Research Notes

No effect of heterozygous *argonaute3* or *caravaggio* mutations on *P*-element regulation in the germ line of *D. melanogaster* males.

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Many of the transposons in *D. melanogaster* are regulated by small RNAs that associate with the Piwi class of proteins. These Piwi-interacting (or pi) RNAs are generated from transposon sequences inserted in special loci scattered about the genome (Brennecke *et al.*, 2007). One such locus is in the Telomere Associated Sequences (TAS) at the left end of the X chromosome. Transposable *P* elements inserted in this locus produce piRNAs that regulate the entire *P*-element family (Brennecke *et al.*, 2008). These telomeric *P* elements are therefore anchors of the P cytotype, the term that Engels (1979) gave to the cellular state that represses *P*-element excision and transposition.

The Piwi-type proteins are encoded by three genes in the *D. melanogaster* genome: *argonaute3* (*ago3*), *aubergine* (*aub*), and *piwi*. In homozygous condition, mutations in these genes are either lethal or sterile. Thus, their possible effects on transposon regulation can only be studied in heterozygous condition. Previously, heterozygous *aub* mutations have been shown to disrupt *P*-element regulation in both males and females (Reiss *et al.*, 2004; Simmons *et al.*, 2007; Simmons *et al.*, 2014), and heterozygous piwi mutations have been shown to disrupt *P* element regulation and the related *trans*-silencing effect in females (Josse *et al.*, 2007; Belinco *et al.*, 2009; Simmons *et al.*, 2014); in addition, one heterozygous *piwi* mutation (*piwi*^{1}, thought to be the more severe of the two alleles tested) was found to disrupt *P* regulation in males (Simmons *et al.*, 2010). Heterozygous mutations in another vital gene, *Suppressor of variegation 205* [*Su(var)205*], also impair *P* regulation—in females as well as males (Ronsseray *et al.*, 1996; Haley *et al.*, 2005; Simmons *et al.*, 2014). This gene encodes heterochromatin protein 1 (HP1), a protein involved in chromatin organization (Eissenberg *et al.*, 1990). HP1 is found in the centric heterochromatin, at telomeres, and at some euchromatic loci (James *et al.*, 1989). Its presence at telomeres suggests that it plays a role in preventing chromosome entanglements and in maintaining chromosome integrity (Savitsky *et al.*, 2002; Perrini *et al.*, 2004). The HP1/ORC associated protein (HOAP), encoded by the *caravaggio* (*cav*) gene, apparently collaborates with HP1 to stabilize telomere structure (Cenci *et al.*, 2003).

We tested heterozygous *ago3* and *cav* mutations for impairment of *P*-element regulation in males that carried *TP5*, an incomplete *P* element inserted in the TAS of XL (Stuart *et al.*, 2002). The strength of regulation was measured by monitoring the excision of *P* elements inserted in *sn*^{w}, a double-*P* insertion mutation of the *singed* bristle gene (Roiha *et al.*, 1988). In hemizygous males, this mutation causes a mild malformation of the bristles. However, when one or the other of the inserted *P* elements is excised from *sn*^{w} in a male's germ line, in the next generation the bristles show a different phenotype—either extreme mutant (sn^{e}) or pseudo-wild type (sn$^{(+)}$). Thus, by counting the frequency of sn^{e} and sn$^{(+)}$ flies among the progeny of each tested male, we could quantify the strength of *P* regulation in its germ line. A high frequency of sn^{e} and sn$^{(+)}$ flies—that is, a high frequency of *P* excisions from *sn*^{w}—implies weak regulation whereas a low frequency of these flies implies strong regulation. Excision of the *sn*^{w} *P* elements was catalyzed by the P transposase produced by *H(hsp/CP)2*, a *hobo* transgene containing a terminally truncated but otherwise complete *P* element (*CP*) that encodes this enzyme (Simmons *et al.*, 2002). *H(hsp/CP)2* is stably located on chromosome II and the *ago3* and *cav* mutations are located on chromosome III; in our experiments these sterile or lethal mutations were balanced over *TM3, Sb Ser*.

To set up the experiments, we crossed *TP5 sn*^{w}; *mutation*/*TM3, Sb Ser* females to *H(hsp/CP)2* males. The mutations tested were *cav* (Cenci *et al.*, 2003) and the *ago3* alleles *t1* and *t3* (Li *et al.*, 2009), all of which behave as nulls. The F_1 *TP5 sn*^{w}; *mutation*/*H(hsp/CP)2* sons were then individually crossed to 3 *C(1)DX, y f*

females. Because of the attached-X chromosomes in these females, the *TP5* sn^w chromosome is transmitted patroclinously. Thus, we scored the F_2 males for the singed bristle phenotypes. The cultures were reared at 25°C and scored on days 14 and 17 after the cultures were established. The excision frequency was calculated for each tested male, and then averaged over all the males in a test group; the standard error (SE) associated with this average was calculated empirically.

Table 1. Effect of heterozygous mutant *ago3* and *cav* alleles on the frequency of transposase-catalyzed *P* excisions from the sn^w allele in the male germ line.

TP5 present or absent[a]	Mutant allele	No. males tested	No. progeny scored	*P* excision rate ± SE
absent	none	29	586	0.518 ± 0.018
absent	$ago3^{t1}$	31	468	0.457 ± 0.023
absent	$ago3^{t3}$	32	827	0.507 ± 0.022
absent	*cav*	32	890	0.427 ± 0.020
present	none	32	493	0.021 ± 0.008
present	$ago3^{t1}$	31	478	0.038 ± 0.018
present	$ago3^{t3}$	29	1017	0.044 ± 0.011
present	*cav*	35	1145	0.031 ± 0.011

[a] The X-linked telomeric element *TP5* anchors the P cytotype, which represses *P* excisions from sn^w.

The results (Table 1) show that in the absence of the cytotype-anchoring regulatory element *TP5*, the *P* excision rate ranged from 0.427 to 0.518. These values are consistent with other published data from similar experiments in which *H(hsp/CP)2* was the transposase source (Simmons *et al.,* 2002). None of the heterozygous mutant alleles significantly altered the unregulated *P* excision rate. In the presence of *TP5* the *P* excision rate ranged from 0.021 to 0.044—an order of magnitude lower than the unregulated excision rates. The *TP5* element therefore strongly represses transposase-catalyzed *P* excisions from the sn^w allele. The similarity of these excision rates indicates that the heterozygous *ago3* or *cav* mutant alleles do not impair *TP5*-anchored cytotype regulation in the male germ line. Furthermore, because these mutant alleles were derived from heterozygous mothers of the tested males, they also do not disrupt the maternal component of *TP5*-anchored regulation. In tests for heterozygous effects on repression of *P*-excisions from sn^w in the male germ line, the mutant *ago3* and *cav* alleles therefore behave like mutant *piwi* alleles, not like mutant *aub* or *Su(var)205* alleles (Simmons *et al.,* 2007). Repression of *P*-excisions from sn^w in males is evidently more sensitive to the depletion of Aub or HP1—achieved by knocking out one copy of the relevant gene—than to the depletion of Piwi, Ago3 or HOAP.

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Impairment of piRNA-mediated regulation of *P*-element mRNAs in *D. melanogaster* females heterozygous for a mutant allele of *argonaute3* or *caravaggio.*

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The proteins encoded by the genes *argonaute3* (*ago3*), *aubergine* (*aub*), and *piwi* are involved in processing small RNAs that regulate transposable elements in the *D. melanogaster* genome. As a group, these proteins are called Piwi-type proteins, and the RNAs associated with them are called Piwi-interacting (or pi) RNAs (Aravin *et al.*, 2007; Brennecke *et al.*, 2007; Li *et al.*, 2009). piRNAs are generated from many different loci, including one in the Telomere Associated Sequences (TAS) at the left end of the X chromosome. This locus has been identified as the source of the piRNAs that regulate the *P* family of transposable elements (Brennecke *et al.*, 2008). *P* elements are mobilized by an enzyme, the P transposase, which is encoded by structurally complete members of the *P*-element family (Engels, 1989). *P*-element activity is restricted to the germ line, because the mRNA for the transposase is produced only in that tissue (Laski *et al.*, 1986); in the soma, the last of the introns—the one denoted as the 2-3 intron because it lies between exons 2 and 3—is not removed from *P* transcripts. When translated, these incompletely spliced *P* transcripts produce a polypeptide that is unable to catalyze transposition.

In the germ line, *P* elements are regulated by piRNAs generated from *P*-elements that have fortuitously inserted into the TAS of XL (Ronsseray *et al.*, 1991; Marin *et al.*, 2000; Stuart *et al.*, 2002). These piRNAs can be passed from mother to offspring through the egg cytoplasm (Brennecke *et al.*, 2008). Thus, a female that carries a telomeric *P* element can endow her offspring with the ability to regulate *P*-element activity, even if the offspring do not inherit the telomeric *P* element itself (Simmons *et al.*, 2012). This maternal effect is one of the hallmarks of *P* regulation. Engels (1979) coined the term "P cytotype" to encompass all the components of *P* regulation—both chromosomal and cytoplasmic. Recent analyses have revealed that the chromosomal component consists of the *P* elements themselves—especially cytotype-anchoring telomeric *P* elements—and the cytoplasmic component consists of maternally transmitted piRNAs.

At the molecular level, *P* regulation is characterized by a reduction in the amount of germ-line *P* mRNA (Jensen *et al.*, 2008). This reduction could either be due to repression of *P* transcription or to post-transcriptional destruction of *P* transcripts; in either case, the mechanism must be mediated by piRNAs generated from a telomeric *P* element. However, *P*-element regulation might also involve other factors. One candidate is heterochromatin protein 1 (HP1), which is encoded by the *Suppressor of variegation 205* [*Su(var)205*] gene (Eissenberg *et al.*, 1990), and another is the HP1/ORC-associated protein (HOAP), which is encoded by the *caravaggio* (*cav*) gene (Cenci *et al.*, 2003). These two proteins are involved in chromatin organization, notably at telomeres, where they could influence the expression of piRNA-generating *P* elements. The proteins Ago3, Aub, and Piwi are thought to be involved directly in the production of piRNAs from these *P* elements. Depleting any of these chromatin-organizing or piRNA-processing proteins might impair the production of piRNAs, and thereby allow *P*-element mRNAs, especially transposase-encoding mRNAs, to accumulate in the germ line.

To test this hypothesis, we used reverse transcription and the polymerase chain reaction (RT-PCR) to assess the levels of mRNAs from a telomeric *P* element, denoted *TP5*, and a transgenic complete *P* element, denoted *H(hsp/CP)2*, in females that were heterozygous for these elements and a mutant allele of the *ago3* or *cav* gene. Females that are homozygous for the $ago3^{t1}$ or $ago3^{t3}$ alleles are sterile (Li *et al.*, 2009), and flies