Mutations to the piRNA Pathway Component *Aubergine* Enhance Meiotic Drive of Segregation Distorter in *Drosophila melanogaster*

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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 2 results in dysfunction of the non–*Sd*-bearing (Sd^+) sperm and almost exclusive 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 2 known as *Responder* (*Rsp*). The *Rsp* locus comprises repeated copies of a satellite DNA sequence and *Rsp* copy number correlates with sensitivity to *Sd*. Under distorting conditions during spermatogenesis, nuclei with chromosomes containing greater than several hundred *Rsp* repeats fail to condense chromatin 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. Here, we evaluate the role of piRNAs in segregation distortion by testing the effects of mutations to piRNA pathway components on distortion. Further, we specifically targeted mutations to the *aub* locus of a Segregation Distorter (*SD*) chromosome, using ends-out homologous recombination. The data herein demonstrate that mutations to piRNA pathway components act as enhancers of *SD*.

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 natural 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 of two alleles alters the gametic ratio to enhance its own representation in the next generation, violating Mendel's first law (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 normal Mendelian segregation (Sandler and Hiraizumi 1959; Sandler *et al.* 1959; Burt and Trivers 2008; Larracuente and Presgraves 2012). The *Segregation distorter* (*Sd*) locus was mapped to the proximal euchromatin of chromosome 2L and identified as a truncated Ran **G**TPase **A**ctivating **P**rotein (RanGAP) (Hartl 1974; McLean *et al.* 1994; Merrill *et al.* 1999). Formed by a tandem duplication event, this C-terminally truncated (shortened by 243 aa) version remains enzymatically active, but lacks part of a nuclear

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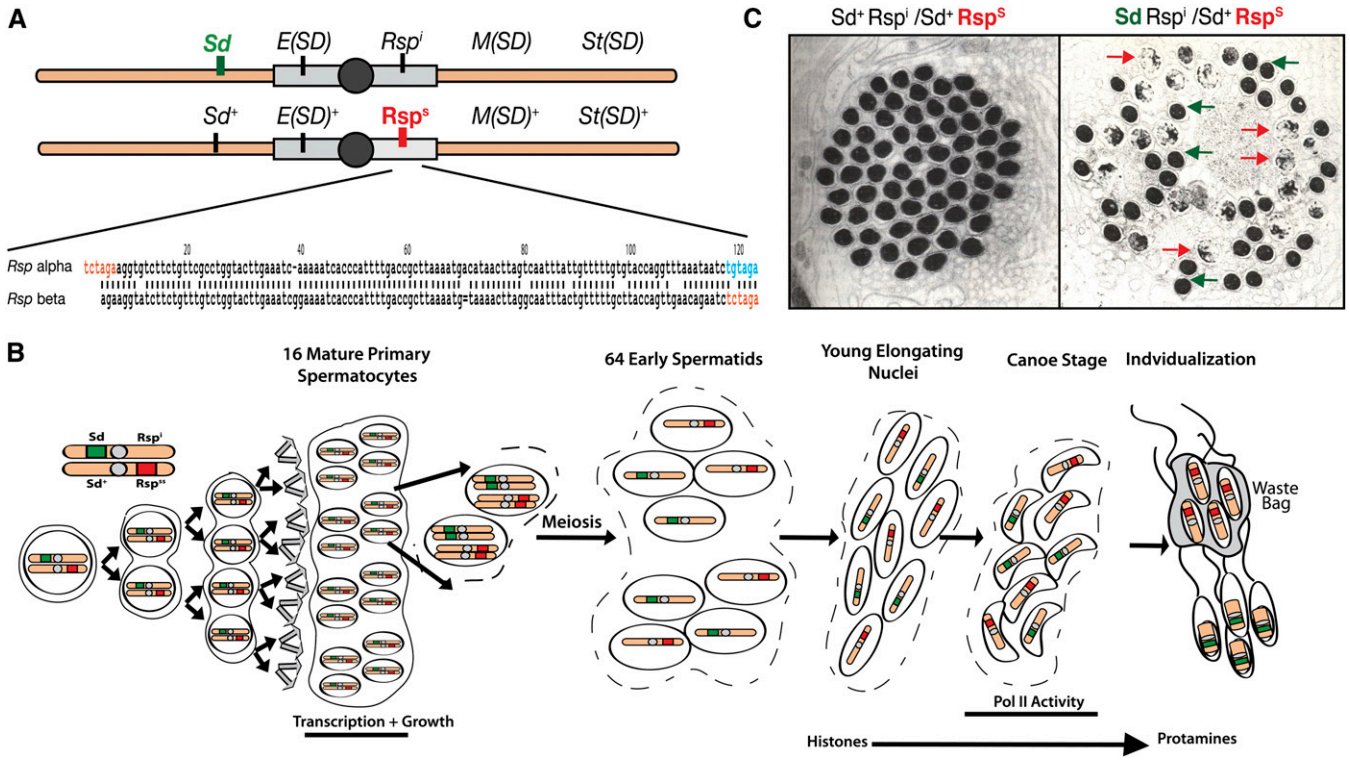

Figure 1 Components of segregation distortion. (A) Top, schematic of *D. melanogaster* second chromosomes showing the relative locations of components of the Segregation Distorter system. *Sd*, *E(Sd)*, *Rsp*^{i}, *M(SD)*, and *St(SD)* all contribute to the drive phenotype while *E(Sd)*$^{+}$, *Rsp*^{s}, *M(SD)*$^{+}$, and *St(SD)*$^{+}$ are wild-type loci and are normally found on nondistorting *SD*$^{+}$ chromosomes. Bottom, an alignment of the α- and β-halves of the canonical *Responder* repeat sequence. The ~120-bp α- and β-sequences are ~87% homologous. In orange are the *Xba*I restriction sites that flank the 240-bp repeat. In blue is a mutated *Xba*I site that is found in at the 3′ end of the α-repeat. (B) An overview of SD in 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 haploid early spermatids. The spermatids mature and condense, exchanging histones for protamines. *Rsp*-bearing nuclei fail to properly condense. The waste bag removes the uncondensed, *Rsp*-bearing spermatids during individualization and only *Sd*-bearing spermatids become mature sperm. (C) Wild-type and dysfunctional chromatin condensation. Left, an electron microscopy image of a cyst of 64 condensing spermatids from a wild-type male. Right, the same stage in the testis of the genotype *Sd Rsp*^{i}/*Sd*$^{+}$ *Rsp*^{s}. Approximately half the nuclei fail to condense. (Reprinted from Tokuyasu *et al.* 1977 with permission from Elsevier.)

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 localizes to the cytoplasm, Sd-RanGAP is retained in the nucleus due to its truncated NES, potentially altering the GTP gradient required for Ran-mediated nuclear transport (Gorlich and Mattaj 1996; Gorlich and Kutay 1999b; 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 nuclear enzymatic activity of RanGAP causes abnormal nuclear retention of Ran cargo and sperm dysfunction in *SD* (Kusano *et al.* 2001, 2002).

The relative strength of Segregation Distorter chromosomes is dependent upon several modifiers distributed along the second chromosome (Figure 1A). The best studied of these, *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 also 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 loci, *Stabilizer of SD* [*St(SD)*] and *Modifier of SD* [*M(SD)*] are located on 2R. Both enhance drive of an *SD* chromosome, although 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, 1974). *Rsp* alleles range from completely insensitive (*Rsp*^{i}), 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 ~700 copies (Wu *et al.* 1988).

No completely insensitive SD^+ chromosomes have been isolated from nature; however, a Rsp^i allele was generated by X-ray ablation of the Rsp^s locus (Ganetzky 1977). This Rsp^i allele is reported to have <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 (Figure 1, B and C) (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, 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 gameteogenesis (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 make an endonucleolytic cut in targeted transposon mRNAs, thus preventing transposition during gameteogenesis (Aravin *et al.* 2004, 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 an unconventional length of small RNA (23–31 nt); and has a specific enrichment for noncoding, 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, 2004; Tao *et al.* 2007a,b).

To evaluate the role of the piRNA pathway in *SD*, we utilized mutations of several components of the piRNA pathway and determined whether 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. These studies reveal that 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 Supporting Information, 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 20–30 males aged <4 days were individually crossed with two Rsp^S *cn bw* virgin females. Crosses were allowed to brood for 4 days at 25° 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 $W = [SD^+$ progeny/*SD* progeny]. The corrected *k* value, k_c, is then $k_c = [SD$ progeny/(*SD* progeny + SD^+ progeny/*W*)] (Figure 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* loci of aub^+Rsp^{mt} recombinants from aub^{HN}, aub^{QC}, and aub^{AWE} chromosomes were verified as wild type

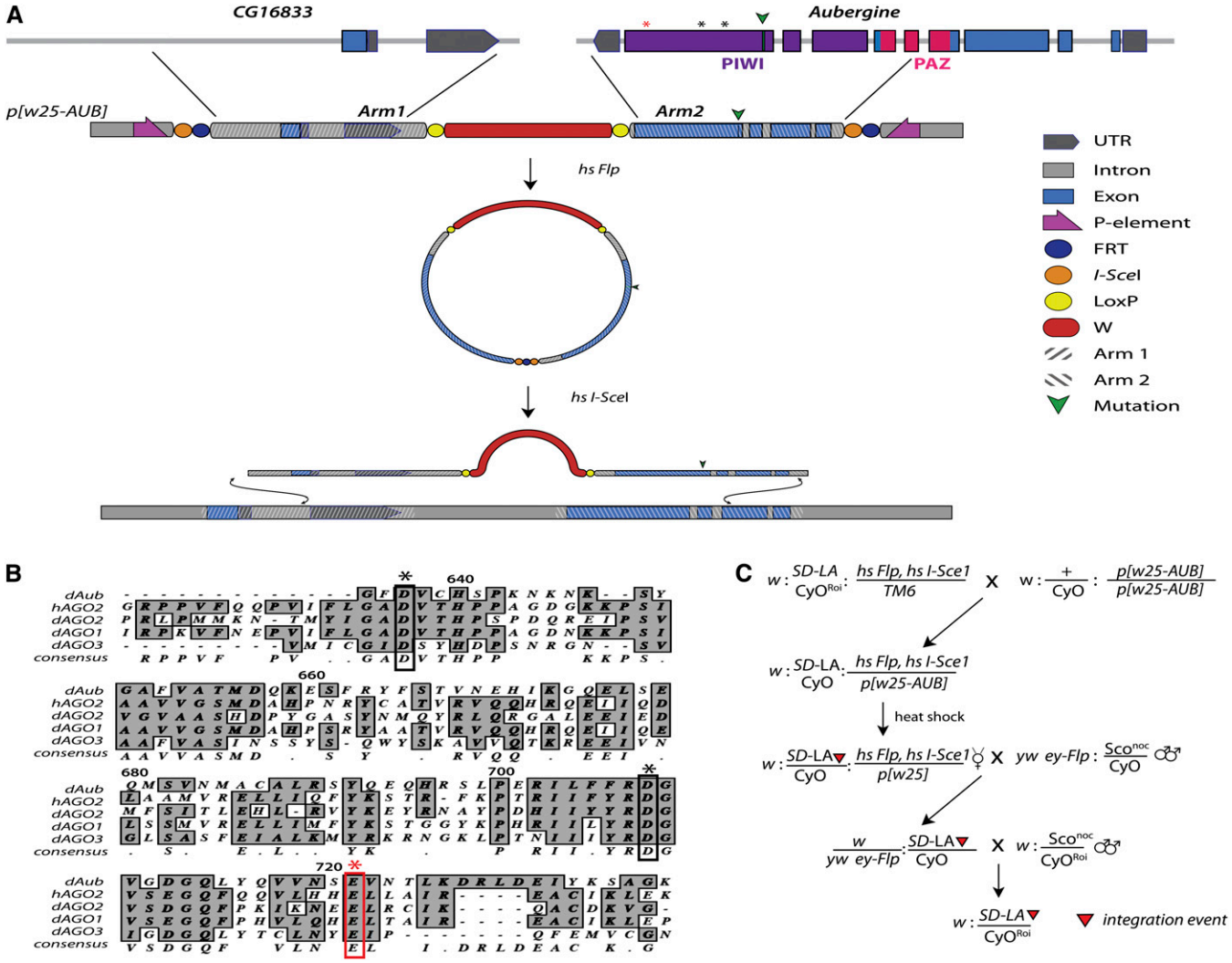

Figure 2 Targeting of the *aub* locus by ends-out homologous recombination (HR). (A) Top, Genomic organization of the *aub* locus and nearby CG16833. Green triangle indicates the location of the Q622* mutation of aub^{HN} and asterisks show the catalytic residues required for nuclease function. The red 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).

(aub^+) by sequence analysis. The aub^{N11} chromosome contains a 110-bp 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 ~30 adult flies, using the Maxwell 16 DNA purification system (no. AS1030; Promega, Madison, WI), eluted in 500 μl of DNase-free water (no. 10977-015; GIBCO, Grand Island, NY), and treated with 20 μg RNAse A (no. AB-0548; Fisher Scientific, Pittsburgh). Samples were then concentrated by centrifugation at 8000 × *g* for 5 min in Microcon Ultracel YM-50 columns (no. 42416; Millipore, Billerica, MA). The final sample concentration was measured using the dsDNA HS assay kit with the Quibit 2.0 fluorometer (no. Q32854; Life Technologies), and 500 ng of DNA per sample was digested for 1.5 hr at 37° 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) (no. R0145L; New England Biolabs, Beverly, MA). Digested samples were run overnight on a 1.5% agarose gel (SeaKem LE Agarose; Lonza, Basel, Switzerland) in 0.5× TBE at 55 mV. The gel was washed for 30 min in denaturation solution (1.5 M NaCl, 0.5 M NaOH), rinsed with dH_2O, 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 (no. RPN203B; GE Healthcare) via capillary action, according to the protocol in *Molecular Cloning: A Laboratory Manual* (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 prehybridized in ECL Gold hybridization buffer (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, La Jolla, CA) vector. The vector-specific primers, T3 and T7, were used to amplify the 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 (no. A9282; Promega). Sample concentration was measured with the dsDNA HS Quibit fluorometer system (Life Technologies) and labeled using the ECL direct labeling kit according to manufacturer's instructions (no. RPN3005; GE Healthcare). Labeled *Rsp* and 100-bp ladder probes (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 0.5× SSC) at 42° and then removed from the hybridization tubes and washed two more times at room temperature on a shaker with 2× SSC. The signal was generated using ECL detection reagents according to manufacturer's instructions (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 minigene flanked by LoxP sites for subsequent removal by Cre-recombinase (Figure 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).

The cloning strategy is as follows, where all genomic coordinates are given by the *D. melanogaster* draft assembly, BDGP Release 5, with release 5.12 annotation provided by FlyBase at the UCSC Genome Browser. Arm1 is the 5′ arm of p[w25-AUB], which contains intronic sequence as well as the 5′-UTR of CG168333 (Figure 2C) and was generated by PCR amplification to incorporate cloning sites as follows: BsiWI-Chr2L:10,995,293–10,997,756-*Asc*I. The last six exons of *aub* are contained in the 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-*Not*I.

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 *Not*I 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*, *hs*Sce-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 2C). Targeted alleles were validated by amplification, using primers outside the region of targeting and primers specific to the *w*$^+$ minigene. All targeted alleles were sequenced to verify no unintended mutations were introduced.

Results

piRNA pathway mutants are genetic enhancers of SD

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 *Rsp^i* chromosomes each exhibit a characteristic banding pattern when digested with the *Xba*I restriction enzyme and probed on Southern blots with labeled *Rsp* sequence (Figure 3A) (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 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 240 bp (Figure 3A).

To determine the *Rsp* repeat status of the mutants used in this study, these chromosomes were compared to the

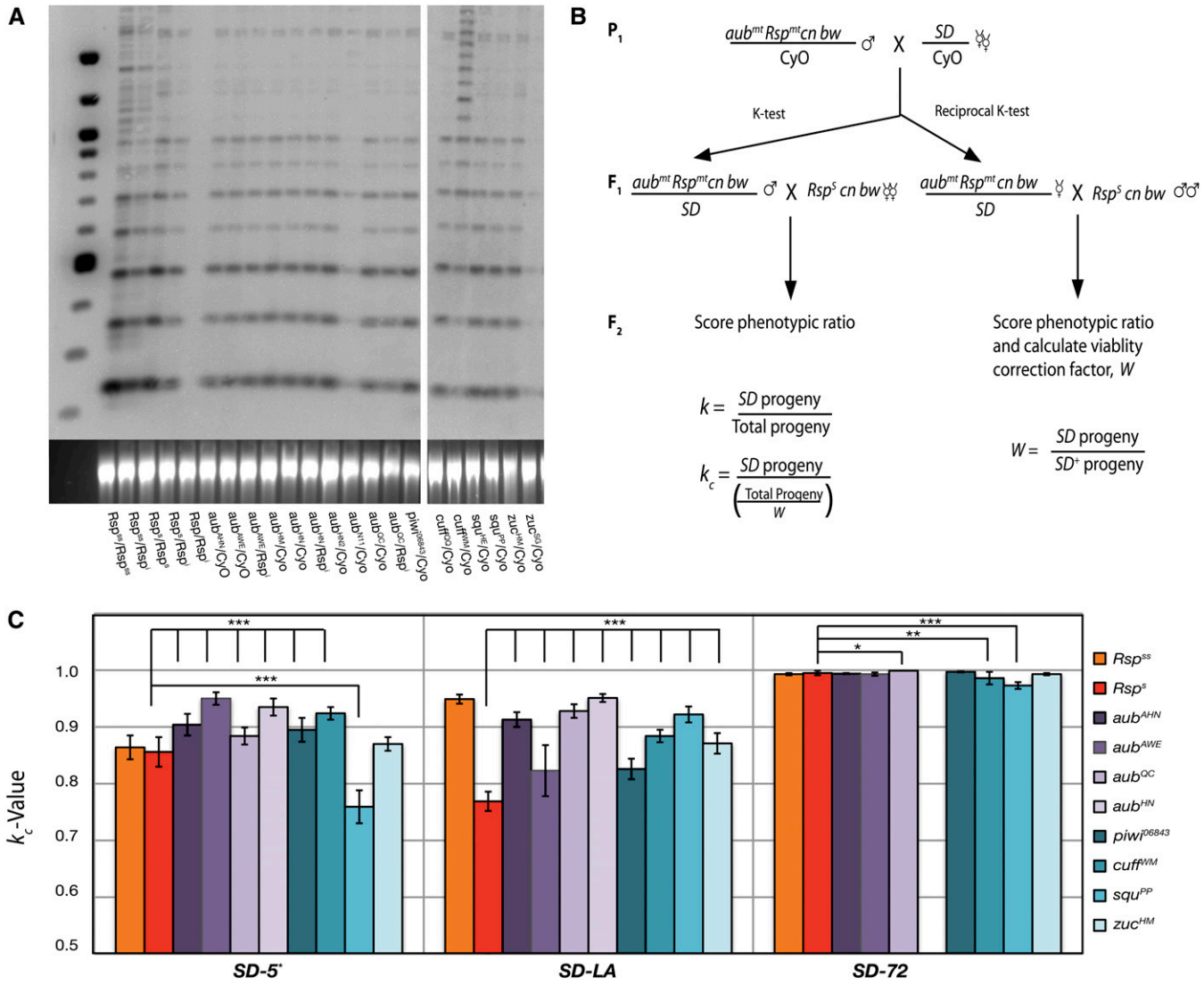

Figure 3 Mutations in *aub* and *piwi* are genetic enhancers of distortion. (A) A representative genomic Southern blot for *Rsp* sequences of indicated genotypes. All lanes contain 500 ng of *Xba*I-digested genomic DNA. The aub^{AHN}, aub^{AWE} , aub^{HM}, aub^{HN}, aub^{HN2}, aub^{QC}, $piwi^{06843}$, $cuff^{QQ}$, 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 detectable *Rsp* repeats. (B) Crosses and calculations for *k* tests. Chromosomes with *aub* mutations were crossed to *SD* stocks. F_1 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 total 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 the standard Rsp^{s} chromosome ($P < 0.0001$). Squ^{PP} showed a significant reduction in drive when paired with either *SD*-72 or *SD*-5* ($P < 0.0001$). 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. Flies of the genotype *SD*-72/aub^{HN} were nonviable and therefore could not be tested. Significance was calculated using a two-tailed *Z*-test (*$P < 0.01$, **$P < 0.001$, ***$P < 0.0001$).

standard Rsp^{ss}, Rsp^{s}, and Rsp^{i} 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^{PP}, and $piwi^{06843}$ displayed a banding pattern identical to that of the standard Rsp^{s} *cn bw* chromosome (Figure 3A). 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 F_1 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 3B). The resulting F_2 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 a single *piwi* mutant chromosome showed a significant increase in distortion over the Rsp^s control with equivalent levels of repeats (Figure 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 (Figure 3C). The aub^{QC} 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 in a manner analogous to increasing *Rsp* repeat copy number.

Mutation of the gene *cutoff* (*cuff*) also significantly enhances distortion (Figure 3C) ($P < 0.0005$). 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* results in the upregulation 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 in piRNA biogenesis (Chen *et al.* 2007; Kibanov *et al.* 2011).

Mutations of *zucchini* (*zuc*) and *squash* (*squ*) have differential effects on distortion (Figure 3C). Zuc is an endoribonuclease thought to be involved in the processing of primary piRNA precursors in ovarian somatic and germline cells (Pane *et al.* 2007; Malone *et al.* 2009; Haase *et al.* 2010; Nishimasu *et al.* 2012). The *zuc* mutant chromosome did not significantly alter distortion in the *SD*-5* and *SD*-72 backgrounds. However, an enhancement was observed in the *SD*-LA background. In the somatic cells of the ovary *Zuc* functions in production of Piwi-associated primary piRNAs and may serve to promote localization of the piRNA processing machinery to mitochondria (Pane *et al.* 2007; Malone *et al.* 2009; Saito *et al.* 2009; Haase *et al.* 2010; Watanabe *et al.* 2011; Nishimasu *et al.* 2012). The role of *Zuc* in the testis has not been extensively studied; however, loss of *zuc* has been shown to have little effect on the abundance of AT-chX-1 and Su(ste)-4, the two most common piRNAs in the *Drosophila* testes (Nagao *et al.* 2010).

The allele squ^{pp}, on the other hand, exhibited suppression of distortion with both *SD*-5* and *SD*-72 chromosomes (Figure 3C). Interestingly, Squ is 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). *Squ* mutant males exhibit a small reduction in levels of At-chX-1 and Su(ste)-4 piRNAs (Nagao *et al.* 2010). In *squ* mutant females overall piRNA levels remain largely the same, with a slight decrease detected in some studies (Pane *et al.* 2007; Malone *et al.* 2009; Haase *et al.* 2010). However, expression of some transposons is increased in these mutants, suggesting that, at least in females, Squ acts downstream of piRNA biogenesis (Pane *et al.* 2007; Haase *et al.* 2010). Unexpectedly, in this study, introduction of a mutant *squ* allele led to a significant suppression of drive compared to the Rsp^s control in two of three genetic backgrounds (Figure 3C) ($P < 0.0005$). However, since only single mutations of *piwi*, *zuc*, and *squ* were tested, we cannot completely rule out the possibility of confounding background influences in our analysis of their effects on distortion.

The contributions of 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 1A). Therefore we wanted to determine whether the enhancement in drive seen in the *aub* mutant chromosomes was specifically due to the introduced mutations *vs.* 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^+ Rsp^i 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 3B.

Four alleles of *aubergine* (aub^{HN}, aub^{QC}, aub^{N11}, and aub^{AWE}) were tested. Most recombinant (aub^+ Rsp^{mt}) chromosomes show a significant decrease in drive relative to the parental chromosome once the *aubergine* mutation is removed. Tests with several *SD* chromosomes and the awe-32 recombinant chromosome, an aub^+ Rsp^{AWE} derivative of the parental aub^{AWE} chromosome, gave k_c values that are between 9.0% and 32.5% lower than the parental chromosome k_c value ($P < 0.00003$) (Figure 4E). Due to its relatively low repeat copy number, the aub^{N11} parental chromosome exhibits only low levels of sensitivity to *Sd* (Figure 3A). However, aub^+ Rsp^{N11} recombinants show a further reduction in drive when tested with *SD*-5 or *SD*-Mad *lt cn* chromosomes (Figure 4D). The aub^+ Rsp^{HN} recombinant chromosome shows a reduction in drive of >50% compared to the aub^{HN} Rsp^{HN} chromosome (Figure 4C). For aub^{QC}, three of four recombinants show a decrease in *k* value with all SD lines tested. Line QC-1 did not show a significant

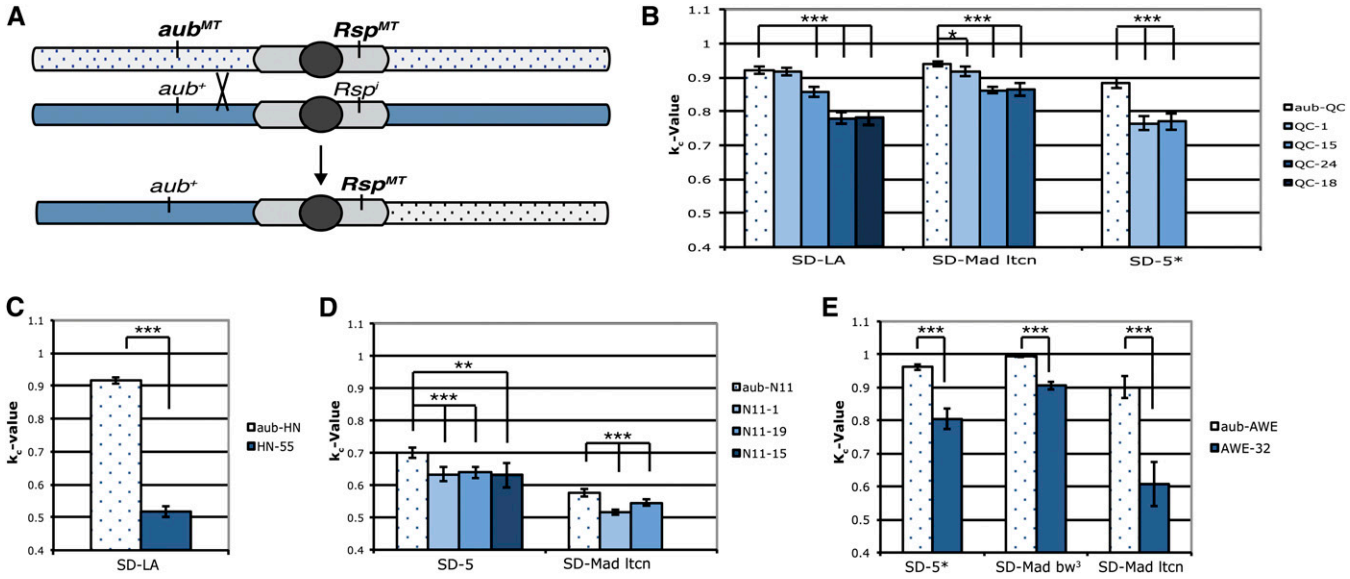

Figure 4 The contributions of *aub* mutants and associated repeats are genetically separable. (A) Recombinant chromosomes of the type *aub*$^{+}$ *Rsp*^{MT} were generated by allowing recombination between the *aub*^{MT} *Rsp*^{MT} and *aub*$^{+}$ *Rsp*^{i} chromosomes. (B) k_c values for recombinant 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 >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 total 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, $P < 0.0001$ (two-tailed *Z*-test).

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 shows a 13% decrease in k_c. (Figure 4B). The cause of this discrepancy is not entirely clear because the break points in these recombination events are unknown. It is possible, 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 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 that of the *Rsp*^{s} control chromosome. This enhancement was observed in two recovered recombinant lines (Figure 5B). Both *aub*^{N11} *Rsp*^{s}*-1* and *aub*^{N11} *Rsp*^{s}*-2* show 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 *Rsp*^{N11} allele leads to a significant increase in drive over that of *Rsp*^{s} alone in three of four independent tests (Figure 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 completely rule out the contributions 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

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) (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. Arm1 contains

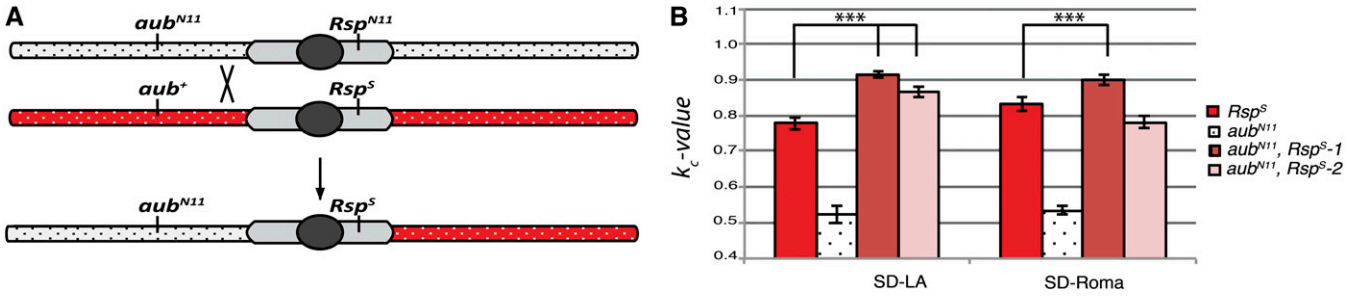

Figure 5 Addition of an *aub* mutation to a *Rsp*^{s} chromosome enhances distortion. (A) Recombinant chromosomes of the type *aub*^{N11} *Rsp*^{s} were generated by allowing recombination between *aub*^{N11} *Rsp*^{N11} and *aub*$^{+}$ *Rsp*^{s} chromosomes. Potential recombinant chromosomes were tested for *Rsp* status by Southern blot and for the presence of the 110-bp deletion in *aub*^{N11} by PCR assay. (B) Both recombinants show 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 total k_c for 20–30 males ($P < 0.00001$, two-tailed *Z*-test).

mostly intronic sequence from the gene *CG16833*, as well as a 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 2B).

In addition to a wild-type construct, two mutations were engineered: an *aub*$^{HR\text{-}HN}$ mutation, which changes Q622 to an amber stop codon (CAG → TAG), and the mutation *aub*$^{HR\text{-}E721A}$, which changes the catalytic glutamic acid required for RNA slicer activity to an alanine (GAG → GCG) (Figure 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*$^{HR\text{-}HN}$ 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 is 0.940, identical to the k_c for the same mutation in *trans* (Figure 6B).

Interestingly the catalytic mutant *aub*$^{HR\text{-}E721A}$ also showed an enhancement of *k* value ($k_c = 0.913$) although this effect was not as strong as that from the premature stop codon in the *aub*$^{HR\text{-}HN}$ 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 shows 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 (our unpublished observation). While the cause of the small alteration in k_c value for *SD*-Los Arrenos *aub*$^{HR\text{-}WT}$ 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.

To further examine the role of the endoribonuclease Zuc and the putative ribonuclease Squ, mutant alleles of each gene were tested in the SD-LA *aub*$^{HR\text{-}WT}$ and SD-LA *aub*$^{HR\text{-}HN}$ backgrounds. SD-LA males carrying either the wild-type or the *aub*$^{HR\text{-}HN}$ alleles showed significant suppression of distortion in the presence of *zuc* or *squ* mutations (Figure S1).

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*^{N11} chromosome, which naturally contains significantly fewer repeats than *Rsp*^{s}, results in a marked enhancement of drive compared with that of the *Rsp*^{s} repeats alone. These independent lines of evidence demonstrate that both mutations to 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

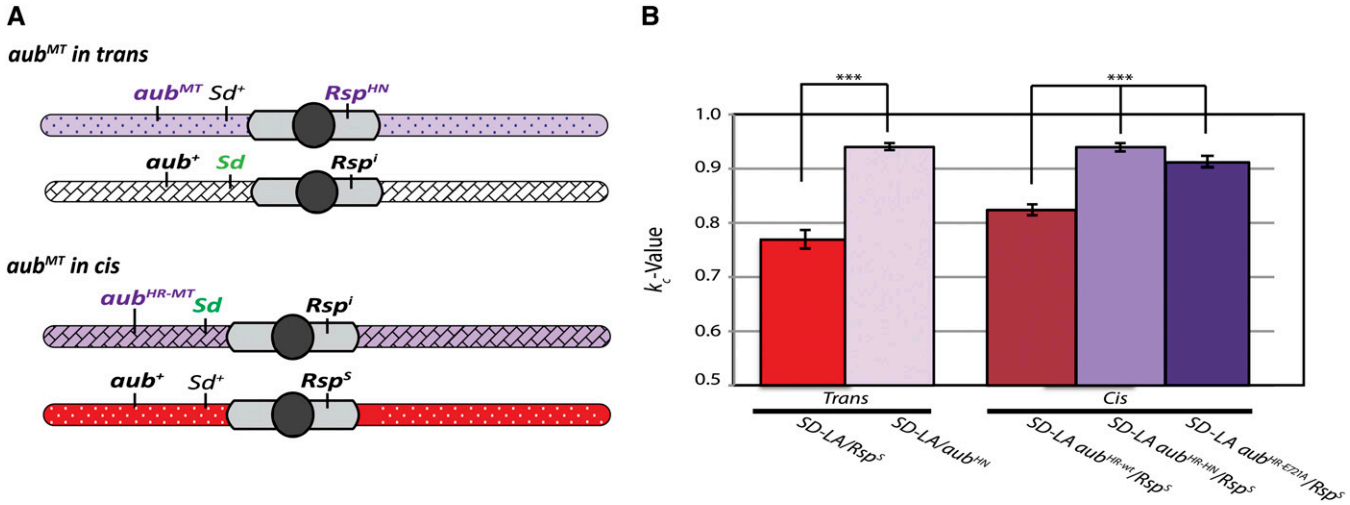

Figure 6 Engineered *aub* mutations on an *SD* chromosome enhance distortion in *cis*. (A) Schematic of chromosomes used to assay the effect of *aub* mutations placed on an *SD* chromosome. (B) The aub^{HN} mutation targeted to an *SD*-Los Arrenos (*SD*-LA) chromosome by HR ($aub^{HR\text{-}HN}$) enhances distortion of a Rsp^{s} compared to a targeted *SD*-LA $aub^{HR\text{-}wt}$ mutation. The $aub^{HR\text{-}E721A}$ 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 $aub^{HN\text{-}wt}$), although the increase is smaller than that seen with the *SD*-LA $aub^{HR\text{-}HN}$ targeted allele. The *SD*-LA $aub^{HR\text{-}HN}$ and *SD*-LA $aub^{HN\text{-}wt}$ k_c values each represent the pooled progeny counts for two independently derived alleles, ~60–70 males total. The *SD*-LA aub^{E721A} data represent a single allele with $n = 27$ males (***$P < 0.00005$, two-tailed *Z*-test).

a significant enhancement in drive, although this effect is not as large as that of the $aub^{HR\text{-}HN}$ allele that contains a premature stop codon, truncating the protein upstream of the catalytic residues. Although we have not completely eliminated the possibility that the effects of *aub* mutations on *SD* are indirect, evidence that disruption of *aub* catalytic activity alters distortion suggests that *aub*'s function as a ribonuclease is required for suppression of *SD*.

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 antisense RNAs by piRNA-guided 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, 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 codependent; 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 wild-type testes. 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 compromise both Aub- and AGO3-dependent processing.

Work in both the *Stellate* and the *Winters* sex ratio drive systems has implicated RNAi in mediating suppression of drive (Aravin *et al.* 2001, 2004; Tao *et al.* 2007a,b). In the *Stellate* sex ratio system antisense 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, 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,b). The two IR copies may be required 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

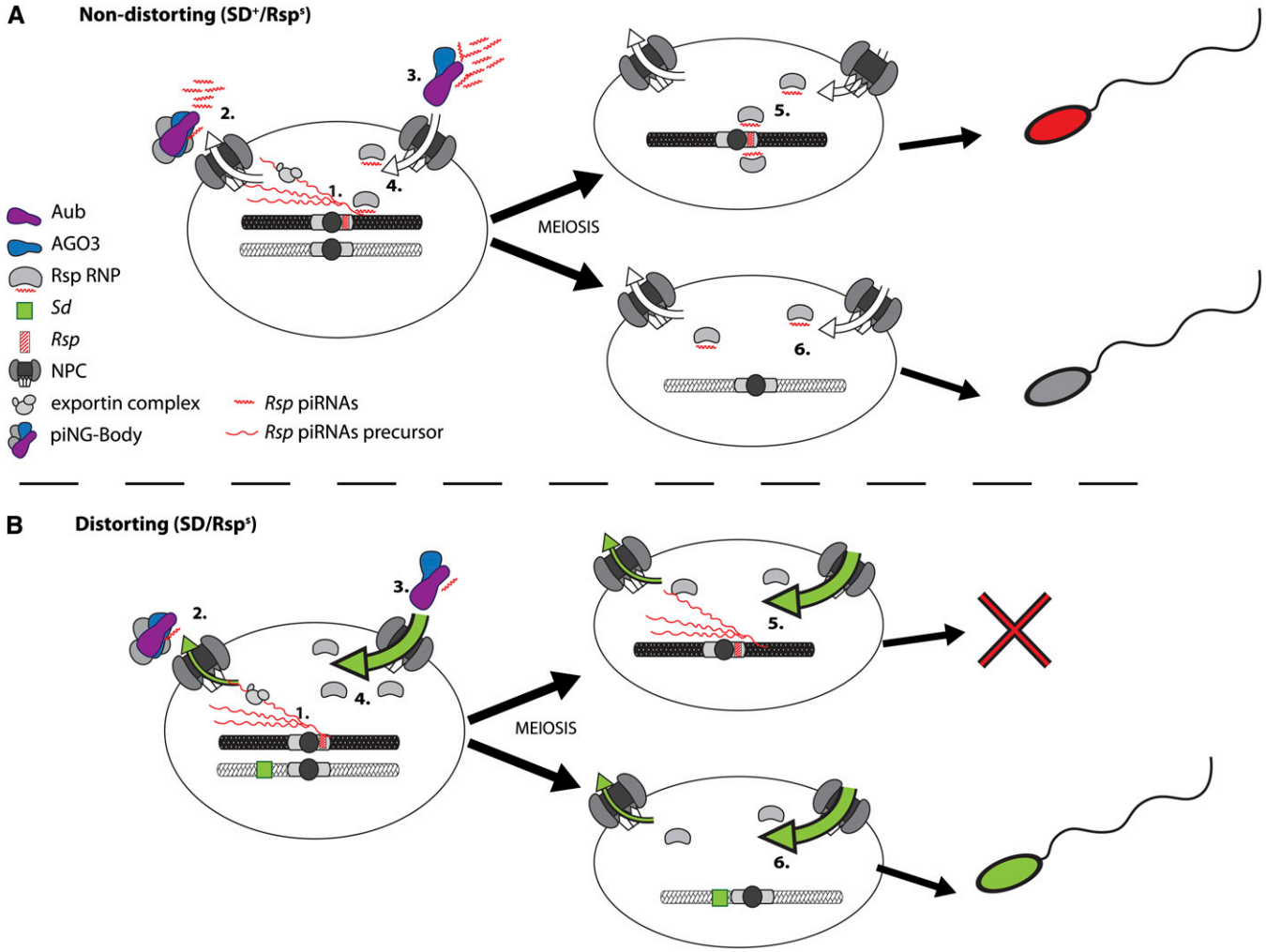

Figure 7 A model for the role of the piRNA pathway in segregation distortion. (A) 1, in *SD*$^{+}$ *Rsp*^{s} nuclei precursor *Rsp* piRNA transcripts are made in spermatocytes; 2, following export through the nuclear pore complex (NPC), these precursor *Rsp* piRNAs are processed into mature piRNAs by Aub and AGO3 in the piNG-body; 3, mature *Rsp* piRNAs reenter 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.

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 mechanisms appear to act protectively to suppress the potentially deleterious effects of these drivers.

In a recent publication, Tao *et al*. (2007a) propose 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; Larracuente and Presgraves 2012). This failure to silence the *Rsp* locus could lead to a generalized 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, 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 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 nuclear pore complex (NPC) (Gorlich and Kutay 1999a; Kohler and Hurt 2007). 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 7B). Interestingly, wild-type RanGAP has been observed to colocalize with piNG-bodies in spermatocytes (Figure S2), suggesting piRNA processing may be tightly coupled with nuclear transport.

In the germline cells of the ovary, Piwi enters the nucleus where it is 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 *Schizosaccharomyces 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; Larracuente and Presgraves 2012). In spermatids bearing 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 mature *Rsp*-piRNA–primed RNP complexes to facilitate effective silencing, leading to defective chromatin compaction and the specific destruction of *Rsp*-bearing spermatids (Figure 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. Still, not all components that influence piRNA biogenesis may have comparable effects on distortion, as our results on the effects of mutations in *zuc* and *squ* demonstrate. However, given the complexities of processing and transport of piRNAs on precursors and final small RNA products, we envision that different mutations could have opposing effects on 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 retrotransposition 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 and the etiology of meiotic drive, as well as the evolution of mechanisms to defend against invasion of the genome by this type of ultraselfish genetic element.

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GENETICS

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Mutations to the piRNA Pathway Component *Aubergine* Enhance Meiotic Drive of Segregation Distorter in *Drosophila melanogaster*

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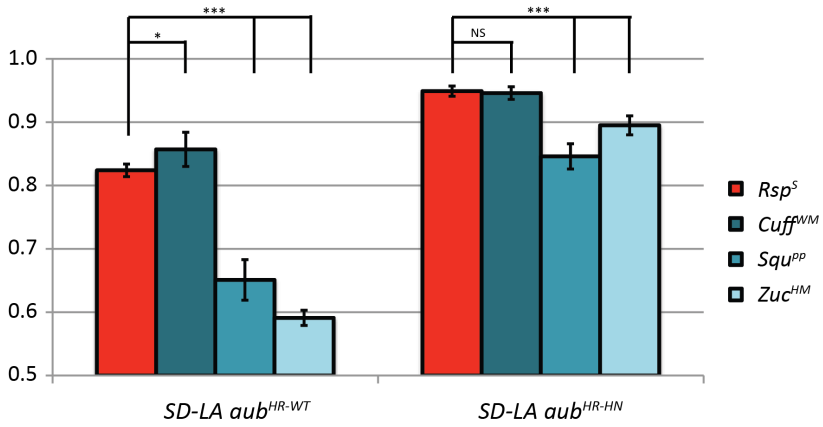

Figure S1 Chromosomes carrying *zuc*^{HM} and *squ*^{PP} suppress distortion by *SD*-LA in both *aub*$^{HR\text{-}wt}$ and *aub*$^{HR\text{-}HN}$ backgrounds. Females of the genotype *SD-LA aub*$^{HR\text{-}wt}$ and *aub*$^{HR\text{-}HN}$ were crossed with males carrying either a mutation in either *zuc, cuff* or *squ.* The 15-30 individual male F1 were then backcrossed to *cn bw* females to test for distortion. The *squ*^{pp} and *zuc*^{HM} chromosomes significantly suppressed distortion when paired with either the *aub*$^{HR\text{-}HN}$ or *aub*$^{HR\text{-}wt}$ chromosome. The *cuff*^{WM} chromosome gave a small but significant enhancement of distortion($p < 0.05$) with the wild type allele and there was no significant change with the *aub*$^{HR\text{-}HN}$ mutant (*** $p < 0.0005$, * $p < 0.05$, 2-tailed Z-test).

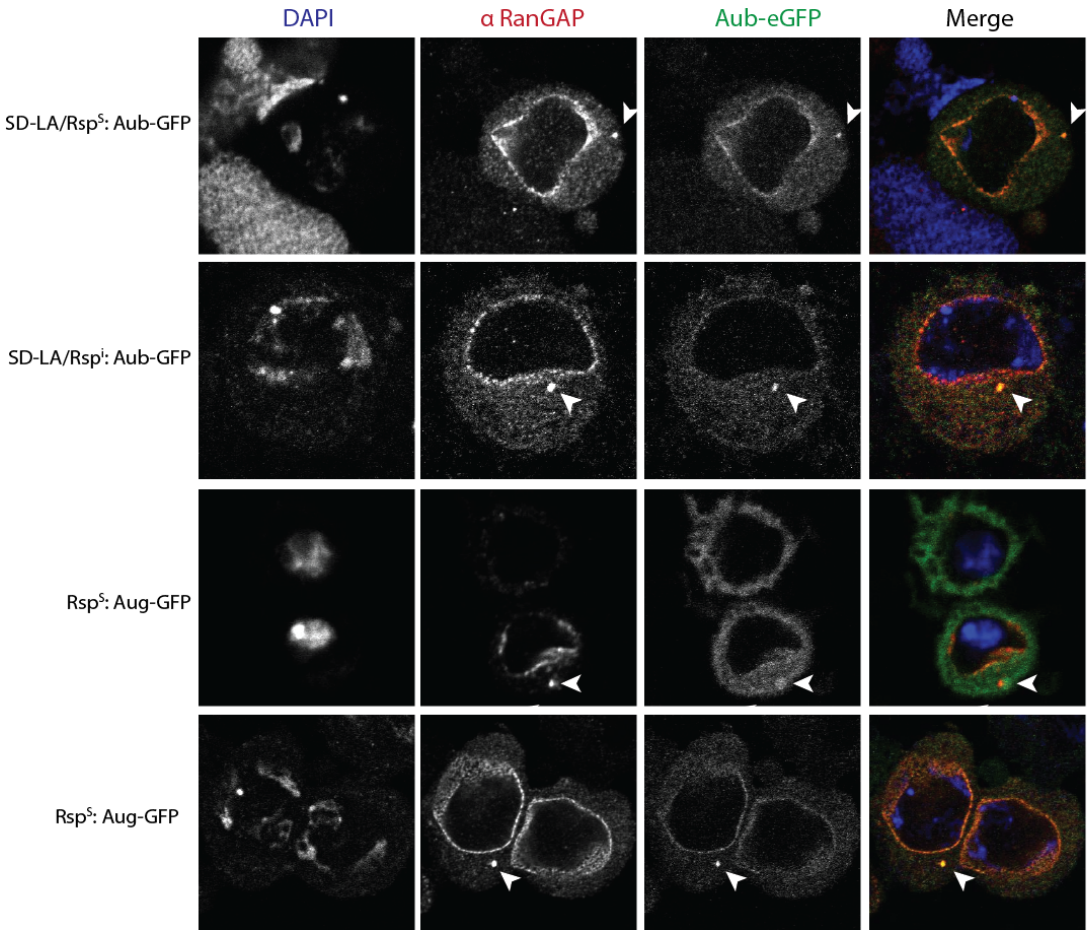

Figure S2 Aub-GFP and RanGAP colocalized in a single large body of primary spermatocytes. Whole mount testis of males expressing Aub-GFP were fixed with paraformaldyhide and stained with anti-RanGAP (1:1000) primary and Alexa Fluor (1:800) secondary antibodies. Confocal imaging was done on a Zeiss LSM 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 colocaliztion between Aub-GFP and Ran-GAP (arrowhead)

Table S1 Genetic stocks used in this study

Line	Notes on genotype	Source	Reference
HR stocks			
y w;FLP-I-SceI/TM6	y^1 w*; P{ $ry^{+t7.2}$=70FLP}11 P{ $v^{+t1.8}$=70I - SceI}2B noc^{Sco}/CyO, S^2	BSC 6930	Staber *et al.* 2011
y w ey-FLP	$y^{d2}w^{1118}$ P{ry[+t7.2]=ey-FLP.N}2	BSC 5580	Staber *et al.* 2011
y w Cre; noc^{Sco}/CyO	y^1 w^{67c23} P{$y^{+mDint2}$=Crey}1b; noc^{Sco}/CyO	BSC 766	Staber *et al.* 2011
w^1		BSC 145	Staber *et al.* 2011
w^{1118}		BSC 3605	Staber *et al.* 2011
Balancer Stocks			
w; TM3 *sb* /TM6 *tb*		B. Ganetzky	
+; $CyO^{lt\ dp\ pr\ cn}$ /Sco		B. Ganetzky	
w: $CyO^{\ cn\ bw\ Roi}$ /Sco		B. Ganetzky	
SD Stocks			
SD-5	Strong distortion phenotype and contains *Sd, E(SD), Rsp^i, M(SD)*and *St(SD) as well as two paracentric inversions on 2R.*	B. Ganetzky	*Sandler and Hiraizumi 1959; Sandler et al. 1959*
*SD-5**	Uncharacterized derivative of *SD-5** which exhibits an intermediate level of distortion	B. Ganetzky	
SD-72	Strong distorter with a both pericentric and paracentric inversions	B. Ganetzky	Sandler and Hiraizumi 1959)
SD-Mad lt cn	Derived from the original *SD-Mad* by recombination with chromosomes carrying the recessive markers *cn, lt*	B. Ganetzky	R.G. Temin 1979
SD-Mad bw^3	Derived from the original *SD-Mad* by recombination with chromosomes carrying the recessive markers *bw^3*	B. Ganetzky	R.G. Temin 1979
SD-Roma	Inversion free moderately distorting SD chromosome isolated in Italy	B. Ganetzky	Nicoletti and Trippa 1967)
SD-Los Arrenos	Weak distorter	B. Ganetzky	
Rsp^S cn bw	[*Sd^+, $E(SD)^+$, Rsp^S*] standard Rsp sensitive chromosome	B. Ganetzky	Lyttle 1991
Rsp^{i16} cn bw	[*Sd^+ $E(SD)^+$ Rsp^i*] Radiation-induced derivative of the Rsps cn bw chromosome where the Rsp locus has	B. Ganetzky	Ganetzky 1977

	been completely deleted		
Rsp^{SS} lt pk cn	[*Sd^{+} E(SD)$^{+}$ Rsp^{SS}*] the canonical RspSS chromosome	B. Ganetzky	Lyttle 1991
RNAi Mutants			
aub^{QC42}cn bw/CyO	EMS; Strong allele	T. Schüpbach	Schupbach and Wieschaus 1991
aub^{HN2} cn bw/CyO	EMS; Strong allele	T. Schüpbach	Schupbach and Wieschaus 1991
aub^{HM23} cn bw/CyO	EMS; Strong allele	T. Schüpbach	Schupbach and Wieschaus 1991
aub^{AHN56} cn bw/CyO	EMS: Strong allele, has secondary mutation	T. Schüpbach	Schupbach and Wieschaus 1991
aub^{AHE13} cn bw/CyO	EMS: Strong allele, has secondary mutation	T. Schüpbach	Schupbach and Wieschaus 1991
zuc^{HM27} cn bw/CyO	EMS: Strong allele	T. Schüpbach	Schupbach and Wieschaus 1991
zuc^{SG63,rec2} pr c px sp/CyO	EMS: homozygotes poorly viable	T. Schüpbach	Schupbach and Wieschaus 1991
squ^{PP32} cn bw/CyO	EMS; Strong allele	T. Schüpbach	Schupbach and Wieschaus 1991
squ^{HE47} cn bw/CyO	EMS; Strong allele	T. Schüpbach	Schupbach and Wieschaus 1991
cuff^{WM25} cn bw/CyO	EMS; Strong allele	T. Schüpbach	Schupbach and Wieschaus 1991
cuff^{QQ37} cn bw/CyO	EMS; Strong allele	T. Schüpbach	Schupbach and Wieschaus 1991
w^{1}; aub^{N11}/CyO	110bp deletion	P. Macdonald	Harris and Macdonald 2001
piwi^{06843} cn /CyO	P{PZ} in first exon	BSC 12225	Cox et al 1998
nosGAL4, Aub-GFP	UAS-Aubergine-GFP recombined on to the same chromosome as nos-GAL4 VP16	P. Macdonald	Harris and Macdonald 2001

Table S2 Primer sequences used in this study

Name	Sequence
Cloning Primers	
AUBarm1-F *Bsi*WI	TCGTACGCTCTCCATGTAGCGAAAAGCGTATAG
AUBarm1-R *Asc*I	TGGCGCGCCAATGCATATATTCGTATGAAATGAGC
AUBarm2-F *Acc*65I	TGGTACCTTCCAGCAACGGAACTTTATAGAC
AUBarm2-R *Not*I	TGCGGCCGCTGGTTACGAAGAGTCGTGCTGGCG
Sequencing Primers	
AUBarm1-s1	GTGACAGTCGCTGCCATCGTATTATTGG
AUBarm1-s2	CTGACAAGTGGACTGTCTGCAGTGGGTG
AUBarm1-s3	CTTTATATCGAAGGGTAAGTACATG
AUBarm1-s4	GACTCGAACGATGAAGAAACAACACCAGCG
AUBarm2-s1	GTAGCGAGAATTAATGCGCTTGGATAC
AUBarm2-s2	GTGTCACCACCTGCGATGGCACCGGTC
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	CCTGTCCTTTAGGGTGTTCACCGCGCTGTTTACCACCTGGTAGAGCTGACC
Arm Specific Validation Primers	
AUBarm2-V1	CTTGTATGAACATAAAGGGATCC
AUBarm2-V2	CGTCGATTTTACGCCTGATGTGG
AUBarm2-V3	GGCACTCATATTACCGTGCAGGC
AUBarm1-V1	GGTTTATAATTCCACGGACTTC
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	CGATCATTCATTATTCGCTGCATGAATTAGC
pW-Acc 1	CATTATACGAAGTTATCTAGACTAGTCTAGGGTAC
pW-Acc 2	GACGCTCCGTCGACGAAGCGCCTC
pW-Acc 3	GCTCAGCTTGCTTCGCGATGTGTTCAC
WTPR	CGCGAACATTCGAGGCGCGCTCTCTCG
Wt SQ10F	GTGACCTGTTCGGAGTGATTAGCG
Southern Blotting	
SP6	GCCAAGCTATTTAGGTGACACTATAG
T7	GAATTGTAATACGACTCACTATAGGG
Rsp13 clone sequence	GGAGCTCCACCGCGGTGGCGGCCGGCCCTGCAGATCTGCGGCCGCTCTAGAAGGTGTCTTCTGTTCGCCT GGTACTTGAAATCGAAAAATCACTCATTTGACCGCTAAAATGACATAACTTAGTCAATTTATTGTTTTTGTG TACCAGGTTTAAATAATCTGTAGAAGGTATCTTCTGTTTGTCTGGTACTTGAAATCGGAAAATCACTCATTT TGACCGCTTAAAATGTAAAACTTAGGCAATTTACTGTTTTTCCTTACCAGTTGAACAGAATCTCTAGAAGG TGTCTTCTGTTCGCCTGGTACTTGAAATCGAAAAATCACTCATTTGACCGCTAAAATGACATAACTTAGTCA ATTTATTGTTTTTGTGTACCAGGTTTAAATAATCTGTAGAAGGTATCTTCTGTTTGTCTGGTACTTGAAATC GGAAAATCACTCATTTTGACCGCTTAAAATGTAAAACTTAGGCAATTTACTGTTTTTCCTTACCAGTTGAAC AGAATCTCTAGAACTAGTGGATCCCCCGGGCTGCAGGAATTCGATATCAAGCTTATCGATACCGTCGACC TCGAGGGGGGGCCCGGTACCAGCTTTTGTTCCCTTTAGTGAGGGTTAATTTCGAGCTTGGCGTAATCATG GTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCAC