Title: Repeated duplication of Argonaute2 is associated with strong selection and testis specialization in *Drosophila*

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Abstract

Argonaute2 (Ago2) is a rapidly evolving nuclease in the *Drosophila melanogaster* RNAi pathway that targets viruses and transposable elements in somatic tissues. Here we reconstruct the history of Ago2 duplications across the *Drosophila obscura* group, and use patterns of gene expression to infer new functional specialization. We show that some duplications are old, shared by the entire species group, and that losses may be common, including previously undetected losses in the lineage leading to *D. pseudoobscura*. We find that while the original (syntenic) gene copy has generally retained the ancestral ubiquitous expression pattern, most of the novel Ago2 paralogues have independently specialised to testis-specific expression. Using population genetic analyses, we show that most testis-specific paralogues have significantly lower genetic diversity than the genome-wide average. This suggests recent positive selection in three different species, and model-based analyses provide strong evidence of recent hard selective sweeps in or near four of the six *D. pseudoobscura* Ago2 paralogues. We speculate that the repeated evolution of testis-specificity in *obscura* group Ago2 genes, combined with their dynamic turnover and strong signatures of adaptive evolution, may be associated with highly derived roles in the suppression of transposable elements or meiotic drive. Our study highlights the lability of RNAi pathways, even within well-studied groups such as *Drosophila*, and suggests that strong selection may act quickly after duplication in RNAi pathways, potentially giving rise to new and unknown RNAi functions in non-model species.

36 Introduction

37 Argonaute genes are found in almost all eukaryotes, where they play a key role in antiviral immune
38 defence, gene regulation and genome stability. They carry out this diverse range of functions through
39 their role in RNA interference (RNAi) mechanisms, an ancient system of nucleic acid manipulation in
40 which small RNA (sRNA) molecules guide Argonaute proteins to nucleic acid targets through base
41 complementarity (reviewed in [1]). Gene duplication has occurred throughout the evolution of the
42 Argonaute gene family, with ancient duplication events characteristic of some lineages – such as
43 three duplications early in plant evolution [2], and multiple expansions and losses throughout the
44 evolution of nematodes (reviewed in [3]) and the Diptera [4]. After duplication, Argonautes have often
45 undergone functional divergence, involving changes in expression patterns and altered small RNA
46 (sRNA) binding partners [5–7]. Duplication early in eukaryotic evolution produced two distinct
47 Argonaute subfamilies, Ago and Piwi, which have since been retained in the vast majority of Metazoa
48 [8]. Members of the Ago subfamily are expressed in both somatic and germline tissue, and variously
49 bind sRNAs derived from host transcripts (miRNAs, endo-siRNAs) or transposable elements (TE
50 endo-siRNAs) and viruses (viRNAs). In contrast, in most vertebrates and arthropods, the Piwi
51 subfamily members are expressed only in association with the germline (reviewed in [9]), and bind
52 sRNAs from TEs and host loci (piRNAs), suggesting that the Piwi subfamily specialised to a germline-
53 specific role on the lineages leading to vertebrates and arthropods.

54 After the early divergence of the Ago and Piwi subfamilies, subsequent duplications gave rise to three
55 Piwi subfamily members (Ago3, Aubergine (Aub) and Piwi) and two Ago subfamily members (Ago1 &
56 Ago2) in *Drosophila melanogaster*. All three Piwi subfamily genes are associated with the germline
57 and bind Piwi-interacting RNAs (piRNAs) derived from TEs and other repetitive genomic elements:
58 Ago3 and Aub amplify the piRNA signal through the "Ping-Pong" cycle (reviewed in [10]), and Piwi
59 suppresses transposition by directing heterochromatin formation [11]. These functional differences
60 are associated with contrasting selective regimes, with Aub evolving under positive selection [12] and
61 more rapidly than Ago3 and Piwi [13]. In contrast, Ago1 binds microRNAs (miRNAs), and regulates
62 gene expression by inhibiting translation and marking transcripts for degradation (reviewed in [14]).
63 This function imposes strong selective constraint on Ago1, resulting in slow evolution and very few
64 adaptive substitutions [12,13,15]. Finally, Ago2 binds small interfering RNAs (siRNAs) from viruses
65 (viRNAs) and TEs (endo-siRNAs), and functions in gene regulation [16], dosage compensation [17],

66 and the ubiquitous suppression of viruses [18,19] and TEs [20,21]. Ago2 also evolves under strong
67 positive selection, with frequent selective sweeps [12,13,15,22,23], possibly driven by an arms race
68 with virus-encoded suppressors of RNAi (VSRs) [15,24,25].

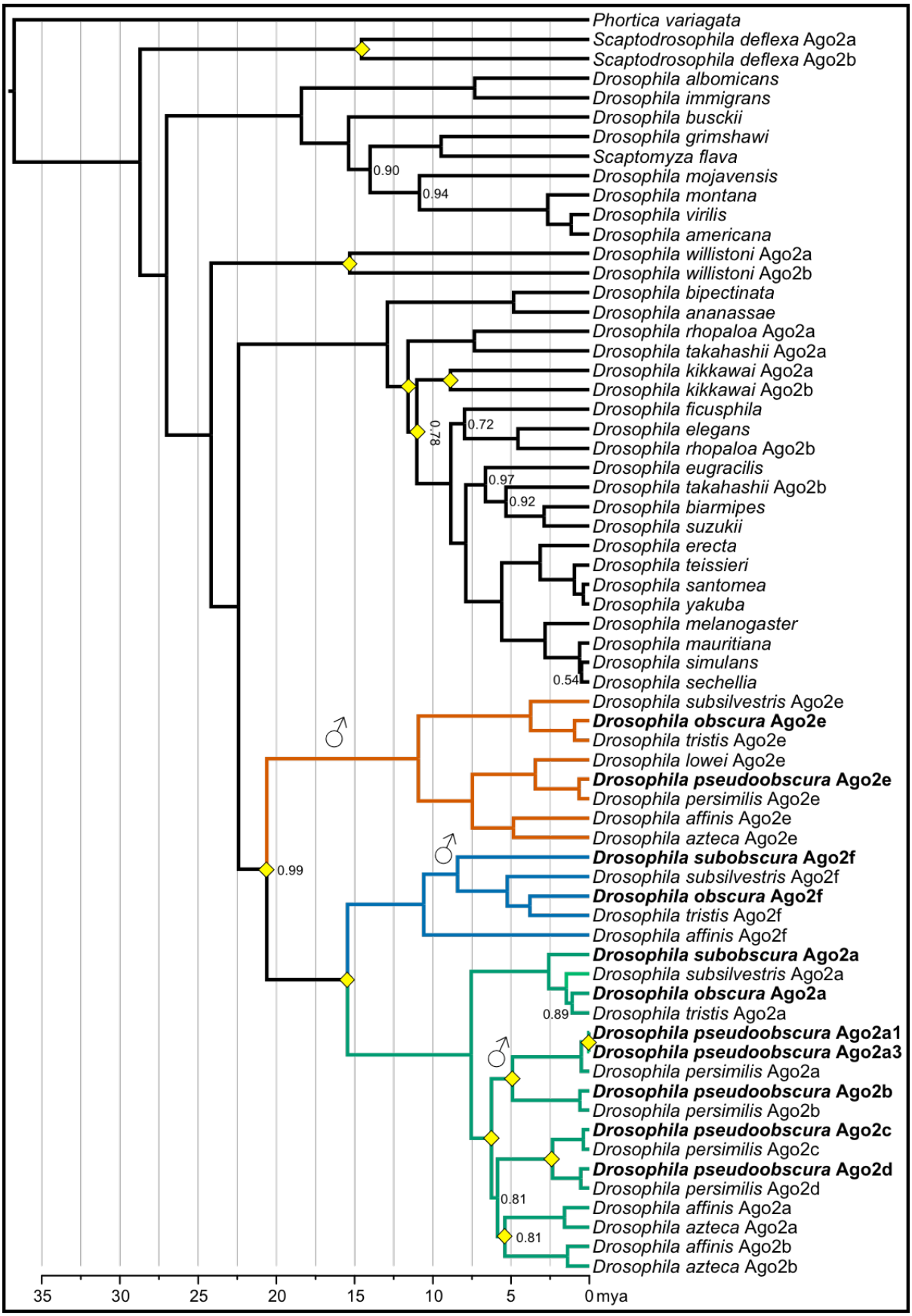
69 In contrast to *D. melanogaster*, from which most functional knowledge of Ago2 in arthropods is
70 derived, an expansion of Ago2 has been reported in *D. pseudoobscura* [26], providing us with an ideal
71 opportunity to study how the RNAi pathway evolves after duplication. Given the roles of *D.*
72 *melanogaster* Ago2 in antiviral defence [18,19], TE suppression [20,21], dosage compensation [17],
73 and gene regulation [16], we hypothesized that duplication in *D. pseudoobscura* may have led to
74 subfunctionalization of Ago2 to a subset of these roles, or even to the evolution of entirely new
75 functions. To elucidate the evolution and function of Ago2 paralogues in *D. pseudoobscura* and its
76 relatives, we identified and dated Ago2 duplication events across available *Drosophila* genomes and
77 transcriptomes, tested for divergence in expression patterns between the Ago2 paralogues in *D.*
78 *subobscura*, *D. obscura* and *D. pseudoobscura*, and quantified the evolutionary rate and positive
79 selection acting on each of these paralogues. We find that testis-specificity of Ago2 paralogues has
80 evolved repeatedly in the *obscura* group, and that the majority of paralogues show evidence of recent
81 positive selection.

82

83 Results

84 Ago2 has undergone numerous ancient and recent duplications in the *obscura* group

85 Ago2 duplications had previously been noted in *D. pseudoobscura* [26], but their age and distribution
86 in other species was unknown. We used BLAST [27] and PCR to identify 65 Ago2 homologues in 39
87 species sampled across the Drosophilidae, including 30 homologues in 9 *obscura* group species. To
88 characterize the relationships between Ago2 homologues in the *obscura* group and the other
89 Drosophilidae, and estimate the date of the duplication events that produced them, we carried out a
90 strict clock Bayesian phylogenetic analysis (Figure 1). This showed that there are early diverging
91 Ago2 clades in the *obscura* group: the Ago2e subclade that diverged from other Ago2 paralogues
92 around 21mya (±10 My), and the Ago2a and Ago2f subclades that were produced by a gene
93 duplication event around 16mya (±7 My). Subsequently there have been a series of more recent
94 duplications in the *D. pseudoobscura* subgroup Ago2a-d lineage. Using published genomes,


Phortica variagata
Scaptodrosophila deflexa Ago2a
Scaptodrosophila deflexa Ago2b
Drosophila albomicans
Drosophila immigrans
Drosophila busckii
Drosophila grimshawi
Scaptomyza flava
Drosophila mojavensis
Drosophila montana
Drosophila virilis
Drosophila americana
Drosophila willistoni Ago2a
Drosophila willistoni Ago2b
Drosophila bipectinata
Drosophila ananassae
Drosophila rhopaloa Ago2a
Drosophila takahashii Ago2a
Drosophila kikkawai Ago2a
Drosophila kikkawai Ago2b
Drosophila ficusphila
Drosophila elegans
Drosophila rhopaloa Ago2b
Drosophila eugracilis
Drosophila takahashii Ago2b
Drosophila biarmipes
Drosophila suzukii
Drosophila erecta
Drosophila teissieri
Drosophila santomea
Drosophila yakuba
Drosophila melanogaster
Drosophila mauritiana
Drosophila simulans
Drosophila sechellia
Drosophila subsilvestris Ago2e
Drosophila obscura Ago2e
Drosophila tristis Ago2e
Drosophila lowei Ago2e
Drosophila pseudoobscura Ago2e
Drosophila persimilis Ago2e
Drosophila affinis Ago2e
Drosophila azteca Ago2e
Drosophila subobscura Ago2f
Drosophila subsilvestris Ago2f
Drosophila obscura Ago2f
Drosophila tristis Ago2f
Drosophila affinis Ago2f
Drosophila subobscura Ago2a
Drosophila subsilvestris Ago2a
Drosophila obscura Ago2a
Drosophila tristis Ago2a
Drosophila pseudoobscura Ago2a1
Drosophila pseudoobscura Ago2a3
Drosophila persimilis Ago2a
Drosophila pseudoobscura Ago2b
Drosophila persimilis Ago2b
Drosophila pseudoobscura Ago2c
Drosophila persimilis Ago2c
Drosophila pseudoobscura Ago2d
Drosophila persimilis Ago2d
Drosophila affinis Ago2a
Drosophila azteca Ago2a
Drosophila affinis Ago2b
Drosophila azteca Ago2b
0.90
0.94
0.78
0.72
0.97
0.92
0.54
0.99
0.89
0.81
0.81
♂
♂
♂
35
30
25
20
15
10
5
0 mya

95

Figure 1: An approximately time-scaled Bayesian gene tree of Ago2 in the Drosophilidae. Duplication events are marked by yellow diamonds, Bayesian posterior support is shown for nodes for which it is less than 100%, and the genes and species that are the focus of the present study are marked in bold. Ago2 has duplicated at least twelve times in the Drosophilidae: seven times in the *obscura* group, twice early in the *melanogaster* group, and once each in the lineages leading to *D. willistoni*, *S. deflexa* and *D. kikkawai*. There has also been a potentially recent duplication of Ago2a on the *D. affinis* / *D. azteca* lineage (~5mya), although the low support for this node may suggest that these paralogues could also nest within the *D. pseudoobscura* / *D. persimilis* expansion, with one paralogue sister to the Ago2a1-Ago2b subclade and the other sister to the Ago2c-Ago2d subclade. After duplication, Ago2 paralogues in the *obscura* group have specialised to the testis three times independently (marked with ♂), and have been retained for an extended period of time (>10 My in the case of Ago2e), suggesting an adaptive basis for testis-specificity. The labelling a-e of paralogous clades corresponds to reference [26], while clade f is newly reported here.

transcriptomes and PCR we were unable to identify Ago2e in *D. subobscura*, Ago2e or Ago2f in *D. lowei*, or Ago2f in *D. pseudoobscura*, *D. persimilis* and *D. azteca*. While some of these losses may reflect incomplete genome assemblies or unexpressed genes in transcriptome surveys, we attempted to validate the losses in *D. pseudoobscura* and *D. subobscura* by extensive PCR, and were again unable to recover these genes.

In release 3.03 of the *D. pseudoobscura* genome Ago2b-Ago2e have confirmed locations, but Ago2a1 and Ago2a3 (the very recent paralogues newly identified here) lie in tandem on an unplaced contig with a third incomplete copy (Ago2a2) between them. We used PCR to confirm the existence, orientation, and relative positioning of these genes, and to identify the location of this contig, which lies in reverse orientation on chromosome XL-group1a (predicted coordinates 3,463,701-3,489,689). We then combined this information with our phylogenetic analysis to reconstruct the positional evolution of *D. pseudoobscura* Ago2 paralogues (Figure 2).

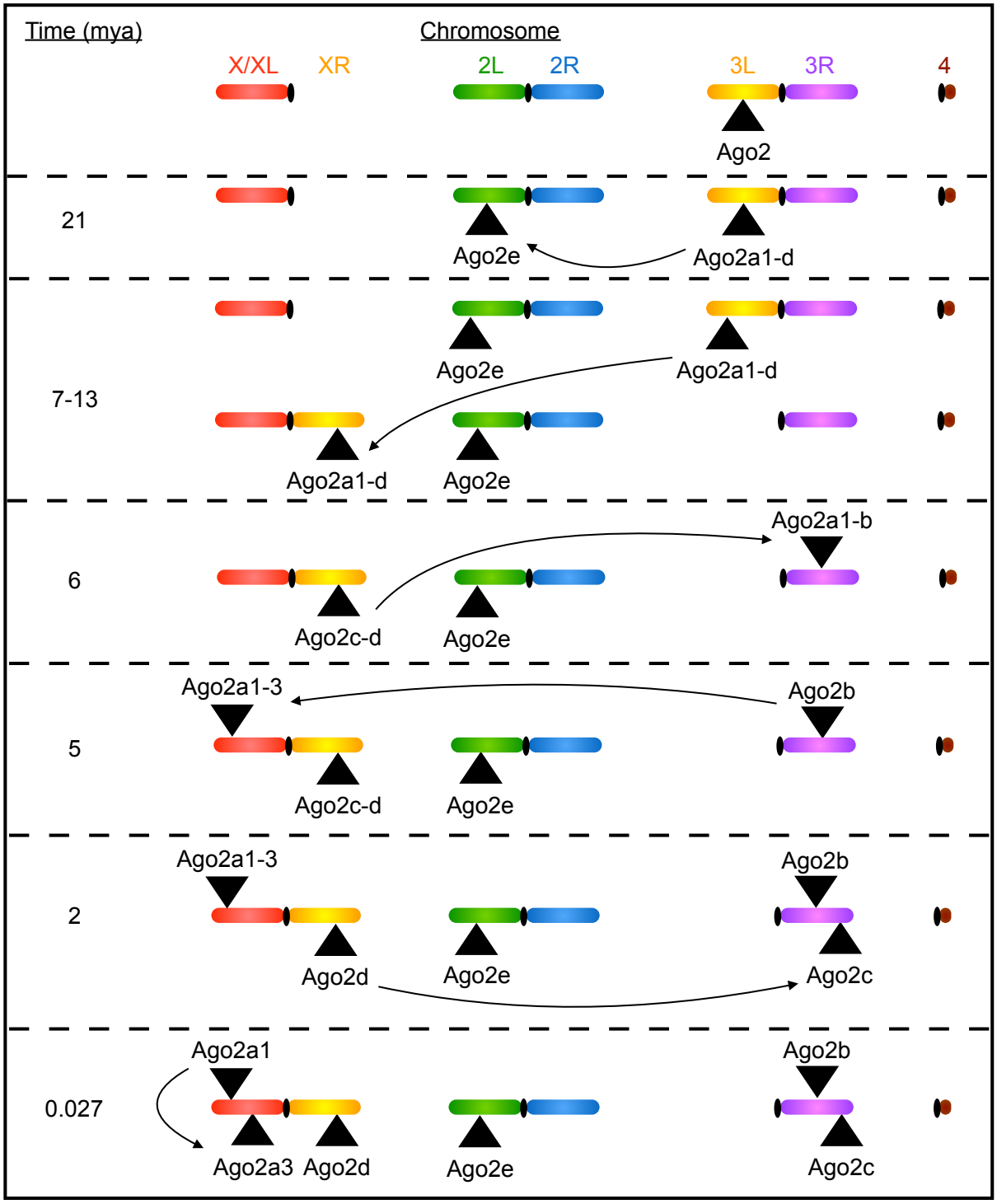


Figure 2: The course of duplications and translocations of Ago2 paralogues in *D. pseudoobscura.* A complex series of duplications and translocations has produced six Ago2 paralogues in *D. pseudoobscura*, located on four different chromosome arms. Chromosome arms correspond to Muller Elements A (X/XL), B (2L), C (2R), D (3L), E (3R) & F (4) (adapted from [91] Fig. 1). Firstly, the Ago2a1-e ancestor duplicated ~21mya to form Ago2a1-d and Ago2e, the latter of which moved onto chromosome 2L. Next, the 3L arm fused with the X chromosome, moving Ago2a1-d onto the X: this happened 7-15mya, after the divergence of the *obscura* group into Palearctic (e.g. *D. subobscura*) and Nearctic (e.g. *D. pseudoobscura*) clades [92]. Ago2a1-d then duplicated ~6mya, forming Ago2c-d and Ago2a1-b, the latter of which moved onto chromosome 2. After this, Ago2a1-b duplicated ~5mya, producing Ago2b and Ago2a1-3, the latter of which moved onto the left arm of the X chromosome. This was followed by a duplication of Ago2c-d ~2mya, forming Ago2d and Ago2c, the latter of which moved onto chromosome 2. Finally, Ago2a1-3 duplicated ~27kya, producing Ago2a1 and Ago2a3 in tandem. Note that due to differences in evolutionary rate between branches, the timings of these events should be treated with caution.

110 Ago2 paralogues in *D. subobscura*, *D. obscura* and *D. pseudoobscura* are probably functional

111 Our phylogenetic analysis (Figure 1) revealed that the Ago2 paralogues in the *obscura* group have
112 retained coding sequences for millions of generations, showing that they have remained functional for
113 this period. They have also retained PAZ and PIWI domains and a bilobal structure (characteristic of
114 Argonaute proteins across the tree of life), suggesting that they are part of a functional RNAi pathway.
115 In *D. melanogaster*, Ago2 plays a key role in antiviral immunity, but is ubiquitously and highly
116 expressed in both males and females, and is not strongly induced by viral challenge (Figure 3a, [28]).
117 To test whether this expression pattern has been conserved after Ago2 duplication, or whether any
118 Ago2 paralogues have become inducible by viral challenge, we measured the expression of each
119 Ago2 paralogue in female and male *D. subobscura*, *D. obscura* and *D. pseudoobscura* after infection
120 with Drosophila C Virus (DCV). These species are separated by ~10My of evolution, and represent
121 the three major clades within the *obscura* group. Members of the *obscura* group are known to be
122 highly susceptible to DCV, supporting high viral titres and displaying rapid mortality [29]. We found
123 that only one paralogue is expressed in both sexes at a high level in *D. subobscura* (Ago2a), *D.*
124 *obscura* (Ago2a) and *D. pseudoobscura* (Ago2c). Unexpectedly, and with only one exception, the
125 other Ago2 paralogues in all species were only expressed in males (Figure 3b-d), raising the
126 possibility that they have specialised to a sex-specific role. The one exception was *D. pseudoobscura*
127 Ago2d, which is the ancestral paralogue in this species (inferred by synteny), and for which we could
128 not detect any expression.

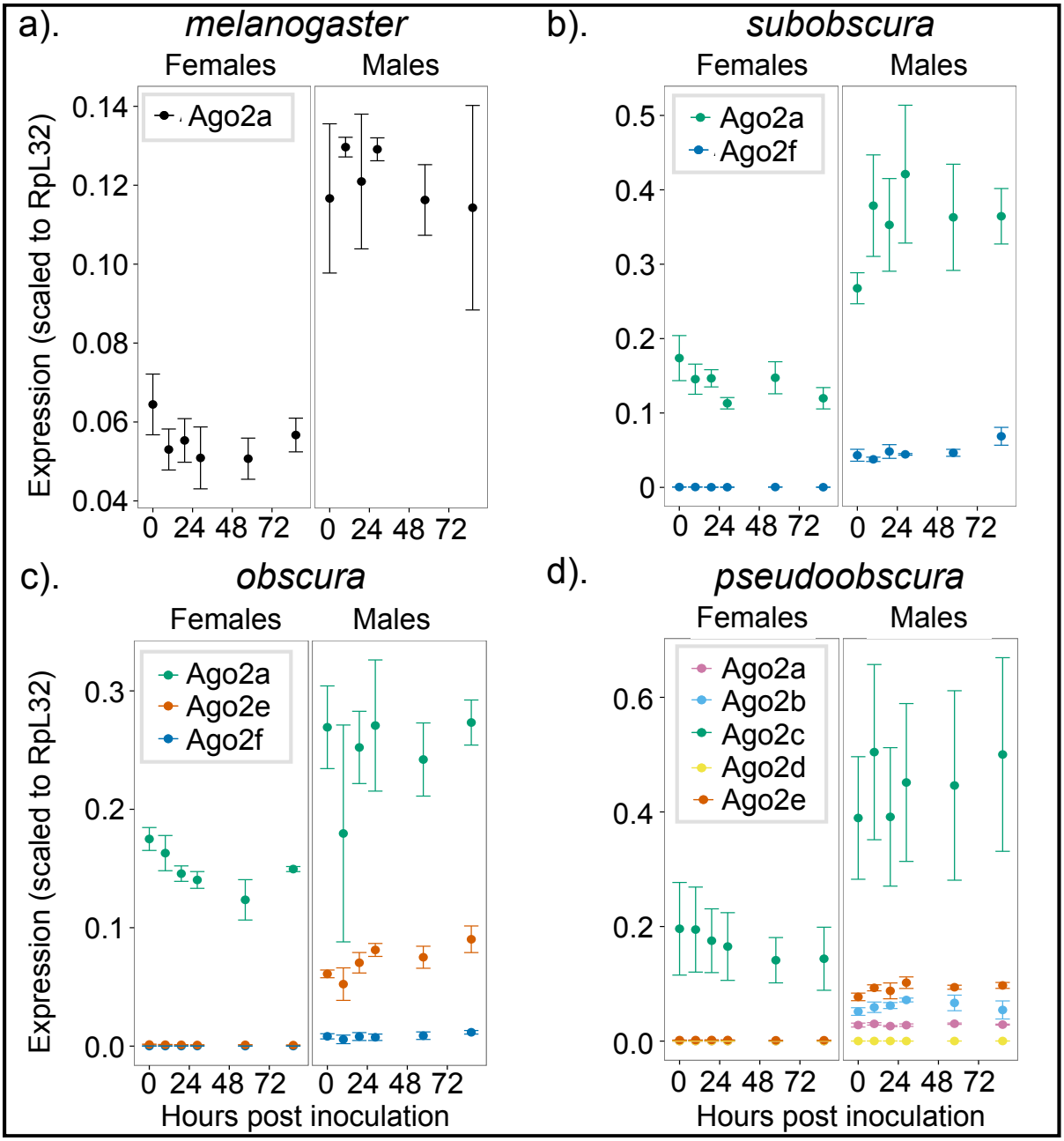


Figure 3: Expression patterns of Ago2 paralogues under challenge with Drosophila C Virus. In each *obscura* group species, only one Ago2 paralogue has retained the ancestral pattern of ubiquitous stable expression in each sex (illustrated by *D. melanogaster*). In contrast, all other paralogues are expressed in males only (apart from *D. pseudoobscura* Ago2d, which is unexpressed in either sex). The high degree of sequence similarity between Ago2a1 and Ago2a3 prevented us from amplifying these genes separately in qPCR, and here they are combined as "Ago2a". Error bars indicate 1 standard error estimated from 2 technical replicates in each of three different genetic backgrounds. Apparent differences in expression between sexes and species should be interpreted with caution, as these may be driven by differences in expression levels of the reference gene (RpL32).

129

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131

132 Ago2 paralogues have repeatedly specialised to the testis

133 To determine whether the strongly male-biased expression pattern is associated with a testis-specific
134 role, we quantified the tissue-specific expression patterns of Ago2 paralogues in *D. subobscura*, *D.*
135 *obscura* and *D. pseudoobscura*. In *D. melanogaster* the single copy of Ago2 was expressed in all
136 adult tissues (Figure 4a), and transcripts were present in the embryo (S1 Figure). In *D. subobscura*,
137 *D. obscura* and *D. pseudoobscura*, we found that the Ago2 paralogues exhibited striking differences
138 in their tissue-specific patterns of expression (Figure 4b-d). In each species, one paralogue has
139 retained the ancestral ubiquitous expression pattern in adult tissues. In contrast, every other
140 paralogue was expressed only in the testis, except for the non-expressed *D. pseudoobscura* Ago2d.
141 None of the testis-specific paralogues in *D. pseudoobscura* was detectable in embryos (S1 Figure).

142 Interestingly, the ubiquitously expressed paralogue in *D. subobscura* and *D. obscura* is the ancestral
143 gene (Ago2a in both cases, as inferred by synteny with *D. melanogaster*), but in *D. pseudoobscura*
144 another paralogue (Ago2c) has evolved the ubiquitous expression pattern, and the ancestral gene
145 (Ago2d) was not expressed at a detectable level in any tissue. When interpreted in the context of the
146 phylogenetic relationships between these paralogues, the most parsimonious explanation is that
147 testis-specificity evolved at least three times: firstly at the base of the Ago2e clade, secondly at the
148 base of the Ago2f clade, and thirdly at the base of the *D. pseudoobscura-D. persimilis* Ago2a-Ago2b
149 subclade (Figure 1).

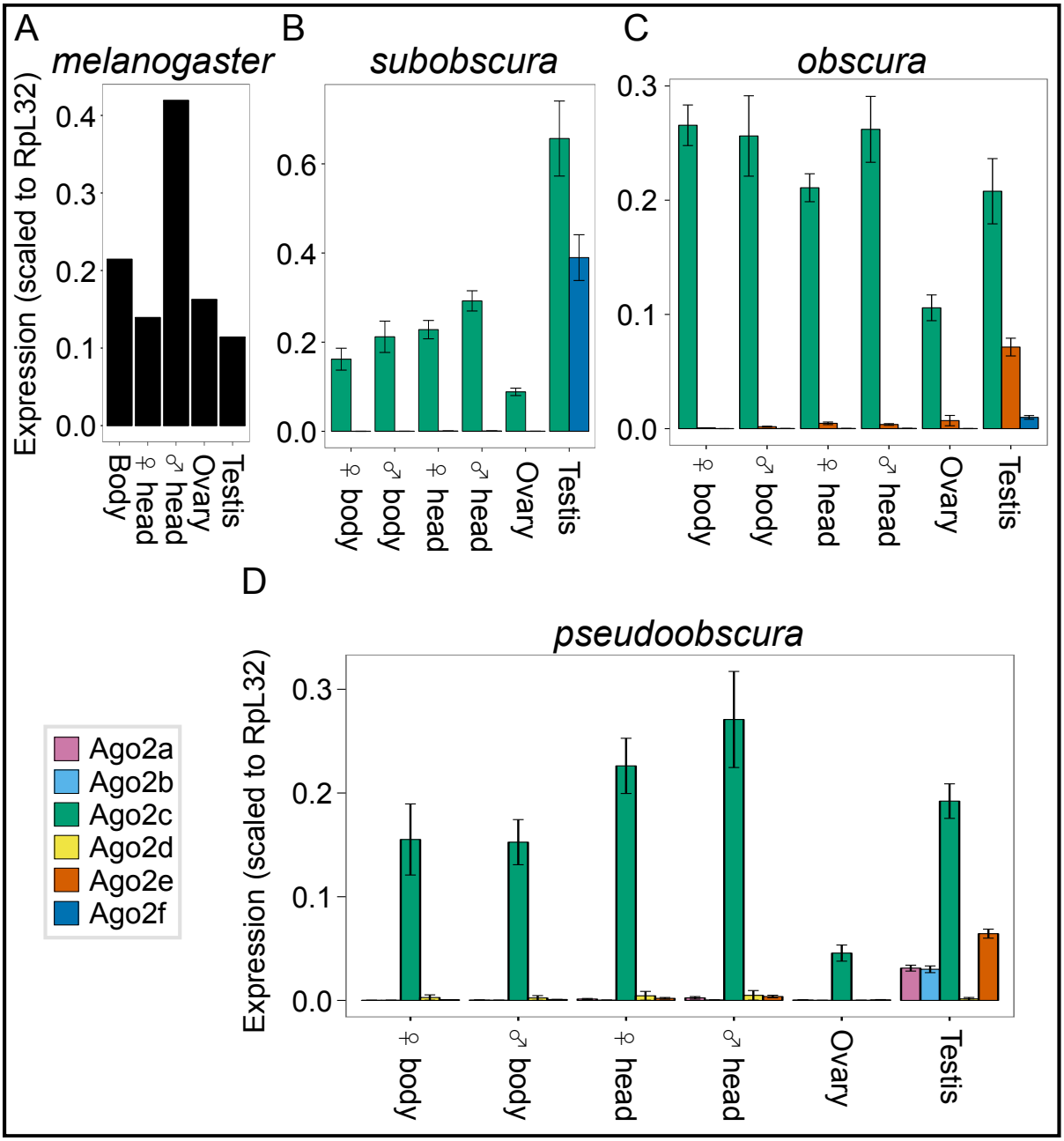

Figure 4: Tissue-specific expression patterns of Ago2 paralogues.
In each of the three *obscura* group species tested, one paralogue has retained the ancestral ubiquitous expression pattern, while the others have specialised to the testis (with the exception of *D. pseudoobscura* Ago2d). The high degree of sequence similarity between Ago2a1 and Ago2a3 prevented us from amplifying these genes separately in qPCR, and here they are combined as "Ago2a". Error bars indicate 1 standard error estimated from 2 technical replicates in each of five different genetic backgrounds. *D. melanogaster* expression levels were taken from a single RNA-seq experiment [71].

150

151 <u>Testis-specificity is associated with faster protein evolution</u>

152 To test for differences in evolutionary rate between testis-specific and ubiquitously expressed Ago2

153 paralogues, we fitted sequence evolution models to the set of drosophilid Ago2 sequences depicted in

154 Figure 1 using codeml (PAML, Yang 1997). These tests estimate separate dN/dS ratios (ω) and
155 likelihoods for different subclades in the gene tree, providing a test for differential rates of evolution.
156 We found that most support (Akaike weight = 0.99) falls behind a model specifying a different ω for
157 each obscura group Ago2 subclade, and another separate ω for the *D. pseudoobscura-D. persimilis*
158 Ago2a-Ago2b subclade. Under this model, the testis-specific *D. pseudoobscura-D. persimilis* Ago2a-
159 Ago2b subclade has the highest rate of protein evolution (ω=0.32±0.047 SE), followed by the testis-
160 specific Ago2f subclade (ω=0.21±0.014), the ubiquitous Ago2a subclade (ω=0.19±0.012), the testis-
161 specific Ago2e subclade (ω=0.16±0.010), and finally the other Drosophilid Ago2 sequences
162 (ω=0.12±0.002). This shows that the evolution of testis-specificity is generally accompanied by an
163 increase in the rate of protein evolution. We also used the Bayes Empirical Bayes sites test in codeml
164 to identify codons evolving under positive selection across the entire gene tree, and the branch-sites
165 test to identify codons under positive selection in the *obscura* group Ago2 subclade. While we found
166 no positively-selected codons with the sites test, we identified three codons under positive selection
167 (297, 338 & 360) in the *obscura* group Ago2 subclade with the branch-sites test (likelihood ratio test
168 M8 vs M8a, $p<0.005$).

169 McDonald-Kreitman tests identify strong positive selection on *D. pseudoobscura* Ago2e

170 This increase in evolutionary rate after the evolution of testis-specificity may have occurred as a result
171 of positive selection, or the relaxation of selective constraint. However, unless there are multiple
172 substitutions within single codons, this will be hard to detect using methods such as codeml.
173 Therefore, as a second test for positive selection on Ago2 paralogues in *D. subobscura*, *D. obscura*
174 and *D. pseudoobscura*, we gathered intraspecies polymorphism data for each Ago2 paralogue in
175 these species (S4 Appendix), and performed McDonald-Kreitman (MK) tests (S1 Table). The MK test
176 uses a comparison of the numbers of fixed differences between species at nonsynonymous (Dn) and
177 synonymous (Ds) sites, and polymorphisms within a species at nonsynonymous (Pn) and
178 synonymous (Ps) sites to infer the action of positive selection. If all mutations are either neutral or
179 strongly deleterious, the Dn/Ds ratio should be approximately equal to the Pn/Ps ratio; however, if
180 there is positive selection, an excess of nonsynonymous differences is expected [31]. The majority of
181 MK tests were non-significant (Fisher's exact test, $p>0.1$), despite often displaying relatively high
182 K_A/K_S ratios e.g. *D. pseudoobscura* Ago2a1 (K_A/K_S =0.34), Ago2b (K_A/K_S =0.43) & Ago2d (K_A/K_S
183 =0.36). However, the low diversity at these loci (<10 polymorphic sites in most cases; see below) will

184 mean that the MK approach has little power, and that estimates of the proportion of substitutions that
185 are adaptive (α) are likely to be poor. In contrast to the other loci, we identified strong positive
186 selection acting on *D. pseudoobscura* Ago2e – which has relatively high genetic diversity – with α at
187 100% (α=1.00; Fisher's exact test, p=0.0004). This result is driven by the extreme skew in the
188 proportion of nonsynonymous to synonymous polymorphisms (0 Pn to 17 Ps), despite substantial
189 numbers of fixed differences (77 Dn to 120 Ds), and is robust to the choice of outgroup (S2 Table).

190 <u>The majority of Ago2 paralogues have extremely low levels of sequence diversity</u>

191 When strong selection acts to reduce genetic diversity at a locus, it can also reduce diversity at linked
192 loci before recombination can break up linkage [32]. Recent positive selection can therefore be
193 inferred from a reduction in synonymous site diversity compared with other genes. Because MK tests
194 can only detect multiple long-term substitutions, and are hampered by low diversity, diversity-based
195 approaches offer a complementary way to detect very recent strong selection. We therefore
196 compared the synonymous site diversity at each Ago2 paralogue in *D. pseudoobscura* with the
197 distribution of genome-wide synonymous site diversity. We found that all paralogues have unusually
198 low diversity relative to other loci: Ago2a1, Ago2b and Ago2c fall into the lowest percentile, Ago2a3
199 and Ago2d into the 2nd lowest percentile and Ago2e into the 8th lowest percentile (S3 Figure). A
200 multi-locus extension of the HKA test (ML-HKA [33]) confirmed that the diversity of Ago2a1-Ago2e is
201 significantly lower than the *D. pseudoobscura* genome as a whole (Akaike weight = 0.98).
202 Unfortunately, population-genomic data are not available for *D. subobscura* and *D. obscura*,
203 preventing a similar analysis. However, we found similar results for Ago2a and Ago2e when
204 comparing the diversity of *D. subobscura* and *D. obscura* Ago2 paralogues to levels of diversity
205 inferred from transcriptome data (data from [34]), suggesting that this effect is not limited to *D.*
206 *pseudoobscura* and these genes may therefore have been recent targets of selection in multiple
207 species. In *D. obscura*, Ago2a and Ago2e fall into the 2nd and 4th lowest diversity percentile
208 respectively, whereas Ago2f falls into the 19th percentile (S3 Figure). In *D. subobscura*, Ago2a falls
209 into the 7th percentile, whereas Ago2f falls into the 16th percentile (S3 Figure). The prevalence of low
210 intraspecific diversity for testis-specific paralogues is consistent with recent selective sweeps,
211 suggesting that positive selection, not merely relaxation of constraint, has contributed to the increased
212 evolutionary rate seen after specialization to the testis.

213 Four out of six *D. pseudoobscura* Ago2 show a strong signature of recent hard selective sweeps

214 The impact of selection on linked diversity (a selective sweep) is expected to leave a characteristic
215 footprint in local genetic diversity around the site of selection, and this forms the basis of explicit
216 model-based approaches to detect the recent action of positive selection [35]. For *D. pseudoobscura*,
217 population genomic data for 11 haplotypes is available from [36], permitting an explicit model-based
218 test for recent hard selective sweeps near to Ago2 paralogues. We therefore combined our Ago2 data
219 with 111kb haplotypes from [36] to analyse the neighbouring region around each paralogue. Ago2a1
220 and Ago2a3 form a tandem repeat, and were therefore analysed together as a single potential sweep.
221 We found strong evidence for recent selective sweeps at or very close to Ago2a1/3, Ago2b and
222 Ago2c, which display sharp troughs in their diversity levels, and large peaks in the composite
223 likelihood of a sweep, which far exceed a significance threshold derived from coalescent simulation
224 ($p<0.01$) (Figure 5). These localised reductions in diversity remain when our own Ago2 haplotype data
225 is removed, showing the results are robust to the fact that our Ago2 sequence data is derived from a
226 different population to the genome-wide data of [36] (S5 Figure; note that sequence data for Ago2
227 paralogues cannot be derived from the data of reference [36], because of their extreme similarity). In
228 addition, there is ambiguous evidence for a sweep at Ago2d, in the form of one significant ($p<0.01$)
229 likelihood peak just upstream of the paralogue, but two other peaks ~1kb and ~3kb further upstream.
230 There is no evidence for a hard sweep at Ago2e, which has no diversity trough or likelihood peak.

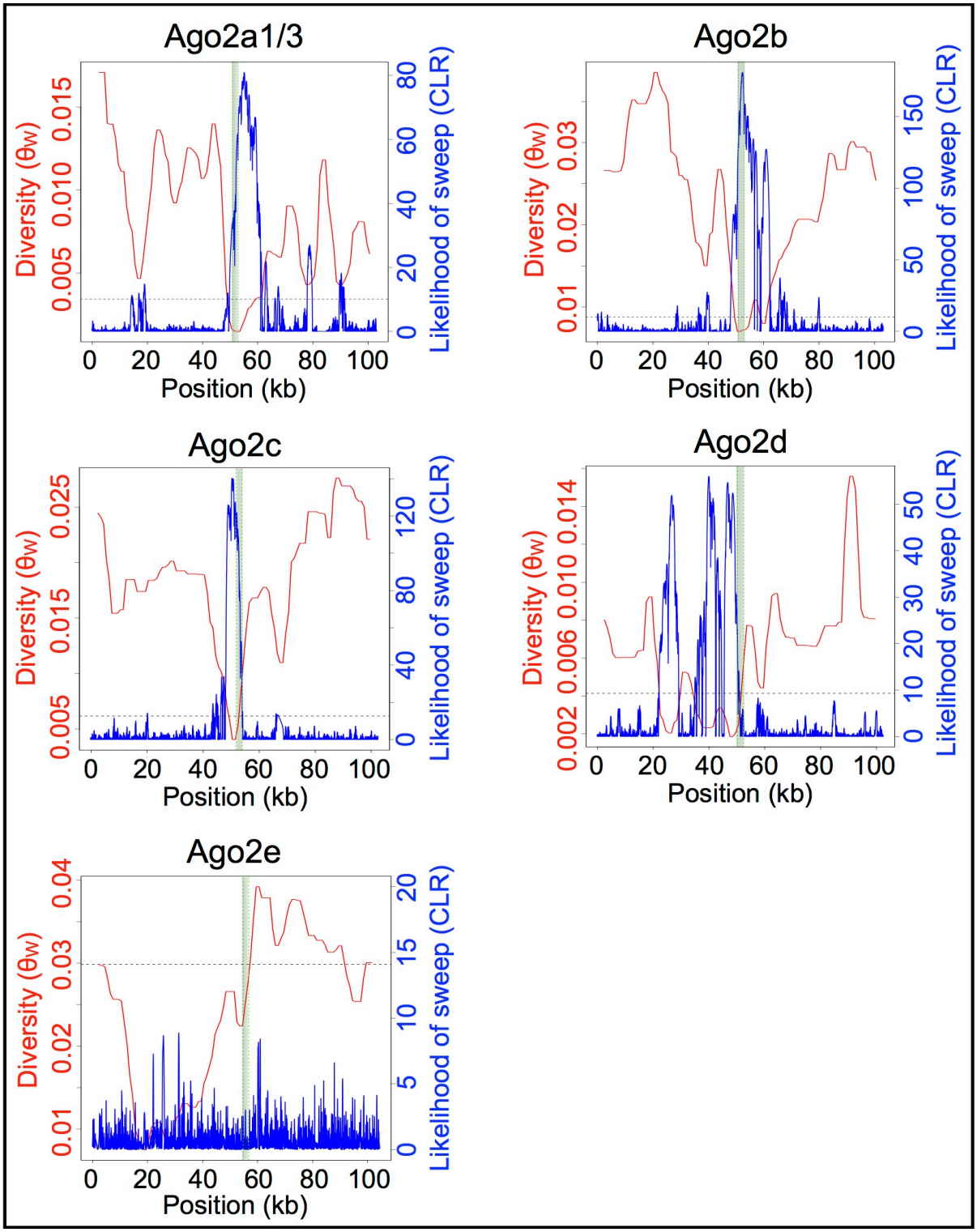

Figure 5: Selective sweeps at *D. pseudoobscura* Ago2 paralogues.
For each paralogue, diversity at all sites (Watterson's θ) is displayed in red, and the likelihood of a sweep centred at that site (composite likelihood ratio, CLR) is displayed in blue. The significance threshold for the CLR is displayed by the horizontal dotted line ($p<0.01$, derived from the 10th-highest CLR out of 1000 coalescent simulations, assuming constant recombination rate and N_e). There is strong evidence for sweeps at Ago2a, Ago2b and Ago2c, indicated by troughs in their diversity levels and peaks in the likelihood of a sweep.

231

232 Discussion

233 Testis-specificity may indicate a loss of antiviral function

234 We have found that Ago2 paralogues in the *obscura* group have repeatedly evolved divergent
235 expression patterns after duplication, with the majority of paralogues specializing to the testis. This is
236 the first report of testis-specificity for any arthropod Ago2, which is ubiquitously expressed in *D.*
237 *melanogaster* [37], and provides a strong indication that these paralogues have diverged in function.
238 This testis-specificity (Figure 4), combined with a lack of upregulation on viral challenge (in contrast to
239 virus-responsive genes in the Toll [38] and Jak-STAT [39] signalling pathways in *D. melanogaster*),
240 suggests that these Argonautes are likely to have lost their ancestral ubiquitous antiviral role. In
241 contrast, one paralogue in each species has retained this ubiquitous expression pattern (*D.*
242 *subobscura* Ago2a, *D. obscura* Ago2a & *D. pseudoobscura* Ago2c, Figure 4), suggesting that these
243 paralogues have retained roles in antiviral defence [18,19], dosage compensation [17] and/or somatic
244 TE suppression [20,21].

245 Both ubiquitous and testis-specific Ago2 paralogues show evidence of recent positive selection

246 We identified selective sweeps at the ubiquitously expressed Ago2 paralogue in *D. pseudoobscura*
247 Ago2c, and very low diversity in the ubiquitously expressed Ago2 paralogues of *D. subobscura* and *D.*
248 *obscura* (Ago2a), suggesting that all of these genes may have recently experienced strong positive
249 selection. This is consistent with previous findings of strong selection and rapid evolution of Ago2 in
250 *D. melanogaster* [15,22,23] which has also experienced recent sweeps in *D. melanogaster*, *D.*
251 *simulans*, and *D. yakuba* [23], and across the *Drosophila* more broadly [12]. It has previously been
252 suggested that this is driven by arms-race coevolution with viruses [12,13], some of which encode
253 viral suppressors of RNAi (VSRs) that block Ago2 function [40]. The presence of VSR-encoding
254 viruses, such as Nora virus, in natural *obscura* group populations [34], combined with the host-
255 specificity that VSRs can display [25], suggest that arms-race dynamics may also be driving the rapid
256 evolution of ubiquitously expressed Ago2 paralogues in the *obscura* group.

257 Potential testis-specific functions

258 In contrast to their ancestral ubiquitous expression pattern, the dominant fate for Ago2 paralogues in
259 the *obscura* group appears to have been specialization to the testis. Paralogues often undergo a brief
260 period of testis-specificity soon after duplication [41,42], and this has given rise to the 'out-of-the-

261 testis' hypothesis, in which new paralogues are initially testis-specific before evolving functions in
262 other tissues [43]. However, two lines of evidence suggest an adaptive basis for the testis-specificity
263 observed for the *obscura* group Ago2 paralogues. First, testis-specificity has been retained for more
264 than 10 million years in Ago2e and Ago2f, in contrast to the broadening of expression over time
265 expected under the out-of-the-testis hypothesis [41,43]. Second, all testis-specific Ago2 paralogues
266 in *D. pseudoobscura* show evidence either of long-term positive selection (MK test for the high-
267 diversity Ago2e) or of recent selective sweeps (in low-diversity Ago2a1/3 and Ago2b), and the testis-
268 specific *D. obscura* Ago2e displays a reduction in diversity, potentially driven by selection.

269 Under a subfunctionalization model for Ago2 testis-specialization, four candidate selective pressures
270 seem likely: testis-specific dosage compensation, antiviral defence, TE suppression, and/or the
271 suppression of meiotic drive. Of these, testis-specific dosage compensation seems the least likely to
272 drive testis-specificity because the male-specific lethal (MSL) complex, which Ago2 directs to X-linked
273 genes to carry out dosage compensation in the soma of *D. melanogaster*, is absent from testis [44].
274 Testis-specific antiviral defence seems similarly unlikely, as the only known paternally-transmitted
275 *Drosophila* viruses (Sigmaviruses; Rhabdoviridae) pass through both the male and female gametes
276 [45], and so the potential benefits of testis-specificity seem unclear. In contrast, the suppression of
277 TEs or meiotic drive seem more promising candidate selective forces. First, numerous TEs transpose
278 preferentially in the testis, such as *Penelope* in *D. virilis* [46] and *copia* in *D. melanogaster* [47,48],
279 which could impose a selection pressure on Ago2 paralogues to provide a testis-specific TE
280 suppression mechanism. Nevertheless, it should be noted that all members of the canonical anti-TE
281 Piwi subfamily (Ago3, Aub and Piwi) are also expressed in *obscura* group testis (S2 Figure),
282 suggesting that if Ago2 paralogues have specialised to suppress TEs, they are doing so alongside the
283 existing TE suppression mechanism. Second, testis-specificity could have evolved to suppress
284 meiotic drive, which is prevalent (in the form of sex-ratio distortion) in the *obscura* group [49–53], and
285 which is suppressed by RNAi-based mechanisms in other species [54–56]. A high level of meiotic
286 drive in the *obscura* group could therefore impose selection for the evolution of novel suppression
287 mechanisms, leading to the repeated specialization of Ago2 paralogues to the testis.

288 <u>Prospects for novel functions during the evolution of RNAi</u>

289 The functional specialization that we observe for *obscura* group Ago2 paralogues raises the prospect
290 of undiscovered derived functions following Argonaute expansions in other lineages. Ago2 has

291 duplicated frequently across the arthropods, with expansions present in insects (*Drosophila willistoni*
292 (Figure 1) & *Musca domestica* [57]), crustaceans (*Penaeus monodon* [6]) and chelicerates
293 (*Tetranychus urticae*, *Ixodes scapularis*, *Mesobuthus martensii* & *Parasteatoda tepidariorum* [58]).
294 The prevalence of testis-specificity in *obscura* group Ago2 paralogues raises the possibility that
295 specialization to the germline may be more widespread following Argonaute duplication. The
296 expression of Ago2 paralogues has previously been characterized in *P. monodon*, and shows that
297 one paralogue has indeed specialised to the germline of both males and females, but not the testis
298 alone [6]. Publicly available RNAseq data from the head, gonad and carcass of male and female
299 *Musca domestica* [59] suggests that neither Ago2 paralogue has specialised to the testis (S6 Figure).
300 However, public data from the head, thorax and abdomen of male and female *D. willistoni* [60] shows
301 that one Ago2 paralogue (FBgn0212615) is expressed ubiquitously, while the other (FBgn0226485) is
302 expressed only in the male abdomen (S6 Figure), consistent with the evolution of testis-specificity
303 after duplication. This raises the possibility that a testis-specific selection pressure may be driving the
304 retention and specialization of Ago2 paralogues across the arthropods.

305 In conclusion, we have identified rapid and repeated evolution of testis-specificity after the duplication
306 of Ago2 in the *obscura* group, associated with low genetic diversity and signatures of strong selection.
307 Ago2 and other RNAi genes have undergone frequent expansions in different eukaryotic lineages
308 [4,61], and have been shown to switch between ubiquitous and germline- or ovary-specific functions
309 in isolated species. This study provides evidence for the evolution of a new testis-specific RNAi
310 function, and suggests that positive selection may act on young paralogues to drive the rapid
311 evolution of novel RNAi mechanisms across the eukaryotes.

312

313 Materials and Methods

314 Identification of Ago2 homologues in the Drosophilidae

315 We used tBLASTx to identify Ago2 homologues in transcriptomes and genomes of 39 species of the
316 Drosophilidae, using previously-characterised Ago2 from the closest possible relative to provide the
317 query for each species. If blast returned partial hits, we aligned all hits from the target species to all
318 Argonautes from the query species, and assigned hits to the appropriate Ago lineage based on a
319 neighbour-joining tree. For each query sequence, we then manually curated partial blast hits into

320 complete genes using Geneious v5.6.2 (http://www.geneious.com [62]) (see Supplementary Materials
321 for sequence accessions).

322 Additionally, we used degenerate PCR to identify Ago2 paralogues in *D. azteca* and *D. affinis*, and
323 paralogue-specific PCR with a touchdown amplification cycle to validate the Ago2 paralogues
324 identified in *D. subobscura*, *D. obscura* and *D. pseudoobscura*. For each reaction, unincorporated
325 primers were removed with ExonucleaseI (New England Biolabs) and 5' phosphates were removed
326 with Antarctic Phosphatase (NEB), the PCR products were sequenced by Edinburgh Genomics using
327 BigDye V3 reagents on a capillary sequencer (Applied Biosystems), and Sanger sequence reads
328 were trimmed and assembled using Geneious v.5.6.2 (http://www.geneious.com [62]). We also used
329 a combination of PCR and blast searches to locate *D. pseudoobscura* Ago2a1 & Ago2a3, which lie on
330 the unplaced "Unknown_contig_265" in release 3.03 of the *D. pseudoobscura* genome (all PCR
331 primers are detailed in S4 Table).

332 Phylogenetic analysis of drosophilid Ago2 paralogues

333 To characterise the evolutionary relationships between Ago2 homologues in the Drosophilidae, we
334 aligned sequences using translational MAFFT [63] with default parameters. We noted that there is a
335 high degree of codon usage bias in *D. pseudoobscura* Ago2e (effective number of codons
336 (ENC)=34.24) and *D. obscura* Ago2e (ENC=40.36), and a lesser degree in *D. subobscura* Ago2f
337 (ENC=45.63) and *D. obscura* Ago2f (ENC=48.39). To reduce the impact of codon usage bias, which
338 disproportionately affects synonymous sites, we stripped all third positions [64]. We then inferred a
339 gene tree using the Bayesian approach implemented in BEAST v1.8.1 [65] under a nucleotide model,
340 assuming a GTR substitution model, variation between sites modelled by a gamma distribution with
341 four categories, and base frequencies estimated from the data. We used the default priors for all
342 parameters, except tree shape (for which we specified a birth-death speciation model) and the date of
343 the *Drosophila*-*Sophophora* split. To estimate a timescale for the tree, we specified a normal
344 distribution for the date of this node using values based on mutation rate estimates in [66], with a
345 mean value of 32mya, standard deviation of 7mya, and lower and upper bounds of 15mya and 50mya
346 respectively. We ran the analysis for 50 million steps, recording samples from the posterior every
347 1,000 steps, and inferred a maximum clade credibility tree with TreeAnnotator v1.8.1 [65]. Note that
348 precise date estimates are not a primary focus of this study, but that other calibrations [67,68] would
349 lead to more ancient estimates of divergence, and thus stronger evidence for selective maintenance.

350 Domain architecture and structural modelling of Ago2 paralogues in the *obscura* group

351 To infer the location of each domain in each paralogue identified in *D. subobscura*, *D. obscura* and *D.*
352 *pseudoobscura*, we searched the Pfam database [69]. To test for structural differences between the
353 *D. pseudoobscura* paralogues, we built structural models of each paralogue based on the published
354 X-ray crystallographic structure of human Ago2 [70]. We used the MODELER software in the
355 Discovery Studio 4.0 Modeling Environment (Accelrys Software Inc., San Diego, 2013) to calculate
356 ten models, selected the most energetically favourable for each protein, and assessed model quality
357 with the 3D-profile option in the software. To assess variation in selective pressure across the
358 structure of each paralogue, we mapped polymorphic residues onto each structure using PyMol
359 v.1.7.4.1 (Schrödinger, LLC).

360 Quantification of virus-induced expression of Ago2 paralogues

361 We exposed 48-96hr post-eclosion virgin males and females of *D. melanogaster*, *D. subobscura*, *D.*
362 *obscura* and *D. pseudoobscura* to Drosophila C virus (DCV), by puncturing the thorax with a pin
363 contaminated with DCV at a dose of approximately $4x10^7$ $TCID^{50}$ per ml. Infection with DCV using this
364 method has previously been shown to lead to a rapid and ultimately fatal increase in DCV titre in *D.*
365 *melanogaster* and *obscura* group species [29]. All flies were incubated at 18C on a 12L:12D light
366 cycle, with *D. melanogaster* on Lewis medium and *D. subobscura*, *D. obscura* and *D. pseudoobscura*
367 on banana medium. We sampled 4-7 individuals per species at 0, 8, 16, 24, 48 and 72 hours post
368 infection. At each time-point we extracted RNA using TRIzol reagent (Ambion) and a
369 chloroform/isopropanol extraction, treated twice with TURBO DNase (Ambion), and reverse-
370 transcribed using M-MLV reverse transcriptase (Promega) primed with random hexamers. We then
371 quantified the expression of Ago2 paralogues in these samples by qPCR, using Fast Sybr Green
372 (Applied Biosystems) and custom-designed paralogue-specific qPCR primer pairs (see Table S6 for
373 primer sequences). Due to their high level of sequence similarity (99.9% identity), no primer pair could
374 distinguish between *D. pseudoobscura* Ago2a1 and Ago2a3, so these two genes are presented
375 together as "Ago2a". All qPCR reactions for each sample were run in duplicate, and scaled to the
376 internal reference gene Ribosomal Protein L32 (RpL32). To capture the widest possible biological
377 variation, the three biological replicates for each species each used a different wild-type genetic
378 background (see S3 Table for backgrounds used).

379 Quantification of Ago2 paralogue expression in different tissues and life stages

380 For *D. subobscura*, *D. obscura* and *D. pseudoobscura*, we extracted RNA from the head,
381 testis/ovaries and carcass of 48-96hr post-eclosion virgin adults, with males and females extracted
382 separately. Each sample consisted of 8-15 individuals in *D. subobscura*, 10 individuals in *D. obscura*
383 and 15 individuals in *D. pseudoobscura*. We then used qPCR to quantify the expression of each Ago2
384 paralogue in each tissue, with two technical replicates per sample (reagents, primers and cycling
385 conditions as above). We carried out five replicates per species, each using a different wild-type
386 background (see S3 Table for details of backgrounds used). To provide an informal comparison with
387 the expression pattern of Ago2 before duplication (an "ancestral" expression pattern), we used the
388 BPKM (bases per kilobase of gene model per million mapped bases) values for Ago2 calculated from
389 RNA-seq data from the body (carcass and digestive system), head, ovary and testis of 4 day old *D.*
390 *melanogaster* adults by [71], scaling each BPKM value to the value for RpL32 in each tissue. Due to
391 the design of that experiment, the body data are derived from pooled samples of males and females
392 [71].

393 To quantify expression of Ago2 paralogues in *D. pseudoobscura* embryos, we collected eggs within
394 30 minutes of laying, and used qPCR to measure the expression of each Ago2 paralogue (reagents
395 and primers as above) in two separate wild-type genetic backgrounds (MV8 and MV10). As above,
396 we estimated an ancestral expression pattern of Ago2 before duplication from the BPKM values for
397 Ago2 in 0-2hr old *D. melanogaster* embryos according to [71], scaled to the BPKM value for RpL32 in
398 embryos. To determine any changes in the expression of other *D. pseudoobscura* Argonautes (Ago1,
399 Ago3, Aub & Piwi) that are associated with Ago2 duplication, we measured their expression in adult
400 tissues and embryos as detailed above, and compared this with the expression of the Argonautes in
401 *D. melanogaster* as measured by [71].

402 Testing for evolutionary rate changes associated with tissue-specificity of Ago2

403 We used codeml (PAML, Yang 1997) to fit variants of the M0 model (a single dn/ds ratio, ω) to the 65
404 drosophilid Ago2 homologues shown in Figure 1. In contrast to the tree topology, which was based on
405 1st and 2nd positions only, the alignment for the codeml analysis included all positions. To compare the
406 evolutionary rates of ubiquitously expressed and testis-specific Ago2 paralogues, we fitted a model
407 specifying one ω for the Ago2 paralogues that were shown to be testis-specific by qPCR, and another

408 ω for the rest of the tree. We also fitted two models to account for rate variation between the *obscura*
409 group Ago2 subclades. The first model specified a separate ω for the Ago2a subclade, the Ago2e
410 subclade, the Ago2f subclade and the rest of the tree. The second model additionally incorporated an
411 extra ω specified for the *D. pseudoobscura-D. persimilis* Ago2a-Ago2b subclade (which is testis-
412 specific, in contrast with the rest of the *obscura* group Ago2a subclade). We used Akaike weights to
413 assess which model provided the best fit to the data, given the number of parameters.

414 Sequencing of Ago2 paralogue haplotypes from *D. subobscura*, *D. obscura* and *D. pseudoobscura*

415 To gain genotype data for the Ago2 paralogues in *D. subobscura*, *D. obscura* and *D. pseudoobscura*,
416 we sequenced the Ago2 paralogues from six males and six females of each species, each from a
417 different wild-collected line (detailed in S3 Table, sequence polymorphism data in S4 Appendix). We
418 extracted genomic DNA from each individual using the DNeasy Blood and Tissue kit (Qiagen), and
419 amplified and Sanger sequenced each Ago2 paralogue from each individual (reagents and PCR
420 primers as above, sequencing primers detailed in S5 Table). We trimmed and assembled Sanger
421 sequence reads using Geneious v.5.6.2 (http://www.geneious.com [62]), and identified polymorphic
422 sites by eye. After sequencing Ago2a (annotated as a single gene in the *D. pseudoobscura* genome),
423 we discovered two very recent Ago2a paralogues (Ago2a1 & Ago2a3), both of which had been cross-
424 amplified. For each *D. pseudoobscura* individual we therefore re-sequenced Ago2a3 using one primer
425 targeted to its neighbouring locus GA22965, and used this sequence to resolve polymorphic sites in
426 the Ago2a1/Ago2a3 composite sequence, thereby gaining both genotypes for each individual. For
427 each Ago2 paralogue, we inferred haplotypes from these sequence data using PHASE [72], apart
428 from the X-linked paralogues (Ago2a1, Ago2a3 & Ago2d) in *D. pseudoobscura* males, for which
429 phase was obtained directly from the sequence data. The hemizygous haploid X-linked sequenced
430 were used in phase inference, and should substantially improve the inferred phasing of female
431 genotypes.

432 To quantify differences between paralogues in their population genetic characteristics, we aligned
433 haplotypes using translational MAFFT [63], and used DnaSP v.5.10.01 [73] to calculate the following
434 summary statistics for each Ago2 paralogue: π (pairwise diversity, with Jukes-Cantor correction as
435 described in [74]) at nonsynonymous (π_a) and synonymous (π_s) sites, Tajima's *D* [75] and the
436 effective number of codons (ENC) [76]. To compare the ENC for each gene with the genome as a
437 whole, we used codonW v1.4.2 [77] to calculate the ENC for the longest ORF from each gene or

438 transcript in the genomes of *D. subobscura*, *D. obscura* and *D. pseudoobscura* (ORF sets detailed
439 below). In each species, we then compared the ENC values of each Ago2 paralogue with this
440 genome-wide ENC distribution.

441 Testing for positive selection on Ago2 paralogues in the *obscura* group

442 We used McDonald-Kreitman (MK) tests [31] to test for positive selection on each Ago2 paralogue.
443 For each paralogue, we chose an outgroup with divergence at synonymous sites (K_S) in the range
444 0.1-0.2 where possible. However, the prevalence of duplications and losses of Ago2 paralogues in the
445 *obscura* group meant that for some tests a suitably divergent extant outgroup sequence did not exist.
446 In these cases, we reconstructed hypothetical ancestral sequences using the M0 model in PAML [30].
447 To assess the effect of these outgroup choices on our results, we repeated each test with another
448 outgroup, and found no effect of outgroup choice on the significance of any tests, and only marginal
449 differences in estimates of α and ω_α (results of tests using primary and alternative outgroups are
450 detailed in S1 & S2 Tables).

451 A complementary approach to identifying positive selection is to test for reduced diversity at a locus
452 compared with the genome as a whole. To compare the diversity of each *D. pseudoobscura* Ago2
453 paralogue with the genome-wide distribution of synonymous site diversity, we used genomic data for
454 12 lines generated by [36]. We mapped short reads to the longest ORF for each gene in the R3.2
455 gene set using Bowtie2 v2.1.0 [78], and estimated synonymous site diversity (θ_W based on fourfold
456 synonymous sites) at each ORF using PoPoolation [79]. We then plotted the distribution of
457 synonymous site diversity, limited to genes in the size range of 0.75kb - 3kb for comparability with the
458 Ago2 paralogues, and compared the fourfold synonymous site diversity levels of each *D.*
459 *pseudoobscura* Ago2 paralogue with this distribution. Some *D. pseudoobscura* paralogues are
460 located on autosomes (Ago2b, Ago2c & Ago2e) and some on the X chromosome (Ago2a1, Ago2a3 &
461 Ago2d). Therefore, because of the different population genetic expectations for autosomal and X-
462 linked genes [80], we examined separate distributions for autosomal and X-linked genes. To provide
463 an additional test for reduced diversity at *D. pseudoobscura* Ago2 paralogues, we performed
464 maximum-likelihood Hudson-Kreitman-Aguadé tests [33], using divergence from *D. affinis* and
465 intraspecific polymorphism data for 84 *D. pseudoobscura* loci generated by [81]. We performed 63
466 tests to encompass all one, two, three, four, five and six-way combinations of the paralogues, and

467 calculated Akaike weights from the resulting likelihood estimates to provide an estimate of the level of
468 support for each combination.

469 To infer a genome-wide distribution of synonymous site diversity for *D. obscura* and *D. subobscura*,
470 for which genomic data are unavailable, we used pooled transcriptome data from wild-collected adult
471 male flies that had previously been generated for surveys of RNA viruses [25,34]. To generate a *de*
472 *novo* transcriptome for each species, we assembled short reads with Trinity r20140717 [82]. For each
473 species, we mapped short reads from the pooled sample to the longest ORF for each transcript,
474 estimated synonymous site diversity at each locus using PoPoolation [79], and plotted the distribution
475 of diversity (as described above for *D. pseudoobscura*). The presence of heterozygous sites in males
476 (identified by Sanger sequencing) confirmed that all Ago2 paralogues in *D. subobscura* and *D.*
477 *obscura* are autosomal: we therefore compared the synonymous site diversity for these paralogues
478 with the autosomal distribution, and do not show the distributions for putatively X-linked genes. Our
479 use of transcriptome data for *D. obscura* and *D. subobscura* will bias the resulting diversity
480 distributions in three ways. First, variation in expression level will cause individuals displaying high
481 levels of expression to be over-represented among reads, downwardly biasing diversity. Second,
482 highly expressed genes are easier to assemble, and highly expressed genes tend to display lower
483 genetic diversity [83,84]. Third, high-diversity genes are harder to assemble, *per se*. However, as all
484 three biases will tend to artefactually reduce diversity in the genome-wide dataset relative to Ago2,
485 this makes our finding that Ago2 paralogues display unusually low diversity conservative.

486 Identifying selective sweeps in Ago2 paralogues of *D. pseudoobscura*

487 To test whether the unusually low diversity seen in the *D. pseudoobscura* Ago2 paralogues is due to
488 recent selection or generally reduced diversity in that region of the genome, we compared diversity at
489 each paralogue to diversity in their neighbouring regions. We obtained sequence data for the 50kb
490 either side of each of these paralogues from the 11 whole genomes detailed in [36]. Note that the very
491 high similarity of these Ago2 paralogues means that they cannot be accurately assembled from short
492 read data, and are not present in the data from [36]. For each genome, we therefore replaced the
493 poorly-assembled region corresponding to the paralogue with one of our own Sanger-sequenced
494 haplotypes, making a set of 11 ca. 102kb sequences for each paralogue. We aligned these
495 sequences using PRANK [85] with default settings, and calculated Watterson's θ at all sites in a
496 sliding window across each alignment, with a window size of 5kb and a step of 1kb. For Ago2a1 and

497 Ago2a3, which are located in tandem, we analysed the same genomic region. Since our Ago2
498 haplotypes were sampled from a different North American population of *D. pseudoobscura* to those of
499 [36], an apparent reduction in local diversity might result from differences in diversity between the two
500 populations. Therefore, we also repeated these analyses on a dataset in which our Sanger
501 sequenced haplotypes were removed, leaving missing data.

502 To test explicitly for selective sweeps at each region, we used Sweepfinder [86] to calculate the
503 likelihood and location of a sweep in or near each Ago2 paralogue. We specified a grid size of 20,000,
504 a folded frequency spectrum for all sites, and included invariant sites. To infer the significance of any
505 observed peaks in the composite likelihood ratio, we used ms [87] to generate 1000 samples of 11
506 sequences under a neutral coalescent model. We generated separate samples for each region
507 surrounding an Ago2 paralogue, conditioning on the number of polymorphic sites observed in that
508 region, the sequence length equal to the alignment length, and an effective population size at 10^6
509 (based on a previous estimate for *D. melanogaster* by [88]). We specified the recombination rate at
510 5cM/Mb, a conservative value based on previous estimates for *D. pseudoobscura* [36], which will lead
511 to larger segregating linkage groups and therefore a more stringent significance threshold.

512

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748 Supporting Information Captions

749 **S1 Figure: The expression of *D. pseudoobscura* Ago2 paralogues in embryos.** Error bars
750 indicate 1 standard error estimated from 2 technical replicates in each of two different genetic
751 backgrounds. *D. melanogaster* expression levels were taken from a single publicly-available RNA-seq
752 experiment [71]. Ago2c is highly expressed in embryos, but none of the testis-specific Ago2
753 paralogues (Ago2a, Ago2b & Ago2e) are expressed.

754 **S2 Figure: The tissue-specific expression patterns of other members of the Argonaute gene**
755 **family (Ago1, Ago3, Aub & Piwi) in *D. melanogaster* and *D. pseudoobscura*.** For *D.*
756 *pseudoobscura* embryo, error bars indicate 1 standard error estimated from 2 technical replicates in
757 each of two different genetic backgrounds. For all other *D. pseudoobscura* tissues, error bars indicate
758 1 standard error estimated from 2 technical replicates in each of five different genetic backgrounds. *D.*
759 *melanogaster* expression levels were taken from a single RNA-seq experiment [71]. In *D.*
760 *pseudoobscura*, Ago1 is expressed in all tissues, but the other genes are only expressed in the
761 embryo and germline.

762 **S3 Figure: The distribution of synonymous site diversity across genes, derived from genome**
763 **(*D. pseudoobscura*) or transcriptome (*D. subobscura* & *D. obscura*) data.** The percentile of the
764 distribution into which each paralogue falls is indicated in brackets under the paralogue name. In each
765 species, members of the Ago2a and Ago2e subclades have very low diversity compared with the
766 genome as a whole.

767 **S4 Figure: The distribution of codon usage bias, derived from genome (*D. pseudoobscura*) or**
768 **transcriptome (*D. subobscura* & *D. obscura*) data.** The percentile of the distribution into which
769 each paralogue falls is indicated in brackets under the paralogue name. Ago2e has a very low
770 effective number of codons (ENC) compared with the genome as a whole, indicating a high degree of
771 codon usage bias.

772 **S5 Figure: Genetic diversity in the regions surrounding each *D. pseudoobscura* Ago2**
773 **paralogue, with Ago2 paralogue haplotype sequences removed.** After specifying Ago2 paralogue
774 sequence data as missing information, sharp troughs in diversity remain at Ago2a, Ago2b and Ago2c,
775 indicating a selective sweep.

776 **S6 Figure: The tissue-specific expression patterns of the Argonaute gene family in *D. willistoni***

and *M domestica*. Transcriptome data for *D. willistoni* were taken from [60], and transcriptome data for *M. domestica* were taken from [59]. For both species, we mapped reads to coding sequences using Bowtie 2.1 [78], counted reads mapping to each coding sequence using HTSeq [89], and converted counts to reads per kilobase per million reads (RPKM [90]) to account for coding sequence length and sequencing depth. For *M. domestica*, error bars indicate two biological replicates, each in a different genetic background.

S1 Table: McDonald-Kreitman test results. Pn & Ps are the number of within-species polymorphisms after singletons have been removed. All values are displayed to 2dp, except ω_a which is displayed to 4dp.

S2 Table: McDonald-Kreitman test results with alternative outgroups. Pn & Ps are the number of within-species polymorphisms after singletons have been removed. All values are displayed to 2dp, except ω_a which is displayed to 4dp.

S3 Table: Genetic backgrounds used in each experiment. Line refers to an individual isofemale line, and Origin refers to the geographic location where the female who founded that line was caught.

S4 Table: Primers used for PCR and qPCR amplification of Ago2 paralogues. All primers are displayed in the 5' to 3' direction.

S5 Table: Primers used for Sanger sequencing of Ago2 paralogue haplotypes. All primers are displayed in the 5' to 3' direction.

S1 Appendix: Sequence alignment of drosophilid Ago2 homologues. This alignment has had all 3rd positions stripped, and was used for time-scaled phylogenetic analysis of drosophilid Ago2 evolution.

S2 Appendix: Sequence alignment of drosophilid Ago2 homologues. This alignment has had all 3rd positions stripped, and was used for model-based analysis of differential evolutionary rate and codon-specific positive selection.

S3 Appendix: Sequence metadata for drosophilid Ago2 homologues.

S4 Appendix: Sequence polymorphism data for *D. subobscura*, *D. obscura* and *D. pseudoobscura* Ago2 paralogues

S5 Appendix: Raw data used to plot Figures 3, 4, 5, S1, S2, S3, S4 & S6.