Modulation of the Activity of a Polycomb-Group Response Element in Drosophila by a Mutation in the Transcriptional Activator Woc

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ABSTRACT Polycomb group response elements (PRE) are cis-regulatory elements that bind Polycomb group proteins. We are studying a 181-bp PRE from the Drosophila engrailed gene. This PRE causes pairing-sensitive silencing of mini-white in transgenes. Here we show that the 181-bp PRE also represses mini-white expression in flies with only one copy of the transgene. To isolate mutations that alter the activity of the 181-bp PRE, we screened for dominant suppressors of PRE-mediated mini-white repression. Dominant suppressors of mini-white repression were rare; we recovered only nine mutations out of 68,274 progeny screened. Two of the nine mutations isolated are due to the same single amino acid change in the transcriptional activator Woc (without children). Reversion experiments show that these are dominant gain-of-function mutations in woc. We suggest that Woc can interfere with the activity of the PRE. Our data have implications for how Polycomb group proteins act to either partially repress or completely silence their target genes.

Polycomb group genes (PcG) encode proteins that mediate transcriptional repression. First identified in Drosophila as genes necessary to maintain the silencing of homeotic genes, it is now evident that PcG proteins have many other targets (reviewed in Simon and Kingston 2007). In genome-wide studies, PREs were identified as binding sites for multiple PcG proteins (Schwartz et al. 2006; Négre et al. 2006; Tolhuis et al. 2006). Two functional assays have also been used to identify PREs. In one assay, the PRE is combined in a transgene with regulatory DNA from a gene normally regulated by PcG proteins, where the PRE is required to maintain the “off” transcriptional state (Müller and Bienz 1991; Hagstrom et al. 1997). In the other assay, PREs are used to repress expression of the mini-white reporter gene in transgenic flies. Because mini-white repression is stronger in flies homozygous for the PRE-mini-white reporter, this latter assay has been called pairing-sensitive silencing (Kassis 1994).

One of the puzzles of the transgene assays for PREs is that silencing does not occur at every chromosomal insertion site. For example, for the four engrailed and inverted PREs, pairing-sensitive silencing was observed at a frequency of 21–62% of insertion sites (Americo et al. 2002; Cunningham et al. 2010). PRE activity is regulated by the expression state of the gene it regulates; thus it follows that PRE activity in transgenes is dependent on the activity of regulatory elements that flank the transgene insertion site.

We have been studying a 181-bp engrailed DNA fragment that acts as a PRE in several different assays: (1) it represses inappropriate expression in both engrailed and Ultrabithorax-reporter genes in embryos (Americo et al. 2002; Devido et al. 2008); (2) PcG proteins are associated with it in tissue culture cells, embryos, larvae, and adults (Strutt and Paro 1997; Négre et al. 2006; Oktaba et al. 2008); and (3) it acts as a pairing-sensitive silencing element (Kassis 1994). This fragment contains...
binding sites for the PRE DNA binding proteins Pho, Pho-like, GAGA factor, and Spps (Americo et al. 2002; Brown et al. 2005; Brown and Kassis 2010). Thus, the 181-bp DNA fragment is clearly a PRE. Therefore, we reasoned that conducting a genetic screen for mutations that alter the activity of this PRE might yield mutations in PcG genes.

We conducted a genetic screen for dominant suppressors of pairing-sensitive silencing by a transgene that contained the 181-bp en PRE and mini-white. These mutations were rare; we only obtained nine suppressors among 68,274 genomes screened. None of the mutations affected mini-white repression of transgenes at all chromosomal insertion sites. This suggests that none of the mutations affects PRE activity directly. Instead, we believe that these mutations affect the expression of genes flanking the transgene insertion site. Consistent with this, two of the dominant suppressors are the same gain-of-function mutation in the gene without children (woc), which encodes a transcriptional activator. Our data suggest that there is a competition between transcriptional activators and PcG repression and that certain types of activators may be better able to overcome PcG repression.

MATERIALS AND METHODS

Mutagenesis

For EMS mutagenesis, adult males were fed EMS as described (Lewis and Bacher 1968; Kennison 1983), and then discarded 3–4 days following treatment to avoid pre-meiotic clusters of mutations. For the X-ray mutagenesis, males were irradiated with 30–40 Gy at 120 keV using a Faxitron Torrex 2800. The irradiated males were discarded 4–5 days following treatment.

Sequencing

DNA was isolated from homozygous or hemizygous mutant adults or larvae, and the entire woc transcription unit was sequenced.

Construction of P[181PRE]

The 181-bp en PRE was amplified with the primers GCGGAATTCCGAATGGGTGCTTACT and GCCGGAATTCGCTGCTGCAAGAGTGTGTGT, cut with EcoRI, and cloned into EcoRI cut, phosphatased EK710, which contains loxP sites on both sides of the EcoRI site (Kuhn et al. 2004). A fragment of DNA containing the 181-bp PRE and flanking loxP sites was cut with NotI and cloned into NotI cut CaSpeR4. The resulting clone was sequenced to determine the orientation of the insert.

Generation and analysis of transgenic lines

P[181PRE] was injected into homozygous Df(1)w67c23, y embryos using standard techniques. Some lines were generated by P-element mobilization by crossing to a strain with the endogenous transposase insertion P[ry+Δ2, 3]99B (Robertson et al. 1988). P[Li] derivative lines lacking the en181bp-PRE were obtained by crossing males with the P[181PRE] insertion to virgin females that carried a constitutively lacking the PRE and mini-pairing-sensitive silencing by a transgene that contained the 181-bp PRE. These mutations were rare; we only obtained nine suppressors among 68,274 genomes screened. None of the mutations affected mini-white repression of transgenes at all chromosomal insertion sites. This suggests that none of the mutations affects PRE activity directly. Instead, we believe that these mutations affect the expression of genes flanking the transgene insertion site. Consistent with this, two of the dominant suppressors are the same gain-of-function mutation in the gene without children (woc), which encodes a transcriptional activator. Our data suggest that there is a competition between transcriptional activators and PcG repression and that certain types of activators may be better able to overcome PcG repression.

qRT-PCR

Flyes of the following genotypes were used: (1) w1118, (2) w1118; P[181PRE]8-10C, (3) w1118; wocD1, and (4) w1118;P[181PRE]8-10C. Total RNA from 3rd instar larvae, 1-day-old pupae, or adult fly heads was prepared (Lorenz et al. 1989) and treated with DNase I before use. qRT-PCR was done with the QuantiTect SYBR Green RT-PCR kit (Qiagen) on the LightCycler 480 real-time PCR system (Roche Applied Sciences) using 0.2 µg total RNA/reaction. The following PCR primers were used: for the BpL32 reference gene, CGGATCGATATGCTAAGCTGT and CGACGCACTCTGGTTGCG, its amplicon is 67 bp; for CG30456, AAAATGGCAAGAATTTCC and AACCTGGCACCACATTGCT, its amplicon is 95 bp; for GstS1, GTCAAGGACACAGTGTCGA and GTGTAGTCGGCAGTAG, its amplicon is 72 bp. Reverse transcription was done at 50°C for 20 min, followed by incubation at 95°C for 15 min to activate the PCR reaction. PCR was for 45 cycles of 94°C, 10 s, 60°C, 20 s, 72°C, 20 s. After PCR, the reactions were heated to 95°C and then cooled to 40°C to analyze the melting temperatures of the PCR products.

RESULTS

Dominant modifiers of mini-white repression

To recover mutations that affect pairing-sensitive silencing, we screened for dominant mutations that suppressed en181bp-mediated mini-white repression. We used the line P[181PRE]8-10C, which contains a P-construct with the 181-bp PRE of en DNA cloned into pCaSpeR (Construct 8 in Kassis 1994; Figure 1). pCaSpeR contains the mini-white gene; a truncated version of the white gene, which contains a promoter fragment that gives expression in the eye but no eye enhancer. The 181-bp PRE is cloned directly adjacent to the mini-white promoter. The w; P[181PRE]8-10C homozygotes have white eyes, and w; P[181PRE]8-10C heterozygotes have orange eyes (Figure 2). For the mutagenesis, we fed w; P[181PRE]8-10C males EMS and crossed to either w; P[181PRE]8-10C or w; P[181PRE]8-10C Sco/CyO females. We looked for mutations that darkened the eye color of either homozygotes or heterozygotes. We recovered nine mutations; one on the X chromosome, four on chromosome 2, and four on chromosome 3. All but two of the mutations darkened the eye color of both P[181PRE]8-10C homozygotes and heterozygotes. These mutations could identify genes involved in repression of mini-white transcription, perhaps via the PRE. One second-chromosome mutation darkened the eye color of heterozygotes only, which suggests that it is not involved in mini-white repression but might be involved in pigmentation. We did not study this mutation further. The sex-linked mutation only darkens the eye color of P[181PRE]8-10C homozygotes. The reason for this is unknown; however, it could mean that the mutation affects the interaction between PREs.

Two mutations cause the same single amino acid change in the transcriptional activator Woc

We mapped the mutations on chromosome 3 using the markers ru, h, th, cu, sr, c, and Pr. Two mutations mapped 2.5 map units distal to Pr. We next tried to recover recombinants between these two mutations. We found no recombinants among 448 progeny, suggesting that the two mutations are very close to each other and might be allelic. We tested whether several overlapping deletions for polytene chromosome region 96F–98B (which should include the mutations) caused a darkening of the P[181PRE]8-10C eye color. As none did, we suspected that both mutations are gain-of-function alleles that produce proteins with altered activities. If so, then a mutation that inactivates the mutant protein should revert the dominant suppression of the P[181PRE]8-10C eye color. Therefore, we tried to revert both mutations.
We used both X-rays and EMS to generate revertants. We recovered three X-ray-induced revertants (from 24,758 progeny) and four EMS-induced revertants (from 5500 progeny). The revertants are lethal over deficiencies for the region 96F1–98A5. By crossing to overlapping deficiencies and lethals in the region, we found that all of the revertants are lethal or semilethal mutations in the gene _woc_.

Sequencing of the _woc_ gene from our original suppressor mutation chromosomes showed that both of these mutations are due to the same single amino acid change in a position evolutionarily conserved throughout the *Drosophila* lineage, as well as in most insects (Figure 3). This amino acid change occurs in a region of the protein with no known domain or function. We named the two original suppressor mutations _wocD1_ and _wocD2_ (for _wocDominant1_ and _wocDominant2_), and we named the revertant alleles based on the allele reverted and the mutagen used (i.e., _wocD1Rev_ was a revertant (rv) generated from _wocD1_ by EMS (E) mutagenesis). The _wocD1/+_ and _wocD2/+_ flies have no phenotypic defects. The _wocD1_ and _wocD2_ homozygotes survive, are fertile, and also show no phenotypic defects.

We also sequenced the revertants. As expected, all revertants contained the mutation present in _wocD1_ and _wocD2_, as well as an additional lesion in the _woc_ transcription unit (Figure 3). With the exception of _wocD2wX1_, all of the mutants were lethal when heterozygous with all other _woc_ mutants. The _wocD2wX1_, which contains a four amino acid deletion in the sixth zinc finger, is a hypomorphic allele. The _wocD2wX1_ survives poorly in combination with the other hypomorphic _woc_ alleles, _wocD0_ and _wocD6_. Transheterozygous _wocD2wX1/wocD0_ and _wocD2wX1/wocD6_ flies have multiple phenotypic defects, including downturned wings, lack of wing veins, slightly rough eyes, and they are sterile.

**_wocD_ suppresses the eye color in a position-specific manner**

_P[181PRE]-8-10C_ is inserted in the genome between the genes _GstS1_ and _CG30456_ (Figure 1). We wanted to know whether _wocD_ modulates the PRE directly or whether it acts through regulatory DNA flanking the insertion site of _P[181PRE]-8-10C_. Importantly, _wocD_ does not darken the eye color of _w_ in the _w_ gene that reduces the amount of _w_ transcript and leads to orange eyes (Pirrotta and Bröckl 1984; Levis et al. 1984). This shows that _wocD_ does not darken eye color indiscriminately. We examined whether the eye colors of flies heterozygous for other mini-white containing transgenes inserted near _GstS1_ were altered by _wocD_. We used a line with a _P[EP]_ element inserted about 1.2 kb away from the insertion site of _P[181PRE]-8-10C_ and six lines with a _P[lacW]_ inserted in the promoter region of _GstS1_ (Figure 1). The eye colors of _P[lacW]_ or _P[EP]/+; w/+/+; +/TM6C_ were compared with the eye colors of _P[lacW]_ or _P[EP]/+; w0D+/+;_ flies; no eye color differences were observed. This suggests that the effect of _wocD_ on the eye color of _P[181PRE]-8-10C_ flies is dependent on the presence of the PRE in the transgene.

We next examined whether _wocD_ could alter the eye color of flies with _P[181PRE]_ inserted at different chromosomal locations. We used the transgene _P[L181PRE]_, which contains the same 181-PRE as _P[181PRE]-8-10C_. In _P[L181PRE]_, the 181-bp PRE is flanked by _loxP_ sites (see below). Because _wocD_ dominantly alters the eye color of _P[181PRE]-8-10C_ heterozygotes, we looked at whether _wocD_ could dominantly alter the eye color of flies heterozygous for _P[L181PRE]_ insertions that show mini-white repression. For 14 out of 15 _P[L181PRE]_ lines tested, _wocD_ does not alter the eye color. However, in _P[L181PRE]-8A_, the eye color was slightly darker in a _wocD_ mutant (data not shown). _P[L181PRE]-8A_ is inserted just upstream of the PcG-regulated gene _OcyA_ (at 3L:11826614). To determine whether the effect on the eye color of _P[L181PRE]-8A_ flies was due to the PRE, we examined the eye color of _P[L]-8A_ flies in which the 181-bp PRE had been removed. We found that _wocD_ had no effect on the eye color of flies that lacked the PRE. This shows that, at least at this chromosomal location, the change in eye color mediated by _wocD_ is dependent on the PRE. However, as _wocD_ does not influence the eye color of most _P[L181PRE]_ lines, we believe that _wocD_ is not working on the PRE directly but on sequences flanking the _P[181PRE]_ insertion sites.

**_wocD_ increases the levels of _GstS1_ RNA in adult heads**

We examined whether the levels of _GstS1_ and _CG30456_ transcripts were altered in _wocD_ mutants, both in the presence and in the absence of the _P[181PRE]-8-10C_ insertion. We examined RNA levels at three developmental stages: 3rd instar larvae, 1-day-old pupae, and adult heads. We saw no significant differences in the expression levels of _GstS1_ and _CG30456_ between _wocD_ and wild-type 3rd instar larvae.
or one-day old pupae (data not shown). However, we saw a twofold increase in the level of GstS1-RNA in the adult heads of homozygous wocD1 mutants compared to wild-type (Figure 4). Flies with the P[181PRE]8-10C insert had about a twofold decrease in the expression levels of CG30456 at all developmental stages, suggesting that the insertion interferes with the transcription of CG30456 (Figure 4 and data not shown). However, wocD2 had no significant effect on the transcription level of CG30456 at any developmental stage either in wild-type or in P[181PRE]8-10C animals (Figure 4 and data not shown). We also examined GstS1 and CG30456 transcript levels in eye-antennal disks from third instar larvae of wild-type and wocD2 mutants with P[181PRE]8-10C and saw no significant differences (data not shown). Finally, we tested whether wocD altered the transcription level of CycA in adult heads. We saw no significant differences in CycA levels between wild-type and wocD2; P[181PRE] heads (data not shown).

Other dominant suppressors do not affect the expression level of GstS1 or CG30456

We tested whether four of our other dominant suppressors of pairing-sensitive silencing of P[181PRE]8-10C effect the expression levels of GstS1 or CG30456 in fly heads by qRT-PCR; we saw no effect on transcript levels of either gene (data not shown). Thus, a change in transcription level of a GstS1 is not required for suppression of the pairing-sensitive silencing of P[181PRE]8-10C. Finally, we examined the effects of three other suppressor mutants on the eye color of P[181PRE] at multiple insertion sites. Like wocD, none of the other suppressor mutations altered the eye color of P[181PRE] at the insertion sites.

A single unpaired copy of the PRE reduces the eye color of mini-white transformants

We flankied the 181-bp PRE by loxP sites and cloned it upstream of the mini-white reporter in pCaSpeR (P[L181PRE]), in the same orientation and position as in the construct P[181PRE] (Figure 5). We recovered 32 lines with insertions of P[L181PRE]. Of the 25 insertion lines that were homozygous viable, 15 exhibited pairing-sensitive silencing (60%). All lines were treated with Cre recombinase to excise the 181-bp PRE, yielding P[I]. None of the lines without the PRE showed pairing-sensitive silencing. In 7 of the 15 pairing-sensitive lines, the eye color of heterozygous flies became darker upon removal of the PRE, showing that some repression of mini-white expression occurred even in the heterozygotes (Figure 5). In contrast, in the lines that did not show pairing-sensitive silencing, the eye colors of flies heterozygous or homozygous for P[L181PRE] did not change after removal of the PRE, with one exception. In line P[L181PRE]11A, the eye color was slightly lighter after removal of the PRE, suggesting that this element was acting as a slight activator of mini-white expression at this location.

This result is consistent with earlier evidence that showed that the 181-bp fragment, in the context of a reporter gene driven by the en promoter, can act as either an activator or repressor of gene expression depending on the context (Devido et al. 2008). This activation activity was weak and only occurred in 1 line out of 32. This shows that, in the mini-white assay, PRE-mediated repression is the usual situation.
wocD1 and wocD2 are gain-of-function mutations

Heterozygosity for either wocD1 or wocD2 darkens the eye color of P[181PRE]8-10C flies, whereas heterozygosity of woc deletions does not. This shows that wocD1 and wocD2 have acquired new activities and are gain-of-function alleles. Our results also show that wild-type Woc protein competes with WocD protein. This is suggested by the observation that P[181PRE]8-10C/P[181PRE]8-10C; wocD/woc− flies have a darker eye color than P[181PRE]8-10C/P[181PRE]8-10C; woc+y flies. Finally, the observation that the activity of WocD is abrogated by mutations that inactivate the Woc protein shows that the wocD mutation alters the activity of the protein.

A model for WocD

The woc gene encodes a zinc-finger transcription factor implicated in transcriptional activation (Wismar et al. 2000; Raffa et al. 2005) that acts, at least in part, through an association with HP1c (Font-Burgada et al. 2008; Able et al. 2009). There are five HP1 isoforms in Drosophila. Of these, HP1a is the best studied and is associated with heterochromatic DNA. In contrast, HP1c is excluded from centromeric heterochromatin and is associated with euchromatin.

As stated above wocD suppresses mini-white expression from P[181PRE] in a position-dependent manner. Therefore, we wanted to know whether Woc binds to the genomic regions near the P[181PRE] insertion sites it regulates. We were not able to obtain Woc antisera, and there is no published data showing where Woc binds in the genome. However, the Drosophila ModENCODE project has mapped the binding sites of HP1c in four different cell culture lines by chromatin immunoprecipitation followed by hybridization to tiling arrays (Kharchendo et al. 2010). Because Woc is often associated with HP1c, we examined whether there was a correlation between HP1c binding and the suppressor activity of wocD. HP1c is bound in a cell-type–specific manner. There is no HP1c associated with the region between GstS1 and CG30456 in S2, Kc167, or BG3 cells; however, HP1c is bound to this region in clone 8 cells. HP1c is not associated with CycA in any cell type. There is no data on HP1c localization in the pigment cells in the eye, so we cannot make any conclusion about whether WocD acts via HP1c in suppressing the PRE activity of P[181PRE]8-10C.

How does wocD affect the eye color of P[181PRE]8-10C? We suggest that the eye colors of P[181PRE]8-10C flies result from a competition of transcriptional repression (caused by the PRE) and transcriptional activation of GstS1 (Figure 6). In the wild-type case, we suggest there is a competition between transcriptional activation of mini-white by flanking regulatory DNA and transcriptional repression mediated by the PRE, leading to an intermediate eye color. One prediction of this model is that if Woc levels are decreased, the PRE upstream of mini-white should be able to work more strongly, and the eye color should be lighter. Consistent with this hypothesis, the eye color of P[181PRE]8-10C; wocD/wocD flies is white (Figure 6). We suggest that the PRE in line P[181PRE]8-10C modulates the levels of mini-white expression in part through a competition with flanking activators.

What determines PRE activity?

PREs have been studied for many years as silencers of homeotic gene expression in Drosophila. Recent genome-wide studies showed that PREs may play an important role in regulating gene expression levels as well. What determines whether a PRE will completely silence a gene or only decrease its expression levels? Our data suggests two things. First, the number of PREs is important. This is evident from the fact that flies homozygous for PRE-mini-white constructs, which have two
Table 1 $P[181PRE]$ insertions

| Line Name | Location | Gene | Distance | Transcript Level in Eye |
|-----------|----------|------|----------|------------------------|
| 2A        | 3R:1982721 | Foxo | +31bp    | 48                     |
| 2B        | X:9581402  | Nej  | +303bp   | NID                    |
| 3A        | X:18398636 | Wnt5 | +800bp   | 18                     |
| 1B        | 3R:9487247 | BS2  | +255bp   | NID                    |
| 1A        | X:19498690 | CG14207 | -501bp | 300                    |
| 12A       | 2L:11106592 | Reps | +60bp    | 27                     |
| 14A       | 3R:21867498 | Gro  | -66bp    | 197                    |
| 24A       | 2R:2051740 | Lbk  | +150bp   | 138                    |
| 15A       | 3L:19922217 | RhoD | +310bp   | 4481                   |
| 23A       | 2L:10414068 | Klp31E | -34bp | 98                     |
| 21B       | 3L:11826614 | CycA | -81bp    | NID                    |

Lines that show pairing-sensitive silencing

| Line Name | Location | Gene | Distance | Transcript Level in Eye |
|-----------|----------|------|----------|------------------------|
| C1        | 2R:3623151 | CG18812 | -27bp | 728                    |
| 2         | 3L:12074850 | Sema-Sc | +77bp | 47                     |
| C1-12A    | 3L:13932279 | CG32137 | -73bp | 146                    |
| 13A       | X:14983581 | rab3-GEF | -134bp | 173                    |
| 16A       | 2L:2753118 | CG9894 | +319bp | 2886                   |
| 17A       | 2R:13435831 | MESR4 | +502bp | 41                     |
| 28A       | 2R:15556892 | Hrg  | -325bp  | 383                    |
| 10        | 2L:3477289 | Thor | -1145bp | 1016                   |
| 21B       | 2R:8475807 | Sin3A | +793bp | NID                    |

Lines that do not show pairing-sensitive silencing

PREs, repress mini-white to a much higher level than flies heterozygous. We also note that increasing the number of PREs in cis, by duplicating a P-construct with PREs, also causes an increase in mini-white repression (Kassis 1994). Second, changes in the chromatin environment, here caused by a gain-of-function mutation in the transcriptional activator Woc, can inactivate a PRE. The dependence of PRE activity in transgenes on chromosomal environment has long been recognized and is dramatically demonstrated in a recent report showing the effect of chromosome environment on the activities of the Abd-B Fab-7 PRE and the $v_g$ PRE (Okulski et al. 2011).

The relationship between transcriptional activation and PRE function is not simple. Addition of an enhancer containing three binding sites for the eye enhancer-activator protein Glass (GBS) to $pCaSpeR$ darkens the eye color of transformants that contain an en PRE (Americo et al. 2002). If the increased transcription driven by the GBS enhancer interferes with PRE activity, one would expect to see a decrease in the number of lines with pairing-sensitive silencing in this vector. However, this did not occur. Thus, increasing the transcription of mini-white itself does not alter PRE activity. In addition, we found that insertion of $P[181PRE]$ into or next to genes expressed ability to obtain enough woc<sup>HyP</sup> adults to perform qRT-PCR, so we do not know the level of CG30456 and GstS1 RNA in these flies. This uncertainty is indicated by the question mark next to the transcription arrows in (C).
in the eye did not prevent pairing-sensitive silencing from occurring (Table 1). Therefore, we propose that it is not transcriptional activation but the actual activators present that determine whether a PRE is active or not. It has previously been suggested that PREs are general silencer elements that could act on any enhancer (Sengupta et al. 2004). The basis for this conclusion was that the Ubx PRE could act as silencers of three enhancers in reporter genes [two vestigial (vg) and one decapentaplegic (dpp) enhancer]. At that time, neither vg nor dpp was thought to be regulated by PcG proteins. However, since then, a vg PRE and a dpp PRE have been identified (Lee et al. 2005; Hauenschild et al. 2008; Okulska et al. 2011). We suggest that PREs may not be able to silence all enhancers, and in some chromosomal locations, they cannot act. It was recently reported that a human tissue-specific enhancer functions in erythroid cells by evicting PcG proteins (Vernimmen et al. 2011). Enhancers with this activity may also be present in Drosophila.

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