in the human genome assembly." PLoS Comput Biol **10**(5): e1003628.

Averbeck, K. T. and T. H. Eickbush (2005). "Monitoring the mode and tempo of concerted evolution in the Drosophila melanogaster rDNA locus." Genetics **171**(4): 1837-1846.

Barbash, D. A. (2010). "Ninety years of Drosophila melanogaster hybrids." Genetics **186**(1): 1-8.

Barbash, D. A. and M. Ashburner (2003). "A novel system of fertility rescue in Drosophila hybrids reveals a link between hybrid lethality and female sterility." Genetics **163**(1): 217-226.

Barbash, D. A., J. Roote and M. Ashburner (2000). "The Drosophila melanogaster hybrid male rescue gene causes inviability in male and female species hybrids." Genetics **154**(4): 1747-1771.

Bayes, J. J. and H. S. Malik (2009). "Altered heterochromatin binding by a hybrid sterility protein in Drosophila sibling species." Science **326**(5959): 1538-1541.

Berloco, M., L. Fanti, F. Sheen, R. W. Levis and S. Pimpinelli (2005). "Heterochromatic distribution of HeT-A- and TART-like sequences in several Drosophila species." Cytogenet Genome Res **110**(1-4): 124-133.

Blum, J. A., S. Bonaccorsi, M. Marzullo, V. Palumbo, Y. M. Yamashita, et al. (2017). "The Hybrid Incompatibility Genes Lhr and Hmr Are Required for Sister Chromatid Detachment During Anaphase but Not for Centromere Function." Genetics **207**(4): 1457-1472.

Bompadre, O. and G. Andrey (2019). "Chromatin topology in development and disease." Curr Opin Genet Dev **55**: 32-38.

Bonaccorsi, S. and A. Lohe (1991). "Fine mapping of satellite DNA sequences along the Y chromosome of Drosophila melanogaster: relationships between satellite sequences and fertility factors." Genetics **129**(1): 177-189.

Bosco, G., P. Campbell, J. T. Leiva-Neto and T. A. Markow (2007). "Analysis of Drosophila species genome size and satellite DNA content reveals significant differences among strains as well as between species." Genetics **177**(3): 1277-1290.

Bouzinba-Segard, H., A. Guais and C. Francastel (2006). "Accumulation of small murine minor satellite transcripts leads to impaired centromeric architecture and function." Proc Natl Acad Sci U S A **103**(23): 8709-8714.

Brideau, N. J., H. A. Flores, J. Wang, S. Maheshwari, X. Wang, et al. (2006). "Two Dobzhansky-Muller genes interact to cause hybrid lethality in Drosophila." Science **314**(5803): 1292-1295.

Bridges, C. B. (1916). "Non-Disjunction as Proof of the Chromosome Theory of Heredity." Genetics **1**(1): 1-52.

Bridges, C. B. (1921). "TRIPLOID INTERSEXES IN DROSOPHILA MELANOGASTER." Science **54**(1394): 252-254.

Bueno, D., O. M. Palacios-Gimenez and D. C. Cabral-de-Mello (2013). "Chromosomal Mapping of Repetitive DNAs in the Grasshopper Abracris flavolineata Reveal Possible Ancestry of the B Chromosome and H3 Histone Spreading." PLoS One **8**(6): e66532.

Busby, S. J. and R. H. Reeder (1983). "Spacer sequences regulate transcription of ribosomal gene plasmids injected into Xenopus embryos." Cell **34**(3): 989-996.

Busch, H., Y. Daskal, F. Gyorkey and K. Smetana (1979). "Silver staining of nucleolar granules in tumor cells." Cancer Res **39**(3): 857-863.

Calo, E., B. Gu, M. E. Bowen, F. Aryan, A. Zalc, et al. (2018). "Tissue-selective effects of nucleolar stress and rDNA damage in developmental disorders." Nature **554**(7690): 112-117.

Carmena, M., J. P. Abad, A. Villasante and C. Gonzalez (1993). "The Drosophila melanogaster dodecasatellite sequence is closely linked to the centromere and can form connections between sister chromatids during mitosis." J Cell Sci **105 (Pt 1)**: 41-50.

Carter, R. and G. Drouin (2009). "The evolutionary rates of eukaryotic RNA polymerases and of their transcription factors are affected by the level of concerted evolution of the genes they transcribe." Mol Biol Evol **26**(11): 2515-2520.

Cassidy, D. M. and A. W. Blackler (1974). "Repression of nucleolar organizer activity in an interspecific hybrid of the genus Xenopus." Dev Biol **41**(1): 84-96.

Caudy, A. A. and C. S. Pikaard (2002). "Xenopus ribosomal RNA gene intergenic spacer elements conferring transcriptional enhancement and nucleolar dominance-like competition in oocytes." J Biol Chem **277**(35): 31577-31584.

Chandrasekhara, C., G. Mohannath, T. Blevins, F. Pontvianne and C. S. Pikaard (2016). "Chromosome-specific NOR inactivation explains selective rRNA gene silencing and dosage control in Arabidopsis." Genes Dev **30**(2): 177-190.

Charlesworth, B., P. Sniegowski and W. Stephan (1994). "The evolutionary dynamics of repetitive DNA in eukaryotes." Nature **371**(6494): 215-220.

Chen, Z. J., L. Comai and C. S. Pikaard (1998). "Gene dosage and stochastic effects determine the severity and direction of uniparental ribosomal RNA gene silencing (nucleolar dominance) in Arabidopsis allopolyploids." Proc Natl Acad Sci U S A **95**(25): 14891-14896.

Chen, Z. J. and C. S. Pikaard (1997). "Transcriptional analysis of nucleolar dominance in polyploid plants: biased expression/silencing of progenitor rRNA genes is developmentally regulated in Brassica." Proc Natl Acad Sci U S A **94**(7): 3442-3447.

Cheng, J., N. Turkel, N. Hemati, M. T. Fuller, A. J. Hunt, et al. (2008). "Centrosome misorientation reduces stem cell division during ageing." Nature **456**(7222): 599-604.

Christophorou, N., T. Rubin and J. R. Huynh (2013). "Synaptonemal complex components promote centromere pairing in pre-meiotic germ cells." PLoS Genet **9**(12): e1004012.

Coen, E., T. Strachan and G. Dover (1982). "Dynamics of concerted evolution of ribosomal DNA and histone gene families in the melanogaster species subgroup of Drosophila." J Mol Biol **158**(1): 17-35.

Coen, E. S. and G. A. Dover (1982). "Multiple Pol I initiation sequences in rDNA spacers of Drosophila melanogaster." Nucleic Acids Res **10**(21): 7017-7026.

Costa-Nunes, P., O. Pontes, S. B. Preuss and C. S. Pikaard (2010). "Extra views on RNA-dependent DNA methylation and MBD6-dependent heterochromatin formation in nucleolar dominance." Nucleus **1**(3): 254-259.

Croce, C. M., A. Talavera, C. Basilico and O. J. Miller (1977). "Suppression of production of mouse 28S ribosomal RNA in mouse-human hybrids segregating mouse chromosomes." Proc Natl Acad Sci U S A **74**(2): 694-697.

Csink, A. K. and S. Henikoff (1998). "Something from nothing: the evolution and utility of satellite repeats." Trends Genet **14**(5): 200-204.

Datta, A. and S. Jinks-Robertson (1995). "Association of increased spontaneous mutation rates with high levels of transcription in yeast." Science **268**(5217): 1616-1619.

De Winter, R. F. and T. Moss (1986). "Spacer promoters are essential for efficient enhancement of X. laevis ribosomal transcription." Cell **44**(2): 313-318.

Defossez, P. A., R. Prusty, M. Kaeberlein, S. J. Lin, P. Ferrigno, et al. (1999). "Elimination of replication block protein Fob1 extends the life span of yeast mother cells." Mol Cell **3**(4): 447-455.

Dernburg, A. F., J. W. Sedat and R. S. Hawley (1996). "Direct evidence of a role for heterochromatin in meiotic chromosome segregation." Cell **86**(1): 135-146.

DiBartolomeis, S. M., K. D. Tartof and F. R. Jackson (1992). "A superfamily of Drosophila satellite related (SR) DNA repeats restricted to the X chromosome euchromatin." Nucleic Acids Res **20**(5): 1113-1116.

Dion-Cote, A. M. and D. A. Barbash (2017). "Beyond speciation genes: an overview of genome stability in evolution and speciation." Curr Opin Genet Dev **47**: 17-23.

Dover, G. (1994). "Concerted evolution, molecular drive and natural selection." Curr Biol **4**(12): 1165-1166.

Dover, G. A. (1987). "DNA turnover and the molecular clock." J Mol Evol **26**(1-2): 47-58.

Dover, G. A. (1989). "Linkage disequilibrium and molecular drive in the rDNA gene family." Genetics **122**(1): 249-252.

Dover, G. A. and R. B. Flavell (1984). "Molecular coevolution: DNA divergence and the maintenance of function." Cell **38**(3): 622-623.

Dover, G. A., A. R. Linares, T. Bowen and J. M. Hancock (1993). "Detection and quantification of concerted evolution and molecular drive." Methods Enzymol **224**: 525-541.

Dover, G. A. and D. Tautz (1986). "Conservation and divergence in multigene families: alternatives to selection and drift." Philos Trans R Soc Lond B Biol Sci **312**(1154): 275-289.

Drygin, D., A. Lin, J. Bliesath, C. B. Ho, S. E. O'Brien, et al. (2011). "Targeting RNA polymerase I with an oral small molecule CX-5461 inhibits ribosomal RNA synthesis and solid tumor growth." Cancer Res **71**(4): 1418-1430.

Durica, D. S. and H. M. Krider (1977). "Studies on the ribosomal RNA cistrons in interspecific Drosophila hybrids. I. Nucleolar dominance." Dev Biol **59**(1): 62-74.

Durica, D. S. and H. M. Krider (1978). "Studies on the Ribosomal RNA Cistrons in Interspecific Drosophila Hybrids. II. Heterochromatic Regions Mediating Nucleolar Dominance." Genetics **89**(1): 37-64.

Earley, K., R. J. Lawrence, O. Pontes, R. Reuther, A. J. Enciso, et al. (2006). "Erasure of histone acetylation by Arabidopsis HDA6 mediates large-scale gene silencing in nucleolar dominance." Genes Dev **20**(10): 1283-1293.

Earley, K. W., F. Pontvianne, A. T. Wierzbicki, T. Blevins, S. Tucker, et al. (2010). "Mechanisms of HDA6-mediated rRNA gene silencing: suppression of intergenic Pol II transcription and differential effects on maintenance versus siRNA-directed cytosine methylation." Genes Dev **24**(11): 1119-1132.

Eickbush, D. G., J. Ye, X. Zhang, W. D. Burke and T. H. Eickbush (2008). "Epigenetic regulation of retrotransposons within the nucleolus of Drosophila." Mol Cell Biol **28**(20): 6452-6461.

Elder, J. F., Jr. and B. J. Turner (1995). "Concerted evolution of repetitive DNA sequences in eukaryotes." Q Rev Biol **70**(3): 297-320.

Elgin, S. C. and G. Reuter (2013). "Position-effect variegation, heterochromatin formation, and gene silencing in Drosophila." Cold Spring Harb Perspect Biol **5**(8): a017780.

Engels, W. R. and C. R. Preston (1979). "Hybrid dysgenesis in Drosophila melanogaster: the biology of female and male sterility." Genetics **92**(1): 161-174.

Feber, A., G. A. Wilson, L. Zhang, N. Presneau, B. Idowu, et al. (2011). "Comparative methylome analysis of benign and malignant peripheral nerve sheath tumors." Genome Res **21**(4): 515-524.

Felle, M., J. H. Exler, R. Merkl, K. Dachauer, A. Brehm, et al. (2010). "DNA sequence encoded repression of rRNA gene transcription in chromatin." Nucleic Acids Res **38**(16): 5304-5314.

Ferree, P. M. and D. A. Barbash (2009). "Species-specific heterochromatin prevents mitotic chromosome segregation to cause hybrid lethality in Drosophila." PLoS Biol **7**(10): e1000234.

Ferree, P. M., K. Gomez, P. Rominger, D. Howard, H. Kornfeld, et al. (2014). "Heterochromatin position effects on circularized sex chromosomes cause filicidal embryonic lethality in Drosophila melanogaster." Genetics **196**(4): 1001-1005.

Frieman, M., Z. J. Chen, J. Saez-Vasquez, L. A. Shen and C. S. Pikaard (1999). "RNA polymerase I transcription in a Brassica interspecific hybrid and its progenitors: Tests of transcription factor involvement in nucleolar dominance." Genetics **152**(1): 451-460.

Fukaya, T. and M. Levine (2017). "Transvection." Curr Biol **27**(19): R1047-r1049.

Ganley, A. R. and T. Kobayashi (2007). "Highly efficient concerted evolution in the ribosomal DNA repeats: total rDNA repeat variation revealed by whole-genome shotgun sequence data." Genome Res **17**(2): 184-191.

Garcia-Muse, T. and A. Aguilera (2016). "Transcription-replication conflicts: how they occur and how they are resolved." Nat Rev Mol Cell Biol **17**(9): 553-563.

Gatti, M., S. Pimpinelli and G. Santini (1976). "Characterization of Drosophila heterochromatin. I. Staining and decondensation with Hoechst 33258 and quinacrine." Chromosoma **57**(4): 351-375.

Gaubatz, J. W. and R. G. Cutler (1990). "Mouse satellite DNA is transcribed in senescent cardiac muscle." J Biol Chem **265**(29): 17753-17758.

Ghoshal, K., S. Majumder, J. Datta, T. Motiwala, S. Bai, et al. (2004). "Role of human ribosomal RNA (rRNA) promoter methylation and of methyl-CpG-binding protein MBD2 in the suppression of rRNA gene expression." J Biol Chem **279**(8): 6783-6793.

Goetze, H., M. Wittner, S. Hamperl, M. Hondele, K. Merz, et al. (2010). "Alternative chromatin structures of the 35S rRNA genes in Saccharomyces cerevisiae provide a molecular basis for the selective recruitment of RNA polymerases I and II." Mol Cell Biol **30**(8): 2028-2045.

Goodrich-Young, C. and H. M. Krider (1989). "Nucleolar dominance and replicative dominance in Drosophila interspecific hybrids." Genetics **123**(2): 349-358.

Greil, F. and K. Ahmad (2012). "Nucleolar dominance of the Y chromosome in Drosophila melanogaster." Genetics **191**(4): 1119-1128.

Grewal, S. S., L. Li, A. Orian, R. N. Eisenman and B. A. Edgar (2005). "Myc-dependent regulation of ribosomal RNA synthesis during Drosophila development." Nat Cell Biol **7**(3): 295-302.

Grimaldi, G. and P. P. Di Nocera (1988). "Multiple repeated units in Drosophila melanogaster ribosomal DNA spacer stimulate rRNA precursor transcription." Proc Natl Acad Sci U S A **85**(15): 5502-5506.

Grimaldi, G., P. Fiorentini and P. P. Di Nocera (1990). "Spacer promoters are orientation-dependent activators of pre-rRNA transcription in Drosophila melanogaster." Mol Cell Biol **10**(9): 4667-4677.

Grummt, I., E. Roth and M. R. Paule (1982). "Ribosomal RNA transcription in vitro is species specific." Nature **296**(5853): 173-174.

Guenatri, M., D. Bailly, C. Maison and G. Almouzni (2004). "Mouse centric and pericentric satellite repeats form distinct functional heterochromatin." J Cell Biol **166**(4): 493-505.

Haider, S., L. Cordeddu, E. Robinson, M. Movassagh, L. Siggens, et al. (2012). "The landscape of DNA repeat elements in human heart failure." Genome Biol **13**(10): R90.

Hamperl, S., M. J. Bocek, J. C. Saldivar, T. Swigut and K. A. Cimprich (2017). "Transcription-Replication Conflict Orientation Modulates R-Loop Levels and Activates Distinct DNA Damage Responses." Cell **170**(4): 774-786.e719.

Hawley, R. S. and K. D. Tartof (1985). "A two-stage model for the control of rDNA magnification." Genetics **109**(4): 691-700.

Heix, J. and I. Grummt (1995). "Species specificity of transcription by RNA polymerase I." Curr Opin Genet Dev **5**(5): 652-656.

Heliot, L., F. Mongelard, C. Klein, M. F. O'Donohue, J. M. Chassery, et al. (2000). "Nonrandom distribution of metaphase AgNOR staining patterns on human acrocentric chromosomes." J Histochem Cytochem **48**(1): 13-20.

Helmrich, A., M. Ballarino, E. Nudler and L. Tora (2013). "Transcription-replication encounters, consequences and genomic instability." Nat Struct Mol Biol **20**(4): 412-418.

Henikoff, S. (1981). "Position-effect variegation and chromosome structure of a heat shock puff in Drosophila." Chromosoma **83**(3): 381-393.

Henikoff, S. (1994). "A reconsideration of the mechanism of position effect." Genetics **138**(1): 1-5.

Henikoff, S., K. Ahmad and H. S. Malik (2001). "The centromere paradox: stable inheritance with rapidly evolving DNA." Science **293**(5532): 1098-1102.

Henikoff, S., J. M. Jackson and P. B. Talbert (1995). "Distance and pairing effects on the brownDominant heterochromatic element in Drosophila." Genetics **140**(3): 1007-1017.

Henikoff, S. and H. S. Malik (2002). "Centromeres: selfish drivers." Nature **417**(6886): 227.

Hey, J. and R. M. Kliman (1993). "Population genetics and phylogenetics of DNA sequence variation at multiple loci within the Drosophila melanogaster species complex." Mol Biol Evol **10**(4): 804-822.

Hoskins, R. A., J. W. Carlson, K. H. Wan, S. Park, I. Mendez, et al. (2015). "The Release 6 reference sequence of the Drosophila melanogaster genome." Genome Res **25**(3): 445-458.

Houchins, K., M. O'Dell, R. B. Flavell and J. P. Gustafson (1997). "Cytosine methylation and nucleolar dominance in cereal hybrids." Mol Gen Genet **255**(3): 294-301.

Hsieh, T. and D. L. Brutlag (1979). "A protein that preferentially binds Drosophila satellite DNA." Proc Natl Acad Sci U S A **76**(2): 726-730.

Hutter, P. and M. Ashburner (1987). "Genetic rescue of inviable hybrids between Drosophila melanogaster and its sibling species." Nature **327**(6120): 331-333.

Iida, T. and T. Kobayashi (2019). "RNA Polymerase I Activators Count and Adjust Ribosomal RNA Gene Copy Number." Mol Cell **73**(4): 645-654.e613.

Ito, H., A. Miura, K. Takashima and T. Kakutani (2007). "Ecotype-specific and chromosome-specific expansion of variant centromeric satellites in Arabidopsis thaliana." Mol Genet Genomics **277**(1): 23-30.

Jacome, A. and O. Fernandez-Capetillo (2011). "Lac operator repeats generate a traceable fragile site in mammalian cells." EMBO Rep **12**(10): 1032-1038.

Jagannathan, M., R. Cummings and Y. M. Yamashita (2018). "A conserved function for pericentromeric satellite DNA." Elife **7**.

Jagannathan, M., R. Cummings and Y. M. Yamashita (2019). "The modular mechanism of chromocenter formation in Drosophila." Elife **8**.

Jagannathan, M., N. Warsinger-Pepe, G. J. Watase and Y. M. Yamashita (2017). "Comparative Analysis of Satellite DNA in the Drosophila melanogaster Species Complex." G3 (Bethesda) **7**(2): 693-704.

Jagannathan, M. and Y. M. Yamashita (2017). "Function of Junk: Pericentromeric Satellite DNA in Chromosome Maintenance." Cold Spring Harb Symp Quant Biol **82**: 319-327.

Jinks-Robertson, S. and A. S. Bhagwat (2014). "Transcription-associated mutagenesis." Annu Rev Genet **48**: 341-359.

Johzuka, K. and T. Horiuchi (2002). "Replication fork block protein, Fob1, acts as an rDNA region specific recombinator in S. cerevisiae." Genes Cells **7**(2): 99-113.

Joyce, E. F., B. R. Williams, T. Xie and C. T. Wu (2012). "Identification of genes that promote or antagonize somatic homolog pairing using a high-throughput FISH-based screen." PLoS Genet **8**(5): e1002667.

Kaeberlein, M., M. McVey and L. Guarente (1999). "The SIR2/3/4 complex and SIR2 alone promote longevity in Saccharomyces cerevisiae by two different mechanisms." Genes Dev **13**(19): 2570-2580.

Kamm, A., I. Galasso, T. Schmidt and J. S. Heslop-Harrison (1995). "Analysis of a repetitive DNA family from Arabidopsis arenosa and relationships between Arabidopsis species." Plant Mol Biol **27**(5): 853-862.

Karpen, G. H., M. H. Le and H. Le (1996). "Centric heterochromatin and the efficiency of achiasmate disjunction in Drosophila female meiosis." Science **273**(5271): 118-122.

Kawabe, A. and D. Charlesworth (2007). "Patterns of DNA variation among three centromere satellite families in Arabidopsis halleri and A. lyrata." J Mol Evol **64**(2): 237-247.

Kawabe, A. and S. Nasuda (2006). "Chromosome-specific satellite sequences in Turritis glabra." Genes Genet Syst **81**(4): 287-290.

Kidd, S. J. and D. M. Glover (1981). "Drosophila melanogaster ribosomal DNA containing type II insertions is variably transcribed in different strains and tissues." J Mol Biol **151**(4): 645-662.

Kim, K., Y. S. Lee, D. Harris, K. Nakahara and R. W. Carthew (2006). "The RNAi pathway initiated by Dicer-2 in Drosophila." Cold Spring Harb Symp Quant Biol **71**: 39-44.

King, T. D., J. E. Johnson and J. R. Bateman (2019). "Position Effects Influence Transvection in Drosophila melanogaster." Genetics **213**(4): 1289-1299.

Kobayashi, T., D. J. Heck, M. Nomura and T. Horiuchi (1998). "Expansion and contraction of ribosomal DNA repeats in Saccharomyces cerevisiae: requirement of replication fork blocking (Fob1) protein and the role of RNA polymerase I." Genes Dev **12**(24): 3821-3830.

Kohorn, B. D. and P. M. Rae (1982). "Accurate transcription of truncated ribosomal DNA templates in a Drosophila cell-free system." Proc Natl Acad Sci U S A **79**(5): 1501-1505.

Kohorn, B. D. and P. M. Rae (1982). "Nontranscribed spacer sequences promote in vitro transcription of Drosophila ribosomal DNA." Nucleic Acids Res **10**(21): 6879-6886.

Koo, D. H., V. K. Tiwari, E. Hribova, J. Dolezel, B. Friebe, et al. (2016). "Molecular Cytogenetic Mapping of Satellite DNA Sequences in Aegilops geniculata and Wheat." Cytogenet Genome Res **148**(4): 314-321.

Kuhn, A. and I. Grummt (1987). "A novel promoter in the mouse rDNA spacer is active in vivo and in vitro." Embo j **6**(11): 3487-3492.

Kuhn, G. C., H. Kuttler, O. Moreira-Filho and J. S. Heslop-Harrison (2012). "The 1.688 repetitive DNA of Drosophila: concerted evolution at different genomic scales and association with genes." Mol Biol Evol **29**(1): 7-11.

Labhart, P. and R. H. Reeder (1984). "Enhancer-like properties of the 60/81 bp elements in the ribosomal gene spacer of Xenopus laevis." Cell **37**(1): 285-289.

Lachaise, D., J. R. David, F. Lemeunier, L. Tsacas and M. Ashburner (1986). "THE REPRODUCTIVE RELATIONSHIPS OF DROSOPHILA SECHELLIA WITH D. MAURITIANA, D. SIMULANS, AND D. MELANOGASTER FROM THE AFROTROPICAL REGION." Evolution **40**(2): 262-271.

Lamb, J. C., J. M. Meyer, B. Corcoran, A. Kato, F. Han, et al. (2007). "Distinct chromosomal distributions of highly repetitive sequences in maize." Chromosome Res **15**(1): 33-49.

Larracuente, A. M. (2014). "The organization and evolution of the Responder satellite in species of the Drosophila melanogaster group: dynamic evolution of a target of meiotic drive." BMC Evol Biol **14**: 233.

Larracuente, A. M. and P. M. Ferree (2015). "Simple method for fluorescence DNA in situ hybridization to squashed chromosomes." J Vis Exp(95): 52288.

Lawrence, R. J., K. Earley, O. Pontes, M. Silva, Z. J. Chen, et al. (2004). "A concerted DNA methylation/histone methylation switch regulates rRNA gene dosage control and nucleolar dominance." Mol Cell **13**(4): 599-609.

Lee, Y. C., C. Leek and M. T. Levine (2017). "Recurrent Innovation at Genes Required for Telomere Integrity in Drosophila." Mol Biol Evol **34**(2): 467-482.

Levesque, M. J., P. Ginart, Y. Wei and A. Raj (2013). "Visualizing SNVs to quantify allele-specific expression in single cells." Nat Methods **10**(9): 865-867.

Lewis, M. S., J. M. Cheverud and C. S. Pikaard (2004). "Evidence for nucleolus organizer regions as the units of regulation in nucleolar dominance in Arabidopsis thaliana interecotype hybrids." Genetics **167**(2): 931-939.

Liao, D. and A. M. Weiner (1995). "Concerted evolution of the tandemly repeated genes encoding primate U2 small nuclear RNA (the RNU2 locus) does not prevent rapid diversification of the (CT)n.(GA)n microsatellite embedded within the U2 repeat unit." Genomics **30**(3): 583-593.

Liu, L. F. and J. C. Wang (1987). "Supercoiling of the DNA template during transcription." Proc Natl Acad Sci U S A **84**(20): 7024-7027.

Lohe, A. R. and D. L. Brutlag (1986). "Multiplicity of satellite DNA sequences in Drosophila melanogaster." Proc Natl Acad Sci U S A **83**(3): 696-700.

Lohe, A. R. and D. L. Brutlag (1987). "Identical satellite DNA sequences in sibling species of Drosophila." J Mol Biol **194**(2): 161-170.

Lohe, A. R., A. J. Hilliker and P. A. Roberts (1993). "Mapping simple repeated DNA sequences in heterochromatin of Drosophila melanogaster." Genetics **134**(4): 1149-1174.

Lohe, A. R. and P. A. Roberts (1990). "An unusual Y chromosome of Drosophila simulans carrying amplified rDNA spacer without rRNA genes." Genetics **125**(2): 399-406.

Lohe, A. R. and P. A. Roberts (2000). "Evolution of DNA in heterochromatin: the Drosophila melanogaster sibling species subgroup as a resource." Genetica **109**(1-2): 125-130.

Long, E. O. and I. B. Dawid (1980). "Repeated genes in eukaryotes." Annu Rev Biochem **49**: 727-764.

Losada, A. and A. Villasante (1996). "Autosomal location of a new subtype of 1.688 satellite DNA of Drosophila melanogaster." Chromosome Res **4**(5): 372-383.

Louzada, S., A. Vieira-da-Silva, A. Mendes-da-Silva, S. Kubickova, J. Rubes, et al. (2015). "A novel satellite DNA sequence in the Peromyscus genome (PMSat): Evolution via copy number fluctuation." Mol Phylogenet Evol **92**: 193-203.

Lower, S. E., A. M. Dion-Cote, A. G. Clark and D. A. Barbash (2019). "Special Issue: Repetitive DNA Sequences." Genes (Basel) **10**(11).

Lu, K. L., J. O. Nelson, G. J. Watase, N. Warsinger-Pepe and Y. M. Yamashita (2018). "Transgenerational dynamics of rDNA copy number in Drosophila male germline stem cells." Elife **7**.

Macleod, D. and A. Bird (1982). "DNAase I sensitivity and methylation of active versus inactive rRNA genes in xenopus species hybrids." Cell **29**(1): 211-218.

Mayer, C., K. M. Schmitz, J. Li, I. Grummt and R. Santoro (2006). "Intergenic transcripts regulate the epigenetic state of rRNA genes." Mol Cell **22**(3): 351-361.

McKee, B. D., K. Wilhelm, C. Merrill and X. Ren (1998). "Male sterility and meiotic drive associated with sex chromosome rearrangements in Drosophila. Role of X-Y pairing." Genetics **149**(1): 143-155.

Menon, D. U., C. Coarfa, W. Xiao, P. H. Gunaratne and V. H. Meller (2014). "siRNAs from an X-linked satellite repeat promote X-chromosome recognition in Drosophila melanogaster." Proc Natl Acad Sci U S A **111**(46): 16460-16465.

Merker, R. J. and H. L. Klein (2002). "hpr1Delta affects ribosomal DNA recombination and cell life span in Saccharomyces cerevisiae." Mol Cell Biol **22**(2): 421-429.

Merrill, C. J., D. Chakravarti, L. Habera, S. Das, L. Eisenhour, et al. (1992). "Promoter-containing ribosomal DNA fragments function as X-Y meiotic pairing sites in D. melanogaster males." Dev Genet **13**(6): 468-484.

Mohan, J. and F. M. Ritossa (1970). "Regulation of ribosomal RNA synthesis and its bearing on the bobbed phenotype in Drosophila melanogaster." Dev Biol **22**(3): 495-512.

Mohannath, G., F. Pontvianne and C. S. Pikaard (2016). "Selective nucleolus organizer inactivation in Arabidopsis is a chromosome position-effect phenomenon." Proc Natl Acad Sci U S A **113**(47): 13426-13431.

Morales-Hojas, R., M. Reis, C. P. Vieira and J. Vieira (2011). "Resolving the phylogenetic relationships and evolutionary history of the Drosophila virilis group using multilocus data." Mol Phylogenet Evol **60**(2): 249-258.

Moss, T. (1983). "A transcriptional function for the repetitive ribosomal spacer in Xenopus laevis." Nature **302**(5905): 223-228.

Moss, T., K. Mitchelson and R. de Winter (1985). "The promotion of ribosomal transcription in eukaryotes." Oxf Surv Eukaryot Genes **2**: 207-250.

Moss, T. and V. Y. Stefanovsky (2002). "At the center of eukaryotic life." Cell **109**(5): 545-548.

Murayama, A., K. Ohmori, A. Fujimura, H. Minami, K. Yasuzawa-Tanaka, et al. (2008). "Epigenetic control of rDNA loci in response to intracellular energy status." Cell **133**(4): 627-639.

Nelson, J. O., G. J. Watase, N. Warsinger-Pepe and Y. M. Yamashita (2019). "Mechanisms of rDNA Copy Number Maintenance." Trends Genet **35**(10): 734-742.

Neves, N., J. S. Heslop-Harrison and W. Viegas (1995). "rRNA gene activity and control of expression mediated by methylation and imprinting during embryo development in wheat x rye hybrids." Theor Appl Genet **91**(3): 529-533.

Nomura, M. (2001). "Ribosomal RNA genes, RNA polymerases, nucleolar structures, and synthesis of rRNA in the yeast Saccharomyces cerevisiae." Cold Spring Harb Symp Quant Biol **66**: 555-565.

Ohta, T. and G. A. Dover (1984). "The cohesive population genetics of molecular drive." Genetics **108**(2): 501-521.

Olavarrieta, L., P. Hernández, D. B. Krimer and J. B. Schvartzman (2002). "DNA knotting caused by head-on collision of transcription and replication." J Mol Biol **322**(1): 1-6.

Oliveira, C. I., H. E. Bicudo and M. M. Itoyama (2006). "New evidence for nucleolar dominance in hybrids of Drosophila arizonae and Drosophila mulleri." Genet Mol Res **5**(4): 632-637.

Orr, H. A. (1995). "The population genetics of speciation: the evolution of hybrid incompatibilities." Genetics **139**(4): 1805-1813.

Paco, A., F. Adega, N. Mestrovic, M. Plohl and R. Chaves (2014). "Evolutionary story of a satellite DNA from Phodopus sungorus (Rodentia, Cricetidae)." Genome Biol Evol **6**(10): 2944-2955.

Peng, J. C. and G. H. Karpen (2007). "H3K9 methylation and RNA interference regulate nucleolar organization and repeated DNA stability." Nat Cell Biol **9**(1): 25-35.

Phadnis, N., E. P. Baker, J. C. Cooper, K. A. Frizzell, E. Hsieh, et al. (2015). "An essential cell cycle regulation gene causes hybrid inviability in Drosophila." Science **350**(6267): 1552-1555.

Pikaard, C. S. (2000). "Nucleolar dominance: uniparental gene silencing on a multi-megabase scale in genetic hybrids." Plant Mol Biol **43**(2-3): 163-177.

Planta, R. J. (1997). "Regulation of ribosome synthesis in yeast." Yeast **13**(16): 1505-1518.

Plohl, M., N. Mestrovic and B. Mravinac (2012). "Satellite DNA evolution." Genome Dyn **7**: 126-152.

Pontes, O., R. J. Lawrence, M. Silva, S. Preuss, P. Costa-Nunes, et al. (2007). "Postembryonic establishment of megabase-scale gene silencing in nucleolar dominance." PLoS One **2**(11): e1157.

Pontes, O., C. F. Li, P. Costa Nunes, J. Haag, T. Ream, et al. (2006). "The Arabidopsis chromatin-modifying nuclear siRNA pathway involves a nucleolar RNA processing center." Cell **126**(1): 79-92.

Pontes, O., N. Neves, M. Silva, M. S. Lewis, A. Madlung, et al. (2004). "Chromosomal locus rearrangements are a rapid response to formation of the allotetraploid Arabidopsis suecica genome." Proc Natl Acad Sci U S A **101**(52): 18240-18245.

Pontvianne, F., T. Blevins, C. Chandrasekhara, W. Feng, H. Stroud, et al. (2012). "Histone methyltransferases regulating rRNA gene dose and dosage control in Arabidopsis." Genes Dev **26**(9): 945-957.

Preuss, S. and C. S. Pikaard (2007). "rRNA gene silencing and nucleolar dominance: insights into a chromosome-scale epigenetic on/off switch." Biochim Biophys Acta **1769**(5-6): 383-392.

Preuss, S. B., P. Costa-Nunes, S. Tucker, O. Pontes, R. J. Lawrence, et al. (2008). "Multimegabase silencing in nucleolar dominance involves siRNA-directed DNA methylation and specific methylcytosine-binding proteins." Mol Cell **32**(5): 673-684.

Probst, A. V., M. Fagard, F. Proux, P. Mourrain, S. Boutet, et al. (2004). "Arabidopsis histone deacetylase HDA6 is required for maintenance of transcriptional gene silencing and determines nuclear organization of rDNA repeats." Plant Cell **16**(4): 1021-1034.

Rabanal, F. A., T. Mandakova, L. M. Soto-Jimenez, R. Greenhalgh, D. L. Parrott, et al. (2017). "Epistatic and allelic interactions control expression of ribosomal RNA gene clusters in Arabidopsis thaliana." Genome Biol **18**(1): 75.

Rasooly, R. S. and L. G. Robbins (1991). "Rex and a suppressor of Rex are repeated neomorphic loci in the Drosophila melanogaster ribosomal DNA." Genetics **129**(1): 119-132.

Reeder, R. H. (1984). "Enhancers and ribosomal gene spacers." Cell **38**(2): 349-351.

Reeder, R. H. and J. G. Roan (1984). "The mechanism of nucleolar dominance in Xenopus hybrids." Cell **38**(1): 38-44.

Reeder, R. H., J. G. Roan and M. Dunaway (1983). "Spacer regulation of Xenopus ribosomal gene transcription: competition in oocytes." Cell **35**(2 Pt 1): 449-456.

Ren, X., L. Eisenhour, C. Hong, Y. Lee and B. D. McKee (1997). "Roles of rDNA spacer and transcription unit-sequences in X-Y meiotic chromosome pairing in Drosophila melanogaster males." Chromosoma **106**(1): 29-36.

Ritossa, F. M., K. C. Atwood and S. Spiegelman (1966). "A molecular explanation of the bobbed mutants of Drosophila as partial deficiencies of "ribosomal" DNA." Genetics **54**(3): 819-834.

Rosic, S., F. Kohler and S. Erhardt (2014). "Repetitive centromeric satellite RNA is essential for kinetochore formation and cell division." J Cell Biol **207**(3): 335-349.

Roussel, P., C. Andre, L. Comai and D. Hernandez-Verdun (1996). "The rDNA transcription machinery is assembled during mitosis in active NORs and absent in inactive NORs." J Cell Biol **133**(2): 235-246.

Roy, V., L. Monti-Dedieu, N. Chaminade, S. Siljak-Yakovlev, S. Aulard, et al. (2005). "Evolution of the chromosomal location of rDNA genes in two Drosophila species subgroups: ananassae and melanogaster." Heredity (Edinb) **94**(4): 388-395.

Salim, D., W. D. Bradford, A. Freeland, G. Cady, J. Wang, et al. (2017). "DNA replication stress restricts ribosomal DNA copy number." PLoS Genet **13**(9): e1007006.

Salim, D. and J. L. Gerton (2019). "Ribosomal DNA instability and genome adaptability." Chromosome Res.

Santoro, R. and I. Grummt (2005). "Epigenetic mechanism of rRNA gene silencing: temporal order of NoRC-mediated histone modification, chromatin remodeling, and DNA methylation." Mol Cell Biol **25**(7): 2539-2546.

Santoro, R., K. M. Schmitz, J. Sandoval and I. Grummt (2010). "Intergenic transcripts originating from a subclass of ribosomal DNA repeats silence ribosomal RNA genes in trans." EMBO Rep **11**(1): 52-58.

Satyaki, P. R., T. N. Cuykendall, K. H. Wei, N. J. Brideau, H. Kwak, et al. (2014). "The Hmr and Lhr hybrid incompatibility genes suppress a broad range of heterochromatic repeats." PLoS Genet **10**(3): e1004240.

Sawamura, K., A. W. Davis and C. I. Wu (2000). "Genetic analysis of speciation by means of introgression into Drosophila melanogaster." Proc Natl Acad Sci U S A **97**(6): 2652-2655.

Sawamura, K., T. Taira and T. K. Watanabe (1993). "Hybrid lethal systems in the Drosophila melanogaster species complex. I. The maternal hybrid rescue (mhr) gene of Drosophila simulans." Genetics **133**(2): 299-305.

Sawamura, K., T. K. Watanabe and M. T. Yamamoto (1993). "Hybrid lethal systems in the Drosophila melanogaster species complex." Genetica **88**(2-3): 175-185.

Sawamura, K. and M. T. Yamamoto (1993). "Cytogenetical localization of Zygotic hybrid rescue (Zhr), a Drosophila melanogaster gene that rescues interspecific hybrids from embryonic lethality." Mol Gen Genet **239**(3): 441-449.

Sawamura, K., M. T. Yamamoto and T. K. Watanabe (1993). "Hybrid lethal systems in the Drosophila melanogaster species complex. II. The Zygotic hybrid rescue (Zhr) gene of D. melanogaster." Genetics **133**(2): 307-313.

Schlotterer, C., M. T. Hauser, A. von Haeseler and D. Tautz (1994). "Comparative evolutionary analysis of rDNA ITS regions in Drosophila." Mol Biol Evol **11**(3): 513-522.

Schmid, M. and C. Steinlein (2015). "Chromosome Banding in Amphibia. XXXII. The Genus Xenopus (Anura, Pipidae)." Cytogenet Genome Res **145**(3-4): 201-217.

Schmitz, K. M., C. Mayer, A. Postepska and I. Grummt (2010). "Interaction of noncoding RNA with the rDNA promoter mediates recruitment of DNMT3b and silencing of rRNA genes." Genes Dev **24**(20): 2264-2269.

Simeone, A., A. La Volpe and E. Boncinelli (1985). "Nucleotide sequence of a complete ribosomal spacer of D. melanogaster." Nucleic Acids Res **13**(4): 1089-1101.

Smetana, K. and H. Busch (1964). "STUDIES ON THE ULTRASTRUCTURE OF THE NUCLEOLI OF THE WALKER TUMOR AND RAT LIVER." Cancer Res **24**: 537-557.

Sofueva, S., F. Osman, A. Lorenz, R. Steinacher, S. Castagnetti, et al. (2011). "Ultrafine anaphase bridges, broken DNA and illegitimate recombination induced by a replication fork barrier." Nucleic Acids Res **39**(15): 6568-6584.

Strachan, T., E. Coen, D. Webb and G. Dover (1982). "Modes and rates of change of complex DNA families of Drosophila." J Mol Biol **158**(1): 37-54.

Strachan, T., D. Webb and G. A. Dover (1985). "Transition stages of molecular drive in multiple-copy DNA families in Drosophila." Embo j **4**(7): 1701-1708.

Sturtevant, A. H. (1920). "Genetic Studies on DROSOPHILA SIMULANS. I. Introduction. Hybrids with DROSOPHILA MELANOGASTER." Genetics **5**(5): 488-500.

Takeuchi, Y., T. Horiuchi and T. Kobayashi (2003). "Transcription-dependent recombination and the role of fork collision in yeast rDNA." Genes Dev **17**(12): 1497-1506.

Tautz, D. and G. A. Dover (1986). "Transcription of the tandem array of ribosomal DNA in Drosophila melanogaster does not terminate at any fixed point." Embo j **5**(6): 1267-1273.

Tautz, D., J. M. Hancock, D. A. Webb, C. Tautz and G. A. Dover (1988). "Complete sequences of the rRNA genes of Drosophila melanogaster." Mol Biol Evol **5**(4): 366-376.

Tautz, D., C. Tautz, D. Webb and G. A. Dover (1987). "Evolutionary divergence of promoters and spacers in the rDNA family of four Drosophila species. Implications for molecular coevolution in multigene families." J Mol Biol **195**(3): 525-542.

Tautz, D., M. Trick and G. A. Dover (1986). "Cryptic simplicity in DNA is a major source of genetic variation." Nature **322**(6080): 652-656.

Ting, D. T., D. Lipson, S. Paul, B. W. Brannigan, S. Akhavanfard, et al. (2011). "Aberrant overexpression of satellite repeats in pancreatic and other epithelial cancers." Science **331**(6017): 593-596.

Tucker, S., A. Vitins and C. S. Pikaard (2010). "Nucleolar dominance and ribosomal RNA gene silencing." Curr Opin Cell Biol **22**(3): 351-356.

Ugarkovic, D. and M. Plohl (2002). "Variation in satellite DNA profiles--causes and effects." Embo j **21**(22): 5955-5959.

Usakin, L., J. Abad, V. V. Vagin, B. de Pablos, A. Villasante, et al. (2007). "Transcription of the 1.688 satellite DNA family is under the control of RNA interference machinery in Drosophila melanogaster ovaries." Genetics **176**(2): 1343-1349.

Viktorovskaya, O. V., K. L. Engel, S. L. French, P. Cui, P. J. Vandeventer, et al. (2013). "Divergent contributions of conserved active site residues to transcription by eukaryotic RNA polymerases I and II." Cell Rep **4**(5): 974-984.

Viktorovskaya, O. V. and D. A. Schneider (2015). "Functional divergence of eukaryotic RNA polymerases: unique properties of RNA polymerase I suit its cellular role." Gene **556**(1): 19-26.

Voelkel-Meiman, K., R. L. Keil and G. S. Roeder (1987). "Recombination-stimulating sequences in yeast ribosomal DNA correspond to sequences regulating transcription by RNA polymerase I." Cell **48**(6): 1071-1079.

Waring, G. L. and J. C. Pollack (1987). "Cloning and characterization of a dispersed, multicopy, X chromosome sequence in Drosophila melanogaster." Proc Natl Acad Sci U S A **84**(9): 2843-2847.

Warsinger-Pepe, N., D. Li and Y. M. Yamashita (2020). "Regulation of Nucleolar Dominance in Drosophila melanogaster." Genetics.

Wilk, R., S. U. M. Murthy, H. Yan and H. M. Krause (2010). "In Situ Hybridization: Fruit Fly Embryos and Tissues." Current Protocols Essential Laboratory Techniques **4**(1): 9.3.1-9.3.24.

Wittkopp, P. J., B. K. Haerum and A. G. Clark (2004). "Evolutionary changes in cis and trans gene regulation." Nature **430**(6995): 85-88.

Wong, L. H., K. H. Brettingham-Moore, L. Chan, J. M. Quach, M. A. Anderson, et al. (2007). "Centromere RNA is a key component for the assembly of nucleoproteins at the nucleolus and centromere." Genome Res **17**(8): 1146-1160.

Yadlapalli, S. and Y. M. Yamashita (2013). "Chromosome-specific nonrandom sister chromatid segregation during stem-cell division." Nature **498**(7453): 251-254.

Yamashiro, H. and M. C. Siomi (2018). "PIWI-Interacting RNA in Drosophila: Biogenesis, Transposon Regulation, and Beyond." Chem Rev **118**(8): 4404-4421.

Yunis, J. J. and W. G. Yasmineh (1971). "Heterochromatin, satellite DNA, and cell function. Structural DNA of eucaryotes may support and protect genes and aid in speciation." Science **174**(4015): 1200-1209.

Zhang, Q., N. A. Shalaby and M. Buszczak (2014). "Changes in rRNA transcription influence proliferation and cell fate within a stem cell lineage." Science **343**(6168): 298-301.

Zhou, J., T. B. Sackton, L. Martinsen, B. Lemos, T. H. Eickbush, et al. (2012). "Y chromosome mediates ribosomal DNA silencing and modulates the chromatin state in Drosophila." Proc Natl Acad Sci U S A **109**(25): 9941-9946.