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A Slicer-Mediated Mechanism for Repeat-Associated siRNA 5′ End Formation in *Drosophila*

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In *Drosophila*, repeat-associated small interfering RNAs (rasiRNAs) are produced in the germ line by a Dicer-independent pathway and function through the PIWI subfamily of Argonautes to ensure silencing of retrotransposons. We sequenced small RNAs associated with the PIWI subfamily member AGO3. Although other members of PIWI, Aubergine (Aub) and Piwi, associated with rasiRNAs derived mainly from the antisense strand of retrotransposons, AGO3-associated rasiRNAs arose mainly from the sense strand. Aub- and Piwi-associated rasiRNAs showed a strong preference for uracil at their 5′ ends, and AGO3-associated rasiRNAs showed a strong preference for adenine at nucleotide 10. Comparisons between AGO3- and Aub-associated rasiRNAs revealed pairs of rasiRNAs showing complementarities in their first 10 nucleotides. Aub and AGO3 exhibited Slicer activity in vitro. These data support a model in which formation of a 5′ terminus within rasiRNA precursors is guided by rasiRNAs originating from transcripts of the other strand in concert with the Slicer activity of PIWI.

Small noncoding RNAs trigger various forms of sequence specific gene silencing, including RNA interference (RNAi), translational repression, and heterochromatin formation in a variety of eukaryotic organisms, commonly referred to as RNA silencing (*1*–*3*). Members of the Argonaute family of proteins are essential components of RNA silencing (*4*, *5*). In *Drosophila*, five genes encode distinct members of the Argonaute family: *AGO1*, *AGO2*, *Aubergine* (*Aub*), *Piwi*, and *AGO3*. *AGO1* and *AGO2* constitute the Argonaute (AGO) subfamily and bind microRNA (miRNA) and small interfering RNA (siRNA), respectively (*6*–*8*). Aub, Piwi, and AGO3 belong to the PIWI subfamily of the Argonaute family (*4*, *5*) and are enriched in germline cells (*9*), and Aub and Piwi have been shown to play important roles in germline cell formation (*10*, *11*). They are involved in silencing retrotransposons and other repetitive elements (*12*–*15*) and exhibit target RNA cleavage (slicing) activity in vitro (*16*). Both Aub and Piwi associate with repeat-associated siRNAs (rasiRNAs) (*15*, *16*). Aub- and Piwi-associated rasiRNAs are derived mainly from the antisense strand of retrotransposons, with little or no phasing, and have a strong preference for uracil (U) at the 5′ end (*15*, *16*). Small RNA processing factors such as Dicer and Drosha are known to cleave preferentially at the 5′ side of U (*17*); however, rasiRNAs are thought to be produced by a Dicer-independent pathway (*15*). The mechanisms governing rasiRNA production remain to be elucidated.

Very little is known about the function of AGO3 (*9*), the third member of the *Drosophila* PIWI subfamily. We isolated a full-length cDNA of AGO3, revealing that the *AGO3* gene is ~83 kb in length (fig. S1). Peptide sequence alignments among *Drosophila* Argonaute proteins revealed that AGO3 is most similar to Piwi (fig. S2A). The Asp-Asp-His motif in the PIWI domain, originally identified as the catalytic center for Slicer activity in human AGO2 (*5*, *18*), is conserved in AGO3 (fig. S2B).

Embryonic RNA expression patterns of *AGO3* are very similar to those of *Piwi* and *Aub*; they are expressed maternally, but their expression disappears by embryonic stages 10 to 12 (*9*). To confirm these results, we produced a monoclonal antibody (mAb) to AGO3 (Fig. 1A), which revealed that AGO3 is strongly expressed in earlier embryonic stages but decreases as development proceeds (Fig. 1B). AGO3 accumulated in the cytoplasm of germline cells including germline stem cells (GSCs), germline cyst cells, nurse cells, and oocytes at earlier stages (Fig. 1C and fig. S3). In testes, AGO3 is expressed in GSC, primary gonial cells, and early spermatocytes (Fig. 1D). Unlike Piwi (*16*), AGO3 expression was undetected in the hub (fig. S3), a tiny cluster of postmitotic somatic cells localized at the apical tip of the testis that functions as a niche for GSC (*19*). Thus, with respect to expression in germline cells, AGO3 is more similar to Aub than to Piwi (*10*, *16*).

All of the other members of the fly Argonautes are specifically associated with a subset of small RNAs: siRNAs, miRNAs, or rasiRNAs (*6*–*8*, *15*, *16*). We therefore investigated whether AGO3 also associates with small RNAs produced in the fly ovary. Immunoprecipitation with AGO3 mAb from ovary lysate revealed small RNAs ~23 to 26 nucleotides (nt) long (Fig. 2A). The size distribution of AGO3-associated small RNAs is similar to that of Aub-associated small RNAs (Fig. 2B); in both cases, the peak is 24 nt and the longest is 27 nt. Small RNAs associated with AGO3 are likely to lack either a 2′ or 3′ hydroxyl group, because they do not migrate faster after β-elimination as opposed to a synthetic siRNA that has 2′ and 3′ hydroxyl groups at the 3′ end, the latter being the hallmarks of Dicer cleavage (Fig. 2C). These results suggest that AGO3-associated small RNAs in the ovary are produced by a pathway similar to those involved in production of rasiRNAs that associate with Aub and Piwi.

We constructed a cDNA library of small RNAs associated with AGO3 in the ovary. Of 420 clones sequenced, 410 matched *Drosophila* genomic sequences in a database search (table S1), and most were rasiRNAs (~86%; 353 of 410), as in the case of Aub and Piwi (table S2). Like rasiRNAs associated with Aub or Piwi (*15*, *16*), rasiRNAs associated with AGO3 included various kinds of transposable elements, both LTR (long terminal repeat) retrotransposons and LINE (long interspersed nuclear element)–like elements (tables S1 and S2). rasiRNAs associated with Aub or Piwi in ovaries are derived mainly from the antisense strand of retrotransposons, and the 5′ end is predominantly U (*15*, *16*). These characteristics were not found for rasiRNAs associated with AGO3. However, AGO3-associated rasiRNAs were derived mainly from the sense strand of retrotransposons (~82%; table S3), and they showed a strong preference for adenine (A) at nucleotide 10, but no preference for U at the 5′ end (Fig. 3A). These results suggest that AGO3-associated rasiRNAs belong to a subset of rasiRNAs that are distinct from Aub- and Piwi-associated rasiRNAs.

Some Argonaute proteins exhibit Slicer activity that directs cleavage of its cognate mRNA target across from nucleotides 10 and 11, measured from the 5′ end of the small RNA guide strand (*20*). Thus, our findings suggest a model for rasiRNA biogenesis, in which the 5′ end of Aub- and Piwi-associated rasiRNAs is determined and cleaved by AGO3-rasiRNA complexes, and the 5′ end of AGO3-associated rasiRNAs is determined by Aub- and Piwi-rasiRNA complexes through a similar rasiRNA-guided cleavage event (Fig. 3B). For instance, AGO3 associated with a rasiRNA with A at nucleotide 10 can target a long RNA molecule by Watson-Crick base pairing and cleave the target RNA, resulting in sliced RNAs with U at the 5′ end. Similarly, when Aub or Piwi associated with

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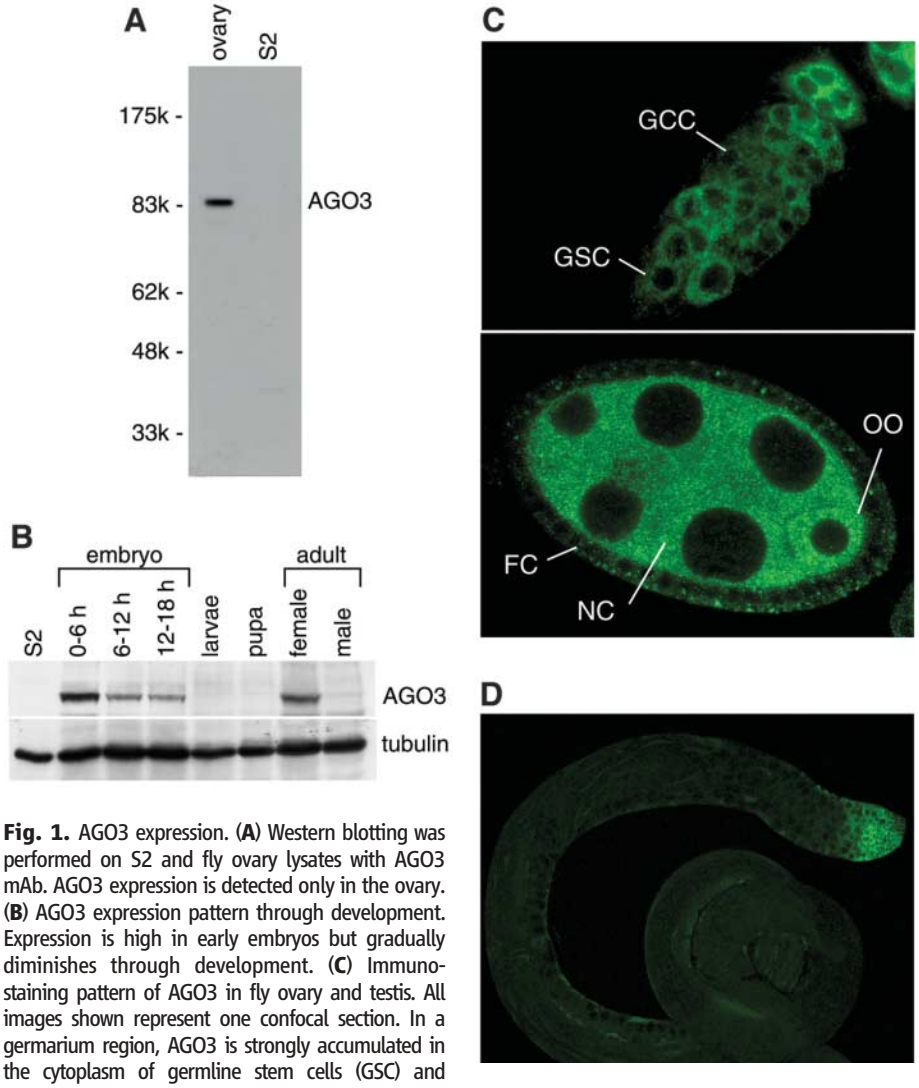

Fig. 1. AGO3 expression. (**A**) Western blotting was performed on S2 and fly ovary lysates with AGO3 mAb. AGO3 expression is detected only in the ovary. (**B**) AGO3 expression pattern through development. Expression is high in early embryos but gradually diminishes through development. (**C**) Immunostaining pattern of AGO3 in fly ovary and testis. All images shown represent one confocal section. In a germarium region, AGO3 is strongly accumulated in the cytoplasm of germline stem cells (GSC) and germline cyst cells (GCC) (upper panel). In an egg chamber at stage 7 (lower panel), AGO3 was found in both oocytes (OO) and nurse cells (NC). A weak signal of AGO3 was also observed in follicle cells (FC). AGO3 is clearly cytoplasmic. (**D**) Fluorescent image of AGO3 in testis. AGO3 expression is detected in germline stem cells, primary gonial cells, and early spermatocytes.

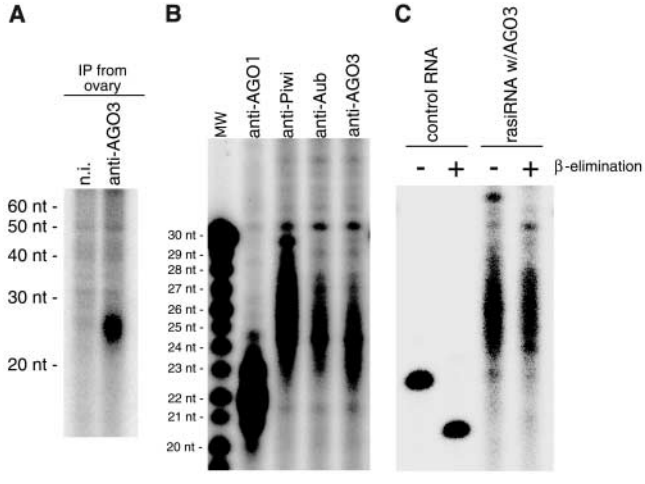

Fig. 2. Small RNAs associated with AGO3 in fly ovary. (**A**) Small RNAs associated with AGO3 immunopurified from ovary are visualized by [^{32}P]adenosine triphosphate labeling. (**B**) Sizes of small RNAs associated with AGO1, Piwi, Aub, and AGO3 are compared. The peak of small RNAs associated with Aub and AGO3 is about 24 nt, whereas that of Piwi is about 26 nt; rasiRNAs with AGO3 contain shorter ones (23 nt) relative to those of Aub. The ~30-nt bands are 2*S* rRNA. (**C**) rasiRNAs associated with AGO3 show resistance to β-elimination treatment, which made 21-nt siRNA (control RNA) without 3′ end modification shorter, indicating that rasiRNAs associated with AGO3 are modified at the 3′ end.

rasiRNAs with U at the 5′ end slices its cognate RNA target, the resulting cleaved RNA will have A at nucleotide 10.

To test this model, we examined AGO3 for Slicer activity by performing in vitro target RNA cleavage assays with glutathione *S*-transferase (GST)–AGO3 fusions (Fig. 4A). The target RNA, *luc* passenger siRNA (21 nt long, 5′ end labeled with ^{32}P) (*8*), was efficiently cleaved by GST-AGO3, as was the case for GST-AGO1 and GST-Aub. The size of the cleaved products (9 nt) indicated that they direct cleavage of target RNA across from nucleotides 10 and 11 as measured from the 5′ end of the small RNA guide strand (Fig. 4A). Both GST-Aub and GST-AGO3 with a longer guide RNA (26 nt) were also able to cleave a long transcript (180 nt) (fig. S4). Long precursors of rasiRNAs both in sense and antisense orientations appear to exist in fly ovaries (fig. S5). These results corroborate the model in which the 5′ end of rasiRNAs within the precursors is determined by rasiRNAs and cleaved by members of PIWI that associate with these rasiRNAs.

Our model predicts that some AGO3-associated rasiRNAs should be complementary to the first 10 nt of Aub- and Piwi-associated rasiRNAs. Sequence comparison between AGO3- and Aub-associated rasiRNAs indeed revealed pairs of rasiRNAs that show complementarities at their first 10 nt (fig. S6). Sixteen of 353 AGO3-associated rasiRNAs had such pairs with 11 of 676 Aub-associated rasiRNAs (fig. S6). However, such pairings were only found between AGO3- and Aub-associated rasiRNAs, and no pairs were observed between AGO3- and Piwi-associated rasiRNAs (353 versus 330). Like Aub-associated rasiRNAs (table S3), Piwi-associated rasiRNAs arise mainly from the antisense strand and their 5′ ends show a strong preference for U (*16*); thus, it is difficult to argue that Piwi is not involved in this type of rasiRNA biogenesis. One possible reason is that Piwi is nuclear, whereas AGO3 and Aub are cytoplasmic (*16*). This type of rasiRNA biogenesis may operate in the cytoplasm. Alternatively, formation of 5′ ends of Piwi-associated rasiRNAs may occur only at an earlier time during germline development.

RasiRNAs are involved in genome surveillance by silencing repetitive elements and controlling their mobilization in the *Drosophila* germ line. It was recently shown that rasiRNAs are produced by a mechanism that requires neither Dicer-1 nor Dicer-2 in flies (*15*). Our data suggest that rasiRNAs in a sense orientation guide formation of the 5′ end of rasiRNAs in an antisense orientation, and vice versa; as well, this cycle of mutual dependency elaborates optimal rasiRNA production (Fig. 4B). In this model, proteins of the PIWI subfamily function as Slicer for formation of the 5′ end during rasiRNA

biogenesis. This model requires that sliced rasiRNA precursors then be cleaved again at the 3′ end by an as yet unidentified endonuclease (or nibbled by exonuclease) to produce mature rasiRNAs before or after loading of the resulting cleavage products onto another member of the PIWI. Once "primary" complexes of rasiRNAs with proteins of PIWI are produced, these complexes will in turn function

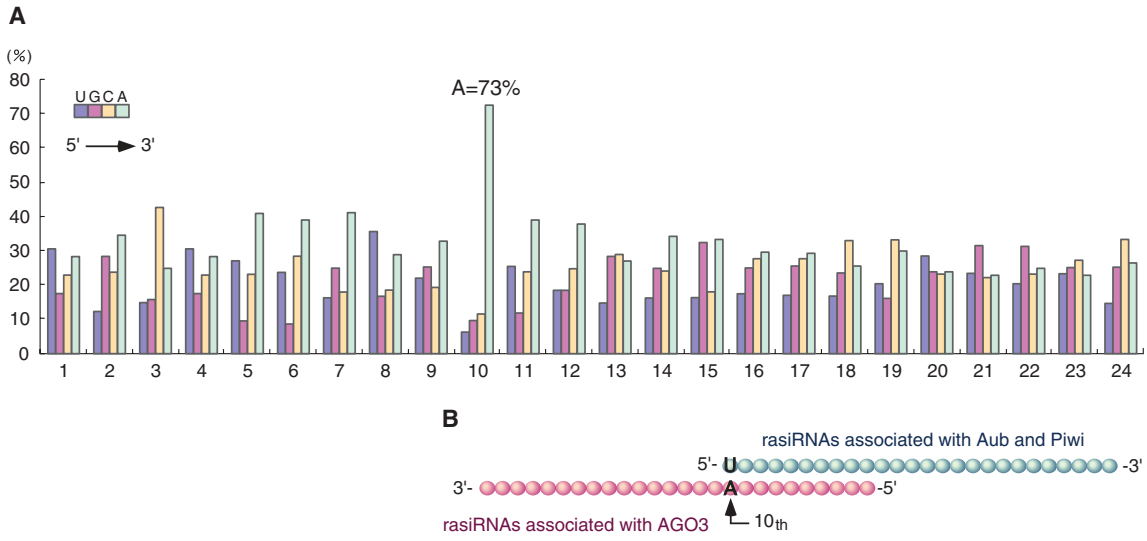

Fig. 3. Characteristics of rasiRNAs associated with AGO3 in the ovary. (**A**) rasiRNAs associated with AGO3 contain A predominantly at nucleotide 10 from the 5′ end. (**B**) A predicted model indicating that the first 10 nt of AGO3-associated rasiRNAs show complementarities to the first 10 nt of Aub- or Piwi-associated rasiRNAs.

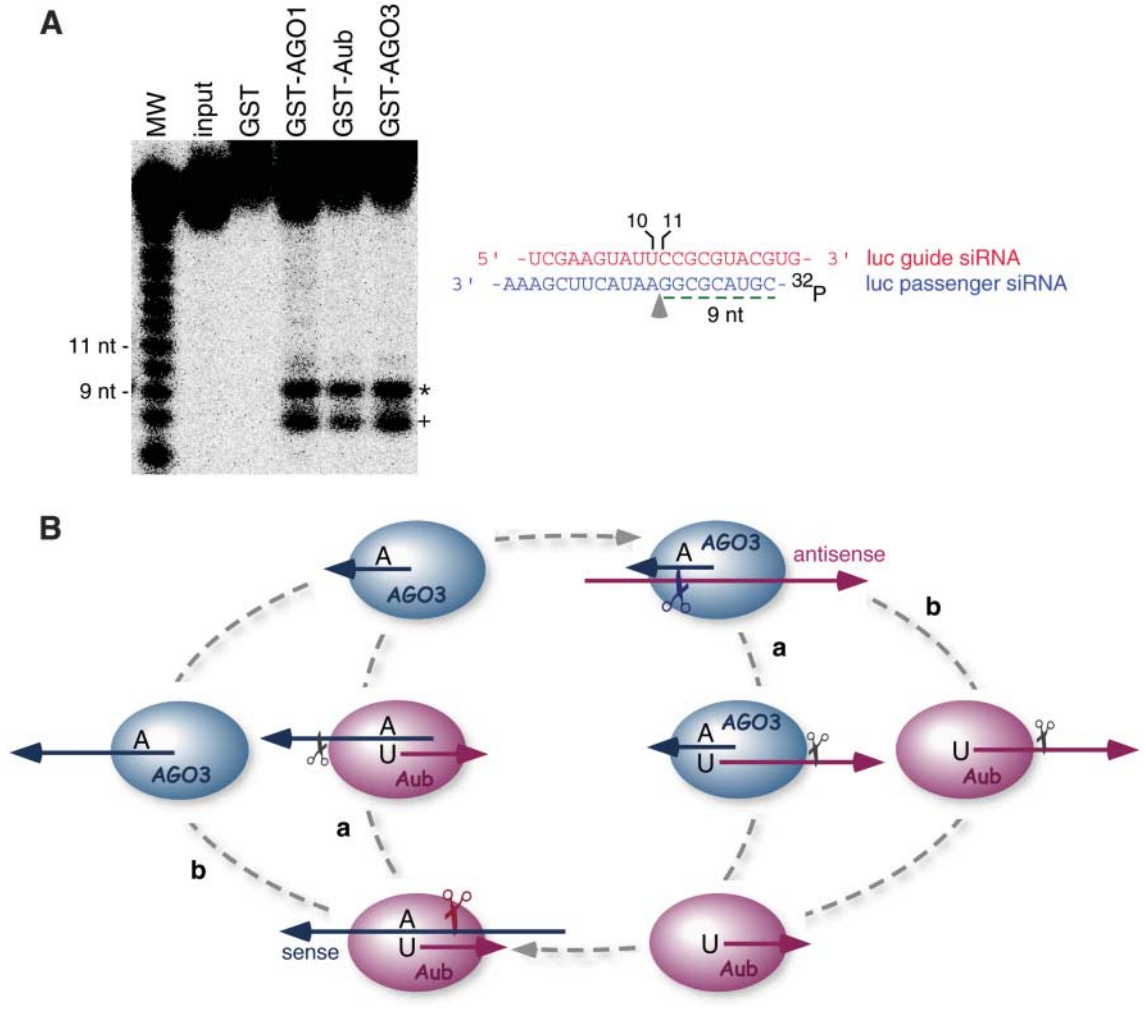

Fig. 4. (**A**) Target RNA cleavage assay using GST-AGO1, GST-AGO3, and GST-Aub produced in *Escherichia coli*. GST-fused proteins were first bound with a single-stranded *luc* guide siRNA; then, target RNA (single-stranded *luc* passenger siRNA labeled with ^{32}P at the 5′ end) was added to the reaction mixture and further incubated. All the proteins, but not GST itself, were able to cleave target RNA across from nucleotides 10 and 11, measured from the 5′ end of the guide RNA. The asterisk indicates cleaved products with the expected size (9 nt); the cross marks by-products of 8 nt in in vitro assays with GST fusions (*8*). (**B**) A model for a rasiRNA biogenesis cycle. AGO3-associated sense rasiRNAs (upper left) guide Slicer-mediated cleavage of primary antisense transcripts, yielding the antisense rasiRNA precursors with U at the 5′ end. Reciprocally, Aub-associated antisense rasiRNAs (lower right) guide Slicer-mediated cleavage of sense transcripts, yielding the sense rasiRNA precursors with A at nucleotide 10. Formation of rasiRNAs at the 3′ end may occur (a) while the precursors still remain in the complexes, or (b) after they are loaded onto the other PIWI by an as-yet-unidentified nuclease.

as the "initiator" of secondary rasiRNA biogenesis, and so nascent rasiRNAs should be continuously supplied in the ovary and testis. Such a process may occur through rasiRNA germline transmission. Of the PIWI members, at least Aub is accumulated to the posterior pole in oocytes and remains in polar granules in early embryos. It is then incorporated in pole cells, the progenitor of the *Drosophila* germ line (*10*).

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Supporting Online Material

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Materials and Methods
Figs. S1 to S6
Tables S1 to S3
References

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Attention-Like Processes in *Drosophila* Require Short-Term Memory Genes

Bruno van Swinderen

Although there is much behavioral evidence for complex brain functions in insects, it is not known whether insects have selective attention. In humans, selective attention is a dynamic process restricting perception to a succession of salient stimuli, while less relevant competing stimuli are suppressed. Local field potential recordings in the brains of flies responding to visual novelty revealed attention-like processes with stereotypical temporal properties. These processes were modulated by genes involved in short-term memory formation, namely *dunce* and *rutabaga*. Attention defects in these mutants were associated with distinct optomotor effects in behavioral assays.

Studies of visual discrimination in flies have revealed sophisticated perceptual effects that are relevant to selective attention (*1*, *2*), such as associative learning (*3*), context generalization (*4*, *5*), cross-modal binding (*6*), and position invariance (*7*). Visual choice behavior in *Drosophila* is correlated with local field potential (LFP) activity in the brain, centered around 20 to 30 Hz (*8*). This activity is transiently increased in amplitude by classical conditioning (*8*), is suppressed during sleep (*8*) or light anesthesia (*9*), and is modulated by dopamine (*10*). Electrophysiological and behavioral measures of visual attention in flies were developed to test whether these short-term processes depend on the effect of genes involved in memory formation and plasticity (*11*–*13*).

LFP responses to two distinct visual objects (a cross or a box, 180° apart, each moving around the fly once every 3 s) were investigated (Fig. 1A). When the objects were presented individually to wild-type flies, they evoked brain responses that were maximal when the single object swept directly in front of the flies (Fig. 1B). In contrast, *dunce* mutants (dnc^1) (*14*), which are defective in short-term memory (*15*, *16*), displayed attenuated and delayed brain responses to each visual object, as compared to wild-type flies (Fig. 1C).

To test for visual selection between these objects, I presented them together after having increased the salience for one object specifically in a recurrent-novelty paradigm (Fig. 1D) (*17*). To measure visual selection, the 20- to 30-Hz brain response (mapped onto the 3-s sequence) (Fig. 1B) was averaged for 10 s (about three rotations) after each transition to novelty, and this was compared to the response for the 10 s before novelty transitions (red versus blue lines, respectively, in Fig. 1D). When wild-type flies were trained with two identical boxes for 100 s (~33 rotations) before one of the boxes changed to a cross, the response mapped selectively to the sectors of the rotation sequence associated with the (novel) cross (100 s, red line in Fig. 1D), and the response for the competing box was significantly suppressed. Converse experiments attaching novelty salience to the alternate image (the box) after 100 s of cross training mapped 20- to 30-Hz responses to the novel box, showing that novelty selection was plastic (fig. S1). Novelty selection was also found to be position-invariant (*7*) in a subset of trials, suggesting a cognitive effect rather than habituation (fig. S2).

By decreasing the time between transitions in otherwise identical experiments, this paradigm provided a way to estimate the minimum exposure required for selection of recurrent novelty. When the training time was decreased to 50 s (~16 rotations), significant selection of the novel object and corresponding suppression of the competing object were still seen in wild-type flies (fig. S3). However, when the training time was further decreased to 25 s (about eight rotations), these novelty effects were lost (Fig. 1D).

To control for the effect of change alone without novelty, I tested transitions from a cross and a box back to two boxes (Fig. 1E). In this case, an object changed to one that was already present during training. Such changes did not produce any selective 20- to 30-Hz responses for any training time in wild-type flies (100 s and 25 s in Fig. 1E). The response is therefore unlikely to emanate from a startle reflex or an electrical artifact.

Salience is a transient phenomenon. To investigate the extinction of novelty, I analyzed the temporal sequence of selective brain responses for successive rotations of a novel panorama after a transition (Fig. 2A). In wild-type flies, 20- to 30-Hz activity was strongly selective for the novel object (the cross) for 9 s (three successive panorama rotations) on average (red lines in Fig. 2A), and this was matched at a lower level for

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