

Genetic Identification of Germline RNAi
Components as Regulators of Segregation Distorter
in *D. melanogaster*

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TABLE OF CONTENTS

Title Page.....	i
Copyright Page.....	ii
Signature Page.....	iii
Curriculum Vitae.....	iv
Acknowledgments.....	vii
Table of Contents.....	ix
List of Tables.....	xi
List of Figures.....	xi
Chapter I: Introduction to Segregation Distortion in <i>D. melanogaster</i>	1
INTRODUCTION	2
Meiotic Drive as an Evolutionary Force	2
Segregation Distorter in <i>D. melanogaster</i>	4
The Molecular Nature of <i>Sd</i> in <i>D. melanogaster</i>	6
Modifiers of Segregation Distorter	9
The Nature of <i>Responder</i>	10
<i>Drosophila</i> Spermatogenesis	12
Mechanisms of Drive in Other Systems	17
Autosomal Drive of the t complex in <i>Mus musculus</i>	17
Stellate Sex Ratio Drive in <i>D. melanogaster</i>	19
Winters Sex Ratio Drive in <i>D. simulans</i>	22
RNAi and Gene Silencing in <i>Drosophila</i>	23
Ovarian piRNA Biogenesis.....	25
Primary piRNA Biogenesis: A Model from Somatic Ovarian Cells	25
Germline piRNA Pathway in Ovaries and the Ping-Pong Mechanism	28
Regulation of PIWIs by Tudor family proteins	32
The piRNA pathway in the <i>Drosophila</i> Testis.....	33
Localization of piRNA Biogenesis in the <i>Drosophila</i> Testis	34
Gene-Specific Regulation by the piRNA Pathway in Testis	35
Transposon Regulation by the piRNA Pathway in Testis.....	37
RNAi and Epigenetic Regulation of Silencing	38
PIWIs as Epigenetic Regulators in <i>Drosophila</i>	39
An Existing Model of SD-piRNA Interaction	40
Open Questions and Current Motivations for This Study	41
FIGURES AND FIGURE LEGENDS	57
Chapter II: Mutations in the piRNA pathway component <i>aubergine</i> enhance meiotic drive of Segregation Distorter in <i>D. Melanogaster</i>	74
ABSTRACT	75
INTRODUCTION	77
MATERIALS AND METHODS	82
Genetic Stocks.....	82
K-tests	82

Generation of Recombinant Chromosomes	83
Southern Blotting	83
Homologous Recombination	85
RESULTS	87
piRNA pathway mutants are genetic enhancers of <i>SD</i>	87
The contributions of the <i>aub</i> and <i>Rsp</i> to <i>SD</i> sensitivity are genetically separable	90
Addition of an <i>aub</i> mutation to the canonical <i>Rsp^s</i> chromosome enhances distortion in trans	91
An <i>aub</i> mutation placed on an <i>SD</i> chromosome by homologous recombination enhances distortion in cis.	92
DISCUSSION	95
REFERENCES	101
FIGURES AND FIGURE LEGENDS	108
SUPPLEMENTAL INFORMATION	114
 Chapter III: Investigating the Molecular Mechanism of piRNA Pathway	
Activity in Segregation Distortion	119
ABSTRACT	120
INTRODUCTION	121
MATERIALS AND METHODS	125
Genetic Stocks	125
Generation of UAS-AGO3 Transgenes	125
Western Blotting	128
Small RNA Polymerase Chain Reaction	129
Reverse-Transcription quantitative PCR	130
RESULTS	131
Chromatin Modifications Are Unaltered in Distorting and Non-Distorting Conditions	131
Staining for mono-methyl H3K9 and HP1 reveals no alterations in heterochromatin related to <i>SD</i>	131
Staining for tri-methyl H3K9 recapitulates patterns seen with mono-me H3K9 staining.	133
RanGAP and Aubergine Colocalize in piNG-bodies of Primary Spermatocytes	134
<i>Rsp</i>-Derived Small RNA Are Detected in the Testes of Both <i>Rsp^s</i> and <i>Rspⁱ</i> Males	135
Additional <i>Rsp</i> loci not detected <i>in vivo</i>	138
<i>AGO3</i> Expression is Unchanged Under Distorting and Non-Distorting Conditions ..	139
Third Chromosomes Carrying TubP-GAL4 Driver Alone Enhance Distortion to a	
Greater Degree than <i>AGO3</i> Overexpression	140
DISCUSSION	141
REFERENCES	148
FIGURES AND FIGURE LEGENDS	152
 Chapter IV: Discussion and Future Directions	
Models for the Role of the piRNA Pathway Activity in Segregation Distortion	173
Nuclear exclusion of <i>Rsp</i> -primed RNP complexes	175
Nuclear accumulation of piRNA pathway components	175
Targeted suppression of <i>AGO3</i> by <i>Rsp</i> -piRNAs	177
The “source” and “sink” model of <i>Rsp</i> -piRNA biogenesis	177
Nuclear retention of piRNA precursors in <i>SD</i>	179
Role of nuclear transport and piRNA components in mitotic chromatin regulation	179
Future Directions for the Study of Segregation Distorter in <i>D. melanogaster</i>	183
FIGURES AND FIGURE LEGENDS	189

LIST OF TABLES

Table 1.1: Effect of modifiers on Segregation Distorter.....	73
Table S2.1 Genetic stocks used in this study.....	115
Table S2.2: Primer sequences used in this study.....	116
Table 3.1: Primers used in this study.....	168
Table 3.2: Genetic stocks used in this study.....	171

LIST OF FIGURES

Figure 1.1: Segregation Distorter in <i>D. melanogaster</i>	57
Figure 1.2: <i>Sd</i> encodes a truncated RanGAP protein.....	58
Figure 1.3: <i>Rsp</i> is a 120 bp AT rich tandem repeat.....	59
Figure 1.4: <i>Drosophila</i> spermatogenesis.....	60
Figure 1.5: The <i>t complex</i> of <i>Mus musculus</i>	61
Figure 1.6: The role of RNAi in <i>Stellate</i> sex-ratio drive.....	62
Figure 1.7: Role of RNAi in <i>Winters</i> sex-ratio drive of <i>D. simulans</i>	64
Figure 1.8: Overview of siRNA and miRNA pathways in <i>Drosophila melanogaster</i>	65
Figure 1.9: Biogenesis of piRNA in the <i>Drosophila</i> Ovary.....	66
Figure 1.10: Biogenesis of piRNA in the somatic cells of <i>Drosophila</i> ovaries.....	67
Figure 1.11: The ping-pong pathway of secondary piRNA biogenesis.....	68
Figure 1.12: Localization of PIWI family proteins in the testis.....	69
Figure 1.12: Gene-specific regulation by piRNAs in the testis.....	70
Figure 1.14: A model of transcriptional gene silencing in <i>S. pombe</i>	71
Figure 1.15: A published model of RNAi involvement in segregation distortion.....	72
Figure 2.1: Components of segregation distortion.....	108
Figure 2.2: Targeting of the <i>aub</i> locus by ends-out homologous recombination.....	109
Figure 2.3: Mutations in <i>aub</i> and <i>piwi</i> are genetic enhancers of distortion.....	110
Figure 2.4: The contributions of <i>aub</i> mutants and associated repeats are genetically separable.....	111
Figure 2.5: Addition of an <i>aub</i> mutation to a <i>Rsp^s</i> chromosome enhances distortion.....	112
Figure 2.6: Engineered <i>aub</i> mutations on an <i>SD</i> chromosome enhance distortion <i>in cis</i>	112
Figure 2.7: A model for the role of the piRNA pathway in segregation distortion.....	113

Figure S2.1 Aub-GFP and RanGAP colocalized in a single large body of primary spermatocytes.....	114
Figure 3.1: HP1 and H3K9 mono-me staining of primary spermatocytes.....	153
Figure 3.2: HP1 and H3K9 mono-me staining of round spermatids.....	154
Figure 3.3: HP1 and H3K9 mono-me staining of elongating spermatids.....	155
Figure 3.4 HP1 and H3K9 tri-me staining does not differ between distorting and non-distorting genotypes.....	156
Figure 3.5: Primary spermatocytes show precise areas of co-localization between Aub-GFP and RanGAP.	157
Figure 3.6: Colocalization of Aub-GFP and RanGAP is specific to a single large perinuclear particle.....	158
Figure 3.7: Only a single piNG-body is visible per cell.....	159
Figure 3.8: RanGAP and Aub-GFP colocalize in early spermatocytes.....	160
Figure 3.9: Experimental design for amplification of <i>Rsp</i> -specific small RNAs.....	161
Figure 3.10 Small RNAs amplified from the testis of <i>Rsp^s</i> males are generated from specific regions of the <i>Rsp</i> repeat.....	162
Figure 3.11: <i>Rsp</i> Small RNAs are amplified from testes of both distorting and non-distorting males.....	163
Figure 3.12: Small RNA PCR amplification of <i>Rsp</i> sequences reveals only minor differences between males and females of <i>Rsp^s</i> and <i>Rspⁱ</i> genotypes.....	164
Figure 3.13: Southern blotting reveals no difference in <i>Rsp</i> repeat arrays of <i>Rspⁱ</i> males and females.....	165
Figure 3.14 <i>Argonaute3</i> (<i>AGO3</i>) expression levels are unchanged between distorting and non-distorting conditions.....	166
Figure 3.15 A third chromosome carrying the TubP-GAL4 driver enhances distortion to a greater degree than AGO3 overexpression.	167
Figure 4.1 The “source” and “sink” model for the role of <i>Rsp</i> piRNAs in segregation distortion.....	189

Chapter I: Introduction to Segregation Distortion in *D.*
melanogaster

INTRODUCTION

Meiotic Drive as an Evolutionary Force

In sexual organisms evolution relies on the faithful segregation and transmission of alleles from one generation to the next, allowing unbiased exposure of these alleles to the effects of natural selection. However, in complex systems, “cheaters” can arise which manipulate the “rules of the game” to best suit their own interests. In the case of evolution, genes that are propagated without consideration of their impact on organismal fitness represent such cheaters. Left unchecked, the propagation of these “ultra-selfish” genetic elements ultimately reduces the success of other, perhaps “more fit” genes (CROW 1988). Multiple examples of these types of elements have been observed including certain plasmids, mitochondrial or plastid organelles, transposable elements and B chromosomes (WERREN *et al.* 1988; JONES 1991; HURST and WERREN 2001). The conflict between the propagation of ultra-selfish genes and overall organismal fitness leads to the evolution of mechanisms to suppress the activity of these cheaters (GINZBURG *et al.* 1984; FRANK 1991; PRUD'HOMME *et al.* 1995). This evolutionary arms race between the host genome and ultra-selfish genetic parasites is hypothesized to lead to hybrid incompatibilities, reproductive isolation and eventually speciation of closely

related but physically isolated populations (DAWKINS 1976; HURST and WERREN 2001; CLARK *et al.* 2007).

Meiotic drive is a type of selfish gene activity that is thought to play a particularly important role in prezygotic species isolation (SANDLER and NOVITSKI 1957; FRANK 1991). Normal sexual reproduction requires segregation of allele pairs ensuring equal representation in the pool of haploid gametes. Meiotic drive occurs anytime one allele type “cheats” by becoming overrepresented in the gamete population. The potential for a meiotic driver to become fixed in a population is dependent on both the fraction of the offspring carrying the favored allele (k) and the fitness of organisms heterozygous or homozygous for the selfish allele. Many meiotic drivers are thought to act by causing the dysfunction or transmission failure of gametes of the alternate genotype. Additionally, meiotic drivers in many organisms are homozygous lethal or infertile due either to their own activity or closely linked mutations (HARTL 1969; HARTL 1972; LYTTLE 1991). Therefore, drivers that carry significant fitness disadvantages remain polymorphic in natural populations (SANDLER and NOVITSKI 1957). Isolated populations may adapt to the fitness burden of a meiotic driver by evolving counter mutations that suppress its deleterious effects. However, these effects can often be uncovered by crossing closely-related species, one of which lacks adaptive defenses against these elements (FRANK 1991; HURST and WERREN 2001).

Further, meiotic drive has been implicated in the establishment of “drive haplotypes” (HEDRICK 1988; LYTTLE 1991). Genes linked to meiotic drivers become genetic hitchhikers leading to linkage disequilibrium. Chromosomal rearrangements that reduce or eliminate recombination can also lead to increases in both disequilibrium and

severity of drive by linking secondary enhancers to the primary drive locus (THOMSON and FELDMAN 1974). These rearrangements enjoy a strong selective advantage and are observed in many meiotic drive systems. In some cases extensive rearrangements lead to *chromosomal* meiotic drive where the haplotype extends the length of the chromosome (LYTTLE 1991). In cases of *sex-ratio* meiotic drive, where one sex-chromosome in the heterogametic sex is preferentially inherited, chromosomal drive can lead to profound alterations in population structure with potentially catastrophic consequences (HAMILTON 1967).

While all genes can be considered selfish units of inheritance, most do so by means that enhance overall organismal fitness (DAWKINS 1976). Ultra-selfish genes operate outside the canonical rules of natural selection, allowing propagation independent of fitness contribution. The competition between genes that benefit from normal Mendelian inheritance and those that cheat the system facilitates an intragenomic arms race. Consequently, these ultra-selfish elements represent both a genetic burden as well as an opportunity to spur evolutionary change and adaptation.

Segregation Distorter in *D. melanogaster*

Although meiotic drive has been studied in systems as diverse as *Neurospora* and mouse, *Drosophila* has proved to be a particularly useful model for the examination of both the evolutionary history and molecular mechanisms of drive systems.

The *Segregation distorter* (*Sd*) locus in *Drosophila* is a classic example of the meiotic drive phenomenon. In 1956, Yuichiro Hiraizumi, a graduate student in the

laboratory of Dr. James F. Crow at the University of Wisconsin noticed an odd phenotypic distribution among progeny from crosses between wild-caught *Drosophila* and an inbred laboratory strain. Eye color markers indicated that a copy of chromosome two from the wild strain was passed from fathers to offspring at a remarkably high frequency, in violation of all the known rules of genetic inheritance (SANDLER *et al.* 1959; GANETZKY 2000). This chromosome was dubbed Segregation Distorter (*SD*) and in the subsequent 50 years much work has been done to understand both its evolution and the molecular mechanism permitting its selfish propagation.

It is now understood that distortion occurs when a male fly carries both a Segregation Distorter (*SD*) chromosome and a sensitive *Responder* (Rsp^S) locus on homologous second chromosomes (Figure 1.1a) (SANDLER *et al.* 1959; SANDLER and HIRAIZUMI 1960b; HARTL 1973; TEMIN *et al.* 1991). Spermatogenesis proceeds normally in these males through the completion of meiotic divisions. Late in spermatogenesis, nuclei containing Rsp^S -bearing chromosomes fail to condense and are removed, ensuring that virtually all gametes carry the *SD* chromosome (Figure 1.1b) (HARTL *et al.* 1967; TOKUYASU *et al.* 1977). The *SD* chromosome carries an insensitive *Responder* (Rsp^I) allele, which is not subject to distortion, thereby ensuring it cannot act upon itself (Figure 1.1a). The degree of distortion is generally recorded as a “*k* value,” which represents the fraction of the progeny that carry *SD* (Figure 1.1c). The expected Mendelian value for *k* in wild type conditions is 0.500. However, strongly distorting *SD* chromosomes can reach $k = 0.999$ (SANDLER and HIRAIZUMI 1959; TEMIN and MARTHAS 1984; TEMIN *et al.* 1991). Interestingly, though *SD* alters the transmission of autosomes, the *SD* phenotype is

male specific; heterozygous females display Mendelian segregation (SANDLER *et al.* 1959).

Most *SD* chromosomes isolated from nature contain multiple inversions linking the drive locus, (*Rspⁱ*) and enhancers of *SD*. These inversions generally include a pericentric inversion, inhibiting recombination between the *Sd* and *Rsp* loci as well as paracentric inversions on 2R that are thought to keep enhancer loci in linkage disequilibrium with *SD* (Figure 1.1a). The extent and locations of these inversions define different *SD* chromosome types and influence their stability as well as the strength of drive they exhibit (TEMIN and MARTHAS 1984; PIMPINELLI and DIMITRI 1989; TEMIN *et al.* 1991). For example, the strong distorter *SD*-5 contains all upwards modifiers of *Sd* as well as two paracentric inversions on the right arm of chromosome two, while *SD*-Roma, a weaker distorter, is inversion free (NICOLETTI and TRIPPA 1967; TEMIN *et al.* 1991)

The Molecular Nature of Sd in D. melanogaster

Recombination experiments between *SD* and wild type (*SD⁺*) chromosomes mapped the selfish activity of the *SD* chromosome to a specific locus (*Sd*) in the proximal euchromatin of 2R (HARTL 1974; GANETZKY 1977). This region contains a truncated Ran GTP Activating Protein (RanGAP) (MERRILL *et al.* 1999). Formed by a tandem duplication event, this 234aa C-terminal truncation remains enzymatically active, but lacks part of a nuclear export signal (NES) as well as a sumoylation site required for proper localization to the nuclear pore (Figure 1.2a, e) (MCLEAN *et al.* 1994; MERRILL *et al.* 1999; KUSANO *et al.* 2001).

Nuclear import or export of most large molecules, including proteins and RNA, requires active transport through Nuclear Pore Complexes (NPCs) (GORLICH and KUTAY 1999b). This movement is facilitated by Ran GTPase, which establishes the GTP gradient required for nuclear transport (GORLICH *et al.* 1996a; GORLICH *et al.* 1996b). RanGDP is imported into the nucleus via the NTF2 nuclear transport receptor (CLARKSON *et al.* 1996; PASCHAL *et al.* 1996). Once inside the nucleus, Ran exchanges GDP for GTP with the help of RanGEF (GDP Exchange Factor) also known as RCC1 (Figure 1.2b) (BISCHOFF and PONSTINGL 1991a; BISCHOFF and PONSTINGL 1991b; GORLICH and KUTAY 1999b). Entry of cargo, such as proteins or RNA-Protein (RNP) complexes, into the nucleus requires binding of an importin nuclear transport receptor, transit through the nuclear pore, and then binding of RanGTP to the importin-cargo complex to facilitate cargo release (GORLICH and KUTAY 1999b). Nuclear export requires the formation of a complex containing an exportin nuclear receptor protein, the cargo to be exported, and RanGTP (GORLICH and KUTAY 1999b). Following transit through the NCP, cytoplasmic release of cargo requires the processing of GTP to GDP, which is facilitated by cytoplasmic RanGAP (BISCHOFF *et al.* 1994).

Many lines of evidence support the conclusion that a defect in nuclear transport underlies segregation distortion. Sd-RanGAP was confirmed as the meiotic driver in segregation distortion by transgenic expression of Sd-RanGAP in an *SD*⁺ background. This construct causes distortion that is greatly enhanced by expression in a background containing upwards modifiers of *SD* (MERRILL *et al.* 1999). A catalytically inactive Sd-RanGAP transgene does not cause drive and recombinant *SD*-RanGAP protein enhances RanGTPase activity *in vitro*, suggesting GTP hydrolysis is necessary for distortion. The

nuclear localization of Sd-RanGAP is also necessary because addition of an NES to an *Sd-RanGAP* transgene suppresses its ability to cause distortion. Altered nuclear transport is not limited to the *SD*-RanGAP protein because a GFP transgene modified to include both a NES and a Nuclear Localization Signal (NLS) is retained in the nucleus in *SD* flies, while the same construct is mostly cytoplasmic in an *SD*⁺ background.

Overexpression of Ran or RanGEF suppresses distortion and mutations to *RanGEF* or *importin* genes act as enhancers (KUSANO *et al.* 2001; MCELROY *et al.* 2008). When overexpressed, wild type RanGAP shows increased nuclear localization and, most strikingly, causes distortion in the absence of Sd-RanGAP (Figure 1.2f) (KUSANO *et al.* 2002). Together these data suggest that the truncated RanGAP encoded by *Sd* lacks proper perinuclear localization signals, allowing it to be ectopically located inside the nucleus (Figure 1.2c). This mislocalization causes a generalized disruption of nuclear transport because Sd-RanGAP hydrolyzes nuclear GTP needed for Ran-mediated export. Additionally, distortion is not a *de novo* function of the Sd-RanGAP protein, but rather the consequence of normal RanGAP present in an improper location (IZAURREALDE *et al.* 1997; GORLICH and KUTAY 1999a; KUSANO *et al.* 2001; KALAB *et al.* 2002).

Although *Sd* has been molecularly identified, how its perturbation of nuclear transport results in its selfish activity during spermatogenesis remains unclear. Since the expression of RanGAP and Sd-RanGAP is not germline specific, the effect of *Sd* on nuclear transport must be present in all cells. However, because *Sd* arose from a duplication event, *SD* animals also carry two wild type copies of *RanGAP* and are clearly viable, so this disruption must not greatly alter essential import or export of most Ran cargo. Instead, it seems that the presence of Sd-RanGAP in developing spermatids may

provide a sensitized environment where underlying problems with *Rsp*-bearing nuclei are exacerbated (KUSANO *et al.* 2003).

Modifiers of Segregation Distorter

Interestingly, chromosomes that contain the *Sd* locus but lack any additional upwards modifiers exhibit only very weak distortion (TEMIN *et al.* 1991). For example, while a chromosome that contains *Sd* and all major modifiers distorts strongly with *k* of up to 0.999, a chromosome containing *Sd* alone distorts weakly with a *k* of only 0.62 (Table 1.1) (TEMIN *et al.* 1991).

The best characterized of these loci, *Enhancer of SD* [*E(SD)*] is located in the h35 heterochromatic region of 2L (Figure 1.1a) (GANETZKY 1977; BRITTNACHER and GANETZKY 1984). This region was identified by careful examination of partial revertant *SD* chromosomes generated through x-ray irradiation (GANETZKY 1977; BRITTNACHER and GANETZKY 1984). Curiously, distortion of the *Rsp* locus can be achieved in the absence of *Sd* if two doses of the *Enhancer* region are present. This effect was demonstrated by translocation of *E(SD)* onto a *Sd*⁺ *Rsp*^S chromosome and onto the Y, showing that *E(SD)* activity is independent of genomic location (SHARP *et al.* 1985; TEMIN 1991). While two doses of *E(SD)* do not lead to an overall increase in wild type RanGAP protein, this condition does result in an accumulation of wild type RanGAP in the nucleus (TEMIN 1991; KUSANO *et al.* 2002). In spite of detailed deficiency mapping of the heterochromatic region containing *E(SD)* no candidate genes have been identified (COULTHARD *et al.* 2003).

Located on 2R, *Modifier of SD* [*M(SD)*] also exhibits independent distorting ability when present in two or more doses (Figure 1.1a, Table 1.1) (C. STABER, personal communication). This locus is associated with a sharp reduction in fertility in *SD* males with copies of *Rsp^s* on both homologs of chromosome two. While these males generally have low fecundity because both second chromosomes are targeted by *Sd*, the presence of *M(SD)* exacerbates this problem, making these males almost completely sterile (HIRAIZUMI *et al.* 1980). While *Stabilizer of SD*, [*St(SD)*] enhances drive, it has not been demonstrated to have independent distorting ability. Loosely mapped to the distal end of 2R, the presence of *St(SD)* is reported to affect the consistency of *k* values for an *SD* chromosome carrying this locus (Figure 1.1a, Table 1.1) (SANDLER and HIRAIZUMI 1960a). Neither *M(SD)* or *St(SD)* loci has been molecularly identified.

The Nature of Responder

The sperm dysfunction observed in *SD* also depends on the allelic state of the target locus known as *Responder* (*Rsp*) (HARTL 1973; HARTL 1974). *Rsp* loci range from completely insensitive (*Rspⁱ*), which show normal segregation in the presence of *Sd*, to supersensitive (*Rsp^{ss}*) alleles, which are almost completely eliminated in the presence of *Sd* (GANETZKY 1977; HIRAIZUMI *et al.* 1980; TEMIN and MARTHAS 1984; LYTTLE *et al.* 1986). Located in the heterochromatin of 2R, *Responder* is an approximately 120bp satellite repeat array. *Rsp* repeat copy number correlates with sensitivity to *SD* (Figure 1.3) (WU *et al.* 1988; PIMPINELLI and DIMITRI 1989; HOUTCHENS and LYTTLE 2003). *Rsp^{ss}* chromosomes are estimated to have several thousand repeats while the *Rsp^s* chromosome is estimated to have approximately 700 hundred copies (WU *et al.* 1988). No completely insensitive *SD⁺*

chromosomes have been isolated from nature, but a *Rspⁱ* allele was generated by x-ray ablation of the *Rsp^s* locus (GANETZKY 1977). This *Rspⁱ* allele is reported to have fewer than 20 remaining copies of *Rsp* (WU et al. 1988). The repeats are organized in tandem arrays in a mostly head to tail orientation and are thought to make up approximately 85 kb of sequence distributed throughout the nearly 600 kb region that makes up the *Rsp* locus on the canonical *Rsp^s* chromosome (WU et al. 1988; CABOT et al. 1993). However a less stringent sequence analysis suggests that more diverged repeats may comprise the entire 600 kb region (HOUTCHENS and LYTTLE 2003).

The function of *Rsp* is not context-dependent because translocation of *Rsp^s* to the Y chromosome will result in the loss of Y-bearing sperm. Further, the sensitivity of *Rsp* is divisible, because partial deletions of this region lead to a corresponding reduction in sensitivity to *SD* (LYTTLE 1989). Lastly, recombination between *Sd Rspⁱ* chromosomes and *Sd⁺ Rsp^s* homologues results in the generation of a “suicide chromosome” of the genotype *Sd Rsp^s* (HARTL 1974). These chromosomes are self-targeting and quickly eliminated from a population.

While copies of *Rsp* are found on all non-*SD* second chromosomes isolated from nature (WU et al. 1988), chromosomes generated in the laboratory with almost complete ablations of the *Rsp* locus remain both viable and fertile (GANETZKY 1977). *Responder* is not predicted to form any protein product, nor have any *Rsp* mRNAs been detected. Interestingly, some *Rsp* sequences have been detected in populations of small RNAs isolated from both ovaries and testis (SAITO et al. 2006; NAGAO et al. 2010). This finding suggests that *Rsp* itself may be a target of the RNAi pathway in these tissues.

Sd chromosomes exist at frequencies between 1% and 5% in most natural populations of *Drosophila* studied (HIRAIZUMI and NAKAZIMA 1967; TEMIN and MARTHAS 1984). Given the great disadvantage *Rsp*-bearing chromosomes would have in these populations, the prevalence of high copy number repeat arrays in nature is truly remarkable. These repeats are not found in sibling *Drosophila* species with the exception of some highly diverged copies detected in *D. simulans* (WU *et al.* 1988; CABOT *et al.* 1993).

In cage competition experiments *Drosophila* bearing *Rspⁱ* chromosomes showed reduced fitness compared to *Drosophila* carrying the *Rsp^s* chromosome from which the *Rspⁱ* chromosomes were derived, suggesting that these *Rsp^s* repeats have some advantageous, if not essential, wild type function (WU *et al.* 1989).

***Drosophila* Spermatogenesis**

The only phenotypic evidence of interaction between *Sd* and *Rsp* exists within the very specific context of spermatogenesis. Unlike other cells in the adult animal, during the course of their development sperm not only become haploid but also undergo a complete remodeling of every cellular organelle including the nucleus and the mitochondria (FULLER 1993b). Understanding the nature of the *Rsp-Sd* interaction requires consideration of the specific environment in which it takes place.

Spermatogenesis is a complex and highly regulated developmental paradigm. It progresses from the apical tip where the stem cells reside to the basal terminus where

mature sperm exit into the seminal vesicles. The entire process is estimated to take ten days and initiates before pupation (LINDSLEY and TOKUYASU 1980; FULLER 1993b).

The apical tip of the *Drosophila* testis contains approximately 20 post-mitotic cells that comprise the “hub”. The hub cells provide an anchoring point and a signaling center that supports the 5-9 germline stem cells (GSCs) found in the adult *Drosophila* male (WHITE-COOPER 2010). These stem cells divide mitotically to produce *primary spermatogonial cells* (Figure 1.4a). One daughter is pushed away from the hub to become a *primary spermatogonium* while the other remains as a GSC attached to the hub (HARDY *et al.* 1979). The primary spermatogonium is enclosed by two somatically derived *cyst cells* (FULLER 1993b). The spermatogonia undergo four synchronous mitotic divisions producing a cyst of 16 *primary spermatocytes*. The cytokinesis of these divisions is incomplete, causing the cohort of cells derived from a single primary spermatogonium to remain connected by cytoplasmic bridges throughout the course of spermatogenesis, permitting a shared cytoplasmic environment. These gonial divisions are analogous to the divisions in *D. melanogaster* females that produce clusters containing 15 nurse cells and one cell that will form the oocyte (FULLER 1993a).

Almost immediately following mitosis these clusters undergo a pre-meiotic S phase and then enter a G₂ extended growth phase in preparation for meiotic divisions. Because *Drosophila* males lack chiasmata, many of the traditional events of meiotic prophase are not distinguishable (FULLER 1993b). This growth stage lasts approximately 90 hours and marks the activation of testis-specific gene expression (FULLER 1993b; WHITE-COOPER 2010). Virtually all of the products needed for the remainder of spermatogenesis are transcribed during this period, ultimately increasing cell volume

approximately 25 fold (OLIVIERI and OLIVIERI 1965; GOULD-SOMERO *et al.* 1974; LINDSLEY and TOKUYASU 1980). Gene products not required until late spermatogenesis or spermiogenesis are stored in a translationally repressed state (BLUMER *et al.* 2002; WHITE-COOPER 2010).

Once growth is complete, these cells undergo meiotic divisions I and II, generating cysts of 64 haploid *spermatids* (Figure 1.4a). As before, incomplete cytokinesis maintains a shared cellular environment via cytoplasmic bridges. These bridges later open into multiple irregular connections whose role is thought to permit each haploid spermatid access to the full diploid complement of gene products (TOKUYASU *et al.* 1972a; FULLER 1993b).

Chromatin of the post-meiotic spermatid exhibits many heterochromatic marks, including H3K9 and H3K27 methylation, consistent with a generally silenced transcriptional state (Figure 1.4 c,d) (RATHKE *et al.* 2007). A double nuclear membrane with nuclear pores located on the innermost layer, forms post-meiotically. At this point the mitochondria migrate to one side of the nucleus and begin to fuse into two highly interleaved mitochondrial derivatives called the Nebenkern (TOKUYASU 1975). This stage is referred to as the “onion stage” because of the prominent layers of mitochondrial membranes visible with phase contrast microscopy (FULLER 1993b).

Next, during the elongation period, the flagellar axonemes are assembled and the Nebenkern elongate with the axoneme until both of these components extend the length of the sperm tail (TOKUYASU *et al.* 1972a; TOKUYASU 1974; TOKUYASU 1975). Profound alterations in nuclear shape are observed; morphing from spherical to a long, needle-like shape, the nucleus loses much its volume through removal of nucleoplasm and nuclear

membrane, as well as chromatin restructuring (Figure 1.4 a, f) (LINDSLEY and TOKUYASU 1980). The resulting nucleus is only 1/200th of its pre-condensation size (LINDSLEY and TOKUYASU 1980; FULLER 1993b).

During the intermediate “canoe stage” in which the nuclei have initiated these morphological changes but not reached their final needle-like state, the core histones are removed from the chromatin and replaced with small arginine-rich protamines (Figure 1.4f) (JAYARAMAIAH RAJA and RENKAWITZ-POHL 2005). The functional significance of the transition from histones to protamines is unknown, though compacted chromatin may be important for generating a hydrodynamic sperm head as well as protecting the genome from damage. During this process transitional proteins replace core histones (BRAUN 2001; RATHKE *et al.* 2007; RATHKE *et al.* 2010). These proteins are only present briefly and are quickly replaced by protamines. Failure to correctly enact this packaging transition results in dysfunctional sperm.

Interestingly, activating chromatin marks, including H3K4 and H4R3 methylation, are present in post-meiotic spermatids and H4 becomes hyper-acetylated at the onset of the canoe stage (Figure 1.4a, f) (RATHKE *et al.* 2007). These activating marks are proposed to facilitate the opening of chromatin required for the deposition of transitional proteins and removal of histones. Further, these events are concurrent with the presence of DNA nicks and the localization of UbcD6, a *Drosophila* homolog of the yeast DNA repair protein Rad6 (RATHKE *et al.* 2007). Taken together, these data suggest a model for chromatin condensation in *Drosophila* spermatogenesis whereby chromatin becomes de-condensed by assuming a more active transcriptional state and breaks in the DNA allow removal of histones and their replacement first by transitional proteins and

then finally by protamines (JAYARAMAIAH RAJA and RENKAWITZ-POHL 2005; RATHKE *et al.* 2007).

The first visible defects in *Rsp*-bearing nuclei appear during condensation. While the *SD*-bearing nuclei condense normally within a developing cyst, their *SD*⁺ siblings remain un-compacted (Figures 1.1b, 1.4a) (TOKUYASU *et al.* 1977; TEMIN *et al.* 1991). Further, these nuclei do not transition from histones to protamines (KETTANEH and HARTL 1976; HAUSCHTECK-JUNGEN and HARTL 1982), suggesting that a chromatin-related defect might be important to the etiology of segregation distortion.

Historically, radiographic studies suggested post-meiotic transcription was non-existent in *D. melanogaster* males (OLIVIERI and OLIVIERI 1965; GOULD-SOMERO *et al.* 1974; FULLER 1993b). However, recent evidence has emerged that at least a small subset of genes is transcribed during the canoe stage. Firstly, phosphorylated RNA PolII, a mark of active transcription, localizes to canoe-stage spermatids (Figure 1.4f) (RATHKE *et al.* 2007). Secondly, single-cyst quantitative RT-PCR and whole testis RNA *in situ* hybridization detected transcription of at least 24 genes in post-meiotic nuclei just prior to the protamine transition. Most of these proteins are not found in mature sperm and their function in the testis is unknown (BARREAU *et al.* 2008a; BARREAU *et al.* 2008b; WHITE-COOPER 2010; ZHAO *et al.* 2010).

Following condensation, elongated spermatids remain syncytial until individualization. In this stage, the individualization complex (IC), composed of membrane and cytoskeletal components, transverses the length of the spermatid bundle, expels most of the cytoplasm, removes connections between spermatids and invests each spermatid in its own plasma membrane (Figure 1.4a) (TOKUYASU *et al.* 1972a). The

components discarded in this process are left in a “waste bag” at the distal tail-end of the bundle. Following individualization, the sperm bundle coils, drawing the mature spermatids into the base of the testis (TOKUYASU *et al.* 1972b). As part of a quality control mechanism, defective sperm within the cyst fail to individualize. These defective sperm, including SD^+ nuclei from SD/SD^+ males are pushed into the waste bag during the coiling process and eliminated (Figure 1.4a) (TOKUYASU *et al.* 1977). Correctly packaged sperm are moved into the lumen and then to the seminal vesicles where they await insemination of a female (FULLER 1993b).

Spermatogenesis is a highly stereotyped complex process with a high degree of genetic regulation. Mutations that affect male fertility have been shown to act at many different points along this pathway. It is interesting to note that though it is classified as a meiotic driver, the effects of SD in spermatogenesis appear to be post-meiotic and related to the improper condensation of haploid SD^+ nuclei. Further, the mis-regulation of chromatin at this juncture suggests that the activity of SD may intersect with factors known to regulate chromatin in gametogenesis (MCELROY *et al.* 2008).

Mechanisms of Drive in Other Systems

Autosomal Drive of the t complex in Mus musculus

Discovered more than 80 years ago, the *tailless* (*t*) locus in mouse shares many similarities with SD in flies (DOBROVOLSKAÏA-ZAVADSKAÏA and KOBOZIEFF 1927). Like SD , the *t complex* is a male-specific meiotic driver that acts to enhance its own transmission by causing dysfunction of the wild type or “+” sperm in heterozygous $+/t$

males (DUNN and MORGAN 1953; BURT and TRIVERS 2008). It is widespread in natural populations of *Mus musculus* worldwide (ARDLIE 1998). Further, *t* is not a simple, one gene locus. Instead, it is part of a larger *t-haplotype* that covers almost a third of chromosome 17, encompassing 1.2% of the entire mouse genome and likely several hundred genes (SILVER 1985; BURT and TRIVERS 2008).

This region is held in tight linkage disequilibrium by multiple inversions, almost completely eliminating recombination between the wild type and *t* chromosomes (Figure 1.5). (LYTTLE 1991). This stability has led to the accumulation of lethal mutations in *t-haplotypes*, making many of these chromosomes homozygous lethal. Those trans-heterozygotes (t^x/t^y) that are viable are completely male sterile. The *t complex* includes at least five identified *T complex distorters* (*Tcds*) and a single *T complex responder* (*Tcr*), which exists as both sensitive and insensitive alleles (Figure 1.5) (LYON 1984; LYON 1991). The distorter loci are thought to act additively or synergistically to impair the motility of wild type sperm. The insensitive allele of *Tcr*, which is found on the *t complex* chromosome alongside the *Tcds*, is thought aid the transmission of the *t complex* by specifically rescuing the sperm motility defect in the *t complex* sperm.

This hypothesis is supported by the findings that at least two *Tcds* encode proteins with homology to Dynein Light Chain proteins of sea urchin sperm (OGAWA and INABA 2003). Further, *Tcr* is a novel protein-coding gene formed by the fusion of *Smok*, a sperm motility kinase, with *Rsk3* (ribosome S6 kinase 3). The *Smok-Rsk3* fusion identified as *Tcr*, likely arose from the duplication of the closely linked *Smok* locus. *In vitro* kinase activity and sequence homology data have suggested that *Tcr* may act as part

of signaling cascade that regulates flagellar function. Transgenic expression of *Tcr* from the Y chromosome in the presence of the *Tcds* results in a excess of sons, suggesting that this locus is sufficient to cause transmission ratio distortion (HERRMANN *et al.* 1999).

In comparison with *SD*, the nomenclature of the *t complex* is somewhat counterintuitive. Unlike *Rsp*, which is the target of *Sd* activity, the dominant allele of *Tcr* is thought to act protectively in *cis*. It can be argued that, in spite of its name, the *Tcr* locus functions more analogously to *Sd* than to *Rsp*. Both *Tcr* and *Sd* are dominant neomorphic mutations, generated through duplication events, whose activity ensures their own transmission. This activity is manifest post-meiotically, when the haploid nuclei are separated. Further, in the absence of *Tcr*, the *Tcds* act only weakly to cause distortion, much like the effect of multiple doses of *E(SD)* in an *Sd*⁺ background (DUNN and MORGAN 1953; LYON 1984). It is also worth noting that the *t complex* carries a number of 75kb repeat elements in the region of *Tcr*. The T66 family elements differ in both copy number and dispersion between wild type and *t complex* chromosomes with the *t complex* containing a higher copy number and more tightly clustered elements. This observation led the authors to speculate “that portions of individual T66 homology units could be involved in *t* haplotype effects on sperm differentiation” (SCHIMENTI *et al.* 1987). However, there is currently no direct evidence implicating these elements in *t complex* transmission ratio distortion.

Stellate Sex Ratio Drive in *D. melanogaster*

The Y chromosome in *D. melanogaster* is not essential for viability. However, X/O males are sterile and accumulate crystalline protein structures in the testes (MEYER *et al.* 1961).

These crystals are composed of a protein encoded by a locus on the X chromosome containing the tandemly repeated *Stellate* (*Ste*) gene. The shape of the crystals corresponds to *Ste* copy number, with high-copy chromosomes producing star-shaped crystals and low copy chromosomes producing needle-like filaments (Figure 1.6d) (HARDY *et al.* 1984). This phenotype is normally suppressed by a locus on the Y, known as *crystal* (*cry*) or *Suppressor of Stellate* [*Su(Ste)*] (LIVAK 1984). The loss of this Y-linked region leads to the de-repression of *Stellate* transcription and crystal formation as well as other phenotypes including reduced fertility, meiotic non-disjunction, and sex-ratio meiotic drive. (Figure 1.6a,c) (HARDY *et al.* 1984; LIVAK 1984; PALUMBO *et al.* 1994).

The *Stellate* locus encodes a protein homologous to the β subunit of the casein kinase 2 (CK2). CK2 is a holoenzyme with known roles in topoisomerase II activity, chromosome condensation and segregation. *In vitro* reconstitution of the enzyme with recombinant *Ste* protein and CK2 α subunit forms an active kinase complex (BOZZETTI *et al.* 1995).

In addition to unique sequence, the *Su(Ste)* region contains repeats of the *Ste* sequence homologous to those found on the X. While *Su(Ste)* is bi-directionally transcribed, it has not been shown to form a functional protein and only the anti-sense transcripts are required for *Ste* silencing (Figure 1.6b,e) (LIVAK 1990; SCHMIDT *et al.* 1999; ARAVIN *et al.* 2001). Further, the silencing of *Stellate* is dependent on the presence of small RNAs homologous to *Su(Ste)* (ARAVIN *et al.* 2001).

The biogenesis of these 25- 27 nucleotide *Su(Ste)* small RNAs requires proteins involved in germline RNAi including Aubergine (*aub*), an Argonaute family slicer

protein, and putative RNA helicases Spin-E (Spn-E) and Armitage (Armi) (SCHMIDT *et al.* 1999; ARAVIN *et al.* 2001; ARAVIN *et al.* 2004; VAGIN *et al.* 2006). Failure to produce these small RNAs through loss of the *Su(Ste)* locus or mutation of these RNAi proteins leads to transcription of *Stellate* and crystal formation (Figure 1.6c,f). In fact, it was the careful analysis of the interaction between *Stellate* and *Su(Ste)* that helped to identify the germline-specific, post-transcriptional silencing pathway known as the piRNA (*Piwi-interacting RNA*) pathway.

These data have led to a model whereby *Su(Ste)* is transcribed bi-directionally, with antisense transcription initiated using the promoter of a nearby *hoppe* (1360) element (Figure 1.6b) (ARAVIN *et al.* 2001; USAKIN *et al.* 2005). In wild type males these transcripts are retained in the nucleoplasm of primary spermatocytes and processed by Aub and Spin-E into primarily anti-sense *Su(Ste)* piRNAs (ARAVIN *et al.* 2004). These anti-sense piRNAs are used to guide post-transcriptional silencing of *Stellate*. Interestingly, *Su(Ste)* piRNAs make up the largest single class of piRNAs associated with Aub in the testes (NISHIDA *et al.* 2007; NAGAO *et al.* 2010). Failure of piRNA-mediated silencing results in the export of *Ste* sense mRNAs to the cytoplasm, production of Ste protein, and the formation of crystals.

The mechanism through which mutations of *Su(Ste)* lead to sex-ratio drive is unclear. The sex-ratio phenotype uncovered in *cry*⁻ males leads to a excess of daughters due to the loss of Y-bearing sperm (HARDY *et al.* 1984). Some have suggested that the severity of distortion is independent of *Ste* copy number on the X chromosome because distortion persists in backgrounds with no detectable X-linked *Ste* copies (BELLONI *et al.* 2002). However, chromosomes with intermediate copy numbers of *Ste* do exhibit more

drive than low copy number chromosomes (PALUMBO *et al.* 1994). It is not possible to assay drive in high copy individuals because they are sterile. While the relationship between *Ste* and sex-ratio drive remains uncertain, it is interesting to note that mutations in *aub* have been specifically shown to enhance sex-ratio drive in *cry*⁻ males (SCHMIDT *et al.* 1999).

Winters Sex Ratio Drive in *D. simulans*

As in the *Stellate* sex-ratio system, silencing of the drive locus by a suppressor in the Winters system in *D. simulans* appears to be mediated by homology of the repetitive sequences transcribed from these loci (Figure 1.7) (TAO *et al.* 2007a; TAO *et al.* 2007b). The drive locus, *Dox* (*Distorter on X*) and an autosomal suppressor, *Nmy* (*Not much Yang*) were identified in flies isolated in Winters, CA. These two loci arose from sequential transposition events from an additional X-linked gene identified as *Mother of Dox* (*MDox*) (Figure 1.7c). Males of the genotype *Dox, nmy* have an active driver and an inactive suppresser and exhibit loss of Y-bearing sperm.

These sperm show post-meiotic defects as early as the onion stage where large vacuoles are observed. These vacuoles persist into the early elongation or canoe stage where they appear to disrupt normal chromatin condensation. Further, during individualization, the progression of the individualization complex appears to be blocked by these vacuoles causing the sperm to remain syncytial. Failure to individualize results in elimination via the waste bag (TAO *et al.* 2007b).

Interestingly, the mutations of *nmy* that fail to suppress drive differ from *Nmy* only in the loss of a single copy of an inverted repeat (IR) (Figure 1.7b) (TAO *et al.*

2007b). The repeat region needed for *Nmy*-based suppression is homologous to a repeat region of *Dox*. Additionally, *Dox*-mediated distortion requires the presence of a subsection of this repeat region which is absent in non-distorting *dox* alleles (TAO *et al.* 2007a; TAO *et al.* 2007b). While transcripts of both *MDox* and *Dox* genes have been identified, they have only very limited coding potential. Although genetic tests for involvement of RNAi pathway machinery have not yet been completed, the lack of coding potential of these genes in conjunction with the significance of inverted repeat structures with the capacity to form dsRNA precursors for the production of small RNAs strongly suggests a role for an RNA-based silencing mechanism.

The loci involved in the Winters sex-ratio system have several interesting similarities with loci in the *SD* system. Like *Sd*, the drive locus in Winters system, *Dox*, arose from a transposition event that resulted in the partial duplication of a neighboring gene, *MDox* (TAO *et al.* 2007a). Further, the disruption of spermatogenesis in the Winters system occurs in late spermatogenesis during the chromatin condensation and individualization phase, the same point at which *Rsp*-bearing spermatids fail (TAO *et al.* 2007b). The implication of RNA-based silencing, both in the *Stellate* and *Winters* sex-ratio systems, makes a strong argument for the involvement of RNAi in segregation distortion.

RNAi and Gene Silencing in *Drosophila*

Classically RNAi included two distinct methods of silencing exogenous and endogenous transcripts through the production of siRNAs and miRNAs, respectively

(Figure 1.8a,b) (ZARATIEGUI *et al.* 2007). These small RNAs, as well as the recently described endogenous siRNAs (endo-siRNAs), are derived from larger precursor dsRNAs that are processed by a Dicer ribonuclease and then loaded onto Argonaute family proteins to form an RNA-induced silencing complex (RISC) (GHILDIYAL and ZAMORE 2009). Argonaute proteins have RNA-slicer ability and use siRNAs and endo-siRNAs as guides for cleavage of homologous RNA transcripts to facilitate post-transcriptional gene silencing (PTGS) (LIU *et al.* 2004). miRNA RISC complexes generally act to guide translational repression of target mRNAs in the cytoplasm. In some cases, Argonaute RNA-protein (RNP) complexes also mediate transcriptional gene silencing (TGS) through alteration of heterochromatic states (HALL *et al.* 2002).

There are five members of the *Drosophila* Argonaute protein family, which are divided into two subfamilies PIWI and Argonaute (AGO). The ubiquitously expressed AGO1 and AGO2 constitute the AGO subfamily and produce miRNA and siRNA, respectively. The other three family members, Piwi, AGO3, and Aubergine (Aub), represent the PIWI subfamily and are specifically expressed in the gonads (CARMELL *et al.* 2002; WILLIAMS and RUBIN 2002). These proteins have recently been implicated in a silencing pathway involving the production of a novel size-class of small RNAs known as Piwi-associated interfering RNAs (piRNAs).

In *Drosophila*, these 22-31 nt piRNAs are largely derived from repetitive sequences including satellite repeats and transposons. Moreover, they specifically associate with proteins in the PIWI subfamily, but not with Ago1 or Ago2. Unlike siRNA and miRNA, piRNA formation does not depend on Dicer1 or Dicer2 activity. Disruption of the piRNA silencing pathway results in increased expression of transposons in the

germline, suggesting a specific role for the piRNA pathway in the maintenance of germline integrity against the deleterious effects of transposable element (TE) activity (KALMYKOVA *et al.* 2005; VAGIN *et al.* 2006; BRENNECKE *et al.* 2007; GUNAWARDANE *et al.* 2007).

Ovarian piRNA Biogenesis

While much progress has been made in the last several years to understand the biogenesis, propagation and targeting of the piRNA pathway, much remains uncertain. The function of the PIWI subfamily proteins seems to be both cell type and compartment specific in *Drosophila*, making de-convolution of the individual functions of these proteins particularly difficult (SENTI and BRENNECKE 2010).

Much work has been done in ovarian tissues to understand the distinct mechanisms of piRNA biogenesis and function in somatic and germline cells. The germline cells of the ovary include a cyst of 16 syncytial cells, 15 of which will differentiate into nurse cells and one which will become the oocyte. The somatic cells are mostly follicular and serve to maintain and protect the germline (Figure 1.9a) (MARGOLIS and SPRADLING 1995; DENG and LIN 2001).

Primary piRNA Biogenesis: A Model from Somatic Ovarian Cells

The piRNA pathway in somatic ovarian cells has several distinct features and is considered the best understood model for primary piRNA biogenesis (Figure 1.9b). Piwi is the only PIWI subfamily member active in ovarian somatic cells and it is also the only one shown to localize to the nucleus (COX *et al.* 2000; BRENNECKE *et al.* 2007; LAU *et al.*

2009; SAITO *et al.* 2009). Seventy percent of somatic ovarian piRNAs map to transposons or transposon fragments while the remaining 30% map to genes or un-annotated regions. (LI *et al.* 2009; MALONE *et al.* 2009; SENTI and BRENNECKE 2010).

Transposons are widely dispersed throughout the genome, making it impossible to determine the origin of most transposon-derived piRNAs. Of those transposon-derived piRNAs that can be uniquely mapped from somatic ovarian tissues (15%), most originate from inactive transposon copies or fragments that are found in a few genomic loci known as piRNA clusters (SAITO *et al.* 2009). These pericentromeric or subtelomeric clusters house many mutated, broken or otherwise inactivated transposon copies (BERGMAN *et al.* 2006; BRENNECKE *et al.* 2007). One of the best-studied examples of these clusters, the X chromosome *flamenco* locus, has provided particularly useful insight into the biogenesis of primary piRNAs (Figure 1.10a). More than 90% of the piRNAs generated from this cluster are in an anti-sense orientation relative to the transposons integrated at this locus (BRENNECKE *et al.* 2007; LAU *et al.* 2009; SAITO *et al.* 2009). Moreover, a single P-element insertion upstream of the cluster virtually eliminates production of piRNAs from the entire 180 kb region. The loss of *flamenco*-derived piRNAs results in a concurrent increase in expression of TEs found in the *flamenco* locus (BRENNECKE *et al.* 2007; MALONE *et al.* 2009).

The observation that a single P-element insertion can completely abolish *flamenco* piRNA production and the anti-sense bias of *flamenco*-derived piRNAs argues for a model of primary piRNA production involving a long, single-stranded, anti-sense precursor RNA that is then processed into mature piRNAs (BRENNECKE *et al.* 2007; MALONE *et al.* 2009). These piRNAs are then able to silence functional copies of

transposable elements located elsewhere in the genome through homology-mediated cleavage of transcripts (Figure 1.10a). This mechanism of piRNA biogenesis represents a departure from the well-understood mechanisms of siRNA and miRNA biogenesis which both require a dsRNA precursor.

Interestingly, this pathway has been shown to depend on proper localization of several piRNA pathway components to newly identified cytoplasmic structures known as Yb-bodies (Figure 1.10b) (SZAKMARY *et al.* 2009; ISHIZU *et al.* 2011). The founding member of this structure, the Tudor domain-containing RNA helicase Yb, is necessary for both germline stem cell and somatic stem cell renewal (KING and LIN 1999). These Yb-containing foci localize in close proximity to mitochondria and do not overlap with other known cytoplasmic granules such as P-bodies or U-bodies (SZAKMARY *et al.* 2009). The other identified components include both Piwi and the RNA helicase Armitage (Armi). In the absence of Armi or Yb, Piwi fails to enter the nucleus, is evenly distributed throughout the cytoplasm and is not loaded with piRNAs, leading to derepression of TEs (MALONE *et al.* 2009; OLIVIERI *et al.* 2010; SAITO *et al.* 2010; QI *et al.* 2011). Interestingly, while nuclear localization of Piwi is required for somatic piRNA formation and silencing of TEs, Piwi slicer activity is not (SAITO *et al.* 2010).

The Zucchini (Zuc) protein, identified as both a homolog of mitochondrial phospholipase D and a putative RNA nuclease, is also required for piRNA production and transposon silencing in the soma (PANE *et al.* 2007; MALONE and HANNON 2009; SAITO *et al.* 2009; WATANABE *et al.* 2011). Without Zuc, Piwi remains unloaded and becomes concentrated at the Yb-bodies, suggesting a model whereby piRNA precursor RNAs are exported from the nucleus, processed and assembled into functional Piwi-

piRNA complexes in the Yb-bodies before entry in the nucleus (Figure 1.10b) (OLIVIERI *et al.* 2010; WATANABE *et al.* 2011). As a putative nuclease, Zuc remains a candidate for Dicer-like activity in processing of long precursor RNAs into piRNAs (PANE *et al.* 2007). However, Zuc may also have a role in the recruitment of piRNA machinery to the surface of the mitochondria where it may act as a phospholipase to generate the active mitochondrial signaling molecule Phosphatidic Acid (PA) (Figure 1.9a) (ARAVIN and CHAN 2011; WATANABE *et al.* 2011). The role of mitochondrial signaling in piRNA biogenesis remains to be investigated.

Germline piRNA Pathway in Ovaries and the Ping-Pong Mechanism

The germline cells of the ovary express all three PIWI family members and utilize both the Piwi-dependent primary biogenesis pathway as well as a secondary mechanism of piRNA amplification known as the ping-pong pathway (Figure 1.11) (BRENNECKE *et al.* 2007; WATANABE *et al.* 2011). This pathway relies primarily on Aub and AGO3, which localize to nuage, an electron dense structure located on the cytoplasmic face of the nuclear envelope in nurse cells, but not the transcriptionally silent oocyte (HARRIS and MACDONALD 2001; BRENNECKE *et al.* 2007; GUNAWARDANE *et al.* 2007; SENTI and BRENNECKE 2010). The DEAD-box helicase Vasa is the major marker of the nuage and is required for germ cell development, GSC maintenance and fertility (SCHUPBACH and WIESCHAUS 1989; LIANG *et al.* 1994). In the absence of Vasa, Aub fails to properly localize to this perinuclear compartment (LIM and KAI 2007). It is thought that in the nuage Aub and AGO3 process piRNAs or target transposons for destruction (Figure 1.9c).

Ovarian piRNA production relies on several other nuage components with known roles in fertility or embryogenesis (Figure 1.9c). These include Spin-E (Spn-E), also known as Homeless, a putative helicase containing a DExH ATPase and a Tudor domain (GILLESPIE and BERG 1995; LIM and KAI 2007); Krimper (Krimp), a Tudor domain protein (LIM and KAI 2007); Armitage (Armi), an RNA-helicase (COOK *et al.* 2004; TOMARI *et al.* 2004); Maelstrom (Mael), a DNA binding protein with a putative nuclease domain and dual nuclear and nuage localizations (CLEGG *et al.* 1997; LIM and KAI 2007); Squash (Squ), a putative nuclease (PANE *et al.* 2007); and Cutoff (Cuff), a protein of unknown function with homology to yeast RAI-1, a protein that binds a nuclease and functions in RNA processing (CHEN *et al.* 2007). Mutations in these proteins lead to diverse phenotypes including loss of GSCs, disruption of embryonic axis formation, loss of pole plasm, nuage malformation, increased transposon activation, and reduced piRNA production (SCHUPBACH and WIESCHAUS 1991; COOK *et al.* 2004; CHEN *et al.* 2007; LIM and KAI 2007; PANE *et al.* 2007).

While both Aub- and Piwi- associated piRNAs have a strong bias for uridine at the 5' position (5' U), AGO3-associated piRNAs have a strong enrichment for an adenosine at the 10th nucleotide (A10). Additionally, many AGO3- and Aub-associated piRNAs have a 10 bp region of homology (Figure 1.11a) (BRENNECKE *et al.* 2007; GUNAWARDANE *et al.* 2007; LIM and KAI 2007; LI *et al.* 2009; LIM *et al.* 2009; SIOMI *et al.* 2011). *In vitro* these proteins cleave RNA target strands at the position across from the 10th nucleotide of a guide-stand piRNA (GUNAWARDANE *et al.* 2007). These data suggest a model whereby an Aub protein loaded with 5'U anti-sense piRNA is able to direct cleavage of a complementary RNA target strand and generate sense piRNAs precursors

with a 10A bias that can then be loaded onto AGO3. Following 3' cleavage through an unidentified mechanism, these AGO3-piRNA RISC (piRISC) complexes can then generate additional 5'U piRNAs through homology directed cleavage of anti-sense transcripts (Figure 1.11). This reciprocal amplification of AGO3- and Aub-associated piRNAs has been coined the “ping-pong” mechanism (BRENNECKE *et al.* 2007; GUNAWARDANE *et al.* 2007). Mutations in Aub lead to a nearly complete loss of the ping-pong piRNA signature, while in AGO3 mutants the presence of some Aub-Aub ping-pong pairs, where Aub associates with both the sense and anti-sense partner, results in reduced, but significant, presence of ping-pong piRNAs (BRENNECKE *et al.* 2007; GUNAWARDANE *et al.* 2007; LI *et al.* 2009).

Unlike primary piRNA biogenesis, the ping-pong pathway requires sense transcription of transposable elements to reach its full amplification potential (LI *et al.* 2009; MALONE *et al.* 2009). Therefore, it is not surprising that germline-specific piRNA clusters are transcribed bi-directionally (Figure 1.11b). Additionally, these dual strand clusters contain remnants of the diverse complement of TEs known to be active in the germline. There is one germline cluster, known as 20A, that is transcribed in a strictly anti-sense orientation and the majority of its piRNAs are loaded onto Piwi in manner similar to the soma-specific *flamenco* locus (Figure 1.11b) (GUNAWARDANE *et al.* 2007).

Recently, the germline specific HP1 homolog, Rhino (Rhi), was shown to be required for piRNA processing from dual strand clusters in ovaries (KLATTENHOFF *et al.* 2009). This nuclear protein localizes to dual strand clusters where it promotes bi-directional transcription of long RNAs that likely represent piRNA precursor molecules. Mutations in *rhi* lead to loss of transposon-derived piRNAs as well as activation of the

same TE families that are expressed in *aub*, *ago3* and *armi* mutants (KLATTENHOFF *et al.* 2007; KLATTENHOFF *et al.* 2009; LI *et al.* 2009). Unexpectedly, although Rhi appears to be restricted to the nucleus, mutants in *rhi* show a loss of Aub and AGO3 localization to the nuage, implying that piRNA-mediated silencing and nuage assembly are codependent. This interdependence suggests a model whereby the nuage functions to scan for piRNA homology in mRNAs exiting the nucleus. In this manner, transposon transcripts can be identified and then destroyed by the ping-pong machinery. The absence of transposon-derived piRNAs in this model would result in the failure of Aub and AGO3 to properly localize to the nuage (KLATTENHOFF *et al.* 2009).

The relationship between primary and secondary (ping-pong) piRNA biogenesis in germline cells is not clear. It has been suggested that Piwi-associated primary piRNAs could serve as a substrate for initiation of the ping-pong cycle. To this end, there is some evidence for a weak AGO3-Piwi ping-pong signature (MALONE *et al.* 2009). However, the ping-pong signature is not found in a vast majority of Piwi-associated piRNAs and the absence of functional Piwi has little effect on the biogenesis of most ping-pong partners (BRENNECKE *et al.* 2007; LI *et al.* 2009).

Maternal loading of transposon-specific piRNAs into the developing embryo may provide the initiator piRNAs needed to kick-start the ping-pong amplification loop. This requirement for maternal deposition of piRNAs is evidenced in hybrid dysgenesis. In hybrid dysgenesis animals that paternally inherit a transposable element that is not present in the maternal genome are sterile because they have not received the appropriate complement of piRNA for efficient silencing (BLUMENSTIEL and HARTL 2005; BRENNECKE *et al.* 2008; ROZHKOV *et al.* 2010).

Each of these mechanisms, Rhi-mediated dual strand transcription, primary piRNA input, and maternal loading of piRNAs, may contribute substrates for ping-pong amplification. How a particular cluster is identified for activation by any of these processes remains unclear. Moreover, ovary piRNAs are frequently derived from many locations across a cluster with little noticeable bias, suggesting that initial processing may involve random cleavage of transcripts 5' to a U (SENTI and BRENNECKE 2010). How such events are regulated to provide specific silencing of multiple targets is unknown.

Regulation of PIWIs by Tudor family proteins

PIWI proteins in *Drosophila*, mouse and *Xenopus* contain symmetrical dimethyl arginines (sDMAs), deposited as post-translational modifications, which allow the binding of Tudor (TUD) family proteins (MALONE *et al.* 2009). TUD family proteins help target and/or regulate the function of proteins containing sDMAs (KIRINO *et al.* 2009). Interestingly, TUD proteins have long been known to play an important role in gametogenesis and are a component of the nuage (BOSWELL and MAHOWALD 1985; SIOMI *et al.* 2010). Further, mutation of the enzyme PRMT5, which has been shown to methylate symmetric arginines of *Drosophila* PIWI proteins, phenocopies *aub* mutations and leads to de-repression of transposons (BOSWELL and MAHOWALD 1985; HARRIS and MACDONALD 2001).

The Tudor protein (Tud) contains 11 highly conserved TUD domains. These domains are thought to mediate interactions with sDMAs and are found in many other proteins including several known piRNA pathway components such as Krimp, Tejas (Tej), Spn-E, and Yb (HARRIS and MACDONALD 2001; KIRINO *et al.* 2009). Tudor, itself

interacts directly with Aub and AGO3, while the Tudor domain protein Tej has the potential to simultaneously interact with Aub, Vas or Spn-E by permitting binding at both its N- and C-termini (PONTING 1997; LIM and KAI 2007; SZAKMARY *et al.* 2009; SIOMI *et al.* 2010). Tej localizes to the nuage in ovaries, in a manner dependent on functional Vas and Spn-E. In the absence of Tej, nuage components Spn-E, Mael, Aub, AGO3 and Krimp are mislocalized, providing evidence that Tej functions at an intermediate step of nuage assembly. *Tej* mutants also show a reduction in total piRNA species and derepression of some TEs, implying a regulatory, but not essential, role in piRNA biogenesis (NISHIDA *et al.* 2009; PATIL and KAI 2010).

The potential of Tudor proteins, which frequently contain several TUD domains, to simultaneously bind multiple sDMAs suggests that these proteins may serve as a scaffold for the assembly of multi-protein complexes (Figure 1.9c). This model is particularly appealing in light of the ping-pong pathway, which requires close associations between different PIWI proteins. The model suggests that Tudor family proteins may synchronize piRISC complex assembly in the nuage by bringing together multiple proteins with sDMAs, allowing coordination of piRNA biogenesis or target recognition in the nuage (PATIL and KAI 2010; SIOMI *et al.* 2010).

The piRNA pathway in the Drosophila Testis

Much of what has been learned through study of piRNA biogenesis in the ovary can be applied to the testis. However there are some significant sex-specific differences. In ovaries, germline cells express all three PIWI family Argonautes. In the testis Piwi expression is limited to the somatic hub cells and GSCs at the apical tip (Figure 1.12b,c)

(BRENNECKE *et al.* 2007; NISHIDA *et al.* 2007; NAGAO *et al.* 2010; SIOMI *et al.* 2011).

Thus, mechanistically AGO3 and Aub are the primary effectors of the piRNA pathway in the testis.

Localization of piRNA Biogenesis in the Drosophila Testis

As in oogenesis, compartmentalization of the piRNA pathway appears to serve an important role in its regulation. Aub and AGO3 localize to the perinuclear nuage in primary spermatocytes (Figure 1.12a,b). This localization is interdependent; loss of one protein leads to mislocalization of the other (COX *et al.* 2000; BRENNECKE *et al.* 2007; NISHIDA *et al.* 2007; LI *et al.* 2009; NAGAO *et al.* 2010).

Recently, a novel, perinuclear, nuage-associated particle, dubbed piRNA Nuage Giant Body (piNG-body) was described. The piNG-body is larger than ordinary nuage granules and exhibits co-localization of Aub, AGO3 and Vasa (Figure 1.12d,e). Several other piRNA pathway proteins are components of this body including Tud, Spn-E, Squ, Cuff, and Belle (Bel). Interestingly, Vasa, Aub and Tud occupy the peripheral region of the piNG-body while AGO3 remains inside the body, co-localizing with Vasa only at the border. Neither piNG-bodies nor nuage granules are detected in post-meiotic round spermatids, suggesting that active transcription during the spermatocyte growth phase may be necessary for particle formation (KIBANOV *et al.* 2011). Whether the piRNA pathway remains active post-meiotically is unknown, though transcription is largely suppressed at this stage.

Yb-bodies in ovarian somatic cells are thought to be sites of primary piRNA processing by Piwi. In testes only the somatic hub and cyst cells show Yb expression

(KIBANOV *et al.* 2011). Interestingly, Piwi expression is also specific to somatic cells in the testis, suggesting that an analogous mechanism of primary piRNA biogenesis to that found in ovarian somatic cells may exist in the testis. The majority of piRNAs associated with Piwi in the testes are derived from transposons and other repetitive sequences (NISHIDA *et al.* 2007). However, little is known about the molecular mechanism of Piwi-mediated piRNA production in the testis.

Together these data suggest that, as in ovaries, proper piRNA processing in the testis requires localization of a variety of factors including Argonautes, Tudor family proteins, and RNA helicases to a particular site on the cytoplasmic edge of the nuclear membrane. Protein-protein interactions at this location may function to regulate formation, processing or targeting of piRNAs.

Gene-Specific Regulation by the piRNA Pathway in Testis

Unlike ovaries, where the majority of piRNAs are derived from transposon sequences, in the testis the most common piRNAs have specific gene targets. The most abundant class of piRNAs associated with Aub in the testis is derived from the *Su(Ste)* gene (NISHIDA *et al.* 2007; NAGAO *et al.* 2010). These transcripts make up 70% of Aub-associated piRNAs, with one sequence [*Su(Ste)*-4] comprising the majority (65%) of the *Su(Ste)* piRNAs (NAGAO *et al.* 2010). Further, of more than 65,000 reads, only 31 Aub-associated *Su(Ste)* piRNAs were sense in orientation, supporting earlier observations that processing of anti-sense *Su(Ste)* transcripts is required for suppression of *Stellate* (ARAVIN *et al.* 2001; ARAVIN *et al.* 2004; NAGAO *et al.* 2010). While far fewer (6.4%) of the total AGO3-associated piRNAs originate from *Su(Ste)*, most of these piRNAs are also anti-sense in

orientation (NAGAO *et al.* 2010). Interestingly, though *Su(Ste)* transcripts make up a small fraction of AGO3-associated piRNAs, *ago3* mutants show a strong reduction in *Su(Ste)* piRNA production as well as *Stellate* derepression, indicating that functional AGO3 is required for biogenesis of anti-sense *Su(Ste)* piRNAs (Figure 1.13a) (LI *et al.* 2009; NAGAO *et al.* 2010; KIBANOV *et al.* 2011).

The second most abundant class (10%) of Aub-associated piRNA is derived from two tiny repetitive regions on the X chromosome located between retrotransposons (Figure 1.13b) (NISHIDA *et al.* 2007; NAGAO *et al.* 2010). This piRNA, termed *AT-chX-1*, shows homology to the *vasa* mRNA. Mutations to *aub*, which eliminate *AT-chX-1* piRNAs, cause a two-fold increase in Vasa protein levels, but have no effect on Vasa localization. The anti-sense *AT-chX-1* piRNA also makes up 20% of the total AGO3-associated piRNAs and its production is sharply reduced in *ago3* mutants (Figure 1.13b) (LI *et al.* 2009; NAGAO *et al.* 2010).

Other piRNA pathway proteins involved in the ping-pong pathway have specific effects on the accumulation of *Su(Ste)* and *AT-chX-1* piRNAs. Testes from *krimp* and *spn-E* mutants show a severe loss of both *AT-chX-1* and *Su(Ste)-4* piRNAs, while *armi* mutants only affected *Su(Ste)* piRNA production. Mutations to *squ*, *mael* and *vasa* had a significant, but lesser, effect on the accumulation of these species, while mutations in *zuc*, thought to be involved in primary piRNA biogenesis, had no effect (NAGAO *et al.* 2010). Mutations to *rhi*, important for production of dual-strand cluster piRNAs in the ovary, also failed to alter *Su(Ste)* or *AT-chX* piRNA expression (KLATTENHOFF *et al.* 2009). Piwi is rarely found to associate with either of these classes of piRNAs and *piwi* mutants actually show an increase in *Su(Ste)* piRNAs (VAGIN *et al.* 2006; NISHIDA *et al.* 2007).

This complement of genes, with the exception of *Rhi*, is similar to those required for the ping-pong pathway in ovaries. However the *AT-chX* and *Su(Ste)*-derived piRNAs are primarily antisense and lack a ping-pong signature even in the few detectable sense piRNAs (NAGAO *et al.* 2010). Because Piwi is not co-expressed with Aub and AGO3 in testis, primary piRNA biogenesis, as occurs in ovaries, is an unlikely source of initiator piRNAs in the testis. Further, because *Su(St)* and *AT-chX* piRNAs are not produced in ovaries they cannot be maternally deposited in developing male embryos. Therefore, the biogenesis of these gene-specific, anti-sense piRNA must utilize a distinct, testis-specific mechanism.

Transposon Regulation by the piRNA Pathway in Testis

While transposon-specific sequences represent a smaller fraction of the total piRNAs in testes, it is likely that these piRNAs participate in the ping-pong amplification loop. Only 7% of Aub-associated and 54% of AGO3-associated piRNAs represent transposon-derived species. However, as in ovaries, these transposon-derived piRNAs show strong strand bias, with Aub primarily associated with anti-sense and AGO3-associated with sense transcripts. Additionally, these piRNAs frequently exhibit the 5'U and 10A bias as well as 10 bps of homology between anti-sense and sense strands, indicative of ping-pong amplification (NISHIDA *et al.* 2007; NAGAO *et al.* 2010) (Figure 1.11a). These observations strongly suggest the presence of an active ping-pong pathway in testes for transposon-derived piRNAs. However, some reports indicate only a moderate increase in transposon expression in testes from *aub* and *ago3* mutants (NAGAO *et al.* 2010).

RNAi and Epigenetic Regulation of Silencing

In addition to post-transcriptional gene silencing (PTGS), RNAi has been implicated in transcriptional gene silencing (TGS). In TGS the RNAi pathway contributes to establishment or maintenance of heterochromatin through recruitment of silencing factors, such as HP1 or histone methyltransferases, to the sites of small RNA production (HUISINGA and ELGIN 2009). In *Schizosaccharomyces pombe* TGS occurs through bidirectional transcription leading to production of dsRNA (Figure 1.14). Following processing by Dicer the siRNA is loaded onto an Argonaute protein and becomes part of the RNA Induced Transcriptional Silencing (RITS) complex (VERDEL *et al.* 2004; HUISINGA and ELGIN 2009; VERDEL *et al.* 2009). The RITS complex binds heterochromatin through interactions with methylated H3K9 (SADAIE *et al.* 2004). Additionally, RNA Pol II transcription controls RITS localization by allowing homology mediated binding between the nascent transcript and the Argonaute bound siRNA (VERDEL *et al.* 2004). The RITS complex also may mediate silencing by directly binding DNA homologous to the siRNA (VERDEL *et al.* 2004; VERDEL and MOAZED 2005). The RITS complex recruits the RNA-Directed RNA Polymerase Complex (RDRC), which contains the RNA-dependant RNA polymerase Rdp1. Rdp1 uses nascent transcripts as templates to generate dsRNA. This synthesis of dsRNAs at the site of RITS complex targeting creates a positive feedback loop for generation of additional siRNAs (Figure 1.14) (MOTAMEDI *et al.* 2004). Disruption of this mechanism by mutation of *ago1* results in a reduction of total heterochromatin and the loss of heterochromatin spreading (IRVINE *et al.* 2006). Further, artificial tethering of the RITS complex to chromatin triggers the deposition of H3K9me and Swi6, the HP1 homolog in *S. pombe*, suggesting that in some

instances RNAi can initiate heterochromatin formation (BUHLER *et al.* 2006; BUHLER and MOAZED 2007).

PIWIs as Epigenetic Regulators in Drosophila

In addition their role in PTGS of transposable elements, PIWI subfamily Argonats have been implicated in aspects of TGS. Mutations to *aub*, *piwi* and *spn-E* have been shown to suppress position effect variegation (PEV) (PAL-BHADRA *et al.* 2002; PAL-BHADRA *et al.* 2004). PEV occurs when a normally euchromatic gene is juxtaposed next to heterochromatin, resulting in incomplete gene expression. Suppressors of PEV [*Su(Var)*] are mutations that relieve gene silencing by shifting the chromatin state from heterochromatic to more euchromatic (DORER and HENIKOFF 1994; MARTIN-MORRIS *et al.* 1997). In addition to increased expression of *white*⁺ PEV reporter genes, mutations to *spn-E* result in a profound loss of global H3K9 methylation and a redistribution of HP1 from the highly heterochromatic chromocenter to the euchromatic arms of *Drosophila* polytene chromosomes. Changes in heterochromatic marks are also observed, albeit less strongly, in both *aub* and *piwi* mutants (PAL-BHADRA *et al.* 2004).

Interestingly, Piwi co-localizes with both H3K9me and HP1a on polytene chromosomes and PIWI localization is dependent on the presence of ssRNA. In fact, Piwi is able to bind HP1a both *in vitro* and *in vivo*. This direct interaction is required for PEV because a *piwi* transgene lacking the HP1a binding domain does not rescue suppression of a *white*⁺ reporter gene in a *piwi* null background (BROWER-TOLAND *et al.* 2007). These data suggest a direct role for the piRNA pathway in establishment and/or maintenance of heterochromatin.

In specific contexts, the piRNA pathway also plays a role in epigenetic activation. The subtelomeric region for chromosome 3R (3R-TAS) is partially euchromatic. However, *piwi* mutants show increased HP1a localization and loss of euchromatic marks. This change in chromatin state accompanies a loss of 3R-TAS piRNAs and silencing of a nearby *white*⁺ reporter gene (YIN and LIN 2007). *Stellate* suppression requires active transcription of the *Su(Ste)* locus (ARAVIN *et al.* 2001) and HP1 homolog Rhi promotes expression of long RNAs at dual-strand piRNA clusters in ovaries (KLATTENHOFF *et al.* 2009). While clearly important for suppression, piRNA pathway components also have a role maintaining appropriate levels of transcriptional activation (SAXE and LIN 2011).

An Existing Model of *SD*-piRNA Interaction

In a recent publication, P. Ferree and R. Barbash propose a model in which RanGAP mislocalization prevents nuclear import of *Rsp*-primed piRNA RNP complexes needed to silence the *Rsp* locus (FERREE and BARBASH 2007). This failure to silence the *Rsp* locus could lead to a defect in chromatin condensation originating from the second chromosome (Figure 1.15). This model, however, has several major flaws. First, the defects caused by Sd-RanGAP are primarily associated with nuclear accumulation and not with exclusion from the nucleus (KUSANO *et al.* 2001; KUSANO *et al.* 2003). Secondly, in the testes of *SD* male flies, the defect in transport is not restricted to *Rsp*^s-bearing nuclei, but should also be the state for *Rsp*ⁱ-bearing nuclei. Thus, if import of piRISC is disrupted by Sd-RanGAP, these complexes would fail to silence targets in all nuclei, both *Rsp*ⁱ and *Rsp*^s, leading to a global failure in spermatogenesis, not failure

specific to *Rsp*^s-bearing nuclei. Although it seems unlikely that this particular model is correct, it is clear that the potential intersection of meiotic drive phenomena and the piRNA pathway is a key area of interest in the field.

Open Questions and Current Motivations for This Study

In spite of decades of effort, the manner in which *Rsp* and *SD* interact to cause the sperm dysfunction in segregation distortion remains elusive. While *SD* is now understood to disrupt nuclear transport, the role of *Rsp* is unknown. However, increasing evidence for the role of RNAi in facilitation of other meiotic drive systems suggests that the piRNA pathway may play a role in mediating *Rsp*'s role in segregation distortion.

There are several key pieces of data that suggest a connection between segregation distortion and the piRNA pathway. First, piRNAs derived from *Rsp* sequences were found in association with both Aub and AGO3 in testis extracts (NAGAO *et al.* 2010). Second, many piRNA pathway components are important for fertility and are found on the second chromosome in *Drosophila* (SCHUPBACH and WIESCHAUS 1991; COX *et al.* 2000; SAITO *et al.* 2006; PANE *et al.* 2007; KLATTENHOFF and THEURKAUF 2008). *SD*-second-chromosomes are associated with reduced fertility and are known to carry an unidentified series of modifiers and enhancers (HIRAIZUMI *et al.* 1980; TEMIN 1991; TEMIN *et al.* 1991). Third, the piRNA pathway specifically suppresses repetitive elements in the germline (BRENNECKE *et al.* 2007; GUNAWARDANE *et al.* 2007). *Responder* is an array of satellite repeats and its effect is manifest during spermatogenesis (WU *et al.* 1988). Fourth, a second array of *Rsp* repeats has been found on chromosome

3L in an intronic region of the *AGO3* gene, suggesting a potential role for the *Rsp* repeat in regulation of *AGO3* expression (R. REENAN, personal communication). Fifth, both the *Winters* and *Stellate* drive systems involve an RNAi mechanism (ARAVIN et al. 2001; ARAVIN et al. 2004; TAO et al. 2007a; TAO et al. 2007b).

To evaluate the role of the piRNA pathway in segregation distortion, I have used several mutations of components of the piRNA pathway and determined if these mutations influence the severity of the meiotic drive phenotype of *SD*. I then used ends-out homologous recombination (HR) to specifically test the effect of mutations to the *Aubergine* locus on an *SD* chromosome. Additionally, I have examined the populations of *Rsp* small RNA present in the testis through a PCR-based assay and studied the localization of RanGAP and piRNA pathway components during spermatogenesis.

The studies reveal that mutations in both *aub* and *piwi* act as enhancers of segregation distortion, suggesting a model whereby the normal function of the piRNA pathway acts to prevent the altered transmission ratios that characterize *SD*. This study represents a major step forward in our understanding the molecular mechanism of an archetypal selfish genetic element.

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FIGURES AND FIGURE LEGENDS

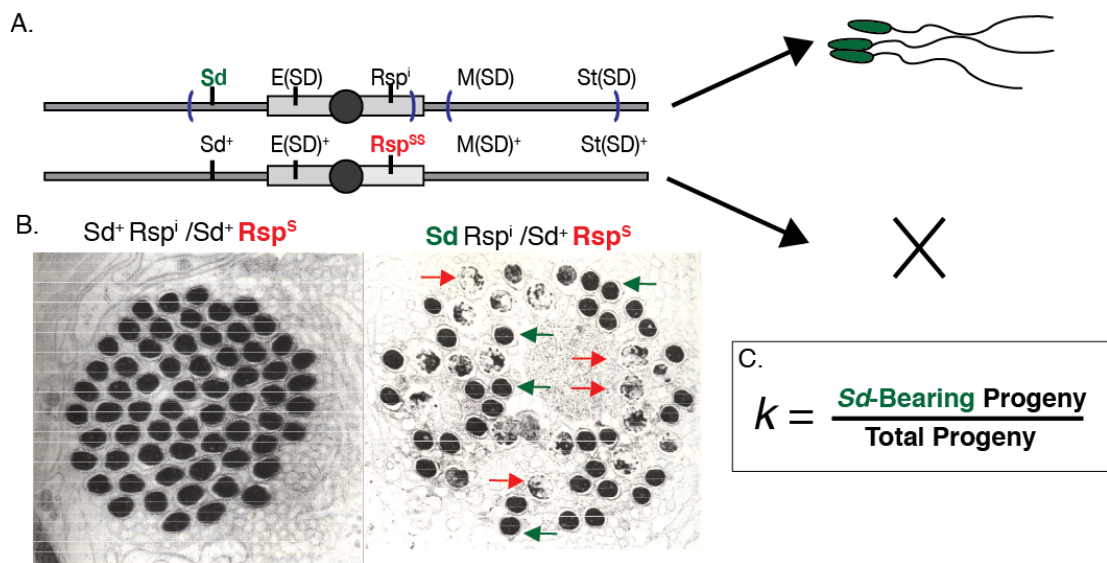


Figure 1.1: Segregation Distortion in *D. melanogaster*. (A) Schematic of chromosome two showing the relative locations of *Sd*, *E(SD)*, *Rsp*, *M(SD)* and *St(SD)*. Thick grey bars represent centromeric heterochromatin. Blue parenthesis represent inversion break points found in some *SD* chromosomes. Only *Sd*, *E(SD)* and *Rsp* have been mapped. Under strongly distorting conditions the *SD* chromosome is packaged into functional sperm and the *Rsp^{ss}* chromosome is lost. (B) Wild type and dysfunctional chromatin condensation. The left panel shows an EM image of a cyst of 64 condensing spermatids from a wild type male. The right hand panel shows the same stage in the testis of the genotype *Sd Rspⁱ / Sd⁺ Rsp^s*. Approximately half the nuclei fail to condense. Adapted from Tokuyasu *et al.*, 1977.

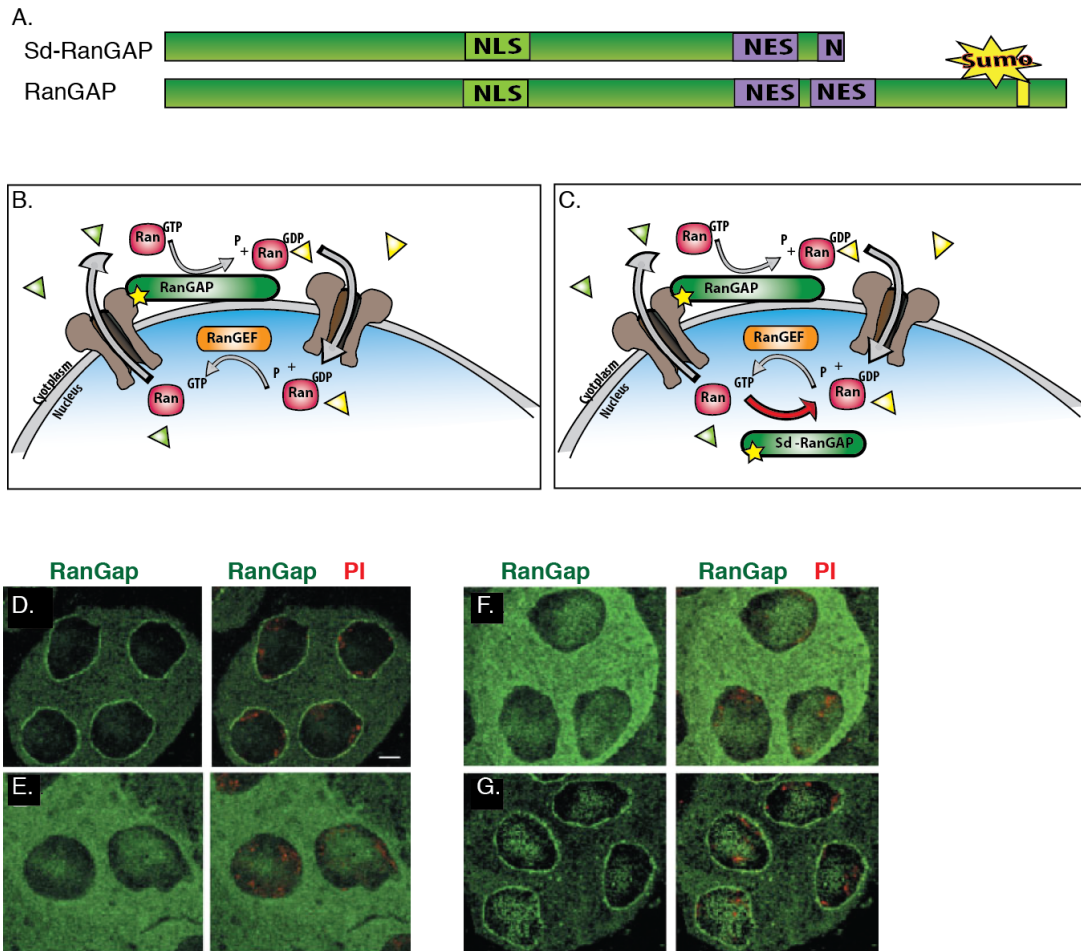


FIGURE 1.2: *Sd* encodes a truncated RanGAP protein. (A) *Sd* encodes a protein that is a 234 aa C-terminal truncation of *Drosophila* RanGAP. This truncation lacks both a Sumoylation site (SUMO), required for localization at the nuclear pore, and part of a Nuclear Export Sequence (NES). (B) A model of wild type nuclear transport. RanGAP is located at the nuclear pore on the cytoplasmic side of the nuclear membrane. There it helps facilitate the hydrolysis of RanGTP to RanGDP required for release of transport cargo from an exportin-Ran complex. RanGDP is then imported into the nucleus via a NTF2 transport receptor. Inside the nucleus RanGEF (RCC1) facilitates Ran exchange of GDP for GTP. GTP bound Ran is then able to bind an exportin-cargo complex and facilitate export. Maintenance of the GTP gradient across the nuclear membrane is therefore required for Ran-mediated nuclear transport. (C) In *SD* flies, Sd-RanGAP is mislocalized to the nucleus where it potentially alters the nuclear GTP concentration, thereby perturbing nuclear transport. The confocal images in D-G are adapted from Kusano *et al.*, 2003. Green shows RanGAP and red is Propidium Iodine (PI) chromatin stain. (D) Anti-RanGAP (green) staining of primary spermatocytes in wild type males shows normal perinuclear RanGAP localization. (E) Anti-HA staining of HA-tagged Sd-RanGAP shows loss of perinuclear localization and increased nuclear staining. (F) Males overexpressing wild type RanGAP from a $\beta 2$ -Tubulin driver exhibit nuclear staining

pattern similar to *SD* males. (G) Males carrying two doses of *E(SD)* also show increased RanGAP localization to the nucleus.

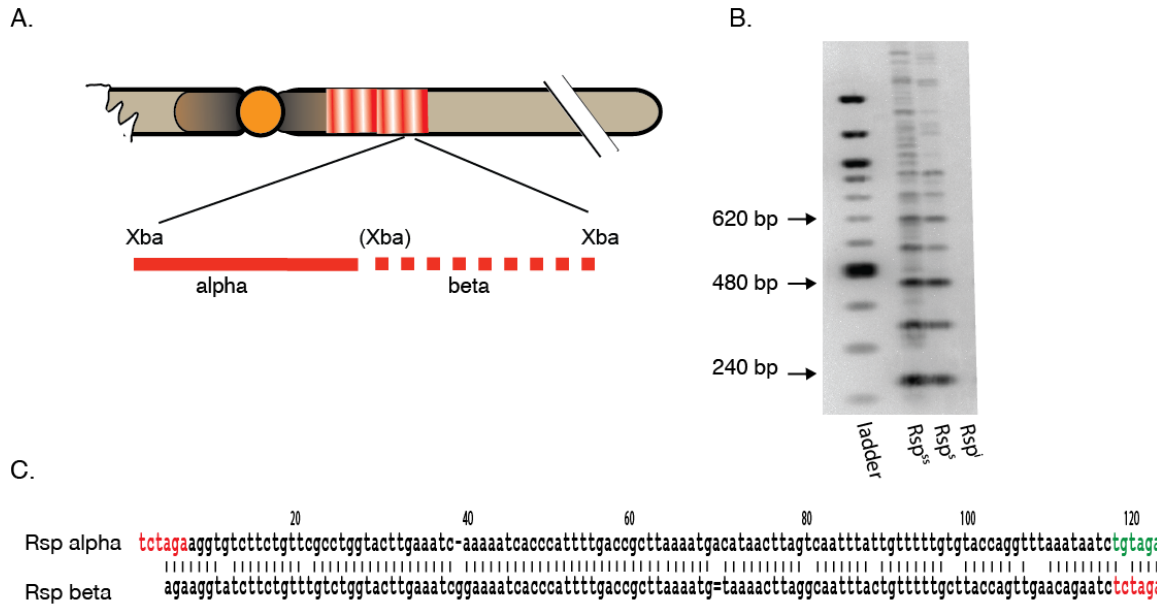


FIGURE 1.3: *Rsp* is a 120 bp AT rich tandem repeat. (A) Top: schematic of the pericentromeric region of chromosome 2R. Bottom: schematic of the *alpha* and *beta* *Rsp* repeats flanked by *Xba*I restriction digest sites. The *Xba*I site between *alpha* and *beta* is mutated in some sequences leading a to ladder effect when genomic *Xba*I digests are analyzed by Southern blot. (B) Example of characteristic banding seen in Southern blots when genomic DNA is digested with *Xba*I and probed with *Rsp* DNA. Bands alternate in intensity representing multiples of 120 and 240 bp repeats. Example of *Rsp*^{SS}, *Rsp*^S and *Rsp*^I are shown. (C) Alignment of the canonical *alpha* and *beta* *Rsp* repeats. *Alpha* and *beta* are 86% homologous. *Xba*I digest sites shown in red.

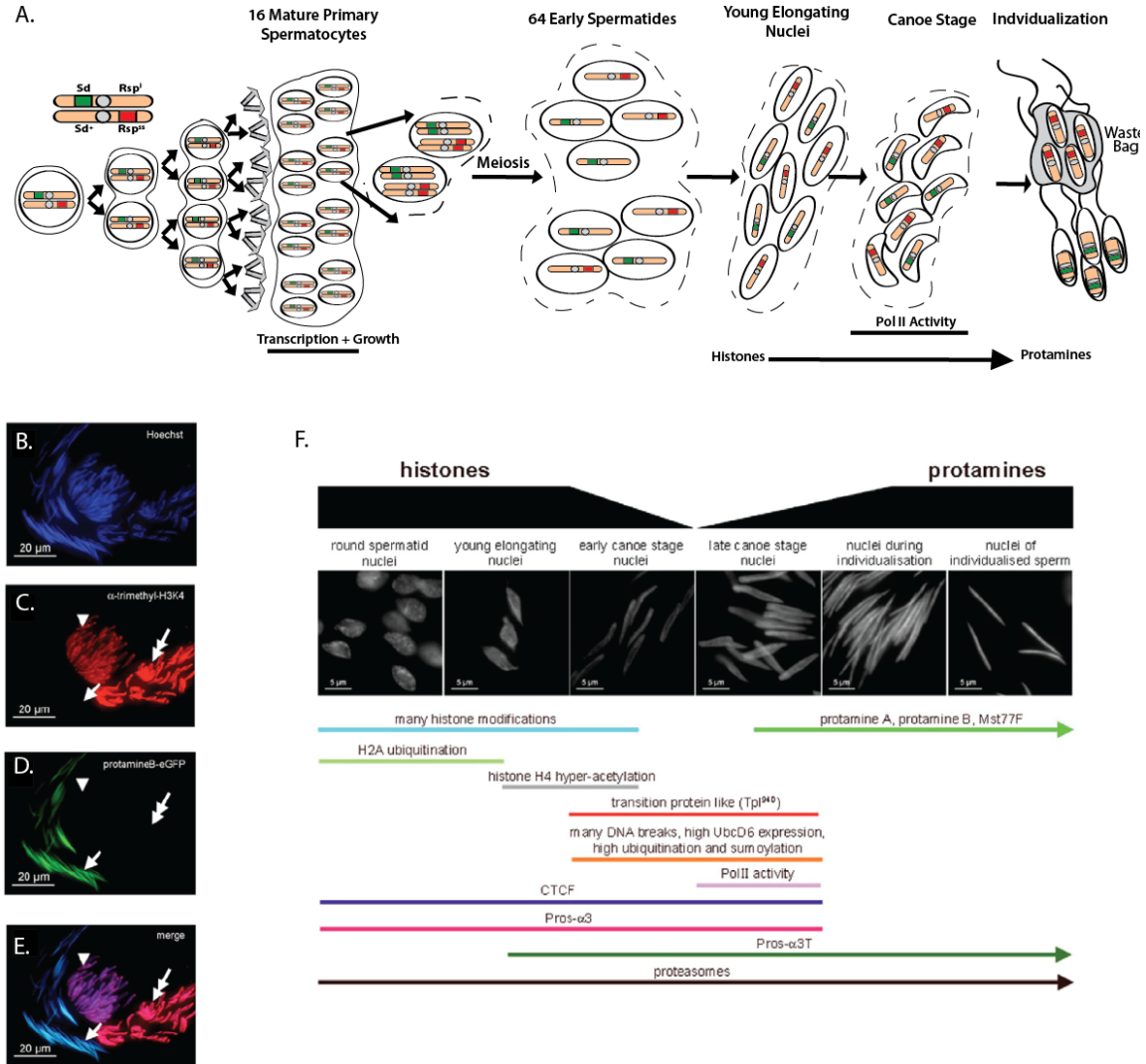


FIGURE 1.4: *Drosophila* spermatogenesis. **(A)** An overview of spermatogenesis: A germline stem cell (GSC) divides mitotically to produce a cyst of 16 mature primary spermatocytes. The spermatocytes enter a period of growth and increased transcription before entering meiosis to become 64 early spermatids. The spermatids mature and condense, exchanging histones for protamines. The waste bag removes *Sd*-bearing spermatids during individualization. **B-F** are reproduced from Rathke *et al.*, 2007. **(B)** DAPI staining of three clusters of spermatids. **(C)** Anti-H3K9me3 strongly stains young elongating nuclei (double arrow) and is reduced during early canoe stage (arrowhead). **(D)** High protamine-eGFP expression is observed in individualizing spermatids (arrow), but not in elongating or canoe stage spermatids. **(E)** Merged image showing three distinct stages of elongation and individualization. **(F)** Overview of histone modifications, histone to protamine transition, Pol II activation, DNA damage, and proteasome subunit activity during spermiogenesis.

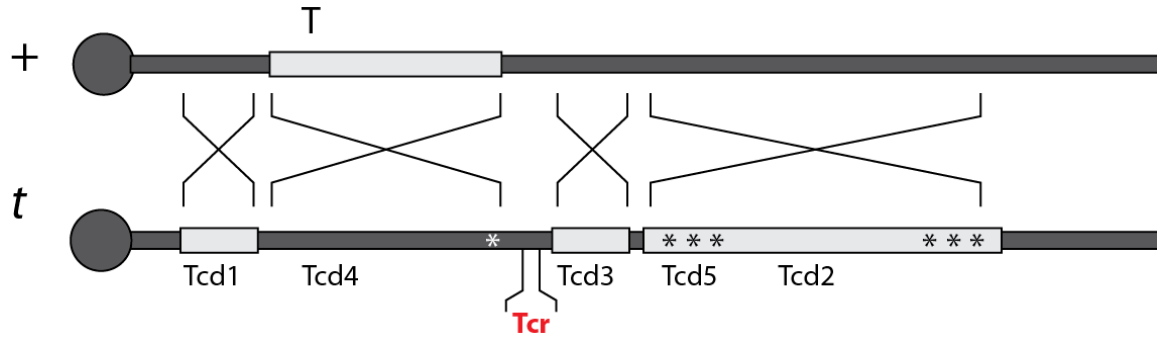


FIGURE 1.5: The *t* complex of *Mus musculus*: There are four inversions (light grey boxes) present in the *t* complex region. One inversion is thought to have arisen on the wild type (+) chromosome and three inversions are carried on the *t* chromosome. The wild type chromosome carries the dominant allele of *Tailless* (*T*) that imparts normal tail size. The *t*-haplotype includes 5 *Tcd* loci as well as the *Tcr*. Stars indicate lethals associated with the *t*-haplotype. Adapted from Burt, A. and R. Trivers, 2006.

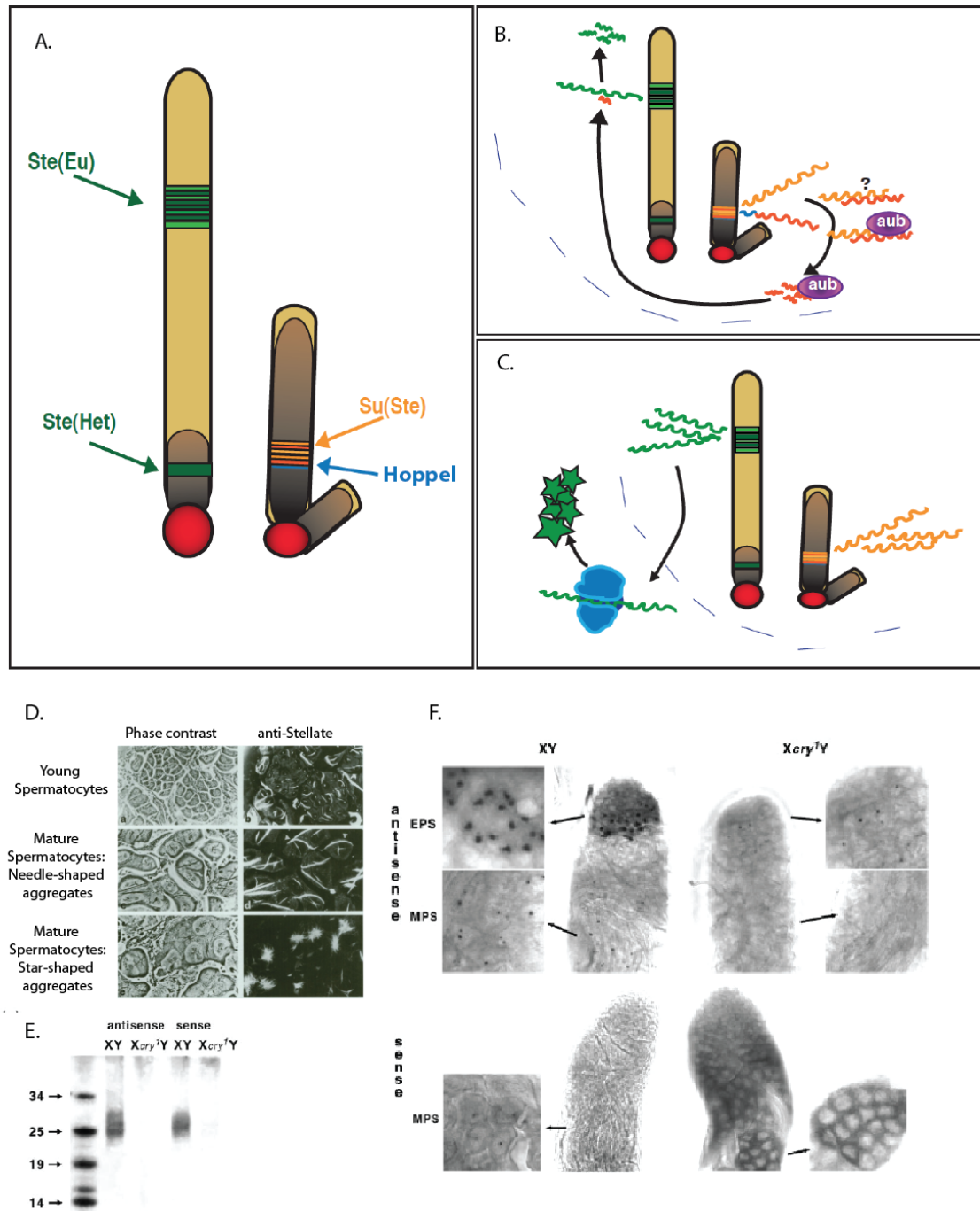


FIGURE 1.6: The role of RNAi in *Stellate* sex-ratio drive. **(A)** The *Stellate* repeat is found at two locations on the X chromosome: a euchromatic location *Ste(Eu)*, which is thought to be the site of most *Ste* transcription, and a location in the pericentromeric heterochromatin that shows only moderate transcriptional activation in X/O or *cry⁻* males. The *Su(ste)* locus on the Y chromosome is adjacent to a *Hoppel* (1360) element with an active promoter. **(B)** In wild type males *Su(Ste)* is transcribed bi-directionally. The transcription in the anti-sense orientation relies on the *Hoppel* promoter. This bi-directional transcription could result in formation of an RNA duplex. The RNA slicer

protein Aub cleaves the anti-sense transcripts into small RNAs that then target homologous *Stellate* sequences for post-transcriptional silencing. (C) In *aub* mutants only sense transcription of *Su(Ste)* is detected. Anti-sense small RNAs are not generated and *Stellate* is transcribed, exported from the nucleus and translated to form crystals. (D) Examples of crystal formation in *cry⁻* males. Left column is phase contrast and right is immunofluorescence staining with an anti-*Stellate* antibody. The top row show crystal formation in young spermatocytes of a male with only moderate *Ste* copy numbers. Middle shows the needle-like crystals in mature spermatocytes of a male of the same genotype. Bottom shows star-like crystals formed in *cry⁻* males with high *Ste* copy number. Reproduced from BOZZETTI *et al.*, 1995 (E) Northern blot for sense and anti-sense small *Su(Ste)* RNAs showing the loss of small *Su(Ste)* RNA in *cry⁻* males. Reproduced from Aravin *et al.*, 2001. (F) RNA *in situ* hybridization of *Stellate* and *Su(Ste)* transcripts in wild type and *cry⁻* males. The top panel shows that the specific expression of antisense *Su(Ste)* transcripts in the early primary spermatocytes of wild type males is lost in *cry⁻* mutants. Note the primarily nuclear localization of the transcript. Bottom panel shows that sense transcript is mostly suppressed in wild type males, and the small amount of sense transcript detected is contained in the nuclei of mature primary spermatocytes. In *cry⁻* animal testis, sense transcript is abundant and primarily cytoplasmic in localization. Reproduced from ARAVIN *et al.*, 2004.

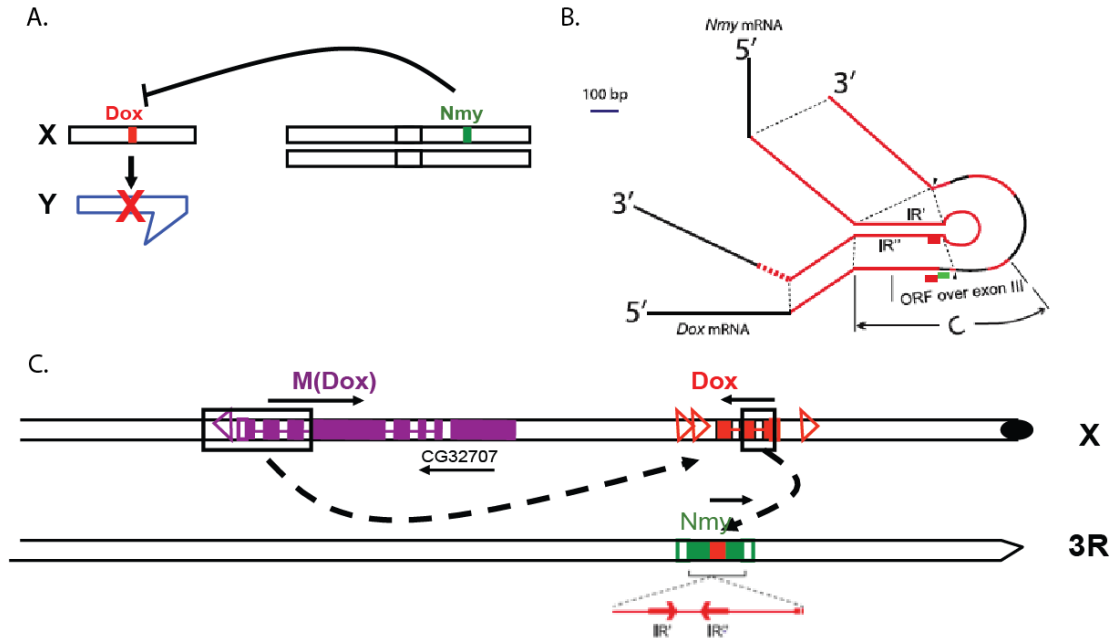


FIGURE 1.7: Role of RNAi in *Winters* sex-ratio drive of *D. simulans*. **(A)** The presence of *Dox* (*Distorter on X*) results in sex-ratio drive and the loss Y-bearing sperm. *Dox* is suppressed by a locus on 3R known as *Nmy* (*Not much yang*). **(B)** A schematic comparison of *Dox* (outer) and *Nmy* (inner) mRNAs. Red lines represent paralogous sequence. Loss of one of the two copies of the inverted repeat (IR) in *nmy* alleles results in a loss of *Dox* suppression. These IRs could fold back to form a hairpin and potentially act as a dsRNA substrate for RNAi. One of the *Nmy* IRs contains one copy of a 42bp repeat also found in *Dox*. The non-driving *dox* allele has lost one of the two copies of the 42 bp repeat found in *Dox*. **(C)** Evolution of *Dox* and *Nmy*. A transposition event inserted a 3833 kb fragment of *M(Dox)* into the a nearby region on the X to generate the *Dox* locus. *M(Dox)* is transcribed in the opposite direction as CG32707. A 360 bp repeat (open triangles) found near both *M(Dox)* and *Dox* may have facilitated this transposition event. A second transposition event inserted 2041 kb of *Dox* cDNA into the gene CG14370 (green) on 3R to form *Nmy* (Red). Adapted from TAO *et al.*, 2007a, TAO *et al.*, 2007 b.

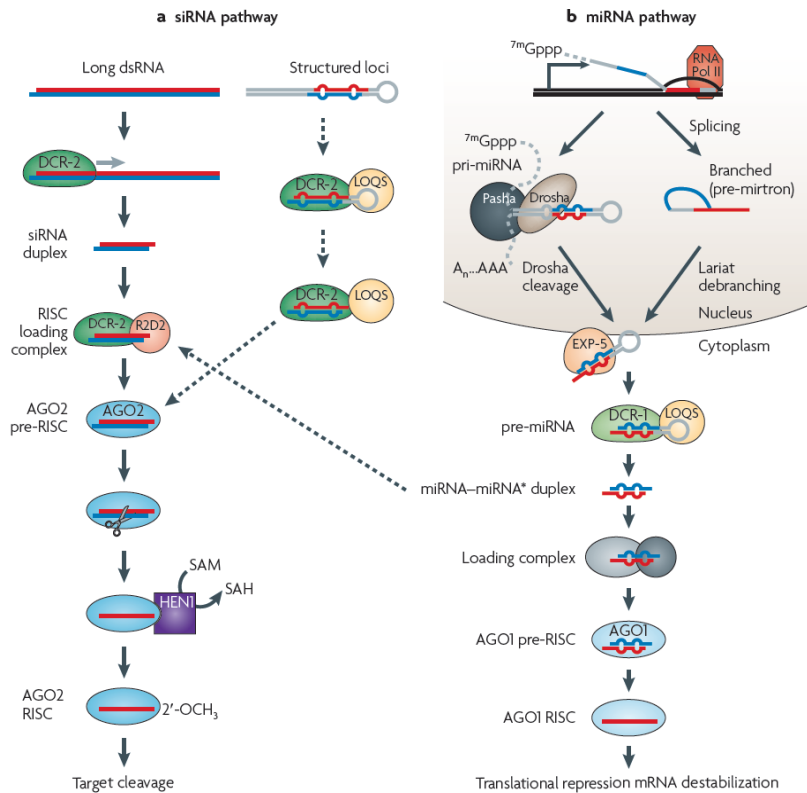


FIGURE 1.8: Overview of siRNA and miRNA pathways in *Drosophila melanogaster*. **(A)** The siRNA pathway requires an exogenous or endogenous dsRNA precursor. The long dsRNA is processed by DCR-2 and R2D2 for perfect duplexes, or DCR-2 and Loquacious (LOQ) in the case of structured esiRNA precursors, and loaded onto AGO2. The Argonaute cleaves the passenger strand and the methyl transferase HEN1 adds a 2' methyl group to the siRNA. Loaded AGO2 forms the RNA induced silencing complex (RISC), which cleaves targets homologous to the guide RNA. **(B)** Transcription of genomically encoded miRNAs generates a primary miRNA (pri-miRNA) which is processed by Pasha and Drosha into a precursor miRNA (pre-miRNA). Alternatively, a class of miRNAs, known as mirtrons, can be processed from introns into pre-miRNAs during splicing. Pre-miRNAs are exported from the nucleus and processed by DCR-1 to generate small duplex RNAs and loaded into AGO1. The AGO-RISC mediates translational repression of miRNA targets. Reproduced from GHILDIYAL, M. and P.D. ZAMORE, 2009.

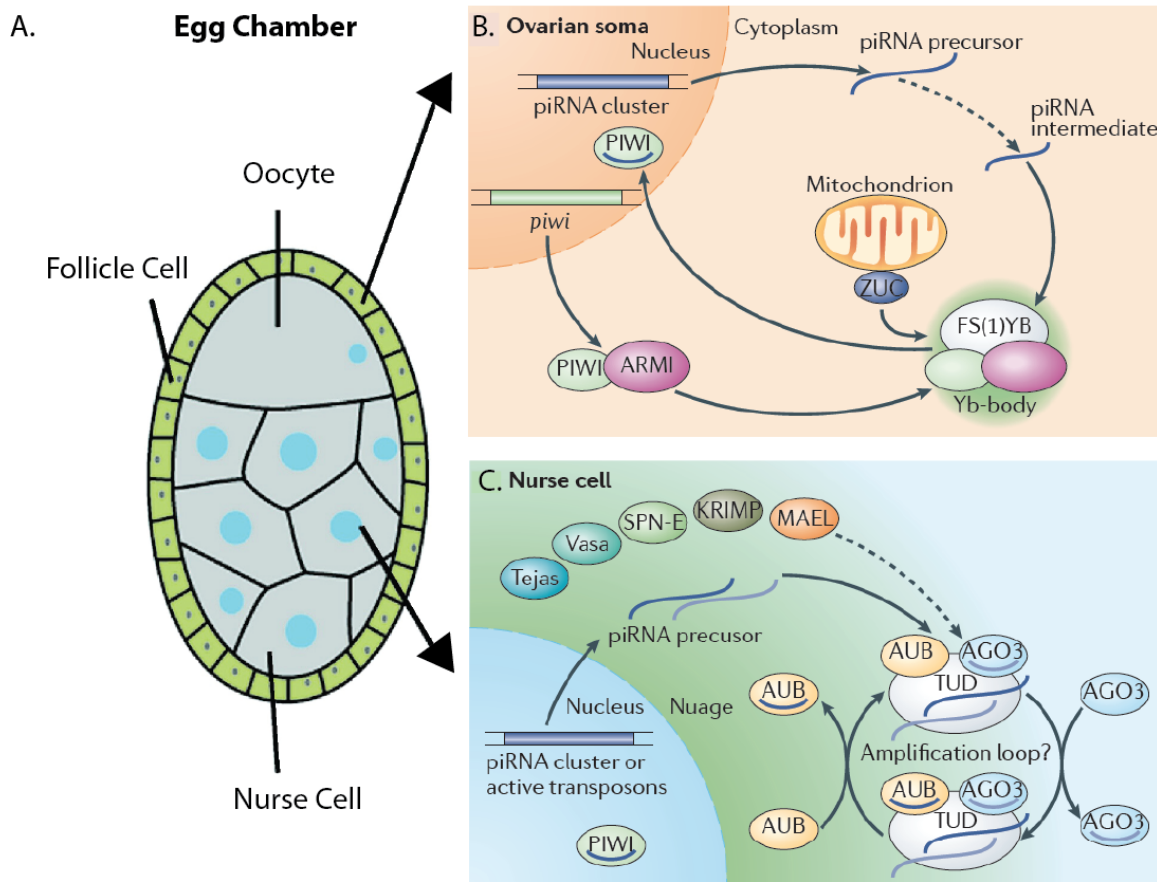


FIGURE 1.9: Biogenesis of piRNA in the *Drosophila* Ovary. **(A)** Schematic of an egg chamber with 15 nurse cells and oocyte. Adapted from ISHIZU *et al.*, 2011. **(B)** and **(C)** were reproduced from SIOMI *et al.*, 2011. **(B)** piRNA biogenesis in the ovarian soma. Primarily antisense transcripts from piRNA clusters are exported to the cytoplasm where they are processed and enter the Yb-body. Here, Piwi is loaded with piRNAs and the Piwi-RISC then returns to the nucleus to initiate gene silencing. **(C)** In germline cells piRNAs are processed in the nuage, an electron-dense perinuclear structure containing multiple protein components. Here the Tudor (TUD) protein may act as a scaffold to bring together Aub and AGO3 which bind anti-sense and sense piRNAs respectively. These proteins work in tandem to generate piRNAs from reciprocal RNA strands of a bi-directionally transcribed piRNA cluster. AGO3 binds piRNAs, which guide cleavage of anti-sense targets bound by Aub. Aub can then reciprocally target AGO3-associated sense RNA, creating an amplification loop known as the ping-pong pathway.

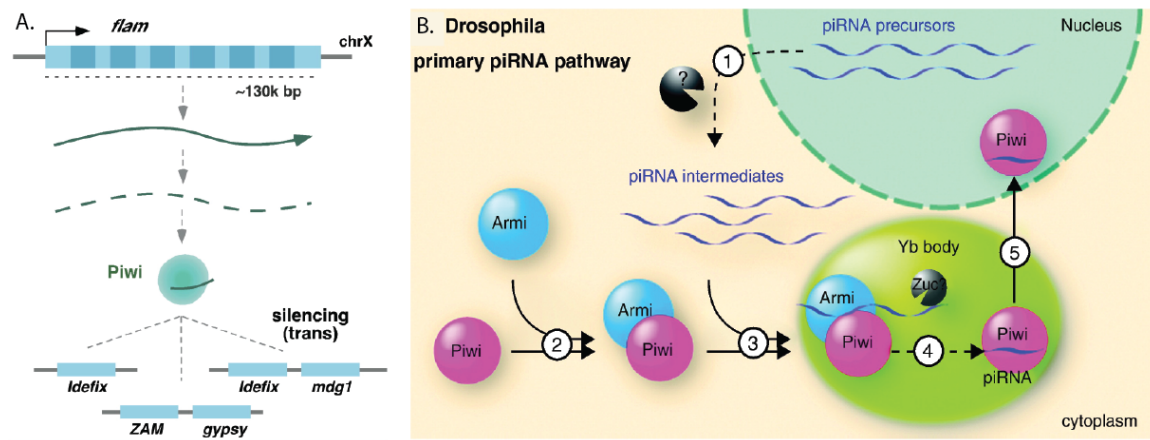


FIGURE 1.10: Biogenesis of piRNA in the somatic cells of *Drosophila* ovaries. **(A)** The *flamenco* locus is transcribed in the anti-sense orientation to generate a long precursor RNA. Piwi processes this RNA and the Piwi-RISC can then silence transposable elements in trans via sequence homology. Reproduced from SIOMI *et al.*, 2011. **(B)** Overview of Piwi mediated silencing in the Yb-body. Long piRNA precursors are processed via an unknown mechanism and then associate with Arm and Piwi in the Yb-body where Zuc could further process them into mature piRNAs. Piwi then returns to the nucleus to facilitate gene silencing. Reproduced from ISHIZU *et al.*, 2011.

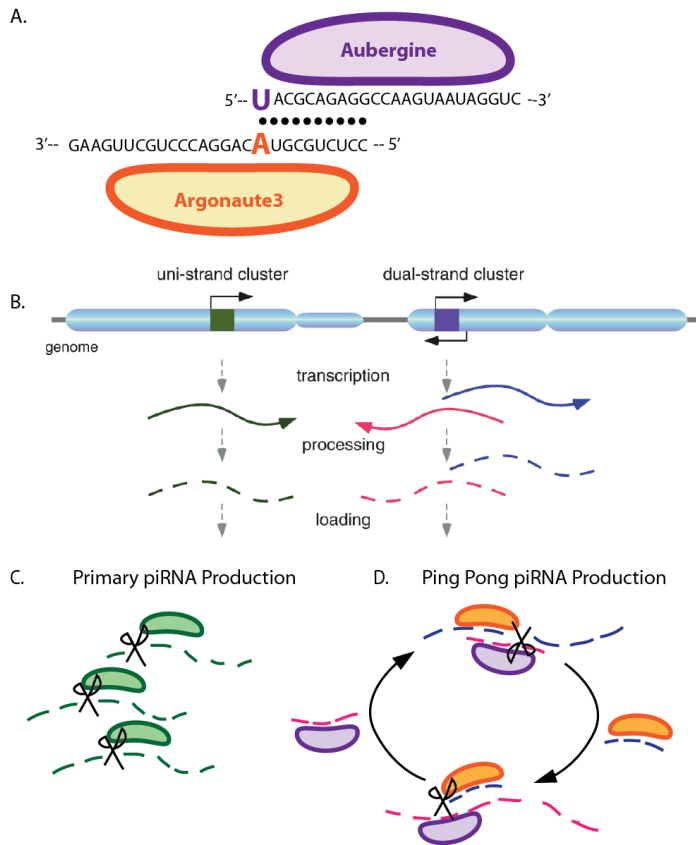


FIGURE 1.11: The ping-pong pathway of secondary piRNA biogenesis. **(A)** Aub is primarily associated with anti-sense piRNAs containing a 5'U while AGO3-associated piRNA are generally sense and show a strong bias for an A at the tenth nucleotide. These sense and anti-sense ping-pong partners frequently show 10 base pairs of homology in their 5' ends. **(B)** Germline piRNAs are produced from both dual-strand and uni-strand piRNA clusters in the germline. These clusters produce long precursor RNAs that require further processing before entering the piRISC complex. **(C)** Primary piRNAs are generally processed by Piwi. **(D)** The ping-pong pathway requires Aub and AGO3 to process reciprocal strands. **A, C and D** are adapted from GUNAWARDANE *et al.*, 2007, **B** is reproduced from SAITO, K. and M. C. SIOMI, 2010.

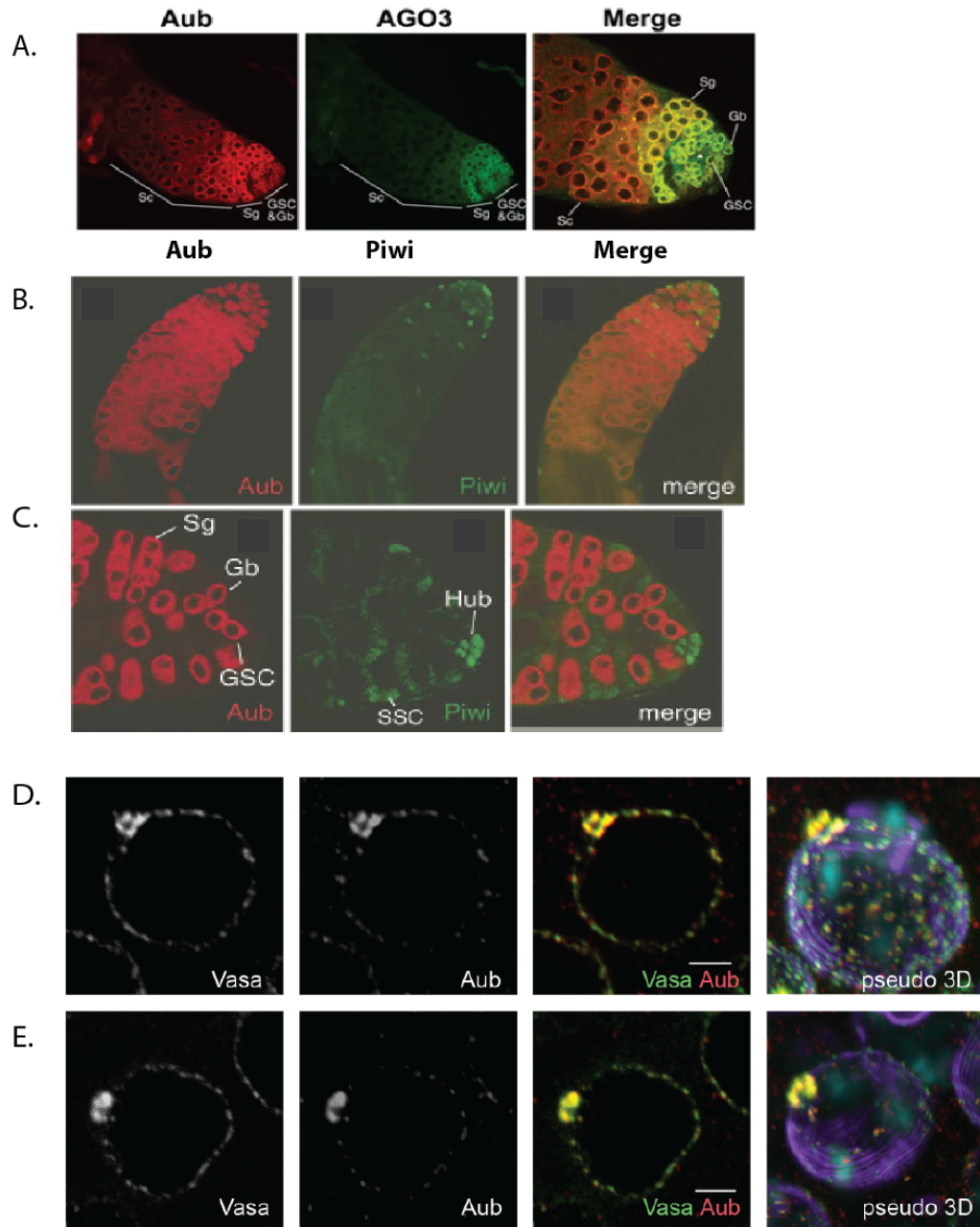


FIGURE 1.12: Localization of PIWI family proteins in the testis. **(A)** Reproduced from NAGAO *et al.*, 2010. Both Aub and AGO3 localize to the perinuclear region of spermatogonia (Sg) and primary spermatocytes (Sc). **(B)** and **(C)** are reproduced from NISHIDA *et al.*, 2007. Aub and Piwi localizations do not overlap in the testis. Piwi shows nuclear localization primarily in hub and somatic stem cells (SSC), while Aub is perinuclear and found in the GSC, spermatogonia (gonial blasts) and spermatocytes. **D** and **E** are reproduced from KIBANOV *et al.*, 2011. The piNG-body is a single large locus showing high levels of Vasa, Aub and AGO3 staining.

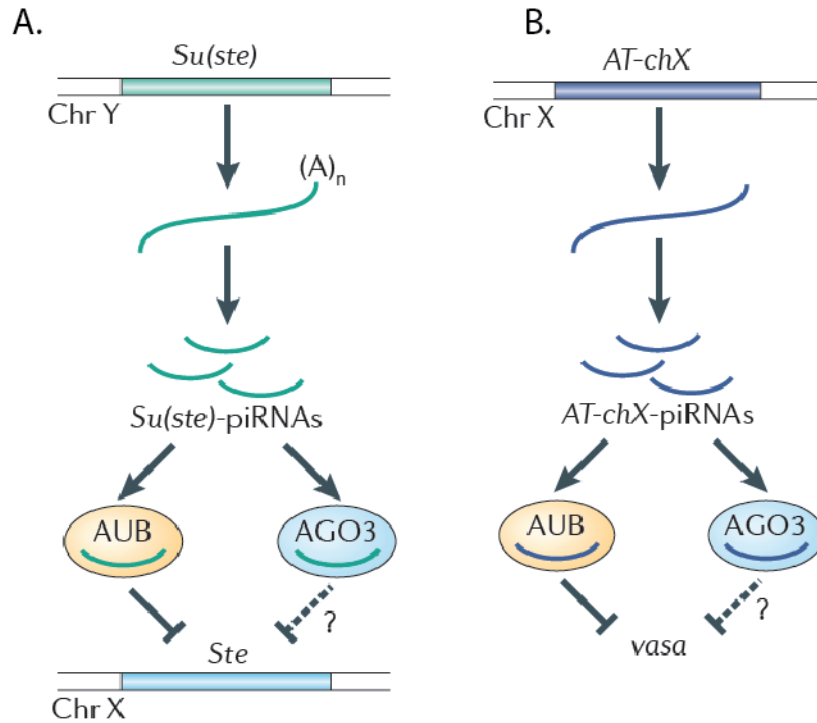


FIGURE 1.13: Gene-specific regulation by piRNAs in the testis. **(A)** Antisense transcription of *Su(ste)* on the Y chromosome leads to processing by Aub and AGO3, allowing post-transcriptional gene silencing of the X-linked *Stellate* locus. **(B)** Transcription of the *AT-chX* region on the X chromosome leads to piRNA processing and homology mediated repression of *vasa*. Reproduced from SIOMI *et al.*, 2011.

A.

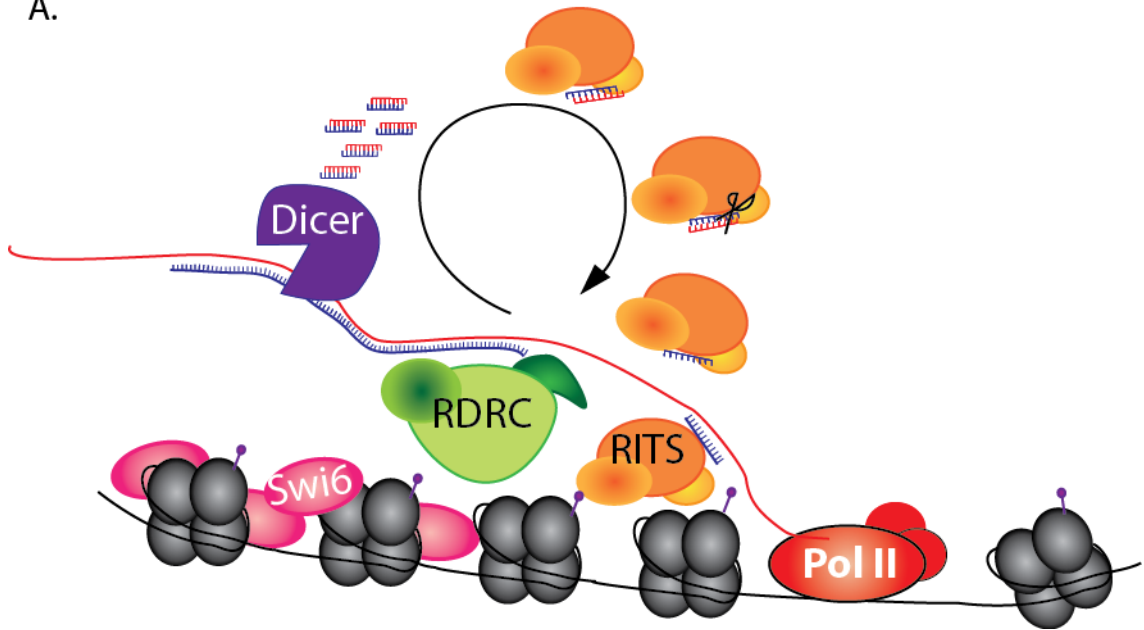


FIGURE 1:14 A model of transcriptional gene silencing in *S. pombe*. siRNA-dependent heterochromatin formation is initiated by Dicer-mediated cleavage of dsRNAs. These RNAs are then loaded onto AGO as the passenger strand is removed (scissors) to form an active RNA-Induced Transcriptional Silencing (RITS) complex. This complex interacts with H3K9me (purple lollipop) allowing RITS to associate with heterochromatin. Additionally, homology between the siRNA and nascent RNA transcripts allows specific targeting. The RITS complex recruits the RNA-Directed RNA polymerase Complex (RDRC), which generates dsRNA. This dsRNA can in turn be processed by Dicer, generating a positive feedback loop. Adapted from VERDEL *et al.*, 2009.

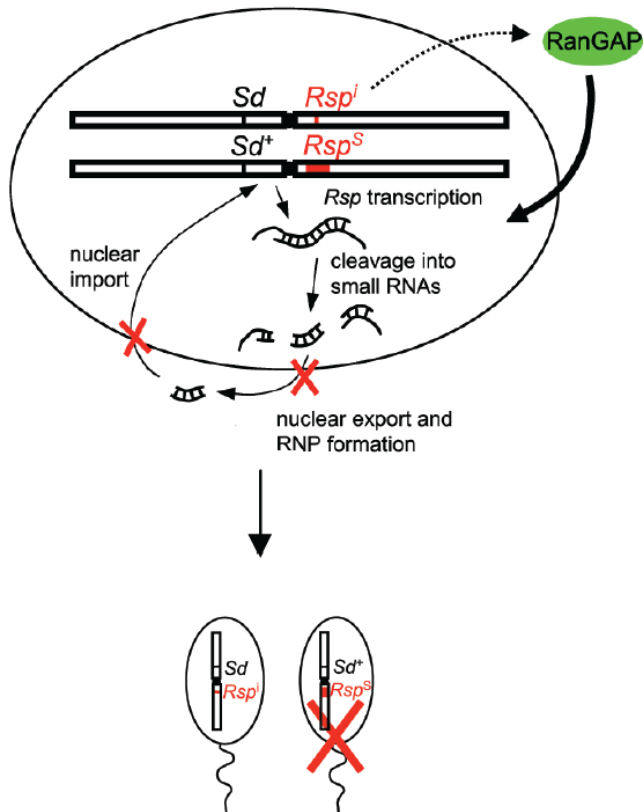


FIGURE 1.15: A published model of RNAi involvement in segregation distortion. The nuclear transport defect caused by *Sd*-RanGAP could block export and formation of *Rsp*-primed RNA-Protein complexes (RNPs) or RISCs. This blockage would prevent import of piRISCs and proper silencing of the *Rsp* locus. Reproduced from FERREE, P M. and D.A. BARBASH, 2007.

	<i>Sd</i>	<i>E(SD)</i>	<i>M(SD)</i>	<i>St(SD)</i>	K-Value
Gene Dosage	1	1	1	1	0.99
	1	1			0.80
	1				0.62
		1	1	1	0.5
		2	1	1	0.74
		1	2	1	0.84

TABLE 1.1: Effect of modifiers on Segregation Distorter. An *SD* chromosome isolated from nature containing all three modifiers exhibits almost complete distortion. Chromosomes with an atypical complement of modifiers were generated through either recombination or X-ray irradiation. These chromosomes show reduced distortion with the loss of *M(SD)* or *St(SD)*. *Sd* alone exhibits only weak distortion with a *Rsp^{ss}* chromosome. In the absence of *Sd*, males with copies of *E(SD)* or *M(SD)* on both homologs show significant distortion. From TEMIN, R. 1991 and C. STABER, unpublished observations.

Chapter II: Mutations in the piRNA pathway component
aubergine enhance meiotic drive of Segregation Distorter
in D. Melanogaster

ABSTRACT

Diploid sexual reproduction involves segregation of allelic pairs, ensuring equal representation of genotypes in the gamete pool. Some genes, however, are able to “cheat” the system by promoting their own transmission. The *Segregation distorter* (Sd) locus in *Drosophila melanogaster* males is one of the best-studied examples of this type of phenomenon. In this system the presence of Sd on one copy of chromosome two results in dysfunction of the non-Sd-bearing (Sd+) sperm and almost complete transmission of Sd to the next generation. The mechanism by which Sd wreaks such selective havoc has remained elusive. However, its effect requires a target locus on chromosome two known as Responder (Rsp). The Rsp locus comprises repeated copies of a satellite DNA sequence and Rsp copy number correlates with sensitivity to Sd. During spermatogenesis, nuclei with chromosomes that contain greater than a several hundred Rsp repeats fail to properly condense and are eliminated. Recently, Rsp sequences were found as small RNAs in association with Argonaute family proteins Aubergine (Aub) and Argonaute3 (AGO3). These proteins are involved in a germline-specific RNAi mechanism known as the Piwi-interacting RNA (piRNA) pathway, which specifically suppresses transposon activation in the germline. To evaluate the role of piRNAs in segregation distortion we have tested the effects of mutations to piRNA pathway components on distortion and specifically targeted mutations to the *aub* locus of an Segregation Distorter (*SD*)

chromosome using ends-out homologous recombination. The data herein demonstrate that mutations to piRNA pathway components act as enhancers of *SD*.

INTRODUCTION

Evolution of sexual organisms relies on the faithful segregation and transmission of alleles from one generation to the next, allowing unbiased exposure of these alleles to selection. Nevertheless, nature contains multiple examples of genes that violate this basic tenet of Mendelian inheritance and act selfishly to ensure their own propagation (LYTTLE 1991). One such phenomenon, known as meiotic drive, occurs when one allele alters the ratio of gamete genotypes to enhance its own transmission into the next generation (SANDLER and NOVITSKI 1957). First discovered more than 50 years ago, Segregation Distorter (*SD*) in *Drosophila melanogaster* is one of the best studied examples of this type of “selfish” genetic behavior (SANDLER *et al.* 1959; TEMIN *et al.* 1991; KUSANO *et al.* 2003).

Segregation Distorter chromosomes contain a dominant gain-of-function mutation that strongly favors the transmission of the *SD* chromosome from [*SD/SD*⁺] heterozygous males by causing dysfunction of wild type (*SD*⁺) sperm (SANDLER *et al.* 1959; SANDLER and HIRAIZUMI 1960b; HARTL *et al.* 1967; TOKUYASU *et al.* 1977; TEMIN *et al.* 1991). While the [*SD/SD*⁺] male transmits the *SD* chromosome to as many as 99% of his progeny, the gametes of heterozygous females show Mendelian segregation (SANDLER and HIRAIZUMI 1959; SANDLER *et al.* 1959; BURT and TRIVERS 2008). The *Segregation distorter* (*Sd*) locus was mapped to the proximal euchromatin of chromosome 2L and identified as a truncated Ran GTPase Activating Protein (RanGAP) (HARTL 1974;

MCLEAN *et al.* 1994; MERRILL *et al.* 1999). Formed by a tandem duplication event, this 234 aa C-terminal truncation remains enzymatically active, but lacks part of a nuclear export signal (NES) and a sumoylation site required for docking at the nuclear pore (MCLEAN *et al.* 1994; MERRILL *et al.* 1999).

While wild type RanGAP is localized to the cytoplasm, Sd-RanGAP is retained in nucleus due to its truncated NES, potentially altering the GTP gradient required for Ran-mediated nuclear transport (GORLICH and MATTAJ 1996; GORLICH and KUTAY 1999a; MERRILL *et al.* 1999; KALAB *et al.* 2002). In the proper genetic background, nuclear localization of even wild type RanGAP is sufficient to cause distortion, suggesting that it is the enzymatic activity of RanGAP in the nucleus that leads to abnormal nuclear retention of Ran cargo and sperm dysfunction in *SD* (KUSANO *et al.* 2001; KUSANO *et al.* 2002).

The relative strength of Segregation Distorter chromosomes is dependent upon several modifiers distributed along the second chromosome (Figure 2.1a). The best studied modifier, *Enhancer of SD* [*E(SD)*], is located in the h35 heterochromatic region of chromosome 2L (GANETZKY 1977; BRITTNACHER and GANETZKY 1984). The presence of the *E(SD)* locus not only strongly enhances drive, but two doses of *E(SD)* result in the accumulation of wild type RanGAP in the nucleus and low levels of distortion even in the absence of *Sd* (TEMIN 1991; KUSANO *et al.* 2002).

The other two modifier loci, *Stabilizer of SD* [*St(SD)*] and *Modifier of SD* [*M(SD)*] are located on 2R. Both enhance drive of an *SD* chromosome, though their mechanisms of action are unknown (SANDLER and HIRAIZUMI 1960a; HIRAIZUMI *et al.* 1980; TEMIN *et al.* 1991).

The sperm dysfunction observed in segregation distortion also depends on the allelic state of the target locus known as *Responder* (*Rsp*) (HARTL 1973; HARTL 1974). *Rsp* loci range from completely insensitive (*Rspⁱ*), which shows normal segregation in the presence of *SD*, to supersensitive (*Rsp^{ss}*), which is almost completely eliminated in the presence of *SD* (GANETZKY 1977; HIRAIZUMI *et al.* 1980; TEMIN and MARTHAS 1984; LYTTLE *et al.* 1986). Located in the heterochromatin of chromosome 2R, *Responder* is composed of an array of 120 bp satellite repeats. Repeat copy number correlates with sensitivity to *SD* (WU *et al.* 1988; PIMPINELLI and DIMITRI 1989; HOUTCHENS and LYTTLE 2003). *Rsp^{ss}* chromosomes are estimated to have several thousand repeats while the *Rsp^s* chromosome is estimated to have approximately 700 hundred copies (WU *et al.* 1988). No completely insensitive *SD⁺* chromosomes have been isolated from nature. However a *Rspⁱ* allele was generated by x-ray ablation of the *Rsp^s* locus (GANETZKY 1977). This *Rspⁱ* allele is reported to have fewer than 20 remaining copies of *Rsp* (WU *et al.* 1988). The loss of *Rsp^s*-containing nuclei occurs during the final stage of spermatogenesis when these nuclei fail to properly condense their chromatin, ensuring that the gametes produced carry the *SD* chromosome (TOKUYASU *et al.* 1977).

The earliest models for the molecular mechanism of *SD* involved direct interaction between the *Sd* protein product and the *Rsp* locus (HARTL 1973). However, following the molecular characterization of these loci, this model appears unlikely. Later it was proposed that the mislocalization of Sd-RanGAP results in a defect in nuclear transport that prevents proper chromatin condensation by upsetting the balance of factors required for the transition from histones to protamines (a sperm specific histone variant) (KUSANO *et al.* 2001; KUSANO *et al.* 2003). In this model the mechanism that specifically

targets only the *Rsp*-bearing sperm for destruction remains unclear. Understanding the function of the *Rsp* repeat in *SD* may be the key to unlocking the molecular mechanism of this phenomenon.

Several recent pieces of data have suggested a possible interaction between the *Rsp* repeat array and the germline-specific small RNA based silencing system, known as the Piwi-interacting RNA (piRNA) pathway. This pathway is facilitated by a distinct subset of Argonaute family RNA slicer proteins in *Drosophila*, known as the PIWI clade (CARMELL *et al.* 2002). These proteins—Piwi, Aubergine (Aub), and Argonaut3 (AGO3)—are specifically expressed in gonads, where they utilize post-transcriptional gene silencing to ensure that transposons and other repetitive elements remain quiescent during gametogenesis (HARRIS and MACDONALD 2001; BRENNECKE *et al.* 2007; GUNAWARDANE *et al.* 2007).

The piRNA pathway acts through a long single-stranded antisense RNA precursor that is cleaved to produce short sequences complementary to transposons or other targets. The Argonaute proteins use these short RNAs as guides to slice transposon mRNAs, thus preventing transposition during gametogenesis (ARAVIN *et al.* 2004; ARAVIN *et al.* 2007; BRENNECKE *et al.* 2007; GUNAWARDANE *et al.* 2007). This mechanism is distinct from both siRNA and miRNA generation in that it is germline specific, involves only Piwi family Argonautes, is Dicer independent, produces a unconventional length of small RNA (23-31 nt) and has a specific enrichment for non-coding, repetitive and transposon-derived small RNAs (BRENNECKE *et al.* 2007; GUNAWARDANE *et al.* 2007; KLATTENHOFF and THEURKAUF 2008; KHURANA and THEURKAUF 2010; SENTI and BRENNECKE 2010).

Given the role of the piRNA pathway in specifically suppressing repetitive elements in the germline, we asked whether the *Responder* array of satellite repeats could be a target of the piRNA pathway. In support of this hypothesis, *Rsp* sequence has been found as piRNA associated with both Aub and AGO3 in *Drosophila* testes (NAGAO *et al.* 2010). Further, two other well studied meiotic drive systems, the *Stellate* system in *D. melanogaster* and the *Winters* system in *D. simulans* are thought to involve small RNA-based silencing mechanisms (ARAVIN *et al.* 2001; ARAVIN *et al.* 2004; TAO *et al.* 2007a; TAO *et al.* 2007b).

To evaluate the role of the piRNA pathway in *SD*, we utilized mutations of several components of the piRNA pathway and determined if these mutations influenced the severity of the drive phenotype of *SD*. In addition, we have used ends-out homologous recombination to introduce mutations into the *Aubergine* locus on an *SD* chromosome and assayed the effects of this mutation on distortion. The studies reveal mutations to both *Aubergine* and *Piwi* act as enhancers of distortion, suggesting a model whereby the normal function of the piRNA pathway acts in part to prevent the altered transmission ratios that characterize *SD*.

MATERIALS AND METHODS

Genetic Stocks

The *Drosophila* stocks were maintained at room temperature on cornmeal molasses food. A complete list of stocks used in this experiment can be found in Table S1.

K-tests

Segregation ratios were measured as described previously (MCLEAN *et al.* 1994). k is the proportion of *SD*-bearing progeny as a fraction of the total progeny (DUNN 1953; GANETZKY 1977). For each cross 20 to 30 males aged less than four days were individually crossed with two *Rsp^s cn bw* virgin females. Crosses were allowed to brood for four days at 25°C and then passed to new food. After an additional 4 days of brooding, the parents were discarded. The progeny classes were counted on days 14, 18, and 22 from the initial cross as in MCLEAN *et al.* 1994. To correct for viability differences between second chromosomes, reciprocal crosses were carried out as previously described (GANETZKY 1977; MCLEAN *et al.* 1994). Single *SD/SD⁺* females were crossed to two *Rsp^s cn bw* males. The progeny counts were used to calculate the viability factor, W , where $W = [SD^{+} \text{ progeny} / SD \text{ progeny}]$. The corrected k value, k_c , is then: $k_c = [SD \text{ progeny} / (SD \text{ progeny} + SD^{+} \text{ progeny} / W)]$ (Figure 2.2b). Significance was calculated using a two-tailed Z-test with a Bonferroni correction.

Generation of Recombinant Chromosomes

Recombinant chromosomes of the genotype $aub^+ Rsp^{mt}$, were generated by allowing exchange between the *aub* mutant (aub^{mt}) chromosomes and the Rsp^{i16} chromosome. Females of the genotype $aub^{mt} Rsp^{mt}/aub^+ Rsp^i$ were crossed with CyO/Sco^{noc} balancer stocks to isolate potential recombinant second chromosomes. Forty isolated second chromosomes were screened by Southern blot for the presence of *Rsp* repeats matching the parental *aub* mutant chromosome.

The *aub* locus of $aub^+ Rsp^{mt}$ recombinants from aub^{HN} , and^{QC} , and aub^{AWE} chromosomes was verified as wild type (aub^+) by sequence analysis. The aub^{N11} chromosome contains an 110bp deletion in the *aub* locus. The presence or absence of this mutation was determined by PCR product size. To generate an $aub^{N11} Rsp^s$ chromosome, males from the line N11-35, previously identified as $aub^{N11} Rsp^i$, were crossed with the standard $aub^+ Rsp^s cn bw$ chromosome and recombinants were isolated and assayed as described above.

Southern Blotting

For Southern analysis genomic DNA was prepared from approximately 30 adult flies using the Maxwell 16 DNA purification system (cat. no. AS1030, Promega, Madison, WI), eluted in 500 µl of DNase-free water (cat. no. 10977-015, GIBCO) and treated with 20 µg RNase A (cat. no. AB-0548, Fisher Scientific). Samples were then concentrated by centrifugation at 8000g for 5 min in Microcon Ultracel YM-50 columns (cat. no. 42416, Millipore, Billerica, MA). The final sample concentration was measured using the

dsDNA HS assay kit with the Quibit 2.0 flourometer (cat. no. Q32854, Life Technologies), and 500ng of DNA per sample was digested for 1.5h at 37°C with *Xba*I, 100 µg/ml bovine serum albumin and NEB Buffer 4 (20 mM Tris-acetate, 50 mM Potassium Acetate, 10 mM magnesium acetate, 1 mM dithiothreitol, pH 7.9) (cat. no. R0145L, New England Biolabs). Digested samples were run overnight on a 1.5% agarose gel (SeaKem LE Agarose, Lonza, Basel, Switzerland) in 0.5x TBE at 55 mV. The gel was washed for 30min in denaturation solution (1.5 M NaCl, 0.5 M NaOH), rinsed with dH₂O and then washed for 30 min in neutralization solution (1.5 M NaCl, 0.5 M Tris-HCl pH 7.5) with gentle shaking. The DNA was transferred overnight to a Hybond-N⁺ membrane (cat. no. RPN203B, GE Healthcare) via capillary action according the protocol in Molecular Cloning (SAMBROOK *et al.* 1989). Following transfer, DNA was fixed to the to the membrane by soaking in 0.4 M NaOH for 2 min. The membrane was pre-hybridized in ECL Gold hybridization buffer (cat. no RPN3006, GE Healthcare) in a Hybaid hybridization oven (Analytical Instruments) at 42° for 30 min.

The *Rsp* probe was generated by PCR amplification of a 238 bp sequence from the canonical *Rsp* repeat cloned into the pBluescript KS⁺ (Stratagene) vector. The vector specific primers, T3 and T7, were used to amplify probe (Table S2). Product was run on a 0.5% agarose gel (SeaKem LE Agarose, Lonza, Basel, Switzerland) and the bands were cut and gel purified using the Wizard SV Gel and PCR Clean-Up System (cat. no. A9282, Promega). Sample concentration was measured with the dsDNA HS Quibit flourometer system (Life Technologies) and labeled using the ECL direct labeling kit according to manufacturer's instructions (cat. no. RPN3005, GE Healthcare). Labeled *Rsp* and 100 bp ladder probes (cat. no N3231L, New England Biolabs) were added to blots at

concentrations of 20 ng/ml and 10 ng/ml respectively in hybridization buffer and incubated overnight at 42°. Following incubation, blots were washed two times for 20 min each with ECL Primary wash buffer (0.4% SDS and 0.5x SSC) at 42°, then removed from the hybridization tubes and washed two more times at room temperature on a shaker with 2x SSC. The signal was generated using ECL detection reagents according to manufacturers instructions (cat. no. RPN3004, GE Healthcare) and detected using the Kodak Image Station 4000R.

Homologous Recombination

We performed ends-out homologous recombination using a similar methodology to that reported previously (STABER *et al.* 2011). Briefly, we utilized the ends-out targeting vector p[w25.2] that contains the *white*⁺ selectable eye color mini-gene flanked by LoxP sites for subsequent removal by Cre-recombinase (Figure 2.2a). Homology arms were cloned and sequenced in pTOPO (Life Technologies) and then shuttled into the multiple cloning sites of the vector to generate p[w25-AUB], which was then introduced into the *Drosophila* genome by standard transgenic methods (Genetic Services Inc.).

The cloning strategy is as follows, where all genomic coordinates are given by the *Drosophila melanogaster* draft assembly, BDGP Release 5, with release 5.12 annotation provided by FlyBase at the UCSC Genome Browser. Arm 1 is the 5' arm of p[w25-AUB], which contains intronic sequence as well as the 5'UTR of CG168333 (Figure 2.2c) and was generated by PCR amplification to incorporate cloning sites as follows: BsiWI-Chr2L:10,995,293-10,997,756-AscI. The last 6 exons of *aub* are contained in 3' arm of p[w25-AUB] (Arm2), which was generated by PCR amplification and

incorporated the following cloning sites; Acc65I- chr2L:10,997,757-11,000,338-NotI (Figure 2.2a).

Mutations were introduced into Arm2 in pTOPO using the Quik-change XL II kit (Agilent Technologies, La Jolla, CA). Once verified, the mutated Arm2 was liberated from pTOPO and ligated into p(w25.2) using the Acc65I and NotI cloning sites. A full list of cloning, mutagenic and sequencing primers can be found in Table S2.

Targeting was performed to generate multiple independent targeting events that incorporate or exclude engineered mutations. To isolate targeting events to an *SD* chromosome, targeting was conducted in flies of the genotype w: *SD*-Los Arrenos/CyO^{*cnbw Roi*}: *hsFlp*, *hsSce*-I/p[w25-AUB]. White or mosaic-eyed females were collected from the heat-shocked vials and then crossed with *yw ey-Flp: noc^{sco}/CyO* males and only red-eyed female progeny were selected for additional validation (Figure 2.2c). Targeted alleles were validated by amplification using primers outside the region of targeting and primers specific to the *w*⁺ mini-gene. All targeted alleles were sequenced to verify that no unintended mutations were introduced.

RESULTS

piRNA pathway mutants are genetic enhancers of SD

In order to test for genetic epistasis between mutations in piRNA pathway components and *SD*, we crossed females carrying an *SD* chromosome with males heterozygous for one of several piRNA pathway mutations including *aub*, *piwi*, *zuc* and *squ*. These loci are located on chromosome 2L (SCHUPBACH and WIESCHAUS 1991; TWEEDIE et al. 2009). Therefore, each mutant chromosome has an associated array of *Rsp* repeats located on chromosome 2R (PIMPINELLI and DIMITRI 1989). Distortion is dependent in part on the number of *Rsp* repeats present, making it necessary to assay the relative number of repeats on these chromosomes (TEMIN and MARTHAS 1984; WU *et al.* 1988). The standard *Rsp^s*, *Rsp^{ss}* and *Rspⁱ* chromosomes each exhibit a characteristic banding pattern when digested with the *Xba*I restriction enzyme and probed on Southern blots with labeled *Rsp* sequence (WU *et al.* 1988). This pattern results from the presence of an *Xba*I digest site at the end of the 240 bp repeat (Figure 2.1b). However, divergence between *Rsp* repeats results in mutations that disrupt the restriction site in some sequences (WU *et al.* 1988; CABOT *et al.* 1993; HOUTCHENS and LYTTLE 2003). These alterations result in larger band sizes increasing in intervals of 240bp (Figure 2.3a).

To determine the *Rsp* repeat status of the mutants used in this study, these chromosomes were compared to the standard *Rsp^{ss}*, *Rsp^s* and *Rspⁱ* chromosomes using an

*Xba*I digest followed by Southern blotting with *Rsp* probe. Strikingly, many second chromosomes mutant for piRNA pathway genes including *aub*^{AHN}, *aub*^{AWE}, *aub*^{HM}, *aub*^{HN}, *aub*^{QC}, *zuc*^{HM}, *squ*^{SG}, and *piwi*⁰⁶⁸⁰³ displayed a banding pattern identical to the standard *Rsp*^s *cn bw* chromosome. These mutant chromosomes also contain the same recessive markers as the canonical *Rsp*^s chromosome, strongly suggesting the original mutagenesis was done in the *Rsp*^s background. We therefore predict that if the mutations do not affect distortion, *SD* should eliminate these mutant chromosomes at a rate equivalent to that of a *Rsp*^s chromosome

To test the sensitivity of the piRNA pathway mutant chromosomes to *SD* we crossed flies carrying an *SD*⁺ chromosome bearing a mutant allele with several well-characterized *SD* chromosomes. Individual F₁ males heterozygous for the *SD*⁺ *aub* mutant chromosome and the *SD* chromosome were then backcrossed with females that contained the same recessive eye color mutations (*cn bw*) that mark the mutant chromosomes (Figure 2.3b). The resulting F₂ progeny were scored by eye color to determine k_c , the fraction of progeny carrying the *SD* chromosome corrected for viability (DUNN 1953; SANDLER and NOVITSKI 1957; MCLEAN *et al.* 1994).

Interestingly, when crossed into weak *SD* backgrounds, all of the *aub* alleles as well as the *piwi* mutant chromosomes showed a significant increase in distortion over the *Rsp*^s control with equivalent levels of repeats (Figure 2.3c) (two tailed Z-test, $p < 0.0001$). In fact, many of these chromosomes were lost at levels equivalent to that seen with the *Rsp*^{ss} chromosome. This result is surprising given that the *Rsp*^{ss} is estimated to contain nearly 10-fold the number of repeats found in the *Rsp*^s background (WU *et al.* 1988). When tested against a strongly distorting *SD* chromosome (*SD*-72), all of the k_c

values were very close to 1.0, making detecting changes in k_c difficult. The *aub^{ec}* chromosome did, however, show a slight enhancement of drive ($K=0.999$, $p < 0.005$) compared to the standard *Rsp^s* chromosome ($K=0.995$). Thus, it appears that *aub* and *piwi* mutants enhance the sensitivity to distortion analogously in increasing *Rsp* repeat copy number.

Mutation of the gene *cutoff* (*cuff*) also significantly enhanced distortion ($P<0.0005$). The function of this gene is unknown. However, it colocalizes with Aub and Vasa in the nuage of both ovaries and testes (CHEN *et al.* 2007). Mutation of *cuff* resulted in the up-regulation of Het-A and Tart retrotransposons but does not affect the production of Het-A- or Tart-derived piRNAs, suggesting a role in targeting or silencing of TEs but not piRNA biogenesis (CHEN *et al.* 2007; KIBANOV *et al.* 2011).

Mutations to the putative RNA nucleases *zucchini* (*zuc*) and *squash* (*squ*) did not enhance distortion. Zuc and Squ have been proposed to have a Dicer-like activity involved in the processing of piRNA precursors in ovarian somatic and germline cells respectively (PANE *et al.* 2007; MALONE *et al.* 2009). In the somatic cells of the ovary *Zuc* functions in production of Piwi-associated primary piRNAs and may serve to help localize the piRNA processing machinery to mitochondria (PANE *et al.* 2007; MALONE *et al.* 2009; SAITO *et al.* 2009; WATANABE *et al.* 2011). *Zuc* is not required for germline piRNA processing in ovaries and its function in the testes is unknown (MALONE *et al.* 2009). In this study, males heterozygous for mutant *zuc* chromosome did not exhibit a significant change in distortion relative to *Rsp^s* controls.

Squ contributes to the production of germline “ping-pong” piRNAs in the ovary (MALONE *et al.* 2009). It is also a component of a testis-specific, electron-dense,

perinuclear structure known as the piRNA Nuage Giant Body (piNG-body), thought to be a major site of piRNA processing and/or targeting in spermatocytes (KIBANOV *et al.* 2011). Unexpectedly, mutation of *squ* led to a significant suppression of drive compared to the *Rsp^s* control ($p < 0.0005$).

The contributions of the aub and Rsp to SD sensitivity are genetically separable

D. melanogaster second chromosomes are known to harbor several unidentified enhancers and suppressors of *SD* (Figure 2.1). Therefore we wanted determine if the enhancement in drive seen in the *aub* mutant chromosomes was specifically due to the introduced mutations versus other properties of the chromosome (SANDLER and HIRAIZUMI 1959; HIRAIZUMI *et al.* 1980; BRITTNACHER and GANETZKY 1984; HIRAIZUMI and THOMAS 1984). We sought to genetically separate the contributions of the *aub* mutation from the *cis* associated *Rsp* repeats. This parsing was achieved by crossing flies with *aub^{mt}* chromosomes containing an associated *Rsp* locus (*Rsp^{mt}*) with an *aub⁺ Rspⁱ* stock. Crossing to a CyO/Sco^{noc} balancer stock isolated individual chromosomes and these isolates were screened by Southern blot for *Rsp* status and by sequencing for the presence or absence of the associated *aub* mutation. Recombinant chromosomes that contained the original *Rsp^{mt}* repeat region, but lacked the associated *aubergine* mutation (*aub⁺ Rsp^{mt}*) were then crossed to *SD* lines and tested for k_c value according to the scheme in Figure 2.3b.

Four alleles of *aubergine* (*aub^{HN}*, *aub^{QC}*, *aub^{NII}* and *aub^{AWE}*) were tested. Most recombinant (*aub⁺ Rsp^{mt}*) chromosomes showed a significant decrease in drive relative to the parental chromosome once the *aubergine* mutation was removed. In tests with several

SD chromosomes and the *awe*-32 recombinant chromosome, an *aub*⁺ *Rsp*^{AWE} derivative of the parental *aub*^{AWE} chromosome, we obtained k_c values between 9.0% and 32.5% lower than the parental chromosome k_c value ($p < 0.00003$) (Figure 2.4e). Due to its relatively low repeat copy number, the *aub*^{N11} parental chromosome exhibited only low levels of sensitivity to *Sd* (Figure 2.3a). However, *aub*⁺ *Rsp*^{N11} recombinants showed a further reduction in drive when tested with *SD*-5 or *SD*-Mad *lt cn* chromosomes (Figure 2.4d). The *aub*⁺ *Rsp*^{HN} recombinant chromosome showed a reduction in drive of more than 50% compared to the *aub*^{HN} *Rsp*^{HN} chromosome (Figure 2.4c). For *aub*^{QC}, three out of four recombinants showed a decrease in k_c value with all *SD* lines tested. Line QC-1 did not show a significant change when tested with *SD*-Los Arrenos and showed only a 2.3% decrease with *SD*-Mad *lt cn*. When tested against *SD* 5*, however, line QC-1 showed a 13% decrease in k_c (Figure 2.4b). The cause of this discrepancy is not known. However, that the recombination event altered the status of another unknown factor that modifies *SD* in some circumstances.

Addition of an aub mutation to the canonical Rsp^s chromosome enhances distortion in trans

The observation that the *aub*^{N11} chromosome has many fewer *Rsp* repeats than the standard *Rsp*^s chromosome provided an opportunity to test whether the addition of the *aub*^{N11} allele to the standard *Rsp*^s repeat is sufficient to increase drive. This experiment was carried out by allowing recombination between the *aub*^{N11} *Rsp*^{N11} chromosome and the *aub*⁺ *Rsp*^s chromosome followed by screening for *aub*^{N11} *Rsp*^s recombinants (Figure 2.5a).

If the *aub* mutation contributes to the degree of drive observed for this chromosome we would expect to see an increase in k_c over the Rsp^s control chromosome. This enhancement was observed in two recovered recombinant lines (Figure 2.5b). Both $aub^{N11} Rsp^s-1$ and $aub^{N11} Rsp^s-2$ showed a statistically significant increase in k -value compared to the Rsp^s control in the *SD*-Los Arrenos background. However, only $aub^{N11} Rsp^s-1$ showed a significant enhancement in the *SD*-Roma background.

Taken together these data strongly argue that the contributions of the specific *aubergine* mutation and its associated repeat array to the overall drive phenotype are genetically separable. Replacement of an *aub* mutation with a wild type allele leads to a reduction in k_c for that chromosome without alteration of its *Rsp* repeat array. Further, placing the Rsp^s array in the context of a aub^{N11} allele leads to a significant increase in drive over that of Rsp^s alone in three out of 4 independent tests (Figure 2.5b).

However, given that multiple known modifiers of *SD*, including $M(SD)$, $E(SD)$, and $St(SD)$ have been mapped to the second chromosome, it is difficult to control for the effect of these unidentified loci using standard genetic recombination (SANDLER and HIRAIZUMI 1959; HIRAIZUMI *et al.* 1980; BRITTNACHER and GANETZKY 1984; HIRAIZUMI and THOMAS 1984).

An aub mutation placed on an SD chromosome by homologous recombination enhances distortion in cis.

In order to remove the confounding effects of unknown modifiers and enhancers from interpretation of genetic experiments, we specifically targeted *aub* mutations to an *SD*-Los Arrenos chromosome using ends-out homologous recombination (HR) (Figure

2.2) (RONG and GOLIC 2000; STABER *et al.* 2011). This technique allows the precise replacement of an endogenous locus with an engineered construct. The p[w25-AUB] construct contains two 2.5 kb “homology arms” cloned from the *aub* locus of *Canton-S* flies. Arm 1 contains mostly intronic sequence from the gene *CG16833*, as well as small portion of the 5’UTR. The *mini-white* selectable marker was placed between the 3’ end of *aub* and the 5’UTR of *CG16833* to reduce the possibility of interference from this insert (Figure 2.2b).

In addition to a wild type construct, two mutations were engineered: an *aub*^{HN} mutation, which changes Q622 to an amber stop codon (CAG → TAG) and the mutation *aub*^{E721A}, which changes the catalytic glutamic acid required for RNA slicer activity to an alanine (GAG → GCG) (Figure 2.2c) (HARRIS and MACDONALD 2001; LIU *et al.* 2004). We selected *SD*-Los Arrenos as the target chromosome because its relatively low *k* value would allow for a sensitized background in which to detect enhancement of distortion.

Following targeting, integration events were verified by PCR followed by sequencing across the length of the arms to ensure no unintended mutations were induced. Additionally chromosomes were tested for the presence of *Sd*-*RanGAP* by genomic PCR across the junction of wild type *RanGAP* and the *Sd*-*RanGAP* duplication (MERRILL *et al.* 1999; ROBINSON *et al.* 2008). Chromosomes identified as carrying both *Sd* and the HR insert were crossed into a *w*⁺ background and then used for subsequent *k* tests.

When placed *in cis* with *Sd* by HR the *aub*^{HN} mutation recapitulated the effect seen *in trans* with males carrying both an *aub*^{HN} mutant second chromosome and an *SD*- Los

Arrenos chromosome. The average k_c for two independent mutations tested against a *Rsp^s* chromosome was 0.940, identical to the k_c for the same mutation *in trans* (Figure 2.6b).

Interestingly the catalytic mutant *aub^{E721A}* also showed an enhancement of k value (0.913), although this effect was not as strong as that from the premature stop codon in the *aub^{HN}* mutant. The average k_c of the wild type construct ($k_c = 0.824$) showed a small but significant enhancement of drive over that of the standard *Rsp^s* chromosome ($k_c = 0.769$, $p < 0.0005$). This enhancement was observed in two independent lines. The *aub* locus of the parental *SD*-Los Arrenos chromosome contains several polymorphisms not found in *Canton-S* or in the *Genbank* reference sequence (BDGP R5.12) (C. STABER, personal communication). Although two of these polymorphisms are intronic and one is a silent mutation, it is possible that replacement of this locus with a wild type *aub* sequence results in this enhancement of drive.

It is also possible that the presence of *mini-white* in the intergenic region 3' to *aub* affects drive. However, removal of the *mini-white* with Cre-recombinase causes a slight increase, not a decrease, in drive (unpublished observation). While the cause of the small alteration in k_c value for *SD*-Los Arrenos *aub^{wt}* chromosomes remains unclear, the change in k_c for the wild type construct is small when compared to the increase caused by the presence of an *aub* mutation.

DISCUSSION

In this study we have shown that mutations to *aubergine* act as genetic enhancers of Segregation Distorter. Chromosomes carrying both a mutation in the *aub* locus and a *Rsp^s* repeat array *in cis* are more strongly distorted than chromosomes with *Rsp^s* alone. Removal of the *aub* mutation by recombination reverses this effect. Further, addition of a *Rsp^s* repeat array to an *aub^{NI}* chromosome, which naturally contains significantly fewer repeats than *Rsp^s*, results in a marked enhancement of drive compared with the *Rsp^s* repeats alone. These independent lines of evidence demonstrate that mutations to both the *aub* locus and the repeat array contribute to the severity of drive, and that these contributions are genetically separable.

Additionally, mutations specifically targeted to the *aubergine* locus of an *SD* chromosome show that the effect of this mutation can be recapitulated *in cis* and is therefore not specific to the *Rsp*-bearing chromosome. A mutation to one of the three catalytic residues of *aub* also produces a significant enhancement in drive, although this effect is not as large as that of the *aub^{HN}* allele, which contains a premature stop codon that truncates the protein upstream of the catalytic residues.

Silencing of repetitive transposon targets in the germline of both testes and ovaries utilizes a mechanism known as “ping-pong” piRNA production because it requires reciprocal cleavage of sense and anti-sense RNAs by AGO3 and Aub proteins respectively (BRENNECKE *et al.* 2007; GUNAWARDANE *et al.* 2007; NAGAO *et al.* 2010).

In the testes these two proteins associate with other piRNA pathway components including Vasa (Vas), Armitage (Armi), and Tudor (Tud) in large electron-dense perinuclear bodies, dubbed piNG-bodies (piRNA Nuage Giant), which are thought to regulate the processing and/or targeting of piRNAs to transposable elements via sequence homology (LIM and KAI 2007; LIM *et al.* 2009; NISHIDA *et al.* 2009; KIBANOV *et al.* 2011). The localization of both Aub and AGO3 to this perinuclear region is co-dependent; loss of one protein through mutation results in mislocalization of the other (COX *et al.* 2000; BRENNECKE *et al.* 2007; NISHIDA *et al.* 2007; LI *et al.* 2009; NAGAO *et al.* 2010).

Interestingly, *Rsp^s* piRNAs have mostly been detected in association with AGO3 in the testis. These piRNAs are derived from a single strand of the *Rsp* repeat sequence (NAGAO *et al.* 2010). The genetic data presented here suggest that wild type function of the piRNA processing machinery suppresses distortion. Hypomorphic mutations, such as *aub^{HN}*, may more profoundly disrupt processing in this pathway than the catalytic mutation *aub^{E721A}*, which could potentially permit proper complex formation, but disrupt piRNA processing or targeting.

This interpretation explains the differential effects of the *aub^{HN}* and the *aub^{E721A}* mutations on drive; the catalytically inactive mutant may allow AGO3-dependent processing at the piNG-body. In contrast the hypomorph, because of the reduced amount of Aub protein or the lack of the C-terminal region, may have a more significant effect on complex formation, and therefore both Aub- and AGO3- dependent processing.

Work in both the *Stellate* and *Winters* sex ratio drive systems has implicated RNAi in mediating suppression of drive (ARAVIN *et al.* 2001; ARAVIN *et al.* 2004; TAO *et*

al. 2007a; TAO *et al.* 2007b). In the *Stellate* sex ratio system anti-sense transcripts of the Y-chromosome *Suppressor of Stellate* [*Su(Ste)*] locus are processed by Aub and AGO3 in the testis of *D. melanogaster* males to generate piRNAs that silence the X-linked *Stellate* (*Ste*) locus by homology-mediated cleavage of sense *Ste* transcripts (ARAVIN *et al.* 2001; ARAVIN *et al.* 2004; NAGAO *et al.* 2010).

In the *Winters* sex ratio system of *D. simulans*, the *Distorter on X* (*Dox*) is suppressed by *Not much yang* (*Nmy*) on chromosome 3R. Suppression requires the presence of two inverted repeats (IRs) in *Nmy*. Loss of a single copy of this 360 bp IR element results in activation of *Dox* and the loss of Y-bearing sperm (TAO *et al.* 2007a; TAO *et al.* 2007b). The two IR copies may be required in order to permit folding of *Nmy* RNA into a dsRNA stem-loop structure that could serve as a substrate for generation of endo-siRNAs or piRNAs (TAO *et al.* 2007b). *Dox* itself carries a single copy of this repeat, suggesting a target for homology-mediated post-transcriptional gene silencing (TAO *et al.* 2007a). Both *Winters* and *Stellate* provide salient examples of systems where RNAi activity is thought to suppress meiotic drive. In line with the observations reported here, the piRNA pathway or other germline RNAi mechanism appears to act protectively to suppress the potentially deleterious effects of these drivers.

In a recent publication, TAO *et al* proposed a model in which RanGAP mislocalization prevents nuclear import of *Rsp*-primed piRNA-induced silencing complexes (piRISC) needed to silence the *Rsp* locus (FERREE and BARBASH 2007; TAO *et al.* 2007a). This failure to silence the *Rsp* locus could lead to a defect in chromatin condensation originating from the second chromosome. While the data presented here certainly support the supposition that the piRNA pathway works to silence *Rsp*

transcription during spermatogenesis, we suggest the following modification to this model.

The defects caused by Sd-RanGAP are primarily associated with nuclear accumulation and not with exclusion of RanGTP-mediated transport cargo from the nucleus (KUSANO *et al.* 2001; KUSANO *et al.* 2003). piRNA biogenesis is thought to require export of a piRNA precursor molecule to the cytoplasm where it is processed into mature piRNAs in the nuage of the ovary or the piNG-body of the testis (Figure 2.7a) (LIM and KAI 2007; KLATTENHOFF and THEURKAUF 2008; KLATTENHOFF *et al.* 2009; KHURANA and THEURKAUF 2010; SAITO *et al.* 2010; KIBANOV *et al.* 2011; WATANABE *et al.* 2011). Nuclear export of RNA-protein complexes (RNPs) requires a class of proteins known as exportins that facilitate directional transit through the NPC (GORLICH and KUTAY 1999b; KOHLER and HURT 2007). In order to export miRNA precursor RNPs from the nucleus, the exportin must also bind RanGTP (YI *et al.* 2003; BOHNSACK *et al.* 2004; LUND *et al.* 2004; KOHLER and HURT 2007). In *SD*, accumulation of nuclear Sd-RanGAP protein depletes nuclear RanGTP through increased GTP hydrolysis, reducing the effective concentration of RanGTP available to bind the exportin-precursor-piRNA complex. Without RanGTP to facilitate exit through the nuclear pore, more RNP complexes are retained in the nucleus, reducing the availability of precursor-piRNA substrates for processing in the cytoplasm (Figure 2.7b). Interestingly, wildtype RanGAP has been observed to co-localize with piNG-bodies in spermatocytes (Figure S2.1), suggesting piRNA processing may be tightly coupled with nuclear transport.

In the germline cells of the ovary, Piwi enters the nucleus where it thought to mediate epigenetic silencing through direct interaction with HP1 and homology-guided

targeting of transposon transcripts (PAL-BHADRA *et al.* 2004; BROWER-TOLAND *et al.* 2007; KLATTENHOFF and THEURKAUF 2008). Further, the role of the interplay between RNAi and maintenance of epigenetic states has been well characterized in *S. pombe* (VOLPE *et al.* 2002; MOTAMEDI *et al.* 2004; VERDEL *et al.* 2004; SUGIYAMA *et al.* 2005; BUHLER *et al.* 2006; VERDEL *et al.* 2009). It is unknown how the generation of piRNAs in the cytoplasm of spermatocytes might contribute to epigenetic silencing. Yet, it seems likely given the known role of RNAi in general, and piRNAs specifically, in chromatin regulation, that mature *Rsp* piRNAs contribute to the epigenetic silencing of the *Rsp* locus through alteration of chromatin states.

Proper silencing of *Rsp* may be required to allow chromatin compaction as well as the transition from histones to protamines, a sperm specific histone variant, necessary for sperm maturation (FERREE and BARBASH 2007; TAO *et al.* 2007a). In spermatids with chromosomes containing large numbers of *Rsp* repeats, silencing may require a significant contribution from the piRNA pathway. The defect in nuclear export caused by Sd-RanGAP may ultimately result in the import of too few *Rsp*-piRNA-primed RNP complexes to facilitate effective silencing, leading to defective chromatin compaction and the specific destruction of *Rsp*-bearing spermatids (Figure 2.7b). Mutation of piRNA pathway components would exacerbate this defect, resulting in a further reduction of the functional pool of piRNAs available for *Rsp* targeting. The data presented here show that as predicted by our proposed model, disruption of piRNA processing through mutation of *aubergine* leads to an enhancement of distortion.

The piRNA pathway is a sophisticated adaptive defense against mobilization of selfish genetic elements in the germline. Thus far, identified targets of this pathway have

mostly been restricted to transposable elements that reproduce through retro-transposition into novel locations in the genome. The data presented here strongly suggest that the piRNA pathway is also able to protect against other types of selfish elements, such as meiotic drivers, which propagate selfishly by destroying gametes of the alternative genotype. Understanding the molecular interaction between piRNA biogenesis and segregation distortion could provide significant insight into the biology of gametogenesis, the etiology of meiotic drive, as well as the evolution of mechanisms to defend against invasion of the genome by this type of ultra-selfish genetic elements.

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FIGURES AND FIGURE LEGENDS

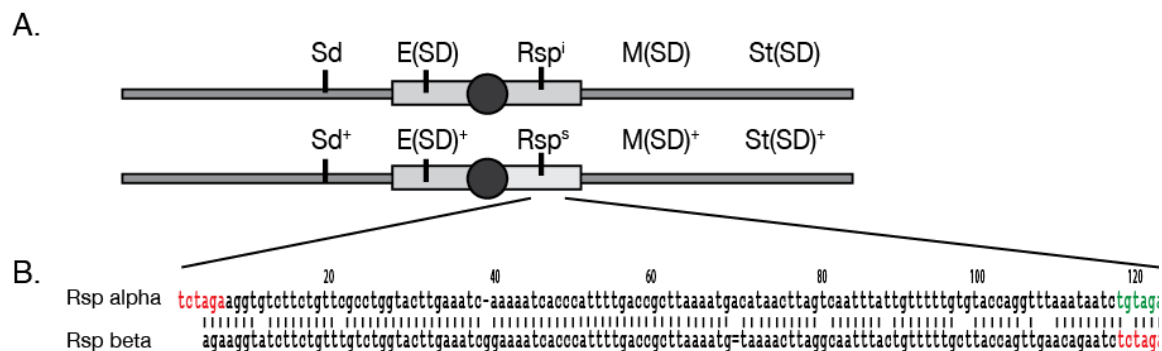


FIGURE 2.1: Components of segregation distortion. (A) Schematic of *D. melanogaster* second chromosomes showing the relative locations of components of the Segregation Distorter system. *Sd*, *E(Sd)*, *Rspⁱ*, *M(SD)* and *Sd(SD)* all contribute to the drive phenotype while *E(Sd)⁺*, *Rsp^s*, *M(SD)⁺* and *Sd(SD)⁺* are the wild type loci that are normally found on non-distorting *SD⁺* chromosomes. (B) An alignment of the alpha and beta halves of the canonical *Responder* repeat sequence. The roughly 120 bp alpha and beta sequences are approximately 87% identical. In red are the *Xba*I restriction sites that flank the 240 bp repeat. In green is a mutated *Xba*I site that is found in at the 3' end of the alpha repeat.

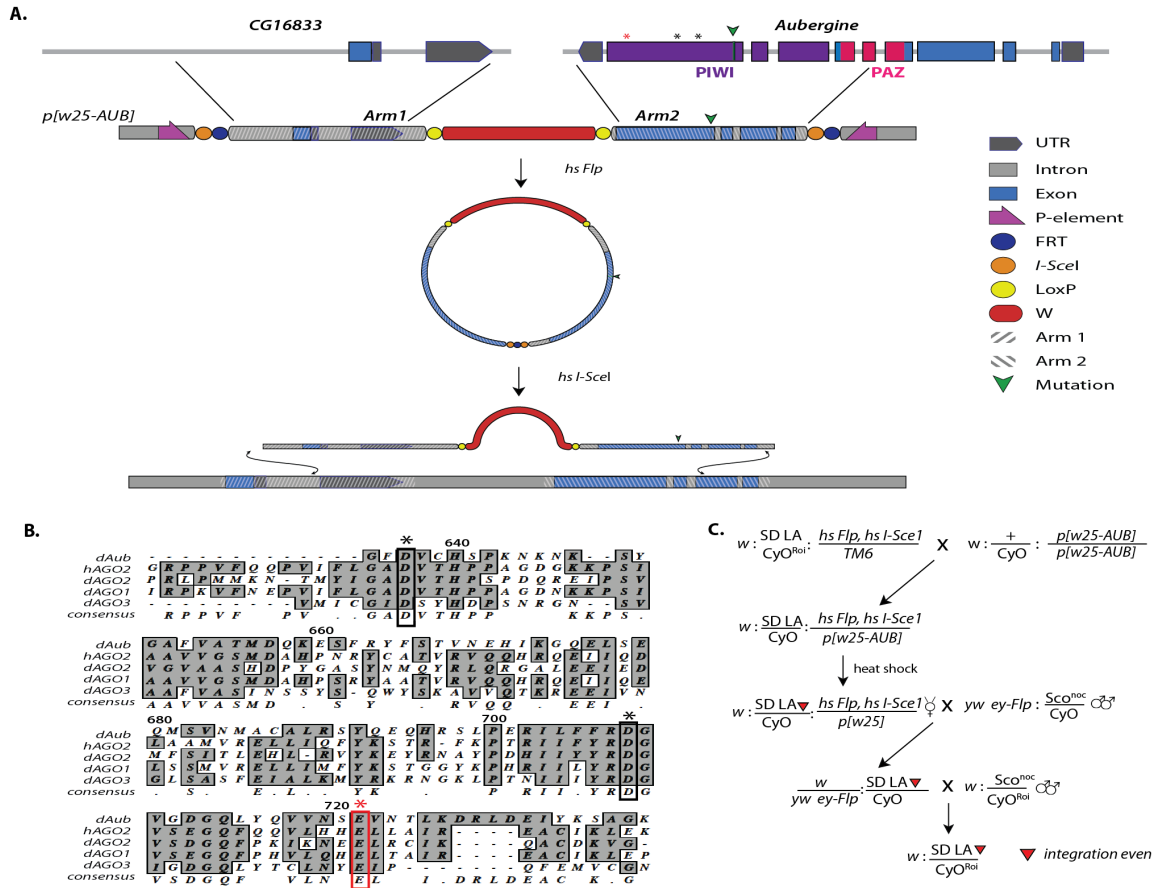


FIGURE 2.2: Targeting of the *aub* locus by ends-out homologous recombination (HR). (A) Top: Genomic organization of the *aub* locus and nearby CG16833. Green triangle indicates the location of the Q622* mutation of *aub*^{HN} and asterisks show the catalytic residues required for nuclease function. The red asterisk is the E721 residue mutated in this study. *Aub* exons 5-9 were cloned from cDNA into arm2 of the p[W25.2] cloning vector. Arm1 contains the 5'UTR and intronic sequences from CG16833. This construct was integrated randomly into the genome using standard transgenic methods. Mobilization and linearization were achieved by heat activation of Flp-recombinase and restriction enzyme *I-SceI* (*hs Flp*, *hs I-SceI*). The linear intermediate is able to recombine at the endogenous locus using native DNA repair machinery, incorporating engineered mutations into the genome. (B) Alignment of human and *Drosophila* Argonaute protein sequences showing the location of the catalytic residues (asterisks). (C) Crossing scheme for specific targeting of the *SD*-Los Arrenos (*SD-LA*) chromosome by HR. Flies containing the transgenic p[w25-AUB] construct on the third chromosome were crossed to doubly-balanced lines containing both *SD-LA* and the *hs-Flp*, *hs I-SceI* chromosome. Embryos from this cross were heat shocked to allow mobilization and linearization of the p[w25-AUB] construct. Following eclosion, white- or mosaic-eyed virgins were crossed to *ey-Flp* males. The progeny were screened for red-eyed females that were then crossed to balancer stocks in preparation for PCR validation as described in STABER *et al.* 2011.

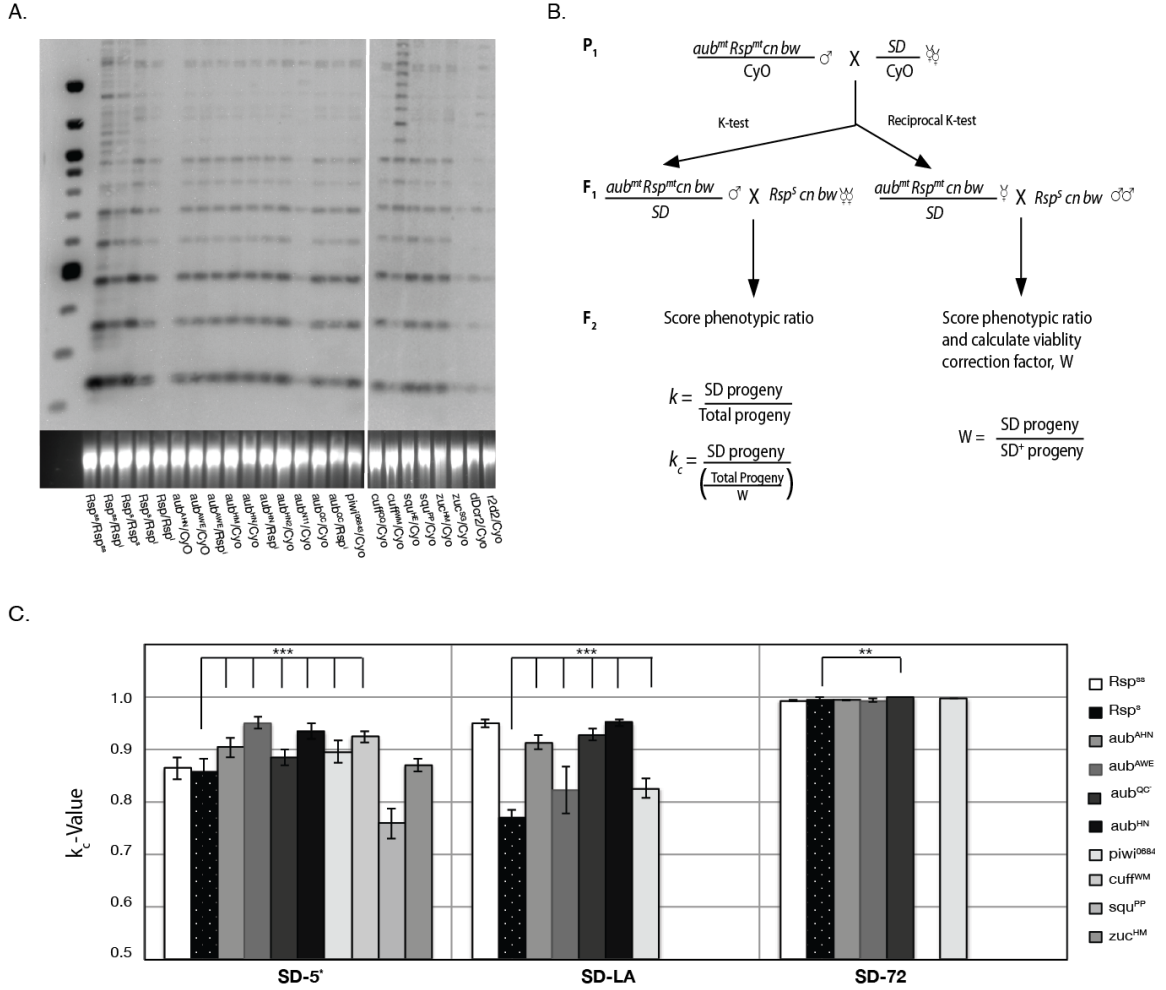


FIGURE 2.3: Mutations in *aub* and *piwi* are genetic enhancers of distortion. (A) A representative genomic Southern blot for *Rsp* sequences of indicated genotypes. All lanes contain 500 ng of *Xba*I digested genomic DNA. The *aub*^{AHN}, *aub*^{AWE}, *aub*^{HM}, *aub*^{HN}, *aub*^{HN2}, *aub*^{N11}, *aub*^{QC}, *piwi*⁰⁶⁸⁴, *cuff*^{QQ}, *squ*^{PP}, *squ*^{PP}, and *zuc*^{HM} chromosomes all show repeat intensity and banding identical to a single copy of the standard *Rsp*^s chromosome (*Rsp*^s/*Rsp*ⁱ). The CyO chromosome has no detectible *Rsp* repeats. (B) Crosses and calculations for *k* tests. Chromosomes with *aub* mutations were crossed to *SD* stocks. F₁ males were then backcrossed to lines carrying recessive eye-color markers *cn* and *bw* to allow scoring of progeny classes by eye color. Reciprocal crosses were carried out to correct for viability differences between the chromosomes. *W*, *k*, and *k_c* were calculated as indicated. (C). piRNA pathway mutants were crossed to three different *SD* chromosomes and *k_c* was determined for each. Each bar represents the sum *k_c* value for the total progeny of 20-40 individual males. All five *aub* mutations tested as well as a *piwi* mutation showed a significant increase in drive over that of standard *Rsp*^s chromosome. *Squ*^{PP} showed a significant reduction in drive. (*P*<0.0005). All genotypes gave *k_c*-values close to 1.0 for the strong driver *SD*-72. However, *aub*^{QC} shows a significant increase in *k_c* (2- tailed Z test, ** *p*<0.001, *** *p*< 0.0001). Flies of the genotype *SD*-72/*aub*^{HN} were non-viable and therefore could not be tested.

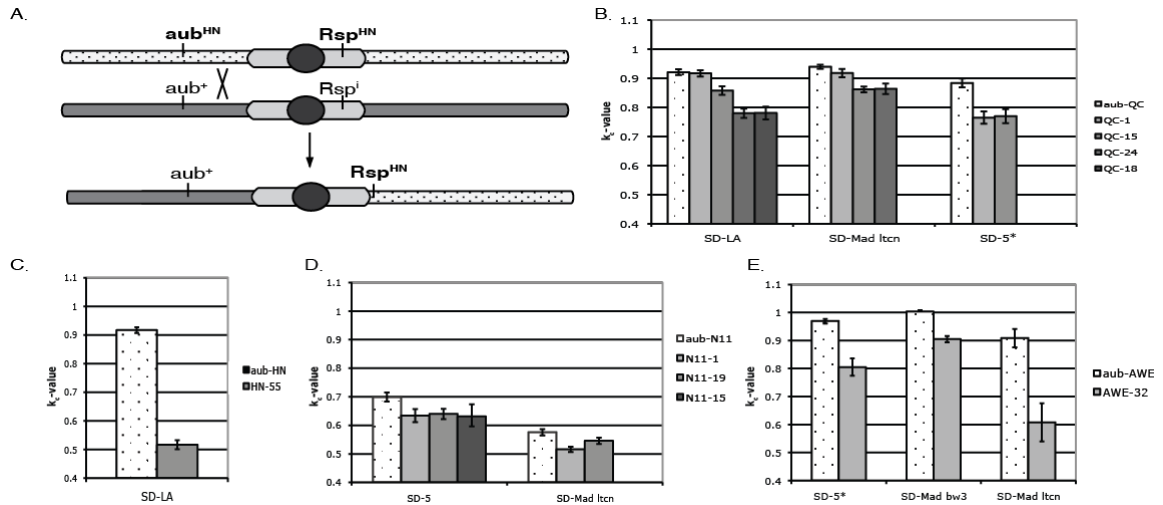


FIGURE 2.4: The contributions of *aub* mutants and associated repeats are genetically separable. (A) Recombinant chromosomes of the type *aub*⁺ *Rsp*^{HN} were generated by allowing recombination between the *aub*^{HN} *Rsp*^{HN} and *aub*⁺ *Rsp*ⁱ chromosomes. (B) *k_c* values for recombinant chromosomes generated from *aub*^{QC}. QC-1 showed no significant reduction in *k* when crossed with SD-Los Arrenos (SD-LA), but showed a significant reduction with both SD-Mad *lt cn* and SD-5*. QC-15, QC-18 and QC-24 showed significant reductions in drive with all SD chromosomes tested. (C) After testing more than 80 individual chromosomes, only one *aub*⁺ *Rsp*^{HN} allele was recovered. This allele showed a significant reduction in drive when tested with SD-LA. (D) *Aub*^{N11} chromosomes contain only an intermediate level of repeats. However, when the *aub* mutation was removed, the amount of drive experienced by these chromosomes was further reduced. (E) AWE-32 showed a reduction in *k_c* compared with the parental chromosome with all three SD lines tested. All tests represent the sum *k_c* value for the total progeny of 20-30 individual males. All recombinants, with the exception of QC-1 crossed with SD-LA, showed a significant reduction in *k_c* value $p < 0.0001$ (2-tailed Z-test).

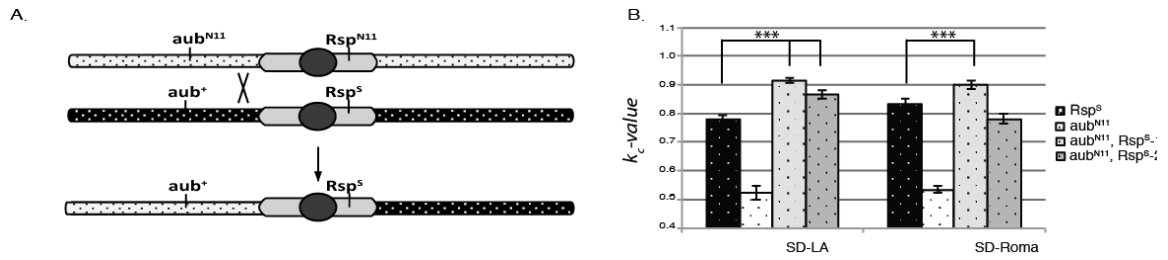


FIGURE 2.5: Addition of an *aub* mutation to a *Rsp*^s chromosome enhances distortion. (A) Recombinant chromosomes of the type *aub*^{N11} *Rsp*^s were generated by allowing recombination between *aub*^{N11} *Rsp*^{N11} and *aub*⁺ *Rsp*^s chromosomes. Potential recombinant chromosomes were tested for *Rsp* status by Southern blot and for the presence of the 110 bp deletion in *aub*^{N11} by PCR-assay. (B) Both recombinants showed a significant increase in distortion over that of a standard *Rsp*^s chromosome when tested against *SD*-Los Arrenos (*SD*-LA). Only *aub*^{N11} *Rsp*^s-2 showed a significant reduction when crossed to *SD*-Roma. All tests represent the sum *k*_c for 20-30 males (*p*<0.00001, 2-tailed Z test).

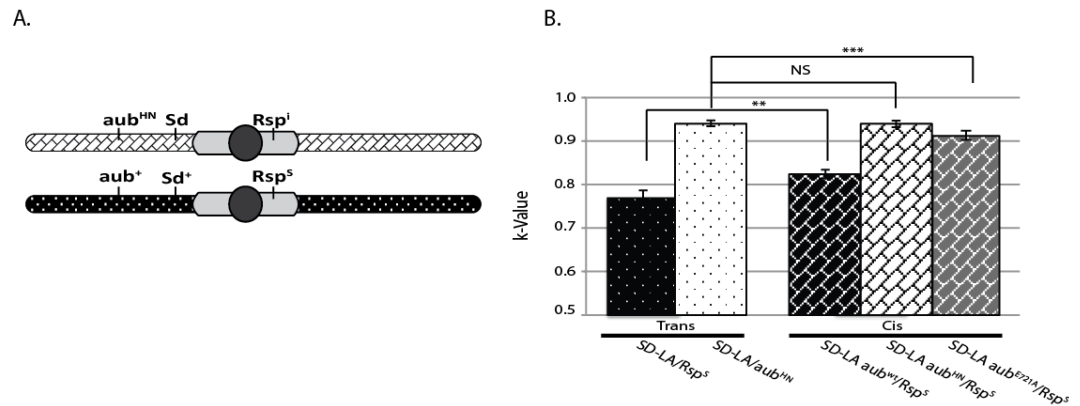
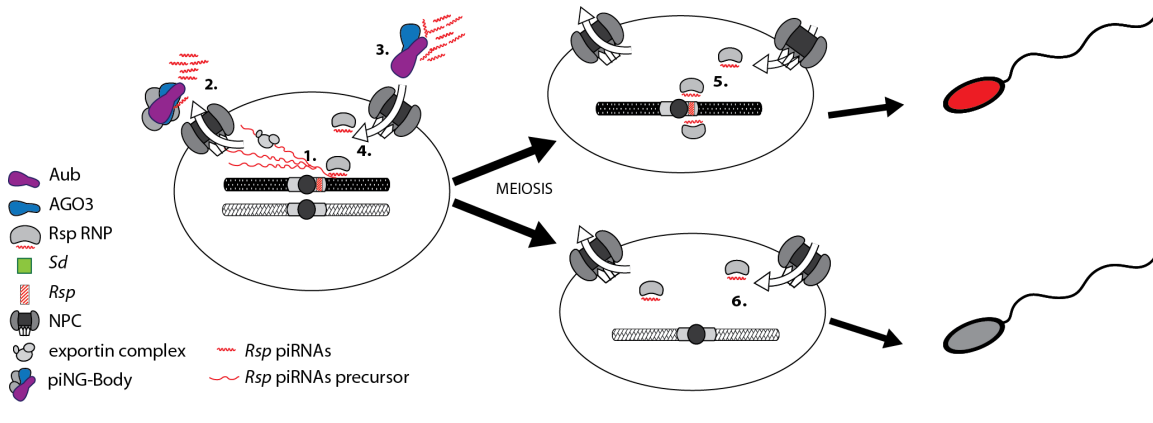


FIGURE 2.6: Engineered *aub* mutations on an *SD* chromosome enhance distortion in *cis*. (A) Schematic of chromosomes used to assay effect of *aub* mutations placed on an *SD* chromosome. (B). The *aub*^{HN} mutation targeted to an *SD*-Los Arrenos (*SD*-LA) chromosome by HR distorts a *Rsp*^s chromosome with a *k*_c of 0.940, the same value as that of the *aub*^{HN} mutation in *trans*. A wild type *aub*⁺ locus placed on an *SD*-LA chromosome by HR shows a small, but significant, increase in drive of the *Rsp*^s chromosome when compared to drive of the parental *SD*-LA chromosome. The *aub*^{E721A} allele, in which a catalytic glutamic acid residue is replaced with alanine, also shows a significant enhancement of drive when compared to a wild type targeted chromosome (*SD*-LA *aub*^{wt}), though the increase is smaller than that seen with the *SD*-LA *aub*^{HN} targeted allele. The *SD*-LA *aub*^{HN} and *SD*-LA *aub*^{wt} *k*_c values each represent the pooled progeny counts for two independently-derived alleles, approximately 60-70 males total. The *SD*-LA *aub*^{E721A} data represent a single allele with *n* = 27 males (***) *p*<0.00005, ***p*<0.0005, 2 tailed Z-test).

A. Non-distorting (SD^+/Rsp^s)



B. Distorting (SD/Rsp^s)

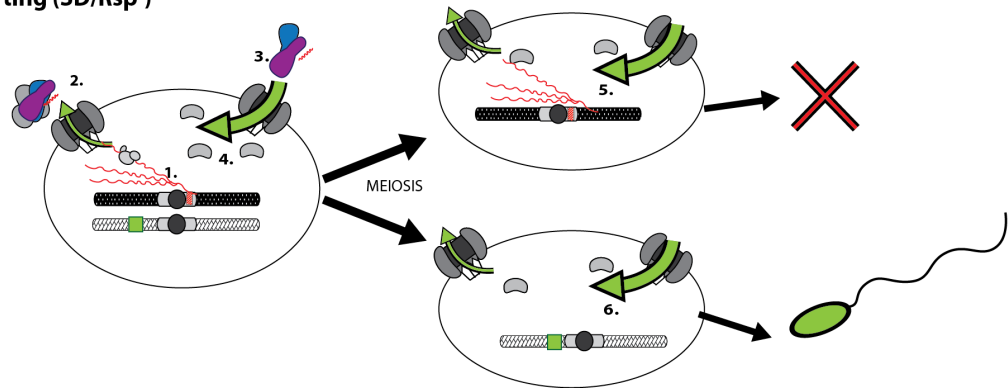


FIGURE 2.7: A model for the role of the piRNA pathway in segregation distortion. (A) 1. In $SD^+ Rsp^s$ nuclei precursor *Rsp* piRNA transcripts are made in spermatocytes. 2. Following export through the Nuclear Pore Complex (NPC), these precursor *Rsp* piRNAs are processed into mature piRNAs by Aub and AGO3 in the piNG-body. 3. Mature *Rsp* piRNAs re-enter the nucleus as part of a RNA-protein silencing complex (*Rsp* RNP) 4. Inside the nucleus *Rsp* piRNAs are then used by the *Rsp* RNP to target the *Rsp^s* locus for silencing. 5. This silencing is maintained through meiosis and is required for proper condensation of *Rsp^s*-bearing spermatids. 6. In Rsp^i spermatids, *Rsp* piRNA complexes have no target. (B) 1. Under distorting conditions, nuclear transport is perturbed (green arrows) causing nuclear retention of *Rsp* precursor piRNAs. 2. This retention prevents cytoplasmic processing of precursors into mature piRNAs. 3. Thus, there is no *Rsp*-piRNA complex primed and ready to enter the nucleus. 4. Therefore, the *Rsp^s* locus is not properly silenced in these cells. 5. As a result, *Rsp^s*-bearing spermatids fail to properly condense following meiosis and are eliminated. 6. Lacking a *Rsp^s* locus, the *SD*-bearing spermatids condense normally and are individualized into sperm.

SUPPLEMENTAL INFORMATION

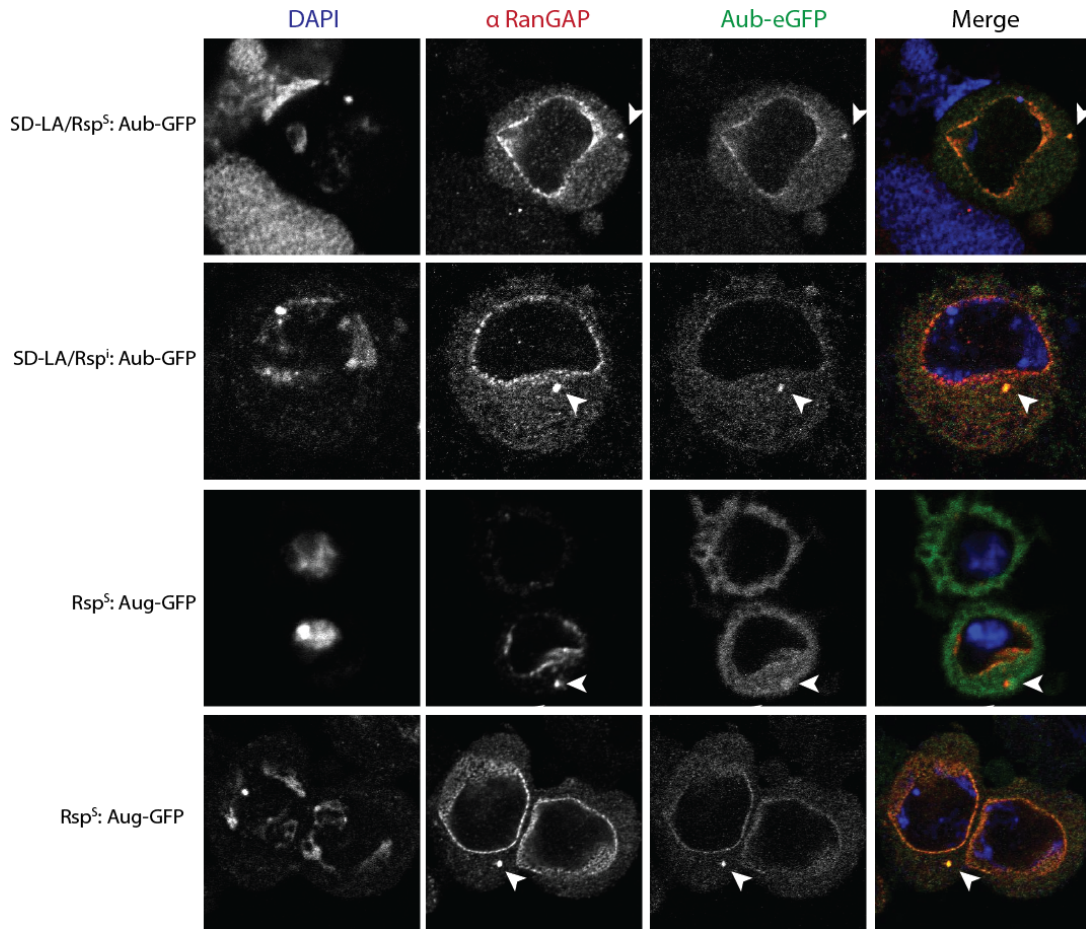


Figure S2.1: Aub-GFP and RanGAP colocalized in a single large body of primary spermatocytes. Whole mount testis of males expressing Aub-GFP were fixed with paraformaldehyde and stained with anti-RanGAP (1:1000) primary and Alexa Fluor (1:800) secondary antibodies. Confocal imaging was done on a Zeiss LSM 510. Spermatocytes in the testis of both distorting (SD-LA/Rsp^s) and non-distorting (SD⁺ Rsp^s) males show a single large point of colocalization between Aub-GFP and Ran-GAP (arrowhead)

TABLE S2.1: GENETIC STOCKS USED IN THIS STUDY			
Line	Notes on genotype	Source	Reference
HR stocks			
<i>y w; FLP-I-Scel/TM6</i>	$y^1 w^*$; $P\{ry^{+17.2}=70FLP\}11 P\{v^{+11.8}=70I-Scel\}2B noc^{ScO}/CyO, S^2$	BSC 6930	Staber <i>et al.</i> 2011
<i>y w ey-FLP</i>	$y^{d2} w^{1118} P\{ry[+t7.2]=ey-FLP.N\}2$	BSC 5580	Staber <i>et al.</i> 2011
<i>y w Cre; noc^{ScO}/CyO</i>	$y^1 w^{67c23} P\{y^{+mDint2}=Cre\}1b; noc^{ScO}/CyO$	BSC 766	Staber <i>et al.</i> 2011
<i>w¹</i>		BSC 145	Staber <i>et al.</i> 2011
<i>w¹¹¹⁸</i>		BSC 3605	Staber <i>et al.</i> 2011
Balancer Stocks			
<i>w; TM3 sb /TM6 tb</i>		B. Ganetzky	
<i>+</i> ; <i>CyO^{lt dp pr cn} /Sco</i>		B. Ganetzky	
<i>w: CyO^{cn bw Roi} /Sco</i>		B. Ganetzky	
SD Stocks			
<i>SD-5</i>	Strong distortion phenotype and contains <i>Sd</i> , <i>E(SD)</i> , <i>Rsp^I</i> , <i>M(SD)</i> and <i>St(SD)</i> as well as two paracentric inversions on 2R.	B. Ganetzky	Sandler and Hiraizumi 1959; Sandler <i>et al.</i> 1959
<i>SD-5*</i>	Uncharacterized derivative of <i>SD-5</i> which exhibits an intermediate level of distortion	B. Ganetzky	
<i>SD-72</i>	Strong distorter with a both pericentric and paracentric inversions	B. Ganetzky	Sandler and Hiraizumi 1959)
<i>SD-Mad lt cn</i>	Derived from the original <i>SD-Mad</i> by recombination with chromosomes carrying the recessive markers <i>cn</i> , <i>lt</i>	B. Ganetzky	R.G. Temin 1979
<i>SD-Mad bw³</i>	Derived from the original <i>SD-Mad</i> by recombination with chromosomes carrying the recessive markers <i>bw³</i>	B. Ganetzky	R.G. Temin 1979
<i>SD-Roma</i>	Inversion free moderately distorting SD chromosome isolated in Italy	B. Ganetzky	Nicoletti and Trippa 1967)
<i>SD-Los Arrenos</i>	Weak distorter	B. Ganetzky	
<i>Rsp^S cn bw</i>	$[Sd^+, E(SD)^+, Rsp^S]$ standard Rsp sensitive chromosome	B. Ganetzky	Lyttle 1991
<i>Rspⁱ¹⁶ cn bw</i>	$[Sd^+ E(SD)^+ Rsp^I]$ Radiation-induced derivative of the Rsp ^S cn bw chromosome where the Rsp locus has been completely deleted	B. Ganetzky	Ganetzky 1977
<i>Rsp^{SS} lt pk cn</i>	$[Sd^+ E(SD)^+ Rsp^{SS}]$ the canonical Rsp ^{SS} chromosome	B. Ganetzky	Lyttle 1991
RNAi Mutants			
<i>aub^{QC42} cn bw/CyO</i>	EMS; Strong allele	T. Schüpbach	Schupbach and Wieschaus 1991

<i>aub</i> ^{HN2} <i>cn bw/CyO</i>	EMS; Strong allele	T. Schüpbach	Schupbach and Wieschaus 1991
<i>aub</i> ^{HM23} <i>cn bw/CyO</i>	EMS; Strong allele	T. Schüpbach	Schupbach and Wieschaus 1991
<i>aub</i> ^{AHN56} <i>cn bw/CyO</i>	EMS: Strong allele, has secondary mutation	T. Schüpbach	Schupbach and Wieschaus 1991
<i>aub</i> ^{AHE13} <i>cn bw/CyO</i>	EMS: Strong allele, has secondary mutation	T. Schüpbach	Schupbach and Wieschaus 1991
<i>zuc</i> ^{HM27} <i>cn bw/CyO</i>	EMS: Strong allele	T. Schüpbach	Schupbach and Wieschaus 1991
<i>zuc</i> ^{SG63,rec2} <i>pr c px sp/CyO</i>	EMS: homozygotes poorly viable	T. Schüpbach	Schupbach and Wieschaus 1991
<i>squ</i> ^{PP32} <i>cn bw/CyO</i>	EMS; Strong allele	T. Schüpbach	Schupbach and Wieschaus 1991
<i>squ</i> ^{HE47} <i>cn bw/CyO</i>	EMS; Strong allele	T. Schüpbach	Schupbach and Wieschaus 1991
<i>cuff</i> ^{WM25} <i>cn bw/CyO</i>	EMS; Strong allele	T. Schüpbach	Schupbach and Wieschaus 1991
<i>cuff</i> ^{QQ37} <i>cn bw/CyO</i>	EMS; Strong allele	T. Schüpbach	Schupbach and Wieschaus 1991
<i>w</i> ¹ ; <i>aub</i> ^{N11} <i>/CyO</i>	110bp deletion	P. Macdonald	Harris and Macdonald 2001
<i>piwi</i> ⁰⁶⁸⁴³ <i>cn /CyO</i>	P{PZ} in first exon UAS-Aubergine-GFP recombined on to the same chromosome as nos-GAL4 VP16	BSC 12225 P. Macdonald	Cox et al 1998 Harris and Macdonald 2001

TABLE S2: PRIMER SEQUENCES USED IN THIS STUDY	
Name	Sequence
Cloning Primers	
AUBarm1-F BsiWI	TCGTACGCTCTCCATGTAGCGAAAAGCGTATAG
AUBarm1-R Ascl	TGGCGCGCCAATGCATATATTCGTATGAAATGAGC
AUBarm2-F Acc65I	TGGTACCTTCCAGCAACGGAACCTTTATAGAC
AUBarm2-R NotI	TGCGGCCGCTGGTTACGAAGAGTCGTGCTGGCG
Sequencing Primers	
AUBarm1-s1	GTGACAGTCGCTGCCATCGTATTATTGG
AUBarm1-s2	CTGACAAGTGGACTGTCTGCAGTGGGTG
AUBarm1-s3	CTTTATATCGAAGGGTAAGTACATG
AUBarm1-s4	GA CTCGAACGATGAAGAAACAACACCAGCG
AUBarm2-s1	G TAGCGAGAATTAATGCGCTTGGATAC
AUBarm2-s2	GTGTCACCACCTGCGATGGCACC GGTC
AUBarm2-s3	CCGGTGGCAACACGCGTGCTGGAATCTC
AUBarm2-s4	CGATTGAAAGTCGACATCGTCAATGCG
aub arm1-RS1	GGGCAACTCAAAAAGTGGTAACAAG
aub arm2-RS1	GATATATGTATGTAGATATGTACATC
AUBarm1-R3	CTTGTTTATTTACATGTAAGTACTGTCC
AUBarm1-R4	CCTGGTCAACCCCTTAAATGCAGATGC
AUBarm1-R5	GCTTAGTAAATAAGTTAACACAATTTAC
AUB HN1 VR	CCGCAACGGCACTTACTCCCAAGCG
Mutagenic Primers	
AUB HN m1 F	GATGGGAGCTCCCTGGTAGGTAGTCATCCCCCTCCACGGTCTGA
AUB HN m1 R	TCAGACCGTGGAGGGGGATGACTACCTACCAGGGAGCTCCCATC
aub E721A R	GGTCAGCTCTACCAGGTGGTAAACAGCGCGGTGAACACCCTAAAGGACAGG
aub E721A F	CCTGTCCTTTAGGGTGTTACCGCGCTGTTTACCACCTGGTAGAGCTGACC
Arm Specific Validation Primers	
AUBarm2-V1	CTTGTATGAACATAAAGGGATCC
AUBarm2-V2	CGTCGATTTTACGCCTGATGTGG
AUBarm2-V3	GGCACTCATATTACCGTGCAGGC
AUBarm1-V1	GGTTTATAATTCCACGGA CTTC
AUBarm1-V2	CCATAGCGAATGTAGTGTG
AUBarm1-V3	CCCGGACCATCGCAAAGTAAACTGC
AUBarm2-VR2	CGGTGAACATGGCGTGCGCCCTGAGG
AUBarm1-R3	CTTGTTTATTTACATGTAAGTACTGTCC
AUBarm1-R4	CCTGGTCAACCCCTTAAATGCAGATGC
AUBarm1-R5	GCTTAGTAAATAAGTTAACACAATTTAC
p[W25.2] Specific Validation Primers	
pW-Not 1	CACTGTTCACGTCGCACTCGAGGGTAC
pW-Not 2	GCACTCGAGAGCTCGTTACAGTCCG

pW-Bsi 1	CGCACCGGACTGTAACGAGCTAC
pW-Bsi 2	GGCGACTCAACGCAGATGCCGTACC
pW-Asc 1	GTATGCTATACGAAGTTATCTAGACTAGTCTAGGGCG
pW-Asc 2	GCTTGGCTGCAGGTCGACTCTAGAGG
pW-Asc 3	CGATCATTCAATTATTCGCTGCATGAATTAGC
pW-Acc 1	CATTATACGAAGTTATCTAGACTAGTCTAGGGTAC
pW-Acc 2	GACGCTCCGTCGACGAAGCGCCTC
pW-Acc 3	GCTCAGCTTGCTTCGCGATGTGTTAC
WTPR	CGCGAACATTCGAGGCGCGCTCTCTCG
Wt SQ10F	GTGACCTGTTGCGAGTGATTAGCG
Southern Blotting	
SP6	GCCAAGCTATTTAGGTGACACTATAG
T7	GAATTGTAATACGACTCACTATAGGG
Rsp13 clone sequence	GGAGCTCCACCGCGGTGGCGGCCGCCCCTGCAGATCTGCGGCCGCTCTAG AAGGTGTCTTCTGTTTCGCCTGGTACTTGAAATCGAAAAATCACTCATTTGACC GCTAAAATGACATAACTTAGTCAATTTATTGTTTTGTGTACCAGGTTAAATA ATCTGTAGAAGGTATCTTCTGTTTGTCTGGTACTTGAAATCGAAAAATCACTC ATTTTGACCGCTTAAAATGTAAAACCTTAGGCAATTTACTGTTTTTCCTTACCAG TTGAACAGAATCTCTAGAAGGTGTCTTCTGTTTCGCCTGGTACTTGAAATCGAA AAATCACTCATTTGACCGCTAAAATGACATAACTTAGTCAATTTATTGTTTTG TGTACCAGGTTTAAATAATCTGTAGAAGGTATCTTCTGTTTGTCTGGTACTTGA AATCGGAAAATCACTCATTTTGACCGCTTAAAATGTAAAACCTTAGGCAATTTAC TGTTTTTCCTTACCAGTTGAACAGAATCTCTAGAACTAGTGGATCCCCGGGC TGCAGGAATTCGATATCAAGCTTATCGATACCGTCGACCTCGAGGGGGGGCC CGGTACCAGCTTTTGTTCCTTTAGTGAGGGTTAATTCGAGCTTGGCGTAAT CATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCAC

Chapter III: Investigating the Molecular Mechanism of piRNA Pathway Activity in Segregation Distortion

ABSTRACT

The molecular mechanism by which Segregation Distorter (*SD*) acts to cause the dysfunction of *Responder*- (*Rsp*-) bearing sperm is not known. Evidence presented here demonstrates that mutations to components of the *Piwi-interacting* RNA (piRNA) pathway enhance the *SD* phenotype. The piRNA pathway is a germline specific RNA interference mechanism that suppresses transposon activity to ensure the fidelity of genetic information passed to the next generation. The *Rsp* repeat array itself is likely a target of this mechanism. In order to study the role of the piRNA pathway in *SD* I have utilized multiple techniques to determine the localization of piRNA pathway components, test for alterations of markers of epigenetic chromatin state, and examine the population of *Rsp*-derived small RNAs found in the testes of distorting and non-distorting males. These observations suggest that *Rsp* piRNAs may be produced from a “source” outside the canonical *Rsp* locus on chromosome 2R.

INTRODUCTION

In spite of almost 60 years of study, the molecular mechanism by which *SD* is able to selectively target *SD*⁺ *Rsp*^s sperm for destruction remains elusive. Like wild type RanGAP, Sd-RanGAP is expressed in all *Drosophila* cells. However, it is only in the specific context of spermatogenesis that the *SD* phenotype is observed (SANDLER *et al.* 1959; TOKUYASU *et al.* 1977; MERRILL *et al.* 1999; CREVEL *et al.* 2001; STEFFENSEN *et al.* 2001). Moreover, the sperm dysfunction in *SD* requires the presence of high copy numbers of the *Rsp* satellite sequence (WU *et al.* 1988). In flies whose genomes carry only a few copies of the *Rsp* repeat, Mendelian transmission ratios are observed (GANETZKY 1977; TEMIN *et al.* 1991). When a *Rsp*^s locus is translocated to the Y-chromosome the sensitivity to *SD* moves with the *Rsp* locus, leading to the loss of Y-bearing sperm, suggesting there is something fundamental about the *Rsp* repeat sequence that leads to sperm dysfunction in *SD* (LYTTLE 1989). Understanding the role of *Rsp* in distortion is clearly key to elucidating its molecular mechanism.

The observation that *Rsp*-bearing spermatids remain uncondensed during chromatin compaction and histone to protamine transition has provided some clues as to the nature of the dysfunction of *Rsp*^s-bearing spermatids (KETTANEH and HARTL 1976; TOKUYASU *et al.* 1977; HAUSCHTECK-JUNGEN and HARTL 1982). This dysfunction suggests that elements involved in regulation of chromatin state are also involved in the

maturation failure of *Rsp^s*-bearing spermatids (KUSANO *et al.* 2003; FERREE and BARBASH 2007; MCELROY *et al.* 2008). In support of this theory, mutation of two genes involved in chromatin condensation: Df31, a decondensation factor which binds the tail of H3, and SMC4, part of the condensin 1 complex which plays a role in mitotic chromosome condensation and sister chromatid resolution, have been reported to enhance distortion (CREVEL *et al.* 2001; STEFFENSEN *et al.* 2001; MCELROY *et al.* 2008).

Rsp transcripts are processed into small RNAs in the testis by a germline-specific small RNA-based silencing pathway known as the Piwi-interacting RNA (piRNA) pathway. In combination with the data reported herein demonstrating that mutations of *aubergine* (*aub*), a component of this pathway, act as enhancers of distortion, these observations strongly suggests that the *Rsp* locus is regulated by the piRNA pathway (ARAVIN *et al.* 2004; BRENECKE *et al.* 2007; GUNAWARDANE *et al.* 2007; NAGAO *et al.* 2010).

While significantly less well understood than piRNA biogenesis in the ovary, production of piRNAs in the *Drosophila* testis is a highly regulated and compartmentalized process. Recently a novel, perinuclear structure, the piRNA Nuage Giant body (piNG-body), was described that houses much of the molecular machinery required for the processing and/or targeting of piRNAs to transposons (KIBANOV *et al.* 2011). Mutation to piRNA pathway components including *aub*, *argonaute3* (*AGO3*), *tudor* (*tud*) and *vasa* (*vas*) results in disruption of piNG-bodies, loss of piRNAs and failure of transposon silencing (NAGAO *et al.* 2010; KIBANOV *et al.* 2011). One model suggests that piRNA precursors are exported from the nucleus, processed in cytoplasmic piNG-bodies and there used to target transposon sequences exiting the nucleus for

homology-mediated cleavage (KLATTENHOFF *et al.* 2009; KHURANA and THEURKAUF 2010). However, it has also been shown that piRNAs mediate chromatin level regulation of gene expression. The Piwi protein specifically binds HP1 and localizes to the heterochromatin of polytene chromosomes (PAL-BHADRA *et al.* 2004; BROWER-TOLAND *et al.* 2007; HUISINGA and ELGIN 2009).

The precise mechanisms, transcriptional or post-transcriptional, of piRNA-mediated silencing in the testis are unknown. The knowledge that small RNAs act as regulators of epigenetic state and that the defect of *Rsp*-bearing sperm is chromatin-related suggests a specific model for regulation of *Rsp* repeat expression by epigenetic modification of this locus by the piRNA pathway.

Rsp repeats have been identified in regions of the genome outside of the canonical repeat locus on chromosome 2R. Interestingly, several *Rsp* repeat copies are located in an intron of the *Argonaute3* gene (R. REENAN and C.J STABER unpublished observation). This observation suggests a potential role for *Rsp* repeats in the regulation of *AGO3* expression, whereby *Rsp* piRNAs could guide homology-mediated post-transcriptional silencing of *AGO3* or regulate transcription through recruitment of epigenetic silencing factors to the genomic *AGO3* locus.

The piRNA pathway has been demonstrated to have a role in silencing of other specific gene targets, including *Vasa* and *Stellate* in the testis and *Fasciclin3* (*Fas3*), a cell adhesion molecule, in the ovary (ARAVIN *et al.* 2004; NISHIDA *et al.* 2007; SAITO *et al.* 2009; NAGAO *et al.* 2010). Additionally, there is precedent for production of piRNAs from untranslated regions of protein coding genes. A large MAF transcription factor known as *traffic jam* (*tj*) has been shown to be a source of piRNAs. Interestingly, the TJ

protein itself regulates *piwi* expression in the somatic cells of the ovary (SAITO *et al.* 2009). Given that mutation of piRNA pathway components enhances distortion, the possibility of a regulatory feedback mechanism that reduces AGO3 expression in *Rsp^s* backgrounds suggests an intriguing model for the activity of *Rsp* repeats in distortion.

In order to further investigate the molecular mechanism of the piRNA pathway in segregation distortion I tested multiple areas of potential interactions between these two pathways. I used immunohistochemical staining of heterochromatic markers to assay for alterations in chromatin state under distorting and non-distorting conditions. I examined the localization of transgenic Aub-GFP in testes of distorting and non-distorting males to determine if the disruption of nuclear transport associated with Sd-RanGAP might perturb Aub localization. I also utilized a PCR-based method to analyze the *Rsp*-derived small RNA population from the testes of flies of both *Rsp^s* and *Rspⁱ* backgrounds. Further, I used RT-qPCR to test for alteration of AGO3 expression under distorting and non-distorting conditions, and I examined the effects of overexpression of a UAS-HA-AGO3 transgene on distortion. Each of these experiments is designed to shed light on the specific mode of interaction between the piRNA pathway and segregation distortion.

MATERIALS AND METHODS

Genetic Stocks

The *Drosophila* stocks were maintained at room temperature on cornmeal molasses food. A complete list of stocks used in this experiment can be found in Table 3.1

Generation of UAS-AGO3 Transgenes

RNA was extracted from testis of Canton-S flies using TriReagent (Molecular Research Center, Inc.) and reverse transcribed with random primers and MMLV-reverse transcriptase (Promega). AGO3 cDNA was amplified with cloning primers that appended 3' *XhoI* and 5' *NotI* cloning sites as well as a 5'HA tag (Table S3.2). The AGO3 construct was ligated into the pTOPO vector (Invitrogen) and mutations were induced using the Quik-change XL II mutagenesis kit (Agilent Technologies). Following sequence verification, wild type and D713A mutant AGO3 constructs were shuttled into the pUAST vector and introduced into the *Drosophila* genome by standard transgenic methods (Genetic Services Inc.).

Generation of UAS-AGO3, TubP-GAL4 recombinants

Females carrying HA-AGO3 wild type or D713A transgene insertions on the third chromosome were crossed with males carrying a TubP-GAL4 driver on the third chromosome. Virgin F₁ females were crossed with a third chromosome balancer stock to isolate individual third chromosomes. Each chromosome was tested for transgene expression by Western blot of testis extract with anti-HA (1:500) and anti-Actin (1:20,000) loading control. Lines with chromosomes containing both transgene and driver were then crossed into a *Rsp^s* or *Rspⁱ* background to test for effects on Segregation Distorter. Each line was validated for *Responder* repeat status by Southern blot.

Southern Blotting

For Southern analysis genomic DNA was prepared from approximately 30 adult flies using the Maxwell 16 DNA purification system (Promega), eluted in 500µl of DNase-free water (GIBCO) and treated with 20µg RNase A (Fisher Scientific). Samples were then concentrated by centrifugation at 8000 g for 5 min in Microcon Ultracel YM-50 columns (Millipore). Final sample concentrations were measured using the dsDNA HS assay kit with the Qubit 2.0 flourometer (Invitrogen), and 1600 ng of DNA per sample was digested for 1.5 h at 37°C with *Xba*I, 100 µg/ml bovine serum albumin and NEB Buffer 4 (20 mM Tris-acetate, 50 mM potassium acetate, 10 mM Magnesium Acetate, 1 mM Dithiothreitol, pH 7.9) (New England Biolabs).

Digested samples were run overnight on a 1.5% agarose gel (SeaKem LE Agarose, Lonza) in 0.5x TBE at 55 mV. The gel was washed for 30 min in denaturation solution (1.5 M NaCl, 0.5 M NaOH), rinsed with dH₂O and then washed for 30 min in

neutralization solution (1.5 M NaCl, 0.5 M Tris-HCl pH 7.5) with gentle shaking. The DNA was transferred overnight to a Hybond-N⁺ membrane (GE Healthcare) via capillary action according to the protocol in Molecular Cloning (SAMBROOK *et al.* 1989). Following transfer, DNA was fixed to the membrane by soaking in 0.4 M NaOH for 2 min. The membrane was pre-hybridized in ECL Gold hybridization buffer (GE Healthcare) in a Hybaid hybridization oven (Analytical Instruments) at 42°C for 30 min.

The *Rsp* probe was generated by PCR amplification of a 238bp sequence from the canonical *Rsp* repeat cloned into the pBluescript KS⁺ (Stratagene) vector. The vector specific primers, T3 and T7, were used to amplify probe (Table S2). Product was run on a 0.5% agarose gel (SeaKem LE Agarose, Lonza,) and the bands were cut and gel purified using the Wizard SV Gel and PCR Clean-Up System (Promega). Sample concentration was measured with the dsDNA HS Quibit flourometer system (Invitrogen) and labeled using the ECL direct labeling kit according to manufacturer's instructions (GE Healthcare). Labeled *Rsp* and 100 bp ladder probes (New England Biolabs) were added to blots at concentrations of 20 ng/ml and 10 ng/ml respectively in hybridization buffer and incubated overnight at 42°C.

Following incubation, blots were washed two times for 20 min each with ECL primary wash buffer (0.4% *SDS* and 0.5x SSC) at 42°C, then removed from the hybridization tubes and washed two more times at RT on a shaker with 2x SSC. The signal was generated using ECL detection reagents according to manufacturer's instructions (GE Healthcare) and detected using the Kodak Image Station 4000R.

Western Blotting

Protein samples were prepared in buffer containing SDS and β -mercaptoethanol and electrophoresed on a 10% gel (Amresco). Anti-HA antibody (Covance) was used at 1:500; anti-Actin (Millipore) was used at 1:20,000. Images were taken on a Kodak Image Station. Each lane represents eight individual testis in 50 μ l of protein sample buffer.

K-tests

Segregation ratios were measured as described previously (MCLEAN *et al.* 1994). k is the proportion of SD -bearing progeny as a fraction of the total progeny (DUNN 1953; GANETZKY 1977). For each cross 20 to 30 males aged less than four days were individually crossed with two Rsp^s cn bw virgin females. Crosses were allowed to brood for four days at 25°C and then passed to new food. After an additional 4 days of brooding, the parents were discarded. The progeny classes were counted on days 14, 18, and 22 from the initial cross as in MCLEAN *et al.* 1994. To correct for viability differences between second chromosomes reciprocal crosses were carried out as previously described (GANETZKY 1977; MCLEAN *et al.* 1994). Single SD/SD^+ females were crossed to two Rsp^s cn bw males. The progeny counts from these reciprocal crosses were used to calculate the viability factor, W , where $W = SD^+$ progeny/ SD progeny. The corrected k value, k_c , is then: $k_c = SD$ progeny/(SD progeny + SD^+ progeny/ W).

Immunohistochemistry

Drosophila testes were harvested and fixed according to the procedure adapted from *Drosophila Protocols* (BONACCORSI *et al.* 2000). Briefly, males less than 48 h post-eclosure were dissected at RT in testis buffer [183 nM KCl, 47 mM NaCl, 10 mM Tris-

HCl, 1 mM EDTA, 1 mM PMSF, pH 6.8] on slides coated with poly-L-lysine (Sigma-Aldrich). Sigmacote-treated (Sigma-Aldrich) coverslips were applied and testes were flattened by wicking with a Kimwipe. Slides were frozen in liquid nitrogen and coverslips removed with a razor blade. The slides were dehydrated for 5min in 100% methanol on dry ice. The slides were then washed at room temperature as follows: 5 min in 66% methanol in PBST, 5 min in 33% methanol in PBST, 5 min in PBST, 5 min in 4% paraformaldehyde in PBS, 3 x 10 min in PBST, 30 min in 1.5% bovine serum albumin in PBS. Slides were incubated overnight at 4°C in primary antiserum [anti-RanGAP at 1:1000, anti-HP1 (DSHB, OCA9-s) at 1:200, anti-H3K9 mono me (Millipore, 07-450) at 1:200, anti-H3K9 tri me (Millipore, 07-442) at 1:200]. Slides were then washed 3x20 min in PBST and incubated for two hours in the dark at RT with Alexa Fluor (AbD) conjugated secondary antibodies at 1:800. Confocal imaging was done on a Zeiss LSM 510. Standard fluorescent microscopy was done on a Zeiss Axio Imager M.1.

Small RNA Polymerase Chain Reaction

The following procedure was adapted from RO and YAN, 2010. Small RNAs were isolated from 100 adult flies using the mirVana small RNA isolation kit (Ambion). Final RNA concentration was measured using the RNA HS kit for the Qubit fluorometer (Invitrogen). 0.5 µg of RNA per sample was incubated with 1.5 U poly(A) polymerase at 37°C for 60min. Contaminants were removed by Acid-Phenol: Chloroform extraction and the aqueous phase was further purified using filter cartridge and wash buffers from mirVana Probe and Marker kit (Ambion). Prior to reverse transcriptions, 2 µg of tailed RNA and 1µg of RTQ primer per sample were melted at 65°C for 10 min and annealed at

4°C for 20 min. Reverse transcription reactions using SuperScript III (Invitrogen) were carried out according to manufacturer's instructions. Reactions were treated with 1.5U RNase H for 20min at 37°C to remove remaining RNA contaminants from the cDNA libraries. Polymerase chain reactions (PCRs) were performed according to manufacturer's instructions using GoTaq polymerase (Invitrogen) and the following cycling parameters: 95°C for 10 min, 40x (95°C for 15s, 60°C for 60s, 72°C for 30s) 72°C for 5min.

Reverse-Transcription quantitative PCR

RNA was extracted from 40 testis using TriReagent (Molecular Research Center, Inc.) according to manufacturer's instructions. Extracted RNA samples were measured on a NanoDrop (Thermo Scientific) and equal amounts of RNA for each sample were used for reverse transcription (RT). The RT reaction was carried out using random primers and MMLV-reverse transcriptase. qPCR reactions [1ul cDNA, 1.5 µl of 5 mM forward and reverse primer mix (Table 3.2) 5 µl of H₂O and 7.5 µl of SYBR green qPCR master mix (Applied Biosystems)] were set up in triplicate in a 96 well MicroAmp optical PCR plate (Applied Biosystems). All samples were run on ABI 7900 Fast Sequence Detection Instrument and normalized to RP49 expression levels. Data represents biological triplicates and error bars show standard error.

RESULTS

Chromatin Modifications Are Unaltered in Distorting and Non-Distorting Conditions

Given that piRNA pathway components are known regulators of epigenetic chromatin state, I sought to examine the chromatin modifications present in the testis of males of distorting and non-distorting genotypes (PAL-BHADRA *et al.* 2004; BROWER-TOLAND *et al.* 2007). To this end, I examined testis of young adult males stained with antibodies against chromatin markers: HP1 and histone H3 either mono- or tri-methylated on Lysine 9 (H3K9 mono-me, H3K9 tri-me) (EISSENBERG *et al.* 1994; NAKAYAMA *et al.* 2001).

Staining for mono-methyl H3K9 and HP1 reveals no alterations in heterochromatin related to SD

Primary spermatocytes doubly stained for H3K9 mono-me and HP1 showed similar staining patterns in males of distorting ($SD^{-5} Rsp^i / SD^{+} Rsp^s$) and non-distorting ($SD^{-5} Rsp^i / CyO Rsp^i$ and $SD^{+} Rsp^s / SD^{+} Rsp^s$) genotypes (Figure 3.1). DAPI-treated primary spermatocytes characteristically show three distinct lobes of darkly stained DNA that represent the three large bivalent chromosomes (FULLER 1993). H3K9 mono-me signal

overlaps the most highly condensed regions of these three lobes, while HP1 staining is more evenly distributed throughout the entirety of the nucleus. There are distinct foci of increased HP1 staining that overlap with the darkest DAPI staining. However, these puncta do not fully overlap with the most intense regions of H3K9 mono-me staining; instead they appear to border the edges of the most intense H3K9 staining. This general staining pattern was observed in the testes of all males, both in the presence and absence of *SD* chromosomes. There was considerable cell-to-cell variation in the precise staining pattern and relative intensities of the signals, likely due to the natural heterogeneity of the tissues, making quantification difficult (Figure 3.1a, b). However, at no time did any single genotype exhibit a reproducible difference in staining pattern for these markers, suggesting H3K9-mono-me and HP1 distributions in spermatocytes do not differ between males of distorting and non-distorting genotypes.

Round spermatids are much more compact than spermatocytes, making visualization of subtle differences in chromatin marks more difficult. Both H3K9 mono-me and HP1 overlapped with areas of strong DAPI stain. However, they did not completely co-localize with each other (Figure 3.2); in general, both H3K9 mono-me and HP1 showed a single strong region of staining that mostly, but not totally, overlapped. Differences between the images in Figure 3.2 for the three genotypes examined may represent slight differences in the developmental stage of the spermatids or in staining of these particular clusters, as overall no difference in HP1 or H3K9 mono-me staining of round spermatids was observed for any genotype examined.

Late in spermatogenesis the nuclei of spermatids begin to condense, first by morphing to a canoe shape and then by further changing into needle-like structures that

become invested in individual membranes (FULLER 1993). During this processes the heads of the spermatids group together to form clusters of elongating nuclei. At this point histones are exchanged for protamines, and a concurrent loss of signal from histone modifications, including H3K9 methylation, is observed (RATHKE *et al.* 2007).

Examination of late, elongating spermatids in distorting and non-distorting conditions confirmed the loss of signal from H3K9 mono-me as the spermatid clusters matured (Figure 3.3). Further, HP1 signal was almost completely lost by the time elongation initiated. Interestingly, the elongating spermatids from males carrying the strong distorting chromosome *SD-5* exhibited a more punctuate or fractured appearance of both the DNA and H3K9 mono-me marks than did the spermatids of *SD⁺ Rsp^s* males (Figure 3.3 top). These atypical elongating clusters were seen in both *SD-5/CyO* and *SD-5/Rsp^s* genotypes, suggesting that they are related to the *SD*-chromosome itself rather than distortion per se. *SD-5* is known to have reduced fertility and so there may be some non-*Sd* specific dysfunction of sperm (SANDLER *et al.* 1959)

Staining for tri-methyl H3K9 recapitulates patterns seen with mono-me H3K9 staining.

Examination of HP1 and H3K9 tri-me staining suggested that there is little significant difference in patterning between mono- and tri-methyl H3K9 in spermatogenesis (Figure 3.1, 3.4). H3K9 tri-me staining matched staining patterns observed for H3K9 mono-me in primary spermatocytes (Figure 3.4), as well as round and elongating spermatids (data not shown) in the testes of both distorting and non-distorting males. Interestingly, cells at the hub of the testis showed both HP1 and H3K9 tri-me marks, but spermatogonial cells

showed only H3K9 tri-me localization. Later, likely following the gonial mitotic divisions, HP1 localization to the nuclei was again observed.

RanGAP and Aubergine Colocalize in piNG-bodies of Primary Spermatocytes

The piNG-body is a prominent perinuclear structure in primary spermatocytes containing Vasa, Aub, AGO3, Cuff, Tud, and Spn-E (KIBANOV *et al.* 2011). This structure is thought to be a major processing center for piRNAs in the testis and is distinguished from typical nuage particles primarily by its size.

I observed a large perinuclear structure in testis of *Drosophila* males expressing an Aub-GFP transgene and stained for RanGAP. These large Aub-GFP foci co-localized with RanGAP staining, suggesting RanGAP may also be present in piNG-bodies (Figures 3.5, 3.6, 3.7, 3.8). These foci were present in spermatocytes from males of both distorting and non-distorting genotypes (Figure 3.5). Interestingly, Aub-GFP and RanGAP did not co-localize throughout most of the perinuclear region. In Figure 3.6 confocal slices taken through a single nucleus showed mostly non-overlapping small RanGAP and Aub-GFP puncta in the perinuclear region. However, one particularly large focus in this nucleus showed strong co-localization of Aub and RanGAP signals. Confocal sections through the thickness of two side-by-side spermatocytes show that each cell contains only one large punctate spot where AubGFP and RanGAP signals overlap (Figure 3.7). Multiple examples of these puncta can be seen in Figure 3.8, which shows clusters of early primary spermatocytes and in Figure 3.5, which shows several examples of more mature

spermatocytes. These puncta were observed in both distorting nuclei, (*SD-LA/Rsp^s*, Figures 3.5 and 3.7) and in non-distorting nuclei (*SD⁺ Rsp^s*, Figures 3.5, 3.6 and 3.8).

***Rsp*-Derived Small RNA Are Detected in the Testes of Both *Rsp^s* and *Rspⁱ* Males**

In order to better understand the role of *Rsp* piRNAs in segregation distortion I utilized a PCR-based method to isolate small *Rsp* RNAs from whole flies (RO and YAN 2010). Briefly, the small RNA fraction (fewer than 100 nt) was isolated and polyadenylated (Figure 3.9a). The poly-A small RNAs were mixed with a 100 nt adapter oligo (miRTQ) containing a 25 nt poly-T sequence. The poly-T region anneals to the poly-A small RNAs and is used as a primer for reverse transcription and generation of a cDNA library. Following RNase H treatment, the library was assayed by PCR using a 20 nt primer specific to the *Rsp* repeat and an adaptor-specific primer (miRTQ-UniR). *Rsp*-specific primers were designed to cover the length of the 240 bp repeat sequence with a 10 bp overlap between oligos (Figure 3.9b). Additional primers, with a 2 bp offset, were designed to a region that had previously been found as a piRNA associated with Piwi in the ovary (SAITO *et al.* 2006).

Walking the length of the *Rsp* repeat by PCR in the cDNA generated from *Rsp^s* animals revealed that small RNAs were generated from specific regions of the repeat in both forward and reverse directions (Figure 3.10a). Most of the *Rsp* small RNAs detected originate from regions of the repeat between nucleotides 10 and 60 in the alpha repeat

and between 130 and 180 in the beta repeat (Figure 3.10a, b). Since the entire 240bp sequence contains alpha and beta halves that are 87% homologous, these two regions share very high sequence similarity. Most primers matching sequences outside of these regions did not generate products. Interestingly, when imported into SFold, a structural prediction program that uses the free energies associated with RNA structures to generate a weighted ensemble of possible folds, the predicted secondary structure of the 240 bp *Rsp* repeat sequences includes fold-back dsRNA in the regions of these small RNAs (Figure 3.10c) (DING *et al.* 2004; CHAN *et al.* 2005).

Small RNA libraries were generated from males of distorting (*SD-72/Rsp^s*) and non-distorting (*SD-72/Rspⁱ*) genotypes and tested by PCR for the presence of *Rsp* sequences. The forward primers showed a similar banding pattern in these two genotypes. However some of the reverse primers gave different products, particularly R9, R11 and R21 (Figure 3.11).

Small RNA libraries were also generated from *Rspⁱ* stocks. The *Rsp^{il6}* chromosome used in this study was generated by x-ray irradiation of the standard *Rsp^s cn bw* chromosome and is thought to contain no more than a dozen responder repeats at the canonical location on chromosome 2R. Surprisingly, the small RNA libraries generated from *Rspⁱ* also contained *Rsp* small RNAs. (Figure 3.11). The same primers that identified *Rsp* sequences in the *Rsp^s* background also yielded hits in the *Rspⁱ* lines. Given that these chromosomes have very few repeats and are insensitive to the effects of *Sd*, it was surprising that these animals contained an equivalent complement of *Rsp*-derived small RNAs as *Rsp^s* flies.

Rsp repeats have been mapped to a few other locations in the genome.

Interestingly, one of these additional loci is in an intronic region of the *AGO3* genomic sequence (REENAN, R., unpublished observation). The presence of approximately five or six of these *AGO3*-associated *Rsp* repeats was experimentally verified by cloning and sequencing of the *AGO3* intronic sequence (S. GELL and C. STABER, unpublished data).

Un-annotated regions of the *Drosophila* genome in Genbank (BDGP R5.12) also contain *Rsp* sequences, but determining their chromosomal location bioinformatically is difficult because of numerous gaps in alignments of these highly repetitive regions. However, while browsing these un-annotated regions of the *Drosophila* genome several areas of *Rsp* sequence were found adjacent to sequences from *Suppressor of Stellate* [*Su(Ste)*], a Y-chromosome locus known to be transcribed and processed into piRNAs.

This observation led to the question: could *Rsp* sequences be transcribed from a piRNA cluster on the Y-chromosome? To look for sex-specific differences in *Rsp* small RNA production I first generated separate small RNA libraries from adult males and females of *Rspⁱ* and *Rsp^s* backgrounds. These libraries were screened by *Rsp*-specific PCR and found to contain *Rsp* sequences generated from the same regions starting approximately 10nt and 130nt from the start of the *Rsp* repeat. Some sex-specific differences were observed, though the differences were mostly in the banding pattern “quality” rather than which primers produced product. PCRs from libraries generated from male tissue had a “smeary” quality indicating products of a wider range of sizes, while the females-derived sequences showed a more precise banding pattern (Figure 3.12). Additionally, products from primers F107 and F151 show a specific enrichment in male tissue.

Additional Rsp loci not detected in vivo

Two approaches were utilized to determine if *Rsp* repeats are present on the Y-chromosome *in vivo*. First, I attempted to look for additional *Responder* loci by using a biotinylated *Rsp* probe for fluorescent *in situ* hybridization (FISH) on mitotic chromosomes. While the endoreduplicated polytene chromosomes of *Drosophila* salivary glands are an easier FISH target because of clear banding patterns and multiple copies of the genome, the Y-chromosome remains in the chromocenter in this preparation, making localization of sequences to the Y impractical (KENNISON 2000). Mitotic spreads, while lower resolution, allow clear separation of both sex chromosomes and autosomes (PIMPINELLI *et al.* 2000). However, following spreading, fixation and FISH, this technique only identified the canonical *Rsp* repeat region on chromosome 2R in *Rsp^s* preparations. No clear FISH signal was observed in a *Rspⁱ* background, suggesting that this assay is not sensitive enough to detect low copy number repeat arrays (data not shown).

Additionally, restriction digests and Southern blots were performed on DNA extracted from both *Rspⁱ* males and females to look for male-specific and, thus, likely Y-associated bands. For each sample approximately 1600ng of DNA was digested with a six-cutter restriction enzyme and blots were probed with labeled *Rsp* sequence (Figure 3.13). In more than 25 restriction enzymes tested only one, *Nde* I, yielded bands of different sizes in males and females (Figure 3.13a). However, this sex-specific difference was not reproducible upon repeated digests.

While a secondary array of *Rsp* repeats has been confirmed experimentally on the third chromosome in an intron of *AGO3* (C. STABER and S GELL, unpublished data),

additional repeat arrays on autosomes or sex chromosomes were not detected in a *Rspⁱ* background.

***AGO3* Expression is Unchanged Under Distorting and Non-Distorting Conditions**

The presence of a small number of *Rsp* repeats (5-6 copies) in the second intron of *AGO3* suggests a potential interaction between this locus and the canonical *Rsp* repeat on chromosome 2R. In *Drosophila* ovaries, the transposon-derived piRNAs generated from the *flamenco* piRNA cluster silence transposons located elsewhere in the genome (BRENNECKE *et al.* 2007). This ability to silence *in trans* led us to hypothesize that *Rsp*-derived piRNAs could potentially act to suppress *AGO3* expression in distorting conditions, thereby disrupting the piRNA processing machinery.

To test this hypothesis, I used quantitative real-time PCR (RT-qPCR) to examine *AGO3* expression levels in the testes of *Rspⁱ* and *Rsp^s* males with and without *SD* chromosomes. Data represent the average of three biological replicates normalized to Rp49 expression. There was no significant difference in fold enrichment of *AGO3* expression in any genotype tested (Figure 3.14). These data suggest that *Rsp* repeats located on 2R do not regulate *AGO3* expression. However, the data do not rule out the hypothesis that piRNAs produced from the introic region of *AGO3* could potentially regulate the 2R *Rsp* repeat cluster.

Third Chromosomes Carrying TubP-GAL4 Driver Alone Enhance Distortion to a Greater Degree than *AGO3* Overexpression

To further examine the role of AGO3 in segregation distortion, two AGO3 transgenes were constructed, a wild type (wt) transgene as well as transgene with a mutation to one of the catalytic triad of amino acids required for RNA slicer activity (D713A). The transgenes were recombined with a TubP-GAL4 on the third chromosome and these driver-transgene lines were used to test the effect of AGO3 overexpression on *SD* (Figure 3.15a).

Three lines of each transgene were tested. Each line represents an independent recombination event between the transgene and the driver on the third chromosome. Overexpression of either the wild type or the D713A transgenes in *SD-LA/Rsp^s* males increased drive over that of the *SD-LA/Rsp^s* alone, with the wild type construct showing higher levels of distortion than the catalytic mutant (Figure 3.15D). However, interpretation of these results is confounded by the fact that in the absence of transgene expression, the TubP-GAL4 driver chromosome increased drive more profoundly than expression of either the wild type or D713A construct. Given the effect on distortion of the TubP-GAL4 third chromosome, little can be extrapolated from this experiment regarding the effects of AGO3 overexpression on *SD*.

DISCUSSION

Both piRNA biogenesis and segregation distortion are highly complex processes that are poorly understood. Given our limited knowledge of each of these systems, discerning the molecular mechanism governing their observed genetic interaction is no trivial task. While it is clear that piRNA pathway components play a role in suppressing the effect of *SD* on *Rsp*, the way in which this activity is facilitated remains mysterious.

The primary defect in spermatogenesis of *SD* flies involves the failure to properly condense chromatin in developing *Rsp^s* spermatids (TOKUYASU *et al.* 1977). This failure suggests that there could be differences in the chromatin state of *Rsp^s* and *Rspⁱ* nuclei in *SD* males. To test this hypothesis, I used immunohistochemical staining of the heterochromatic markers HP1 and methylated H3K9 in whole mount testis from *SD* and *SD⁺* flies.

While there exist cell stage-specific differences in heterochromatic marks, there are no apparent differences between the heterochromatic marks in the testes of flies from distorting and non-distorting genotypes (Figures 3.1, 3.2, 3.3, 3.4). Males carrying the *SD-5* chromosome do show an unusual condensation and fragmentation of nuclei of late elongating spermatids. However, this fragmentation is visible in the testes of both distorting (*SD-5/Rsp^s*) and non-distorting (*SD-5/CyO*) males, suggesting that this defect is related to features of the *SD-5* chromosome not specific to distortion. The *SD-5*

chromosome contains two paracentric inversions on 2R and is not homozygous viable, suggesting the possibility that other mutations affecting fertility could be in linkage disequilibrium with *Sd* (SANDLER and HIRAIZUMI 1959; SANDLER *et al.* 1959; TEMIN *et al.* 1991). The heterogeneity of testis tissues makes a quantitative analysis of heterochromatic markers difficult. However, qualitatively, the lack of prominent differences in heterochromatic marks early in spermatocytes fits with the lack of any observable defects in spermatogenesis of distorting males until after the meiotic divisions. While no reproducible differences in heterochromatic marks were observed in round spermatids, the small size and condensed nature of these cells makes discerning subtle differences difficult.

Additional examination of other chromatin modifications, such as H3K9 acetylation or H3K27 methylation, could potentially reveal differences in the development of *Rsp^s* and *Rspⁱ* nuclei of *SD* males. However, under the conditions tested there are no apparent differences in chromatin marks between developing sperm of distorting and non-distorting males.

Expression of *Sd-RanGAP* disrupts nuclear transport and leads to the nuclear accumulation of targets of RanGTP-mediated transport. However, the contribution of this transport defect to the dysfunction of *Rsp^s* sperm is unclear. While both AGO3 and Aub are primarily cytoplasmic in the testis, it remains possible that small amounts of these proteins could enter the nucleus to facilitate silencing (GUNAWARDANE *et al.* 2007; NISHIDA *et al.* 2007; NAGAO *et al.* 2010). Piwi localizes to both cytoplasm and nucleus, and likely shuttles between the two compartments (NISHIDA *et al.* 2007; KLATTENHOFF and THEURKAUF 2008; MALONE *et al.* 2009). It is therefore possible that Aub or AGO3

could accumulate in the nucleus in the presence of *Sd* even if these proteins are primarily cytoplasmic under wild type conditions.

To test this hypothesis, I examined the testes of flies expressing Aub-GFP in the testes under distorting and non-distorting conditions. However, after examining confocal sections of nuclei from multiple genotypes, Aub-GFP is never observed in the nucleus of spermatocytes from distorting or non-distorting males (Figures 3.5, 3.6, 3.7, and 3.8).

While examining the testes of Aub-GFP transgenic flies, I observed an interesting colocalization of Aub-GFP and RanGAP in a single prominent perinuclear body, likely the piNG-body. Aubergine is a primary marker of piNG-bodies and only a single piNG-body is observed for each nucleus. Similarly, only a single large body containing both Aub and RanGAP was observed per nucleus (Figure 3.7). Because RanGAP associates with the nuclear pore, this observation suggests that piNG-bodies may also be localized to nuclear pore complexes. At the nuclear pore, the piNG-body would be particularly well positioned to process newly exported piRNA precursors and to allow rapid nuclear entry of piRNA-primed RNP complexes that could facilitate transcriptional silencing.

While a few *Rsp*-derived piRNAs have been identified, primarily in association with AGO3 (NAGAO *et al.* 2010), determining the role of *Rsp* piRNAs in *SD* requires a more detailed analysis of the complement of piRNAs associated with distorting and non-distorting genotypes. Using a PCR-based method I have analyzed the population of *Rsp*-associated small RNAs found in the testes of flies from a variety of genetic backgrounds. *Rsp* small RNAs are produced from specific regions of the *Rsp* repeat, initiating from a region starting 10 nt from the start of either the alpha or beta 120 bp repeat (Figure 3.10). These regions are highly homologous; primers to one region may amplify sequences

transcribed from the other. Interestingly, males of both distorting (*SD-72/Rsp^s*) and non-distorting (*SD-72/Rspⁱ*) genotypes showed a very similar complement of *Rsp* small RNAs. With the exception of primers R9 and R21, patterns of *Rsp* expression were quite similar even though the associated repeat arrays in *Rspⁱ* and *Rsp^s* backgrounds are quite different (Figure 3.11). This unexpected similarity in the populations of *Rsp* small RNAs from *Rspⁱ* and *Rsp^s* backgrounds suggests that these *Rsp*-derived small RNAs are generated from a locus other than the canonical repeat on chromosome 2R. The same pattern is observed in *Rsp* small RNAs derived from non-distorting *SD⁺ Rspⁱ* and *SD⁺ Rsp^s* lines, suggesting that generation of these *Rsp* small RNAs is not dependent on expression of *SD*-RanGAP (Figure 3.12).

piRNA clusters function as “master regulators” of transposon activity and facilitate silencing of homologous elements even in distal regions of the genome. The loss of piRNAs generated from a particular cluster, such as *flamenco*, leads to a global activation of those families of transposable elements contained in the cluster (BRENNECKE *et al.* 2007). The ubiquitous presence of *Rsp* small RNAs in all genotypes tested suggests a model whereby these small RNAs could represent a distal source of initiator piRNAs that act on the canonical locus on chromosome 2R to mediate appropriate silencing.

Rsp repeats are found in multiple non-canonical locations in the *Drosophila Melanogaster* BDGP R5 draft genome assembly. In addition to the experimentally confirmed second intron of AGO3 on chromosome 3R, examination of the un-annotated heterochromatic regions of the *D. melanogaster* genome assembly reveals several additional *Rsp* repeat regions. Manual inspection of these repeat regions using the UCSC

genome browser suggests that a few of these repeats align in proximity to sequence from the *Suppressor of Stellate* [*Su(Ste)*] locus.

Su(Ste) is a Y-linked gene, known to be transcribed and processed into piRNAs, suggesting the presence of a Y-linked piRNA cluster (ARAVIN *et al.* 2001; ARAVIN *et al.* 2004). The *in vivo* organization of the *Su(Ste)* sequences listed in the extrachromosomal sequence of the *D. melanogaster* BDGP R5 assembly is not known. However, this observation suggests that *Rsp* repeats might be present on the Y-chromosome in proximity to a known piRNA cluster and provides a mechanism for *Rsp* piRNA transcription. In light of the equivalent complement of *Rsp* small RNAs isolated from *Rspⁱ* and *Rsp^s* genotypes, this hypothesis provides a plausible explanation for the origin of *Rsp*-derived small RNAs.

To test this hypothesis, small RNA populations were isolated from males and females of *Rspⁱ* and *Rsp^s* backgrounds. These small RNA libraries showed similar patterns of *Rsp* repeat amplification (Figure 3.13). There were, however, a few sex specific differences; primers F107 and F151 amplified sequence much more robustly from males than females, and overall the sequences amplified from males showed a greater variety of product lengths and a less definite banding pattern. Further testing of *Rspⁱ* genomic DNA for sex-specific differences by Southern blot and FISH on mitotic chromosomes did not reveal any Y-associated piRNA repeats (Figure 3.14 and data not shown). It is possible that small numbers of repeats are below the sensitivity threshold for these assays. The FISH assay, in particular, did not even detect the *Rsp* repeats located on 3R or the remaining copies of *Rsp* on 2R of the *Rspⁱ* chromosome suggesting additional sensitivity or signal amplification may be needed. An assay such as primed *in situ*

labeling (PRINS), which permits PCR-based amplification of fluorescent signal on fixed chromosomes may provide the sensitivity needed to detect low levels of repeats (SPEEL *et al.* 2003).

The presence of *Rsp* repeats in the second intron of *AGO3* provides another interesting possibility for a “source” of *Rsp* small RNAs from outside the canonical locus on chromosome 2R. piRNAs have been shown to be processed from untranslated regions of several genes in the *Drosophila* ovary including *traffic jam*, CG32000, *brat* and *Klp10A* (ROBINE *et al.* 2009; SAITO *et al.* 2009). However, *Rsp* piRNAs specifically derived from the *AGO3* locus have not yet been detected.

These *Rsp* repeats in the *AGO3* locus also suggest an alternative mechanism in which piRNA production from the canonical *Rsp* locus could regulate *AGO3* expression through homology-mediated silencing. To test this possibility, *AGO3* expression levels were examined in the testes of males of *SD⁺ Rspⁱ*, *SD⁺ Rsp^s*, *SD/Rspⁱ* and *SD/Rsp^s* genotypes. However, no significant change in fold enrichment of *AGO3* RNA was detected in any of these conditions, suggesting that *AGO3* expression is not specifically down-regulated by *Rsp* small RNAs generated from the canonical locus.

Lastly, in order to explore the role of *AGO3* in segregation distortion I generated two UAS-HA-*AGO3* transgenes: a wild type construct and one carrying a mutation to a catalytic aspartic acid (D713A) of *AGO3*. These transgenes were driven in male flies carrying both *SD* and *Rsp^s*. While overexpression of either wild type or catalytically inactive *AGO3* transgenes leads to an enhancement of distortion, control experiments show that the TubP-GAL4 driver alone had an even larger effect, clouding interpretation of these results.

The mechanism by which the piRNA pathway acts to suppress segregation distortion remains unclear. However, the data presented here have eliminated some possible mechanisms and suggested others. Steps in piRNA biogenesis or targeting that require passage through the nuclear membrane represent obvious points of intersection between the piRNA pathway and Segregation Distorter. The co-localization of Aub-GFP and RanGAP in piNG-like bodies strongly suggests nuclear transport and piRNA processing are tightly coupled. Further, the data here have suggested a model whereby *Rsp* piRNAs may be generated from an as yet unidentified “source” distinct from the canonical *Rsp* locus on 2R, exported from the nucleus for processing or maturation and then imported as an RNP complexes primed for silencing of the canonical *Rsp* locus. Proper silencing of *Rsp* may be required for chromatin condensation and maturation of *Rsp*-bearing spermatids. While much of this model awaits experimental confirmation, these data represent a new chapter in our understanding of the etiology of Segregation Distorter.

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FIGURES AND FIGURE LEGENDS

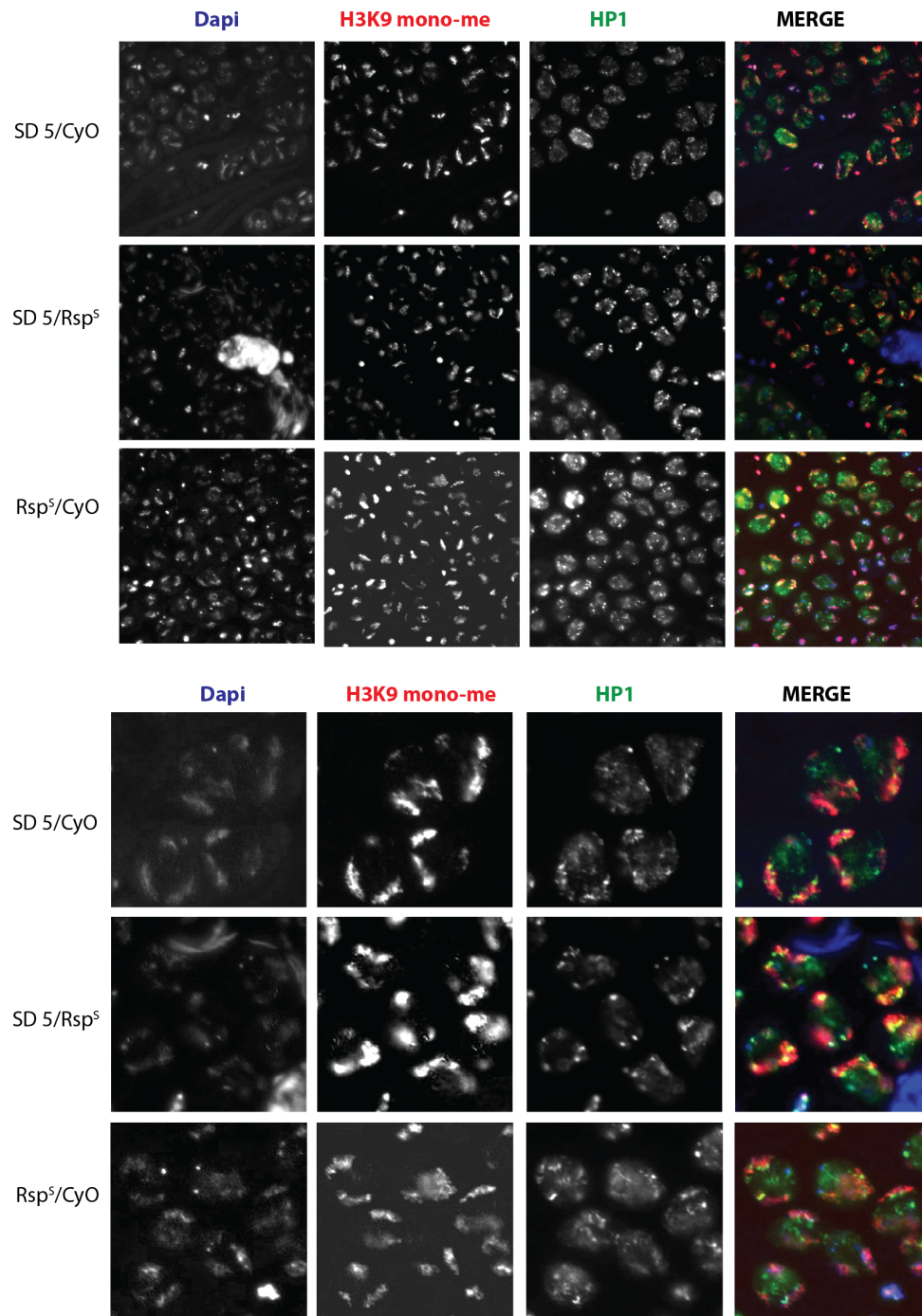


FIGURE 3.1: HP1 and H3K9 mono-me staining of primary spermatocytes. (A) DAPI, HP1 and H3K9 mono-me staining of primary spermatocytes from distorting (*SD-5/Rsp^S*) and non-distorting (*SD-5/CyO* and *SD-5/Rsp^I*) genotypes. (B) Enlargement of a section of each image in (A).

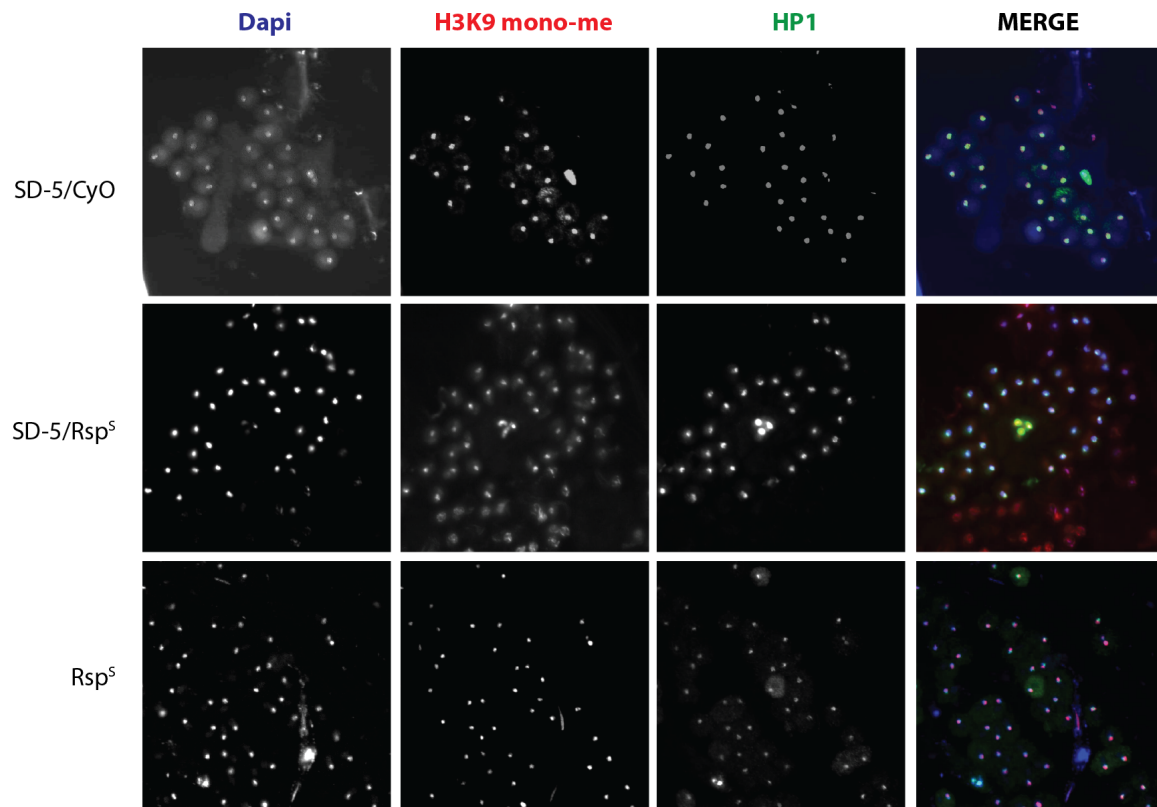


FIGURE 3.2: HP1 and H3K9 mono-me staining of round spermatids. Distorting and non-distorting genotypes both show some, but not complete, overlap of DAPI, H3K9-mono-me and HP1 signal.

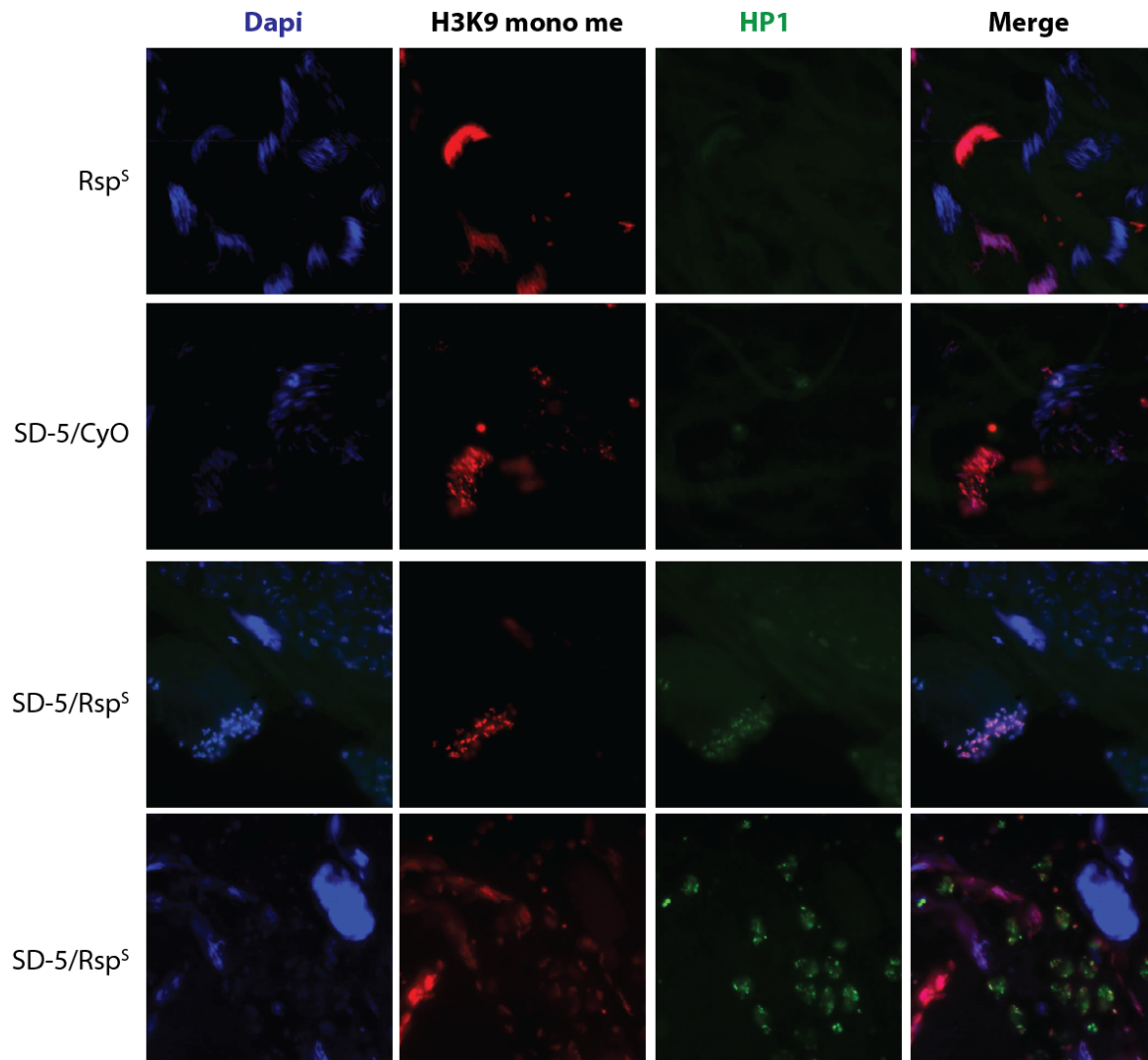


FIGURE 3.3: HP1 and H3K9 mono-me staining of elongating spermatids. The top row shows normal loss of H3K9 histones as spermatids mature. Males containing *SD-5* under both distorting (*SD-5/Rsp^s*) and non-distorting (*SD-5/Rspⁱ*) conditions show an atypical fragmentation of elongating spermatids. This disruption is not present in all clusters of spermatids from *SD-5* males, as seen in the bottom row.

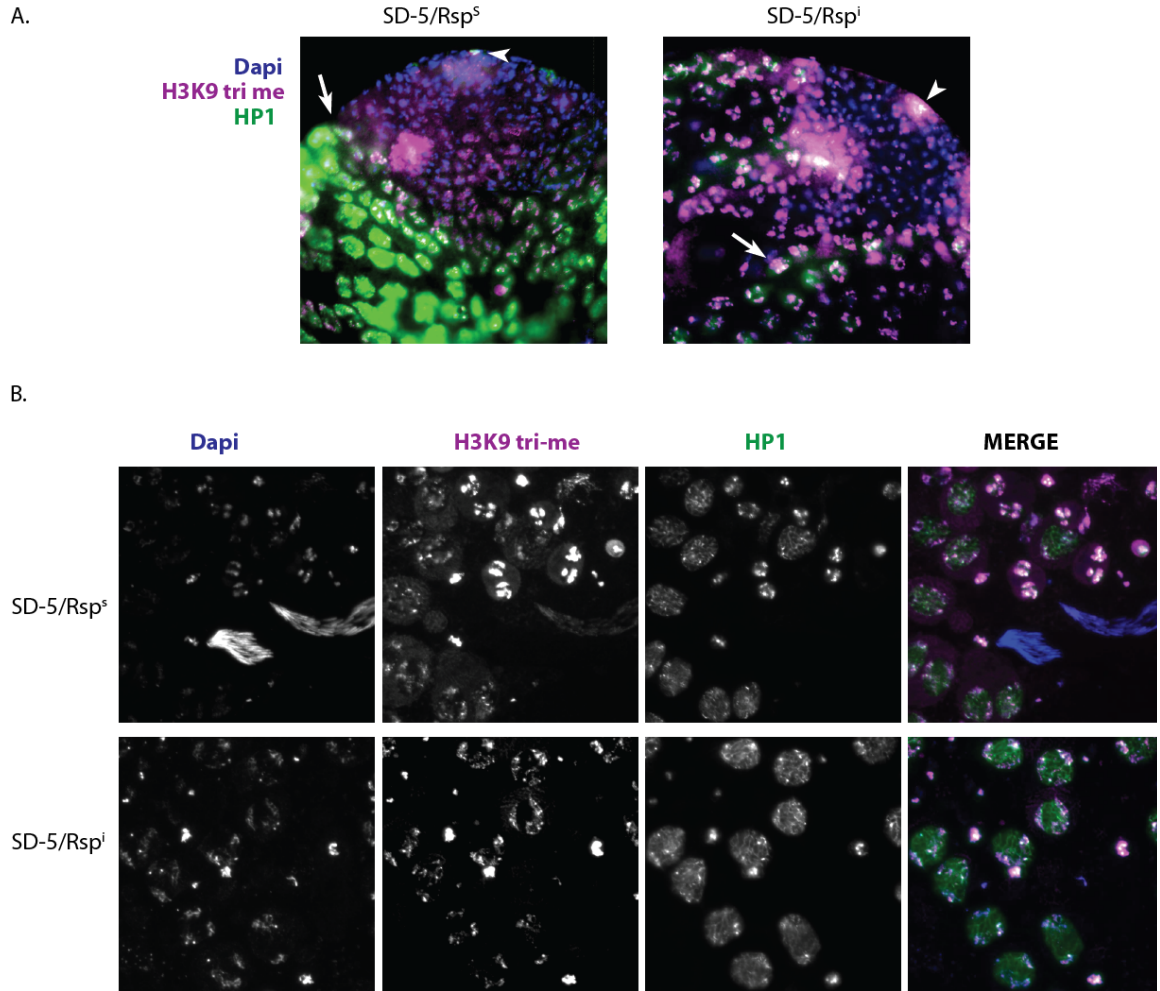


FIGURE 3.4: HP1 and H3K9 tri-me staining does not differ between distorting and non-distorting genotypes. (A) Apical tip of the testis from distorting and non-distorting genotypes. The arrowhead marks the hub region, which shows both H3K9 mono-me and HP1 staining. The spermatogonia show only H3K9 tri-me staining but HP1 marks return as the cells mature into spermatocytes (arrow). (B) Primary spermatocytes show punctate H3K9 tri-me staining and more dispersed HP1 staining in both genotypes examined.

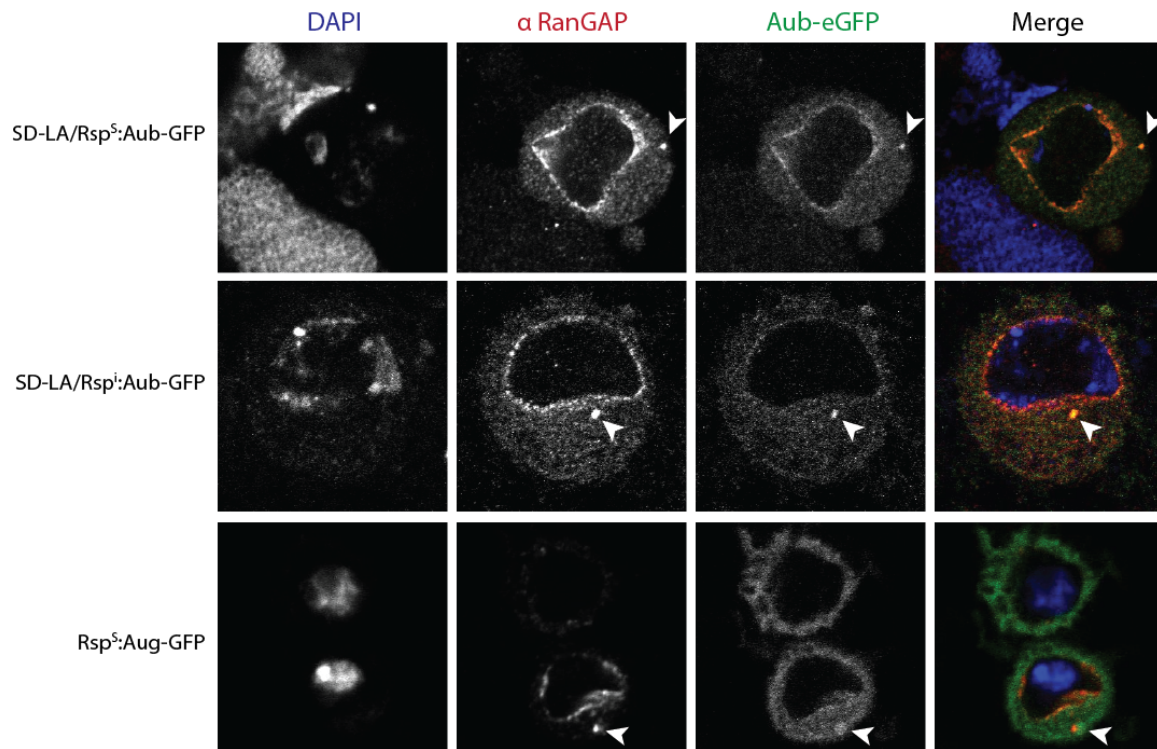


Figure 3.5: Primary spermatocytes show precise areas of co-localization between Aub-GFP and RanGAP. The Aub-GFP construct is driven by a nos-GAL4 VP16 driver recombined on the same chromosome. Nuclei of primary spermatocytes from distorting and non-distorting males each show a single locus of Aub-GFP and RanGAP localization (arrowhead). The anti-RanGAP antibody detects both wild type and Sd-RanGAP.

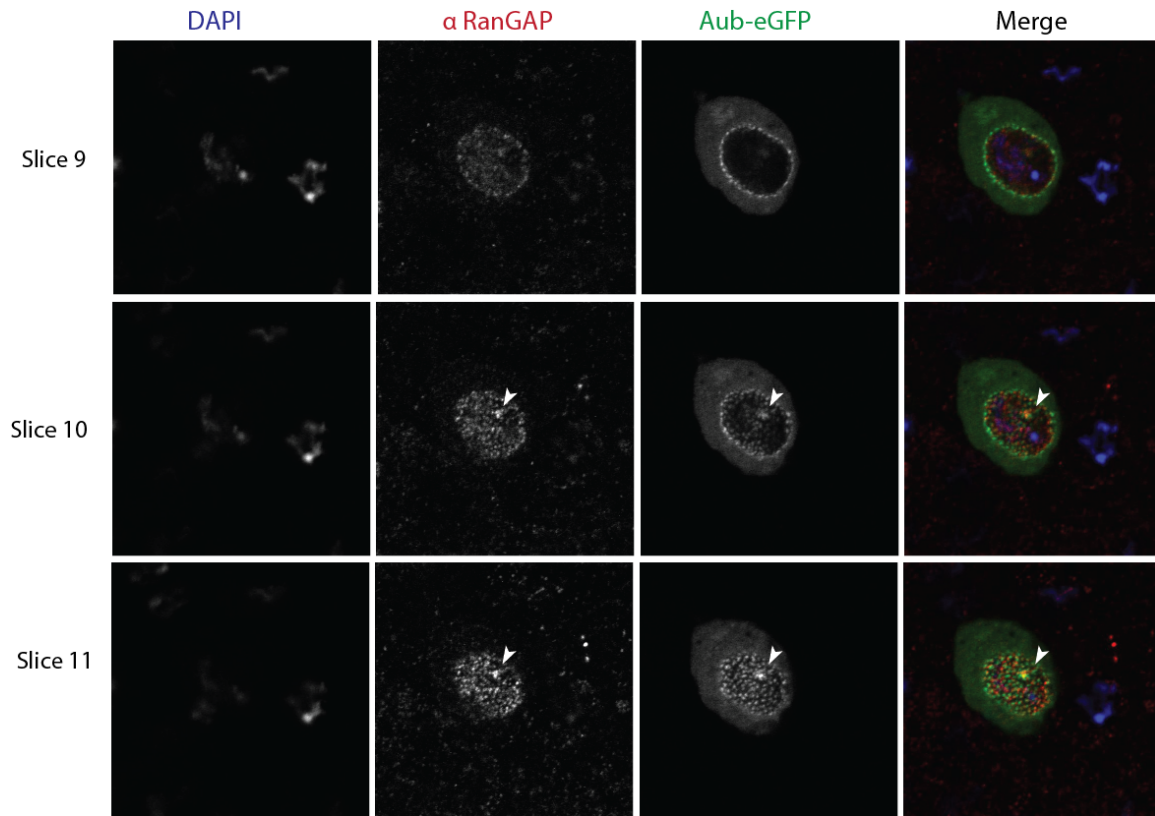


FIGURE 3.6: Colocalization of Aub-GFP and RanGAP is specific to a single large perinuclear particle. Slices from the middle (slice 9) to the top (slice 11) of this spermatocyte from the testis of a *Rsp^s:aub-GFP* male show many non-overlapping, punctate foci of Aub-GFP and RanGAP. Specific co-localization is only observed at one point on the surface of the nucleus. Arrowhead shows point of co-localization.

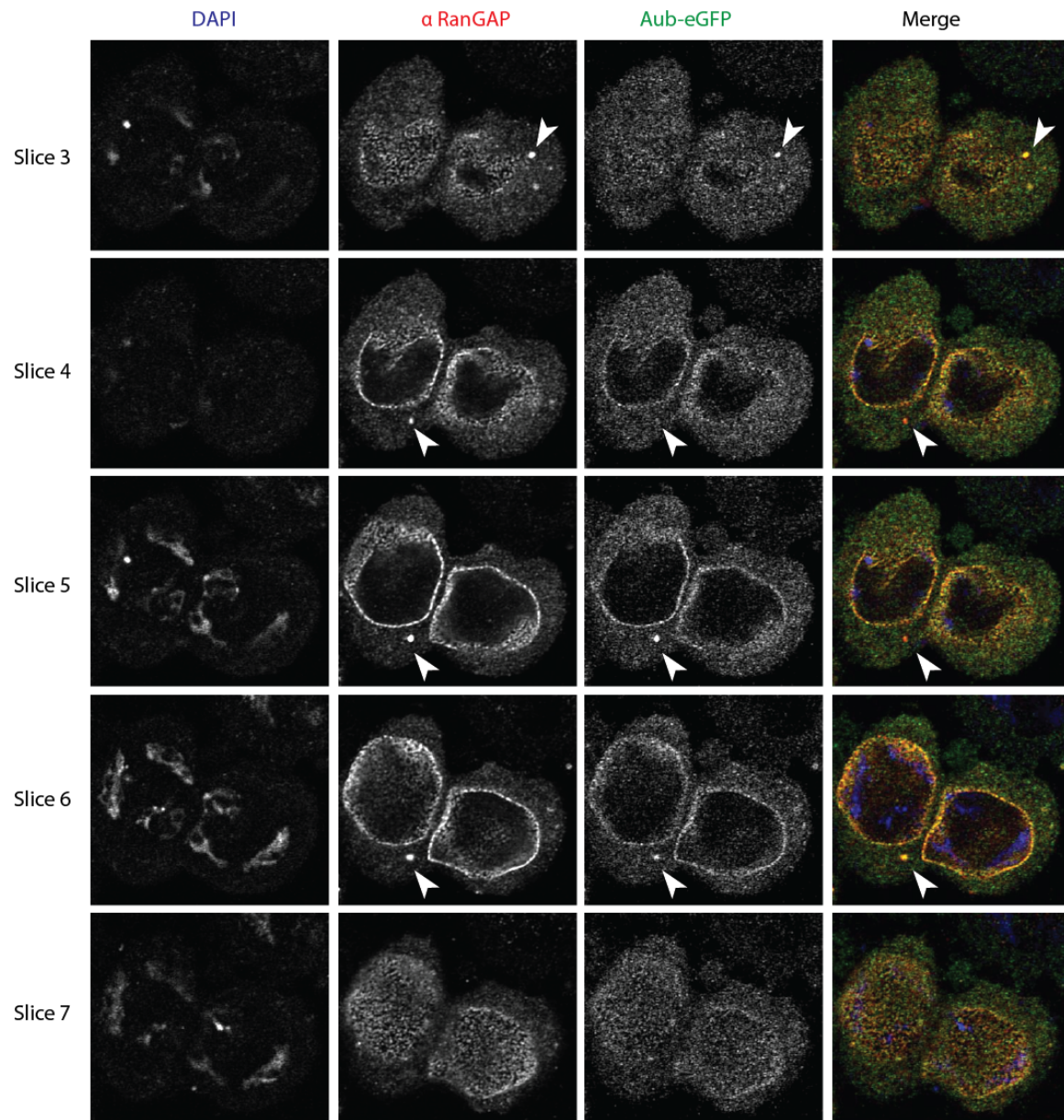


FIGURE 3.7: Only a single piNG-body is visible per cell. Confocal slices through the nuclei of two primary spermatocytes from the testis of an *SD-LA/Rsp^s*: *aub*-GFP male stained for RanGAP show only a single large focus of RanGAP and Aub-GFP co-localization in each cell.

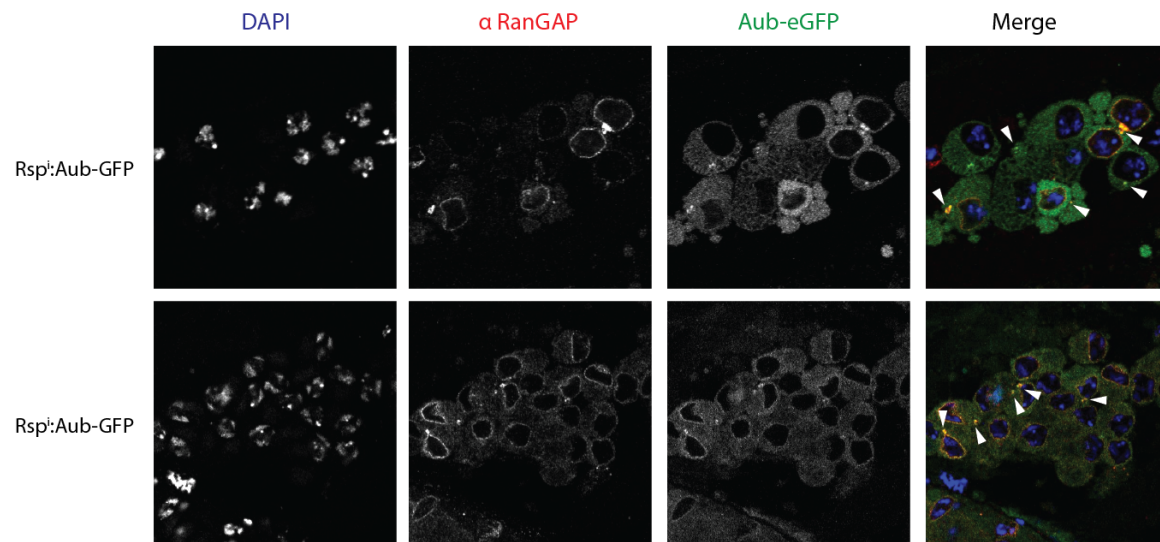
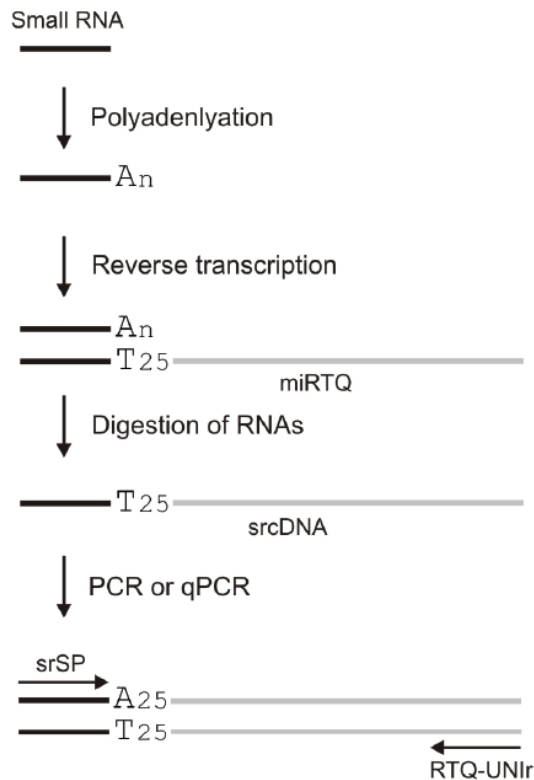


FIGURE 3.8: RanGAP and Aub-GFP colocalize in early spermatocytes. Specific points of colocalization can be seen in the perinuclear region of many of the primary spermatocytes in these clusters.

A.



B.

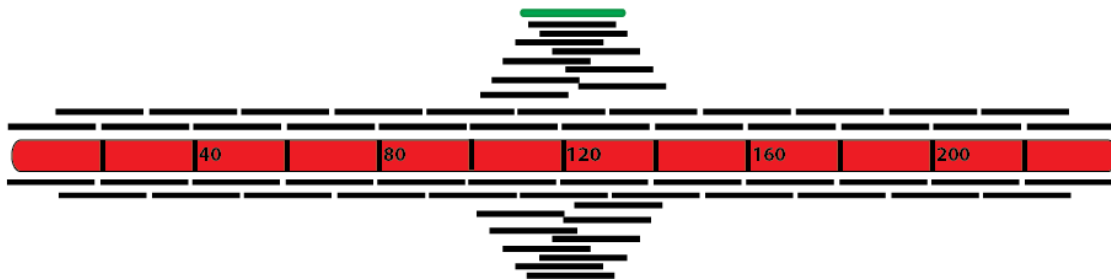


FIGURE 3.9: Experimental design for amplification of *Rsp*-specific small RNAs. (A) Following isolation of the small RNA fraction, the RNAs are polyadenylated. The miRTQ adapter is annealed using homology between the poly-A tail and 25 Ts in the 3' end of the adaptor. The miRTQ adaptor is then used to prime the reverse transcription (RT) reaction and generate the small RNA cDNA (srcDNA) library. Following RT, the sample is treated with RNase H to remove any remaining RNA. Next PCR or qPCR can be performed using a primer specific to the adaptor (RTQ-UNIr) and a small RNA-specific primer (srSP) (Reproduced from Ro *et al.*, 2010). (B) Primers were designed to the *Rsp* repeat covering the 240 bp canonical repeat in both the forward and reverse directions with a 10 nt degeneracy. Additional primers were designed to a region that has been found as a piRNA in ovaries (Nishida *et al.* 2007).

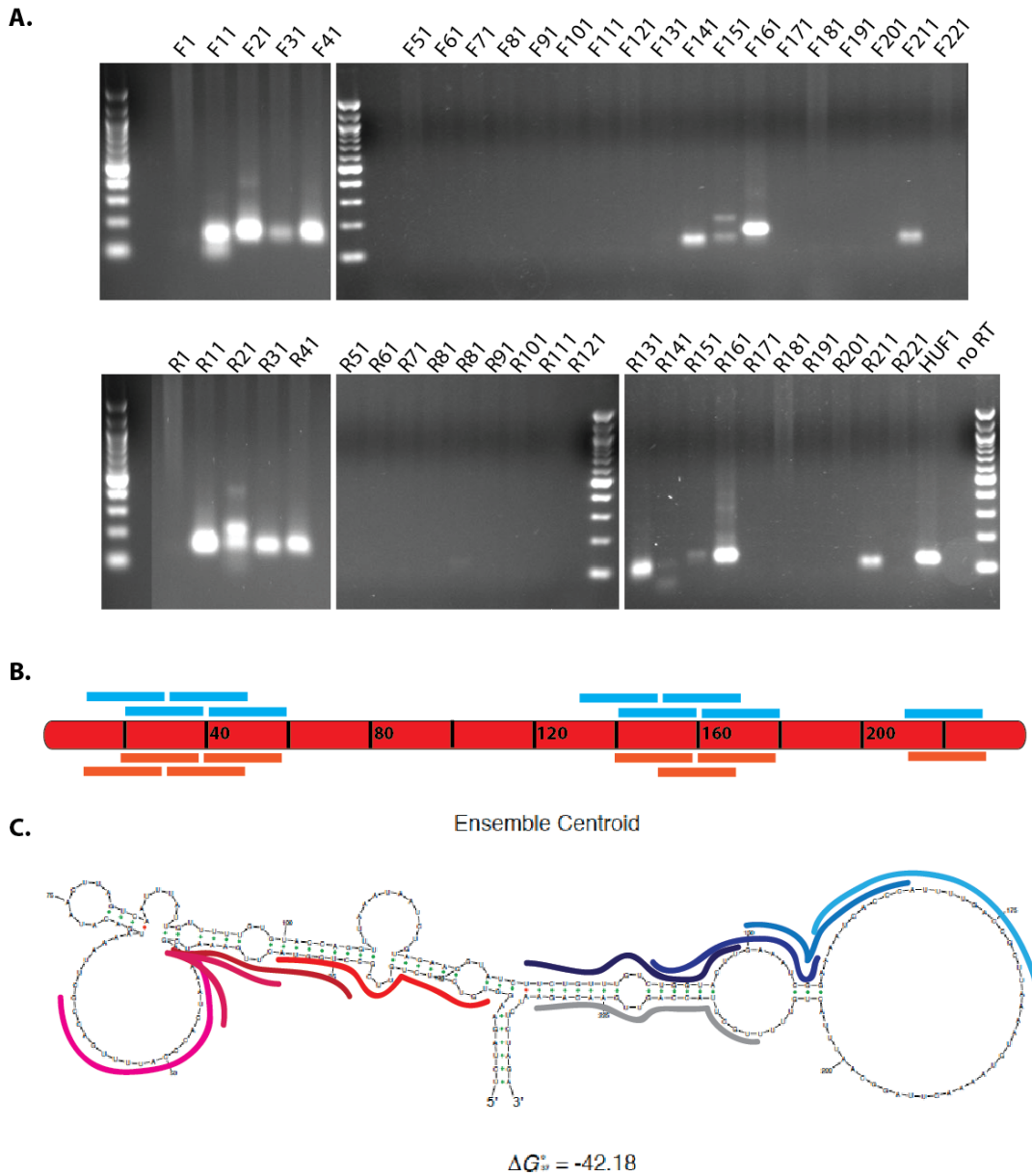


FIGURE 3.10: Small RNAs amplified from the testis of *Rsp^s* males are generated from specific regions of the *Rsp* repeat. (A) PCR products from forward (F) and reverse (R) primers designed to cover the length of the *Rsp* repeat with a 10 nt overlap. The specific products are amplified from a region 10nt and 130 nt from the start of the 240 nt repeat. The hairpins of unknown function (HUF) primer is specific to an abundant small RNA species in the gonad (Y. SAVVA, unpublished observations) and serves as a positive control. No RT reactions contained the HUF-specific primer. (B) A schematic of the *Rsp* repeat showing the locations of products amplified by reverse (blue) and forward primers (orange). (C) The ensemble secondary structure for one copy of the *Rsp* 240 bp repeat as predicted by Sfold. Red lines indicate the position of PCR products from the alpha half of the repeat; blue lines represent the locations of PCR products on the beta half.

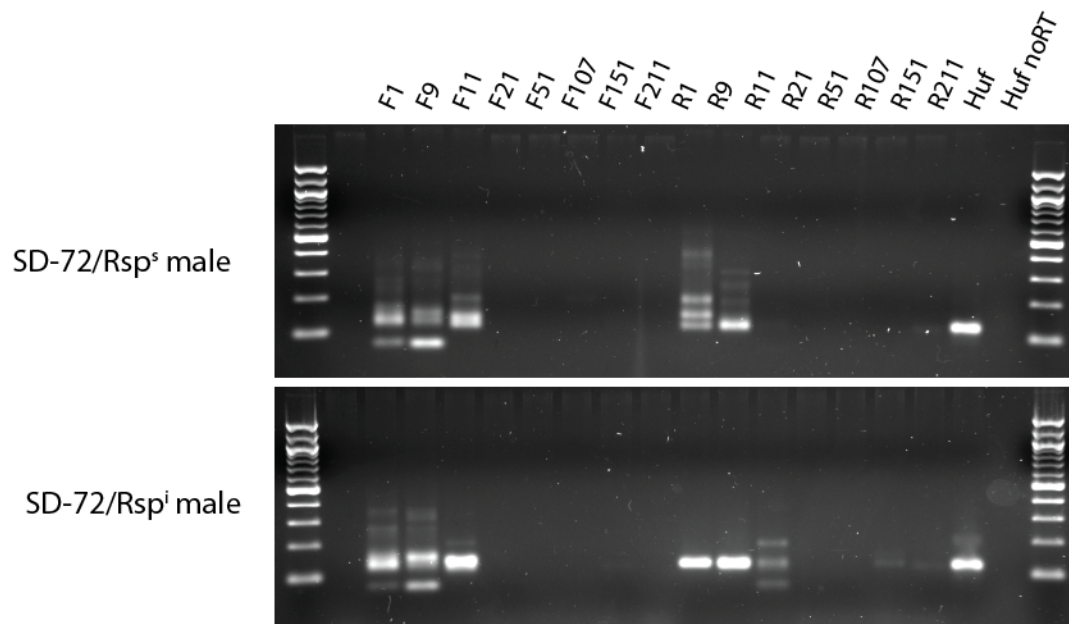


FIGURE 3.11: *Rsp* Small RNAs are amplified from testes of both distorting and non-distorting males. With the exception of primers R21 and R151, the same primers amplified products in non-distorting (*SD-72/Rspⁱ*) and distorting (*SD-72/Rsp^s*) genotypes.

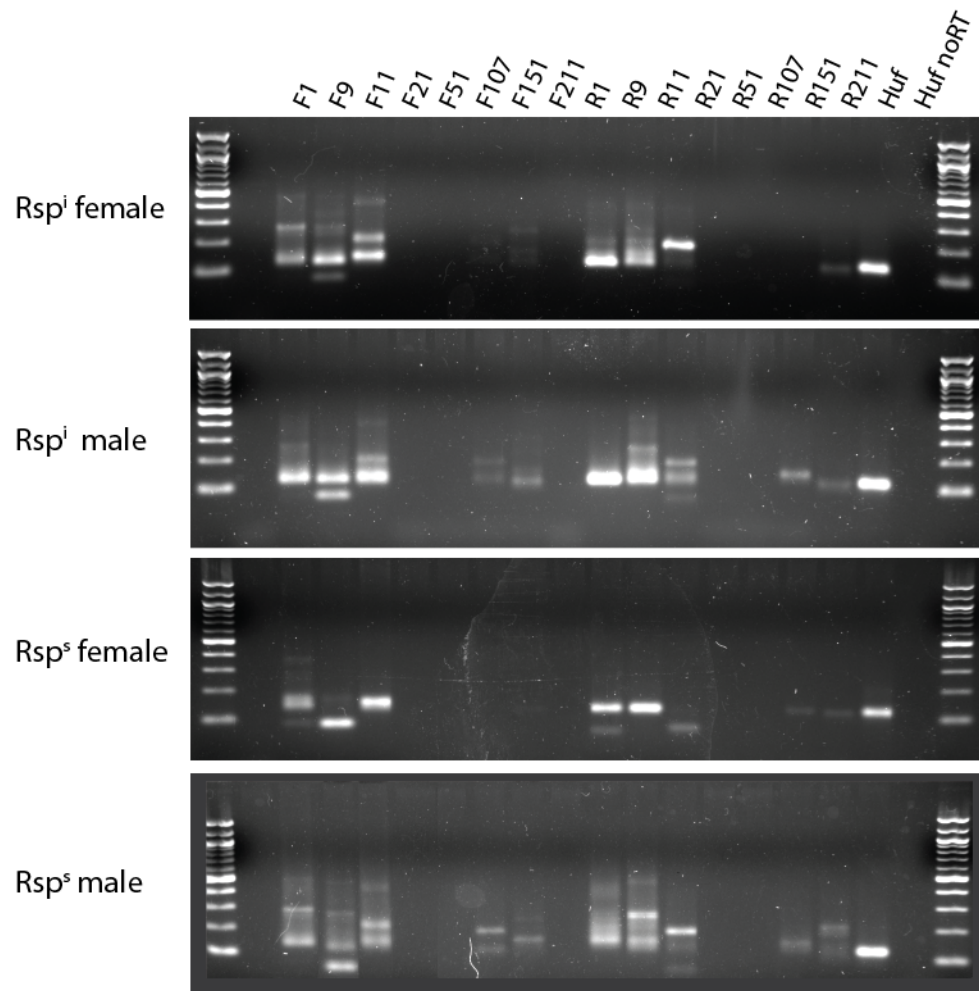


FIGURE 3.12: Small RNA PCR amplification of *Rsp* sequences reveals only minor differences between males and females of *Rsp^s* and *Rspⁱ* genotypes. Forward primers F1, F9, and F11 and reverse primers R9, R11 and R21 as well as the HUF positive control primer yielded product in all genotypes. Products from F107 and F151 are enriched in males.

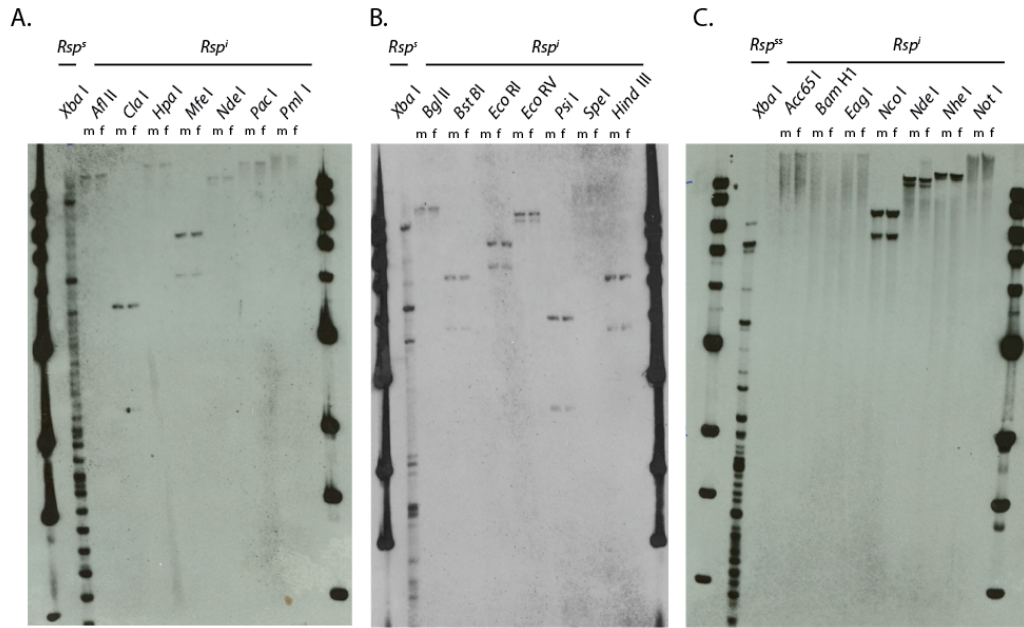
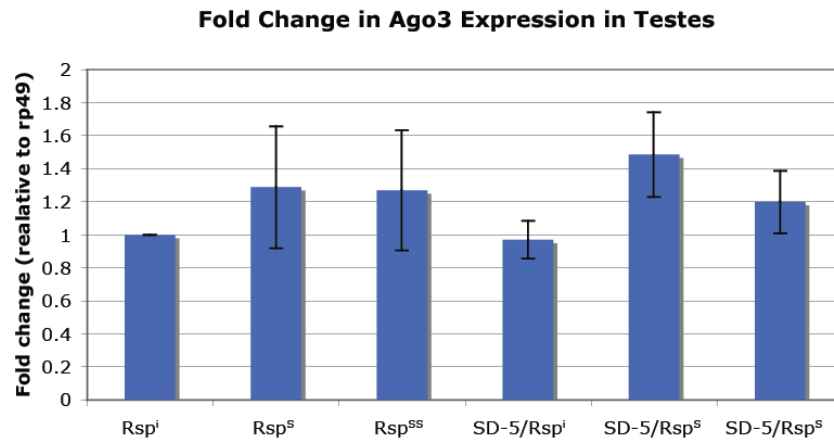


FIGURE 3.13: Southern blotting reveals no difference in *Rsp* repeat arrays of *Rsp^j* males and females. Approximately 1600 ng of genomic DNA extracted from female or male whole flies was digested by a six-cutter restriction enzyme, run overnight and probed for *Rsp*. No repeatable differences in band sizes between male and female genomic DNA digests were observed.

A.



B.

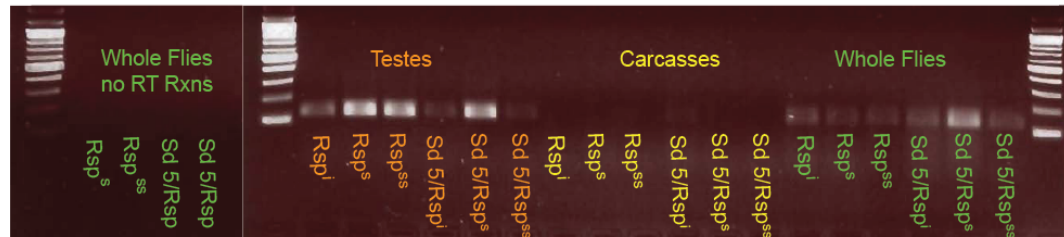


FIGURE 3.14: *Argonaute3* (*AGO3*) expression levels are unchanged between distorting and non-distorting conditions. (A) Average fold change of *AGO3* expression in the testis normalized to *rp49* for three biological replicates in 6 different genotypes. Error bars represent standard error. (B) PCR using *AGO3* primers shows enrichment for *AGO3* in the testis and no expression in reactions lacking reverse transcriptase (RT).

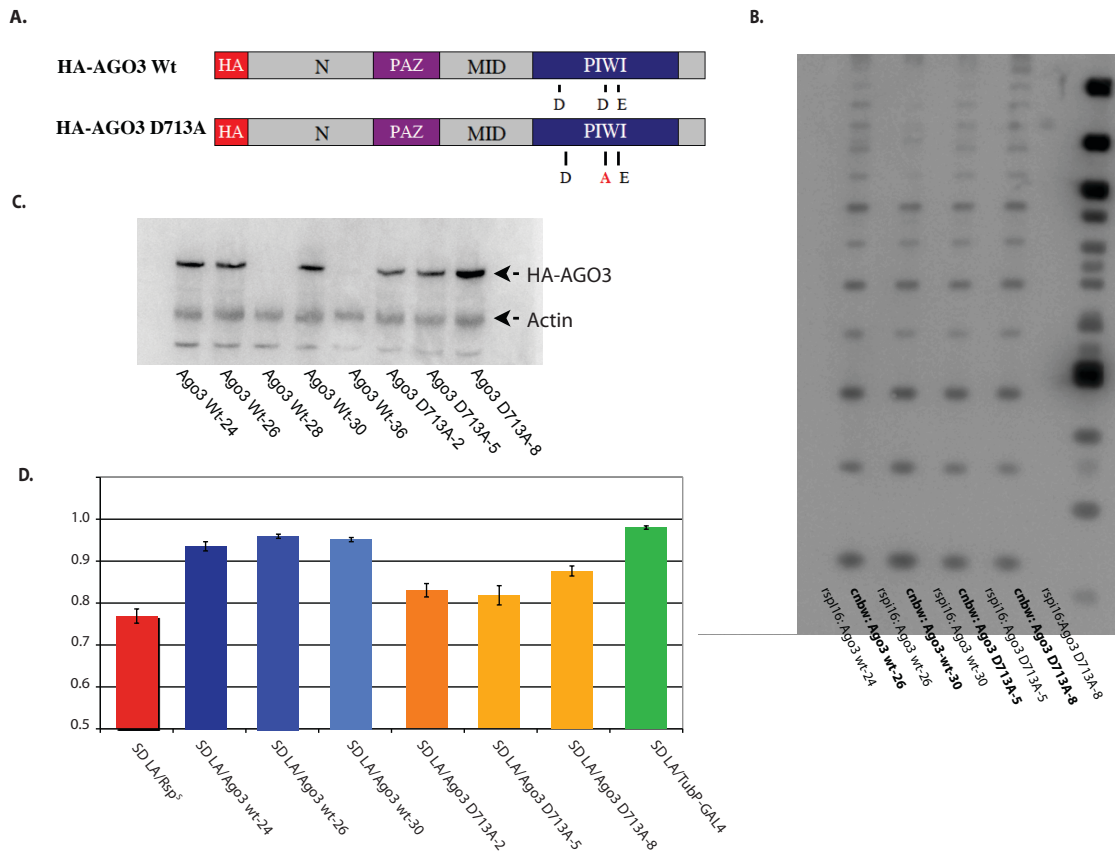


FIGURE 3.15: A third chromosome carrying the TubP-GAL4 driver enhances distortion to a greater degree than AGO3 overexpression. (A) A schematic showing the organization HA-AGO3 transgenes. The HA-AGO3 D713A transgene replaces the second catalytic aspartic acid in the PIWI domain with an alanine. (B) Southern blot for *Rsp* sequence of *Xba*I digested genomic DNA from lines containing TubP-GAL4 driver and HA-AGO3 transgenes crossed into either *Rsp*ⁱ or *Rsp*^s backgrounds demonstrating that these lines have the correct *Rsp* locus. (C) Western blot for HA and Actin showing equal expression levels of HA-AGO3 wild type and mutant transgenes. Lines expressing HA-AGO3 protein represent recombinant third chromosomes carrying both UAS-HA-AGO3 and TubP-GAL4. Lines without the HA-AGO3 band represent chromosomes lacking either the driver or the transgene. (D). Corrected values (k_c) for males carrying both *SD-LA* and *Rsp*^s second chromosomes as well as expressing either wt or D713A HA-AGO3 transgenes. Each bar represents the sum k_c value for 20-30 individual males. Error bars represent the standard error. The TubP-GAL4 line carrying the driver alone enhances distortion more strongly than any of the driven lines.

Table 3.1: Primers used in this study	
Name	Sequence
AGO3-FHAnot	TGC GGC CGC AAA ACA TGT ACC CTT ACG ATG TTC CTG ATT ACG CCT CTG GAA GAG GAA ATT TGT TGA GC
AGO3-Txho	ACT CGA GTC AAA GCG CTC TAG TAA AGT CGT TAA TGG
AGORAG-F	CTC CCC ACA AAT ATT ATA ATA TAC AGA GCC GGA ATT GGA GAT GGA CAG C
AGORAG-R	GCT GTC CAT CTC CAA TTC CGG CTC TGT ATA TTA TAA TAT TTG TGG GGA G
AGOFseq1	GCG GAA GAT GTT GAG TTG CAG ATT CG
AGOFseq2	CTG GGC TGC GAG ACG AAG TAC GGT C
AGOFseq3	GTT CTA CAA ATG AAC TGT AAA CTC GGA G
AGORseq1	GCC GCT ACC CGA GAA GAT GCG AGT G
Ago3qPCR.F4	GAAATGGTGTGTGGAAATCG
Ago3qPCR.R4	TCCTGGCAATGGGTTTTCTA
SP6	GCCAAGCTATTTAGGTGACACTATAG
T7	GAATTGTAATACGACTCACTATAGGG
Small RNA QPCR PRIMERS	
miRTQ	CGAATTCTAGAGCTCGAGGCAGGCGACATGGCTGGCTAGTTAAGCTTGGTACCGAG CTCGGATCCACTAGTCCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN
RTQ-UNlr	CGAATTCTAGAGCTCGAGGCAGG
HUF 1	TTGACTCCAACAAGTTCGCT
Rsp PCR F1	TCTAGAAGGTGTCTTCTGTT
Rsp PCR F11	GTCTTCTGTTGCGCTGGTAC
RSp PCR F21	CGCCTGGTACTTGAAATCGA
Rsp PCR F31	TTGAAATCGAAAAATCACCC
Rsp PCR F41	AAAATCACCCATTTTGACCG
Rsp PCR F51	ATTTTGACCGCTTAAAATGA
Rsp PCR F61	CTTAAATGACATAACTTAG
Rsp PCR F71	CATAACTTAGTCAATTTATT
Rsp PCR F81	TCAATTTATTGTTTTGTGT
Rsp PCR F91	GTTTTTGTGTACCAGGTTTA
Rsp PCR F101	ACCAGGTTTAAATAATCTGT
Rsp PCR F111	AATAATCTGTAGAAGGTATC
Rsp PCR F121	AGAAGGTATCTTCTGTTTGT
Rsp PCR F131	TTCTGTTTGTCTGGTACTTG
Rsp PCR F141	CTGGTACTTGAAATCGGAAA
Rsp PCR F151	AAATCGGAAAAATCACCCATT
Rsp PCR F161	ATCACCCATTTTGACCGCTT
Rsp PCR F171	TTGACCGCTTAAAATGTAAA

Rsp PCR F181	AAAATGTAAACTTAGGCAA
Rsp PCR F191	ACTTAGGCAATTTACTGTTT
Rsp PCR F201	TTTACTGTTTTTGCTTACCA
Rsp PCR F211	TTGCTTACCAGTTGAACAGA
Rsp PCR F221	GTTGAACAGAATCTCTAGA
Rsp PCR F220	AGTTGAACAGAATCTCTAGA
Rsp PCR R1	AACAGAAGACACCTTCTAGA
Rsp PCR R11	GTACCAGGCGAACAGAAGAC
Rsp PCR R21	TCGATTTCAGTACCAGGC
Rsp PCR R31	GGGTGATTTTCGATTCAA
Rsp PCR R41	CGGTCAAAATGGGTGATTTT
Rsp PCR R51	TCATTTTAAGCGGTCAAAAT
Rsp PCR R61	CTAAGTTATGTCATTTTAAG
Rsp PCR R71	AATAAATTGACTAAGTTATG
Rsp PCR R81	ACACAAAAACAATAAATTGA
Rsp PCR R91	TAAACCTGGTACACAAAAAC
Rsp PCR R101	ACAGATTATTTAAACCTGGT
Rsp PCR R111	GATACCTTCTACAGATTATT
Rsp PCR R121	ACAAACAGAAGATACCTTCT
Rsp PCR R131	CAAGTACCAGACAAACAGAA
Rsp PCR R141	TTTCCGATTTCAAGTACCAG
Rsp PCR R151	AATGGGTGATTTTCCGATTT
Rsp PCR R161	AAGCGGTCAAAATGGGTGAT
Rsp PCR R171	TTTACATTTTAAGCGGTCAA
Rsp PCR R181	TTGCCTAAGTTTTACATTTT
Rsp PCR R191	AAACAGTAAATTGCCTAAGT
Rsp PCR R201	TGGTAAGCAAAAACAGTAAA
Rsp PCR R211	TCTGTTCAACTGGTAAGCAA
Rsp PCR R221	TCTAGAGATTCTGTTCAAC
Rsp PCR R220	TCTAGAGATTCTGTTCAACT
Rsp PCR F103	CAGGTTTAAATAATCTGTAG
Rsp PCR F105	GGTTTAAATAATCTGTAGAA
Rsp PCR F107	TTTAAATAATCTGTAGAAGG
Rsp PCR F109	TAAATAATCTGTAGAAGGTA
Rsp PCR F111	AATAATCTGTAGAAGGTATC
Rsp PCR F113	TAATCTGTAGAAGGTATCTT
Rsp PCR F115	ATCTGTAGAAGGTATCTTCT
Rsp PCR F117	CTGTAGAAGGTATCTTCTGT
Rsp PCR F119	GTAGAAGGTATCTTCTGTTT
Rsp PCR R103	CTACAGATTATTTAAACCTG
Rsp PCR R105	TTCTACAGATTATTTAAACC

Rsp PCR R107	CCTTCTACAGATTATTTAAA
Rsp PCR R109	TACCTTCTACAGATTATTTA
Rsp PCR R111	GATACCTTCTACAGATTATT
Rsp PCR R113	AAGATACCTTCTACAGATTA
Rsp PCR R115	AGAAGATACCTTCTACAGAT
Rsp PCR R117	ACAGAAGATACCTTCTACAG
Rsp PCR R119	AAACAGAAGATACCTTCTAC

TABLE 3.2: GENETIC STOCKS USED IN THIS STUDY			
Line	Notes on genotype	Source	Reference
HR stocks			
Balancer Stocks			
w; TM3 <i>sb</i> /TM6 <i>tb</i>		B. Ganetzky	
+; <i>CyO</i> ^{<i>lt dp pr cn</i>} / <i>Sco</i>		B. Ganetzky	
w: <i>CyO</i> ^{<i>cn bw Roi</i>} / <i>Sco</i>		B. Ganetzky	
SD Stocks			
<i>SD-5</i>	Strong distortion phenotype and contains <i>Sd</i> , <i>E(SD)</i> , <i>Rsp</i> ^{<i>l</i>} , <i>M(SD)</i> and <i>St(SD)</i> as well as two paracentric inversions on 2R.	B. Ganetzky	<i>Sandler and Hiraizumi 1959; Sandler et al. 1959</i>
<i>SD-72</i>	Strong distorter with a both pericentric and paracentric inversions	B. Ganetzky	Sandler and Hiraizumi 1959)
<i>SD-Roma</i>	Inversion free moderately distorting SD chromosome isolated in Italy	B. Ganetzky	Nicoletti and Trippa 1967
<i>SD-Los Arrenos</i>	Weak distorter	B. Ganetzky	
<i>Rsp</i> ^{<i>S</i>} <i>cn bw</i>	[<i>Sd</i> ⁺ , <i>E(SD)</i> ⁺ , <i>Rsp</i> ^{<i>S</i>}] standard <i>Rsp</i> sensitive chromosome	B. Ganetzky	Lyttle 1991
<i>Rsp</i> ^{<i>i16</i>} <i>cn bw</i>	[<i>Sd</i> ⁺ <i>E(SD)</i> ⁺ <i>Rsp</i> ^{<i>l</i>}] Radiation-induced derivative of the <i>Rsp</i> <i>cn bw</i> chromosome where the <i>Rsp</i> locus has been completely deleted	B. Ganetzky	Ganetzky 1977
<i>Rsp</i> ^{<i>SS</i>} <i>lt pk cn</i>	[<i>Sd</i> ⁺ <i>E(SD)</i> ⁺ <i>Rsp</i> ^{<i>SS</i>}] the canonical <i>Rsp</i> SS chromosome	B. Ganetzky	Lyttle 1991
RNAi Mutants			
<i>aub</i> ^{<i>QC42</i>} <i>cn bw</i> / <i>CyO</i>	EMS; Strong allele	T. Schüpbach	Schupbach and Wieschaus 1991
<i>aub</i> ^{<i>HN2</i>} <i>cn bw</i> / <i>CyO</i>	EMS; Strong allele	T. Schüpbach	Schupbach and Wieschaus 1991
nosGAL4, Aub-GFP	UAS-Aub-GFP recombined on to the same chromosome as nos-GAL4 VP16	P. Macdonald	Harris and Macdonald 2001

Chapter IV: Discussion and Future Directions

Models for the Role of the piRNA Pathway Activity in Segregation

Distortion

While much is now known about the molecular nature of both *Rsp* and *SD*, the mechanism by which they act to cause the dysfunction of *Rsp*-bearing sperm remains mysterious. The recent detection of *Rsp* sequences as piRNAs, as well as increasing evidence that other meiotic drive systems in *D. melanogaster* and *D. simulans* are likely regulated by this germline-specific RNA interference pathway, focused my attention on evaluating the potential role of the piRNA pathway in regulating distortion.

These studies have demonstrated that mutations to the piRNA pathway components *aubergine* and *piwi* act as genetic enhancers of distortion. However, the mechanism governing their interaction with *SD* remains uncertain. *Sd* encodes a truncated RanGAP protein that mislocalizes to the nucleus, potentially altering the RanGTP gradient required for nuclear transport (MERRILL *et al.* 1999). *Responder*, the target of *Sd*, is an array of satellite repeat sequences (WU *et al.* 1988). The greater number of repeats that comprise *Rsp*, the more sensitive a chromosome is to *SD*-mediated distortion.

The genetic data presented here in combination with the observation that *Rsp* sequences have been recovered as piRNAs associated with Aub, AGO3 and Piwi demonstrate that *Rsp* is a target of the piRNA pathway (SAITO *et al.* 2006; NAGAO *et al.* 2010). Additionally, because mutations to *aub* and *piwi* enhance distortion, it can be

hypothesized that wild type activity of the piRNA pathway works in opposition to *SD* and helps to prevent the dysfunction of *Rsp*-bearing sperm.

There are multiple ways these two pathways could interact. However, the transport defect caused by *Sd-RanGAP* suggests that points of nuclear entry or exit of piRNA machinery are particularly strong candidates. The current model of piRNA processing in the testis involves nuclear export of a piRNA precursor molecule to the cytoplasmic piNG-body (KIBANOV *et al.* 2011). Here, Aub, AGO3, Tud, Vasa and other piRNA pathway components work to process piRNA precursors into mature piRNAs (Figure 1.9c). These piRNA-protein complexes can then screen exiting RNAs for homology to piRNAs, allowing specific targeting of transposons (KLATTENHOFF *et al.* 2009; ISHIZU *et al.* 2011; KIBANOV *et al.* 2011). In some contexts, piRNA pathway components, such as Piwi, have also been shown to enter the nucleus and facilitate transcriptional silencing via epigenetic regulation of chromatin state (PAL-BHADRA *et al.* 2004; BROWER-TOLAND *et al.* 2007; YIN and LIN 2007; HUISINGA and ELGIN 2009). The observation that *RanGAP* colocalizes with Aub in a large, perinuclear piNG-like body suggests that piRNA mediated silencing may be tightly coupled with nuclear transport. Interestingly, these *RanGAP*- and Aub- containing bodies are present under both distorting and non-distorting conditions. Because the *RanGAP* antibody does not distinguish between wild type and *Sd-RanGAP* protein, the specific localization of *Sd-RanGAP* in this context is not known (KUSANO *et al.* 2001). While the gross organization of the piNG-bodies appears to be unchanged in the testes of distorting males, it is possible that the dynamics of nuclear transport of piRNA pathway components are altered.

Nuclear exclusion of Rsp-primed RNP complexes

There are several points in the piRNA processing pathway where Sd-RanGAP could perturb the wild type dynamics of piRNA processing. TAO *et al* have proposed that nuclear re-entry of a piRNA-primed RNP silencing complex could be disrupted by the Sd-RanGAP transport defect (Figure 1.15) (FERREE and BARBASH 2007; TAO *et al.* 2007). However, the presence of Sd-RanGAP is associated with nuclear accumulation rather than exclusion from the nucleus (KUSANO *et al.* 2001). Therefore, this model may not be the most likely intersection of these two pathways.

Nuclear accumulation of piRNA pathway components

Another possibility for the way Sd-RanGAP could interact with the piRNA pathway is through nuclear accumulation of piRNA pathway proteins, leading to a disruption of normal silencing. While Piwi has been shown to enter the nucleus, Aub and AGO3, the primary effectors of piRNA biogenesis in the testis, are cytoplasmic in localization (BRENNECKE *et al.* 2007; NISHIDA *et al.* 2007; NAGAO *et al.* 2010; ISHIZU *et al.* 2011). Further, examination of Aub-GFP under distorting and non-distorting conditions did not reveal any alteration in localization of this protein (Figures 3.1, 3.2, 3.3, 3.4). It remains possible that an as yet unidentified piRNA pathway component required for epigenetic silencing could accumulate in the nucleus. However, until more is understood about this mechanism in the testes, suggestion of candidate genes is difficult. Additionally, if the dysfunction caused by Sd-RanGAP was related to nuclear accumulation of piRNA-RISC,

this would predict that mutation of piRNA pathway components would reduce the number of functional piRNA complexes and suppress, rather than enhance, distortion.

Epigenetic activation of the Rsp locus

In some contexts, Piwi proteins have been shown to act as epigenetic activators (YIN and LIN 2007). Mutations to Piwi have been shown to lead to a loss of euchromatic marks and reduced expression of the telomere-associated sequences (TAS) on chromosome 3R (YIN and LIN 2007; LIN and YIN 2008). The idea that the piRNA pathway could have an activating effect on *Rsp* leads to two distinct models. In the first case, nuclear accumulation of piRNA pathway components leads to increased activation of the *Rsp* locus and thereby failure of chromatin compaction (YIN and LIN 2007). However, this model predicts that mutations to piRNA pathway components reduce *Rsp* activation and therefore suppress, rather than enhance distortion. On the other hand, a certain amount of epigenetic activation is required to facilitate the histone to protamine transition (RATHKE *et al.* 2007). In this model, disruption of the piRNA pathway leads to over-silencing, rather than loss of silencing. Yet, the end consequence would be the same as that from the first model: a failure of the histone to protamine transition and uncondensed nuclei. Given that mutations of piRNA pathway components enhance distortion, models that suggest a specific nuclear accumulation of piRNA pathway components, thereby increasing targeting of the *Rsp* locus, do not fit the experimental data.

Targeted suppression of AGO3 by Rsp piRNAs

The presence of *Rsp* repeats in the second intron of *AGO3* suggests another possible mechanism of activity for *Rsp*. *Rsp* piRNAs produced from the canonical locus on chromosome 2R could potentially guide post-transcriptional silencing of *AGO3* via homology-mediated cleavage of transcripts or mediate transcriptional-level regulation by facilitating epigenetic silencing of the *AGO3* locus. However, expression levels of *AGO3* in the testis remain unchanged in *SD⁺ Rsp^s* and *SD⁺ Rspⁱ*, distorting and non-distorting genotypes, suggesting that *Rsp* does not act to regulate *AGO3* expression.

The “source” and “sink” model of Rsp-piRNA biogenesis

Rsp small RNA profiles from the testes of both *Rsp^s* and *Rspⁱ* males reveal that remarkably similar species of *Rsp* small RNAs are present in each of these genotypes. Given that *Rspⁱ* is thought to have fewer than a dozen repeats on chromosome 2R while *Rsp^s* has several hundred, the similarity of *Rsp* RNA products is unexpected. This observation suggests that *Rsp* piRNAs could be produced from a locus distal to the canonical repeat array on 2R. This piRNA “source” could provide the initiator piRNAs, that then act at the canonical *Responder* locus, or “sink,” to facilitate silencing. While the full location of all *Rsp* repeats present in the *Drosophila* genome is not known, two regions present themselves as particularly interesting possibilities for a *Rsp* small RNAs source. One is the *Rsp* repeat locus on chromosome 3L in intron two of the *AGO3* gene. piRNAs processed from this intronic region, which houses many other transposable elements, could specifically target the piRNA silencing machinery to *Rsp*. This

hypothesis is supported by the observation that piRNAs are derived from untranslated regions of several other protein-coding genes (ROBINE *et al.* 2009; SAITO *et al.* 2009).

The Y-chromosome is another possible source of *Rsp*-piRNAs distinct from the 2R *Responder* locus. This model is particularly appealing because of the sex-specific nature of the phenotype of segregation distortion. Additionally, manual inspection of the un-annotated regions of the Berkeley *Drosophila* Research Project *D. melanogaster* genome assembly R5.12 suggests that some responder repeats are in proximity to sequences from *Suppressor of Stellate* [*Su(Ste)*], a known Y-linked gene (LIVAK 1990; BALAKIREVA *et al.* 1992). Further, *Su(Ste)* is known to be transcribed and processed into piRNAs (ARAVIN *et al.* 2001; ARAVIN *et al.* 2004). In fact, *Su(Ste)*-derived piRNAs are by far the most abundant small RNA species in the testis (NISHIDA *et al.* 2007; NAGAO *et al.* 2010). Even a few *Rsp* repeats located in this piRNA cluster could provide ample substrate for processing into active *Rsp* piRNAs.

While each of these loci provides an interesting potential source for *Rsp* piRNAs, experimental evidence does not yet support either of these hypotheses. Given the repetitive nature of the *Rsp* sequence, mapping the specific source of piRNAs to the genome is difficult. If there are unique sequences present in the *Rsp* repeats of *AGO3*, it would be possible to test for piRNAs derived specifically from this locus either by small RNA PCR or Northern blot.

Due to its repetitive nature, Y-chromosome genome assembly is incomplete, making targeted cloning of Y-chromosome *Rsp* repeats impractical. Several methods, including FISH on mitotic chromosomes and sex-specific Southern blotting, were used to detect the presence of *Rsp* repeats on the Y-chromosome specifically. However, neither

of these experiments detected any Y- associated *Rsp* repeats. If these repeats are present in very low copy number, it is possible that an assay with increased sensitivity may be able to detect them. At this time it is not possible to conclude from where in the *Drosophila* genome *Rsp* small RNAs are generated.

Nuclear retention of piRNA precursors in SD

Whatever the source of *Rsp* piRNAs, it remains clear that a mutation of piRNA pathway components that reduces formation or targeting of piRNA sequences to the *Responder* locus exacerbates the loss of *Rsp*-bearing sperm and increases distortion. Given the evidence for nuclear accumulation as the primary defect in cells expressing Sd-RanGAP, piRNA precursors may be subjected to nuclear retention. Failure to export piRNA precursors for cytoplasmic processing would result in a failure to target the canonical *Responder* locus for silencing. This model fits with the experimental data that mutations to piRNA processing machinery enhance distortion; in this model reduction in Aub concentrations would further disrupt normal silencing, leading to increased *Rsp* expression and failure of chromatin compaction (Figure 4.1).

Role of nuclear transport and piRNA components in mitotic chromatin regulation

While the RanGTP gradient is essential for spatial regulation of the nuclear and cytoplasmic compartments during interphase, this barrier dissolves during mitosis and meiosis, enabling mixing of nuclear and cytoplasmic components. Interestingly, the RanGTP system has distinct roles during mitosis in spindle formation, chromatin

regulation and sister chromatid separation (HETZER *et al.* 2002; WEIS 2003; KUSANO *et al.* 2004).

During mitosis, human RanGAP1 concentrates at the kinetochores of chromosomes in a SUMO dependent manner (JOSEPH *et al.* 2002). Further, mutation of SpRna1, the *S. pombe* homolog of RanGAP, leads to defective chromosome segregation (KUSANO *et al.* 2004). This defect in segregation is rescued by overexpression of Clr4, the *S. pombe* H3K9 methyltransferase, or Snf2, a chromatin remodeling factor. The RanGAP mutants also show reduced H3K9 methylation and reduced centromeric localization of Swi6, a homolog of HP1. Additionally, RanGAP binds histone H3 and enhances Clr4-mediated methylation (NISHIJIMA *et al.* 2006). These data suggest that RanGAP regulates proper silencing at centromeres required for kinetochore attachment and chromosome segregation in mitosis.

Interestingly, the piRNA pathway components also play a role in mitotic regulation in the germline (PEK and KAI 2011b). During mitotic divisions of the cytotblast cells of the *Drosophila* ovary, Vasa, Aub and Spn-E form mitotic bodies that bind peri-centromeric chromatin at the sites of piRNA clusters (PEK and KAI 2011c). *Vasa* mutants exhibit delayed onset of chromatin condensation during prophase and an increased frequency of lagging chromosomes in anaphase. Additionally, mutations of Aub or Spn-E lead to similar defects in chromosomal condensation and a concurrent loss of Vasa localization to mitotic chromosomes. Further, condensin I complex components Barren (Barr or CAP-H) and CAP-D2 directly interact with Vasa and fail to properly localize to mitotic chromosomes in *vasa* mutant ovaries (PEK and KAI 2011c). Condensin

I components, generally cytoplasmic during interphase, are recruited to chromosomes during prophase to facilitate initiation of condensation (OLIVEIRA *et al.* 2007).

These data suggest a model whereby Aub, primed with piRNAs, directs Vasa-Spn-E complexes to the peri-centromeric piRNA clusters. There, Vasa could promote CAP-H loading onto the chromosome, which is known to initiate at the centromere and spread distally towards the arms promoting condensation (OLIVEIRA *et al.* 2007; PEK and KAI 2011b). Interestingly, Belle (Bel), a paralog of Vasa, has been shown to play a similar role in regulation of chromosome condensation in somatic cells via the endo-siRNA pathway (PEK and KAI 2011a).

Specific mitotic or meiotic defects have not been observed in *SD/Rsp^s* males. However, it remains possible that the presence of high *Rsp* repeat copy number recruits piRNA pathway components normally targeted to centromeres during chromatin condensation, thereby altering the chromatin state of the *Rsp* locus or upsetting the balance of chromatin regulators elsewhere. This effect could take place early during either mitotic or meiotic divisions, altering the normal chromatin marks on *Rsp*-bearing chromosomes that could be carried throughout spermatogenesis. Further, Sd-RanGAP, which lacks the proper SUMO signal for localization to the centromere in mitotic cells, may also contribute to altered chromatin dynamics during cell division in *SD* males, further sensitizing the background to disruptions caused by *Rsp* (JOSEPH *et al.* 2002). In support of the model, mutations to *gluon*, which encodes SMC4, part of the condensation I complex, enhance distortion (MCELROY *et al.* 2008).

Evidence provided herein suggests that RanGAP colocalizes with Aub in cytoplasmic piNG-bodies. These bodies contain other piRNA pathway components,

including Vasa, Spn-E and Bel. While the localization of RanGAP and piRNA pathway proteins in mitotic cells has not been specifically examined, this observation suggests that these players could associate in mitotic nuclei to regulate chromatin state, centromere function or spindle assembly (KIBANOV *et al.* 2011; PEK and KAI 2011b). The presence of Sd-RanGAP, which lacks proper chromatin localization signals, or mutations to pathway components required for proper chromosomal targeting to piRNA clusters could lead to miss-regulation of chromatin condensation. In light of these recent findings, detailed examination of mitotic and meiotic chromosomal state in the testes of *sd* males might reveal deviations from wild type organization not visible at the level of chromatin staining with DAPI or PI. While the disruption of nuclear transport caused by Sd-RanGAP clearly contributes to the *SD* phenotype, alteration of chromatin level regulation, either early in cellular divisions or later in chromatin condensation, could also propagate dysfunction resulting in the loss of *Rsp*-bearing nuclei.

The precise mode of interaction between the piRNA pathway and segregation distortion is not known. However, both *Sd*-mediated disruption of nuclear transport as well as potential misregulation of chromatin state during mitosis, meiosis or nuclear chromatin compaction, represent potential points where these mechanisms might intersect to cause dysfunction of *Rsp*-bearing sperm. Further investigation of the role of the piRNA pathway in *SD* will likely reveal important clues regarding the molecular mechanism leading to the dysfunction of *Rsp*-bearing sperm.

Future Directions for the Study of Segregation Distorter in *D. melanogaster*

The demonstrated genetic involvement of piRNA pathway components has provided a guidepost for continued studies on the molecular mechanism of *SD*. There are many additional experiments that could shed light on this complex process.

While outside the scope of this work, further investigation of both the complement of piRNAs present during *SD* and potential for alterations of chromatin state in *Rsp*-bearing nuclei represent obvious avenues of investigation.

Deep sequencing of the population of small RNAs in the testes of distorting and non-distorting males could provide important insight into the role of *Rsp* piRNAs in *SD*. This experiment would provide detailed data on the alterations, if any, found in the global population of piRNAs as well as specific changes to the *Rsp*-derived piRNA population. It remains possible that the defect of *Rsp*-bearing sperm in *SD* flies is related to improper silencing of the normal targets of the piRNA pathway, leading to an increase in transposon activity, DNA damage and failure of chromatin compaction. Deep sequencing of small RNAs from distorting and non-distorting males could determine if any species of piRNAs normally present in the testis are lost or altered in *SD*.

Additionally, if the fraction of *Rsp*-derived piRNAs in the testis is increased or decreased in distorting conditions, this change would demonstrate altered *Rsp* regulation during distortion. Further, deep sequencing could reveal the presence of uniquely-mapping *Rsp* piRNA sequences that could be used to unambiguously identify the

genomic sources of *Rsp* piRNAs. These data would be particularly useful in ascertaining the role, if any, of *Rsp* piRNAs produced from the AGO3 locus.

In the ovary, mutations to *aub*, *armi* or *spn-E* lead to increased transposon mobilization as well as DNA damage in germline cell nuclei (THEURKAUF *et al.* 2006; KLATTENHOFF *et al.* 2007; KHURANA and THEURKAUF 2010). It is currently unknown whether or not transposon activity is altered in the testes of *SD* males. Altered transposon expression levels could be assayed by RT-qPCR for specific transposon sequences. Additionally, immunohistochemical staining for markers of DNA damage such as phosphorylated H2Av, binding of the DNA repair protein UbcD6, or a directly assaying damage using a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay could determine if DNA damage plays a role in the dysfunction of *Rsp*-bearing spermatids.

It would also be informative to follow the localization of piNG-body components through mitotic and meiotic divisions and determine if RanGAP colocalizes with other markers of piNG-bodies. Further, analysis using *aub*, *ago3 vas*, *bel*, or *spn-E* mutations in flies expressing HA-tagged wild type or Sd-RanGAP could reveal if the localization any of these proteins is interdependent in either interphase or mitotic germline cells. These data could provide evidence suggesting precisely how RanGAP interacts with piRNA pathway components and what steps of the pathway are altered in the presence of Sd-RanGAP.

Lastly, a more detailed comparison of chromatin state in the testes of distorting and non-distorting males may reveal the precise nature of the nuclear condensation defect in *Rsp*-bearing spermatids. Investigating the staining of additional epigenetic chromatin

markers, such as histone acetylation, phosphorylation, sumoylation and ubiquitination, could reveal alterations in *SD* males. Examination of centromere, kinetochore or condensin components might reveal alterations in regulation of cell division or nuclear compaction in distortion.

For the past 50 years Segregation Distorter has provided an intriguing puzzle for *Drosophila* geneticists. How does an allele on one chromosome determine the fate of the homologous chromosome in an entirely separate nucleus? While the molecular identification of *Sd* and *Responder* provided great steps forward in our understanding of this system, the mechanism of *Sd* activity on *Rsp* has remained mysterious. The work contained in this study has illuminated a new component of this puzzle by providing genetic evidence for the involvement of the piRNA pathway in preventing the loss of *Rsp*-bearing sperm in *SD* males. While the many details of this complex mechanism remain enigmatic, this study provides a stepping-stone on the pathway towards understanding this archetypal selfish genetic element.

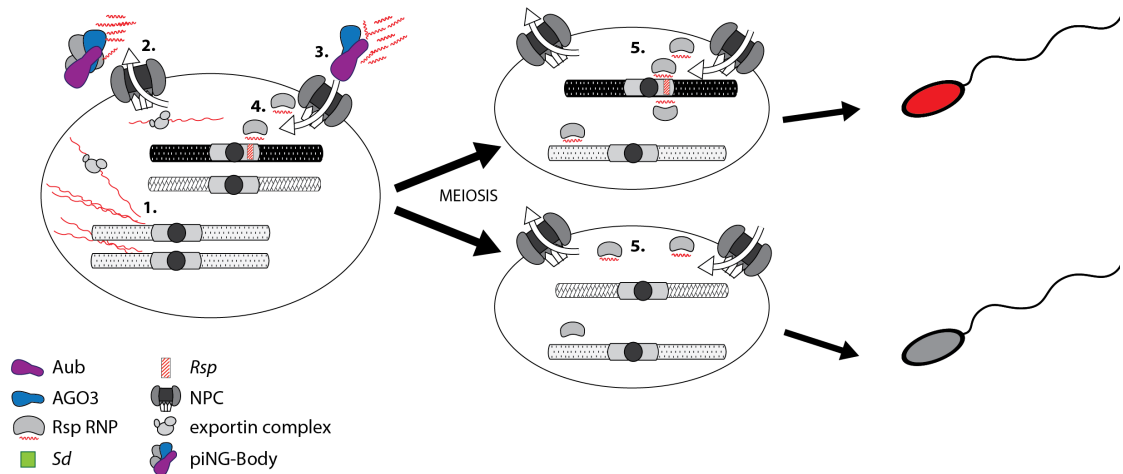
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FIGURES AND FIGURE LEGENDS

A. Non-distorting (SD^+/Rsp^s)



B. Distorting (SD/Rsp^s)

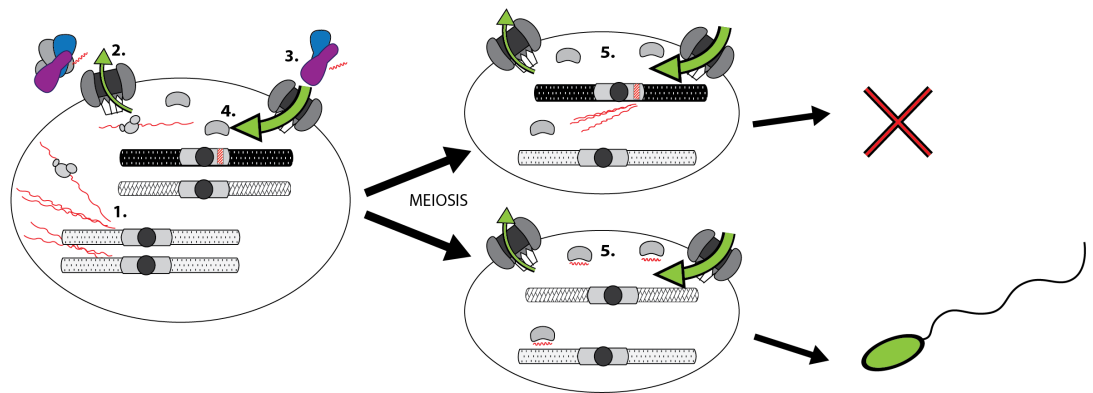


FIGURE 4.1: The “source” and “sink” model for the role of *Rsp* piRNAs in segregation distortion. (A) 1. Under non-distorting conditions, *Rsp* is transcribed from a piRNA cluster distal from the canonical 2R locus. 2. These precursor piRNAs are exported from the nucleus via the nuclear pore. 3. The precursor piRNAs are processed into mature *Rsp* piRNAs in cytoplasmic piNG-bodies. 4. These mature *Rsp* piRNAs are then imported into the nucleus where, as part of RNA-protein (RNP) complex they regulate proper

epigenetic silencing of the canonical *Rsp^s* locus on chromosome two. 5. These epigenetic marks are carried through meiosis and facilitate proper silencing of the *Rsp^s* locus in spermatids, allowing chromatin condensation and sperm maturation. 6. In *Rspⁱ* nuclei there is no target for *Rsp* primed RNP complexes. (B) 1. Under distorting conditions, the disruption of nuclear transport (green arrows) in *SD* males reduces export of *Rsp* piRNA precursor transcripts. 2. Lacking precursor molecules, *Rsp* piRNAs are not made in the cytoplasm. 3. Therefore, *Rsp* piRNA RNP complexes cannot enter the nucleus. 4. *Rsp* transcription is not properly silenced 5. Reduced epigenetic silencing results in inappropriate *Rsp* expression in the nuclei of *SD⁺ Rsp^s*-bearing spermatids causing failure of chromatin condensation and sperm dysfunction. 6. In *SD Rspⁱ* nuclei, which lack the repeat array on 2R, sperm maturation proceeds normally.