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2

3 **FUNCTIONAL DIVERGENCE OF THE MIRNA TRANSCRIPTOME AT THE ONSET**
4 **OF *DROSOPHILA* METAMORPHOSIS**

5

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19 miRNA-mRNA associations

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21 Running head: Evolution of miRNA expression levels

22

24MicroRNAs (miRNAs) are endogenous RNA molecules that regulate gene expression
25post-transcriptionally. To date, the emergence of miRNAs and their patterns of
26sequence evolution have been analyzed in great detail. However, the extent to
27which miRNA expression levels have evolved over time, the role different
28evolutionary forces play in shaping these changes, and whether this variation in
29miRNA expression can reveal the interplay between miRNAs and mRNAs remain
30poorly understood. This is especially true for miRNA expressed during key
31developmental transitions. Here, we assayed miRNA expression levels immediately
32before (≥ 18 BPF) and after (PF) the increase in the hormone ecdysone responsible
33for triggering metamorphosis. We did so in four strains of *Drosophila melanogaster*
34and two closely related species. In contrast to their sequence conservation, $\sim 25\%$
35of miRNAs analyzed showed significant within-species variation in male expression
36levels at ≥ 18 BPF and/or PF. Additionally, $\sim 33\%$ showed modifications in their
37pattern of expression bias between developmental timepoints. A separate analysis
38of the ≥ 18 BPF and PF stages revealed that changes in miRNA abundance
39accumulate linearly over evolutionary time at PF but not at ≥ 18 BPF. Importantly,
40 ≥ 18 BPF-enriched miRNAs showed the greatest variation in expression levels both
41within and between species, so are the less likely to evolve under stabilizing
42selection. Functional attributes, such as expression ubiquity, appeared more tightly
43associated with lower levels of miRNA expression polymorphism at PF than at
44 ≥ 18 BPF. Furthermore, ≥ 18 BPF- and PF-enriched miRNAs showed opposite patterns
45of covariation in expression with mRNAs, which denoted the type of regulatory
46relationship between miRNAs and mRNAs. Collectively, our results show contrasting

47 patterns of functional divergence associated with miRNA expression levels during
48 *Drosophila* ontogeny.

49

51 Precise regulation of gene expression is instrumental for proper execution of the
52 majority of biological processes including cell differentiation and homeostasis. A
53 key parameter of this regulation is mRNA abundance, which is influenced post-
54 transcriptionally by microRNAs (miRNAs) . MiRNAs are small (~22 nt) non-coding
55 RNA trans-acting factors that induce mRNA decay or translation inhibition by base
56 pairing with complementary regions on the mRNA molecule . This complementarity
57 involves Watson-Crick pairing with particular motifs at the 3' untranslated region
58 (UTR) of the mRNA, although it can also occur at the 5' UTR or coding region .
59 Importantly, miRNAs found in distantly related taxa tend to exhibit a remarkable
60 degree of sequence conservation, especially in the so-called "seed" motif, which is
61 close to the 5' end of the miRNA and is critical for the interaction with the targeted
62 mRNAs .

63 MiRNAs are thought to ameliorate expression noise in expression networks and,
64 consequently, increase the robustness of developmental systems contributing to
65 phenotypic stability . It is therefore of special interest to determine the limits to
66 which changes in miRNA expression attributes such as the expression level can be
67 accommodated over evolutionary time. Changes in miRNA abundance can
68 contribute to inter-individual variation in expression of miRNA-regulated targets
69 ultimately impacting on protein levels and distribution . Some changes in miRNA
70 abundance have been shown to account for variability in platelet reactivity and drug
71 sensitivity in humans , elicit common disorders such as cancer , or underlie the
72 intraspecific variation of morphological characters . Among closely related species,
73 divergence in miRNA abundance has also been linked to important functional and
74 phenotypic consequences. In the natural occurring hybrid of two sister *Arabidopsis*

75species, miRNA expression diversity from the parental species results in novel
76phenotypes that contribute to adaptation . In primates, it has been proposed that
77miRNA-mediated differences in mRNA abundance underlie partly the evolution of
78human cognitive functions . To date, the malleability in miRNA expression levels
79during key developmental transitions requiring precise regulation of gene
80expression remains largely unexplored both at the intra- and interspecific levels.

81Metamorphosis is an intricate biological process in which large-scale tissue
82remodeling and organogenesis are orchestrated . In *Drosophila*, fluctuation of 20-
83hydroxyEcdysone (20E) level induces the transition from larva to immobile pupa .
84This transition is accompanied by multiple changes in mRNA abundance , with some
85occurring in a sex-dependent fashion . Evolutionary changes in mRNA abundance
86have been reported both within and between closely related species of the
87*Drosophila melanogaster* species subgroup at the onset of metamorphosis . MiRNAs
88play critical roles during insect metamorphosis and in fact their expression profiles
89have also been characterized at the onset of *D. melanogaster* metamorphosis .
90However, neither the extent to which miRNAs can accommodate intra- and inter-
91specific changes in abundance nor the interplay of these evolved changes with
92fluctuation in target mRNA abundance have been elucidated. This is especially
93relevant when considering the contrast between the two stages that define this
94transition: late third instar larva stage, mostly characterized by the transition into a
95post-feeding stage, wandering, and finding a place to glue; and puparium formation,
96mostly characterized by an effective deployment of part of the developmental
97blueprint that leads to the formation of an adult individual. Here, we address these
98issues by examining miRNA expression profiles and their evolutionary patterns
99within the *D. melanogaster* species group. We generate a portrait of how miRNA

100expression levels have been shaped by different evolutionary mechanisms at larva
101and pupa stages and how they have been impacted by different factors during this
102key organismal transition.

103

104

RESULTS AND DISCUSSION

105**MiRNA expression profiles at the onset of metamorphosis**

106***Expressed miRNAs at the onset of metamorphosis.*** We surveyed miRNA
107expression at late third instar larvae (18 hours before puparium formation; ≥ 18 BPF)
108and white prepuparium (at puparium formation; PF) in both sexes of *D.*
109*melanogaster* Oregon-R and in males of its close relatives *D. simulans* and *D.*
110*yakuba* using Illumina RNA-seq (Material and Methods; supplementary table S1 and
111dataset S1, Supplementary Material online). These two species shared ancestor
112with *D. melanogaster* ~ 5.4 and ~ 12.8 million years ago . We found reads
113corresponding to sequences of $\sim 76\%$ (130 out of 171) of the miRNAs registered in
114miRBase release 15 for *D. melanogaster* , which is comparable to other surveys
115(supplementary figure S1A, Supplementary Material online). Of the miRNAs with
116evidence of expression, $\sim 73\%$ (94 out of 130) were supported by sequence reads
117both in our Illumina dataset and in previously generated sequence reads by 454
118and J.M. Ranz and M. Ashburner, unpublished results; supplementary table S2 and
119alignments, Supplementary Material online). Interestingly, $\sim 38\%$ (41 out of 107)
120and $\sim 29\%$ (35 out of 120) of the miRNA genes with sequence reads in the ≥ 18 BPF
121and PF Illumina datasets, respectively, do so in one sex only, suggesting some
122potential sex-biased expression (supplementary figure S1B, Supplementary Material
123online).

124In the case of *D. simulans* and *D. yakuba*, we documented the expression of 81 and
12595 orthologous miRNAs, respectively (supplementary dataset S1, Supplementary
126Material online). Among these orthologs, 9 in *D. simulans* and 33 in *D. yakuba* were
127either not listed in miRBase release 15 or listed with an associated hairpin sequence
128for which we found no support due to nucleotide differences relative to the
129reference genomes sequence of these species (e.g. *miR-277-3p* and *miR-305-5p* in
130*D. simulans*; supplementary figure S2, Supplementary Material online).

131As a preamble to our analyses on polymorphism and divergence (see below), we
132assayed levels of miRNA expression at ≥ 18 BPF and PF in males using a microarray
133platform that included: i) reporters for miRNAs registered in miRBase release 15
134across insect species; ii) 100 additional reporters based on previous deep-
135sequencing results from several *Drosophila* species; and iii) several controls
136(Material and Methods; supplementary figure S3 and table S3, Supplementary
137Material online). We examined four strains of *D. melanogaster*, including one
138African strain to better reflect the recent demographic history of the species, and
139one strain of each of its relatives *D. simulans* and *D. yakuba* (Material and Methods;
140supplementary table S1, Supplementary Material online). A total of 280 reporters
141representing 132 and 148 sequences of the 5' and 3' arms, respectively, of different
142miRNA genes relevant to the six strains of the *D. melanogaster* species subgroup
143were subject to downstream analyses. The potential impact of nucleotide
144mismatches on expression estimates across *D. melanogaster* strains was found to
145be limited to two reporters (supplementary text, table S4, and figure S4,
146Supplementary Material online). Within the subset of reporters considered, 120
147were identical in sequence for the three species while the rest provided reliable
148information for two or one of the species only. Per species, we found similar counts

149of expressed miRNA reporters: 77 in *D. melanogaster*; 73 in *D. simulans*; and 70 in
150*D. yakuba*. Among the 67 miRNA reporters that are conserved in sequence across
151the three species and could be assayed in our arrays, 55 (83%) were confirmed to
152be expressed at either ≥ 18 BPF, PF, or both in all four *D. melanogaster* strains
153(supplementary dataset S1, Supplementary Material online). The reliability of our
154expression measures using an array platform was supported by the high positive
155correlations between biological replicates (supplementary figure S3, Supplementary
156Material online), by replicating some experiments with qRT-PCR (supplementary
157text, figures S5-S6, and table S5, Supplementary Material online), and by the good
158agreement between the expression estimates obtained between microarrays and
159deep-sequencing experiments (Spearman's $\rho = 0.6171$, $P < 0.0001$; supplementary
160figure S7, Supplementary Material online).

161**MiRNA expression variability during male development.** Microarray profiling
162across males from six *Drosophila* strains revealed the miRNA reporters differentially
163expressed between ≥ 18 BPF and PF (*i.e.* ≥ 18 BPF- and PF-enriched; figure 1A).
164Concordant with early single-strain studies, known ecdysone-induced miRNAs *let-7-*
1655*p*, *miR-125-5p*, and *miR-100-5p*, were PF-enriched across *D. melanogaster* strains,
166as they were in *D. simulans* and *D. yakuba*. Conversely, *miR-34-5p*, which is down-
167regulated by the transcription factor Broad in high ecdysone titer conditions, was
168found to be ≥ 18 BPF-enriched across strains, a pattern also displayed by *miR-8-5p*.
169Nevertheless, the precise fraction of developmentally regulated miRNA reporters
170varied from strain to strain ranging from 32% in *D. yakuba* to 62% in the *D.*
171*melanogaster* strain Zimbabwe-109, being the median 42% (table 1; supplementary
172figure S8, Supplementary Material online).

173 Among *D. melanogaster* males, we confirmed several types of variation associated
174 with miRNA expression levels. ~25% (15 out of 60) of the expressed miRNAs
175 suitable for comparison across the four *D. melanogaster* strains showed significant
176 differences in expression levels at ≥ 18 BPF (6), at PF (4), or at both (5) ($P_{\text{adj}} < 0.01$;
177 Material and Methods). Further inspection revealed that only ~66% (42 out of 64)
178 of the miRNA reporters with detectable level of expression in at least three strains
179 showed consistency in the type of developmental expression pattern, e.g. PF
180 enrichment across all the strains (supplementary table S6, Supplementary Material
181 online). The remaining 33% of miRNAs (22 out of 64) harbored differences in their
182 developmental expression pattern among strains. This is the case of *miR-956-3p*,
183 which is ≥ 18 BPF-enriched in all strains of *D. melanogaster* but in Zimbabwe-109
184 (figure 1B). Only the reporter corresponding to *miR-289-5p* showed opposite
185 developmental regulation, i.e. ≥ 18 BPF or PF enrichment depending on the strain.
186 No difference in the ratio of developmentally to non-developmentally regulated
187 miRNAs (21:21) was observed among reporters categorized as consistent.

188 Among the strains assayed, Zimbabwe-109 showed the largest proportion of PF-
189 enriched miRNAs among those expressed (supplementary figure S8, Supplementary
190 Material online), a difference that is statistically significant (randomization test of
191 goodness-of-fit, $P_{\text{adj}} = 2.9 \times 10^{-2}$ when Zimbabwe-109 is included and $P_{\text{adj}} = 3.1 \times 10^{-1}$
192 when excluded; $P_{\text{adj}} < 0.05$ when any other strain is omitted). To determine whether
193 the lineage leading to Zimbabwe-109 or that leading to the other three strains of *D.*
194 *melanogaster* accumulated the most alterations in developmental expression
195 pattern, we included the strains of *D. simulans* and *D. yakuba* to phylogenetically
196 polarize the differences recorded (supplementary figure S9, Supplementary Material
197 online). Assuming maximum parsimony, the branch associated with Zimbabwe-109

198 appears to have accumulated more differences in developmental expression
199 pattern than that leading to the other three *D. melanogaster* strains.

200 Consistency in the developmental expression pattern across strains does not
201 preclude more subtle changes in miRNA abundance. For example, *miR-1012-5p*
202 was categorized as non-developmentally enriched across all strains, however it
203 entailed statistically significant differences at PF among some of them (ANOVA,
204 $P_{\text{adj}} < 0.01$; figure 1B). To evaluate the extent of these more subtle changes in
205 expression, we focused on those miRNAs showing consistent patterns of expression
206 not only within *D. melanogaster* but also across *D. simulans* and *D. yakuba*. Thirty
207 such miRNA reporters were documented (8 ≥ 18 BPF-enriched; 5 PF-enriched; 18
208 non-developmentally enriched) of which 17% (2 ≥ 18 BPF-enriched, 2 PF-enriched,
209 and 1 non-developmentally enriched) showed significant differences at ≥ 18 BPF, PF,
210 or both ($P_{\text{adj}} < 0.01$; supplementary dataset S2, Supplementary Material online).
211 Taken together, all these forms of variation pointed towards a malleable miRNA
212 transcriptome at the onset of metamorphosis.

213 **Gender differences in miRNA expression.** We assayed miRNA expression levels
214 in females from two strains of *D. melanogaster* and one of *D. simulans* finding a
215 similar fraction of developmentally regulated miRNAs to that in males (table 1;
216 supplementary figure S10, Supplementary Material online). 78% (54 out of 69) of
217 miRNAs with detectable level of expression in females of the two *D. melanogaster*
218 strains showed a consistent expression pattern between developmental stages with
219 24 of them (45%) exhibiting developmental regulation (11 ≥ 18 BPF-enriched and 13
220 PF-enriched). Unlike in males, the proportion of miRNAs displaying different
221 developmental expression patterns did not differ across strains (supplementary

222figure S8, Supplementary Material online; randomization test of goodness-of-fit,
223 $P_{\text{adj}}=1.5\times 10^{-1}$; males -including the same three strains as in females-, $P_{\text{adj}}=6.0\times 10^{-3}$).
224Sex-biased gene expression for the protein-coding fraction of the genome has been
225examined at PF , but no equivalent analysis has been performed for miRNAs.
226Although limited, we did find evidence of miRNA sex-biased expression occurring as
227early as ≥ 18 BPF (supplementary figure S11A, Supplementary Material online). Most
228miRNAs though, whether developmentally regulated or not, showed no evidence of
229sex-bias in expression (supplementary figure S12A-B, Supplementary Material
230online). At $P_{\text{adj}}<0.01$, 10 miRNA reporters showed significant sex-biased expression
231in at least one of the six strains by sex combinations assayed, a number that
232increased up to 22 at $P_{\text{adj}}<0.05$ (supplementary figure S11B and dataset S2,
233Supplementary Material online). Among miRNA reporters showing sex-bias in
234expression, we found cases such as *miR-964-5p*, which displayed differences in
235expression between the genders at a single developmental stage, and others like
236*miR-312-3p*, which did at both ≥ 18 BPF and PF (supplementary figure S12C-D,
237Supplementary Material online). No case involving reversal in the pattern of
238developmental enrichment between the sexes, e.g. from ≥ 18 BPF enrichment in
239males to PF enrichment in females, was detected.

240Among the miRNA reporters showing sex-biased gene expression, the cluster *miR-*
241*310 - miR-313* stood out. Each of the four constituent miRNA genes showed
242statistically significant male-biased expression in at least two of the six strains by
243developmental stage combinations assayed (supplementary figure S12D,
244Supplementary Material online). Although the sequence similarity of some of the
245miRNA genes in the cluster (*miR-310-3p*, *miR-311-3p*, and *miR-312-3p*) could give
246rise to some apparent co-expression, inspection of the deep-sequencing data for the

247strain Oregon-R (supplementary alignments, Supplementary Material online) and
248the inferred co-expression of the mir-310 cluster based on deep-sequencing data
249ruled out this possibility. Therefore, the observed sex-bias expression pattern
250strongly suggests a sex-dependent co-regulation of the mir-310 cluster at PF. This
251interpretation agrees well with the influence of this cluster on male fertility by
252modulating the Wingless signaling pathway, which is required for cell differentiation
253of the somatic and germline tissues in testis . Intriguingly, the expression of *miR-*
254*313-5p* could represent a case of sex-dependent arm-switching.

255

256**Evolution of miRNA expression profiles**

257***Differentiation of expression levels at ≥ 18 BPF and PF.*** The multiple
258developmental changes occurring during early pupation compared to late instar
259larvae parallel changes in mRNA abundance of many genes during this
260developmental transition . Expression profiles of regulatory genes at PF should be
261especially refractory to change due to potential detrimental effects. We tested
262whether this hypothesis was reflected in miRNA expression levels by examining the
263way significant differences have accumulated over evolutionary time and by
264comparing the magnitude of expression differences between stages.

265We calculated expression distances among the males of the six strains surveyed at
266 ≥ 18 BPF and PF separately as well as the divergence time for each strain pair
267(Material and Methods; supplementary text and table S7, Supplementary Material
268online). At PF, unlike at ≥ 18 BPF, expression divergence in miRNA abundance did
269correlate with divergence time (figure 2A and supplementary figure S13,

270Supplementary Material online). This result suggests that the way changes in
271miRNA abundance accumulate over evolutionary time differs between the stages.

272We subsequently estimated the magnitude of miRNA expression differences across
273the six strains as the coefficient of variation (CV), *i.e.* the ratio of the standard
274deviation to the mean. We compared the log₁₀-transformed CV at ≥ 18 BPF and PF
275finding no significant difference (average CV: ≥ 18 BPF, 0.761; PF, 0.729; one-way
276ANOVA, $P=0.630$). Nevertheless, absence of significant differences in the global
277levels of variation does not rule out more subtle patterns of differentiation among
278particular groups of miRNAs based on their expression attributes. According to this,
279we tested for differences in expression levels among miRNAs showing consistent
280developmental expression patterns in *D. melanogaster*. Both at ≥ 18 BPF (one-way
281ANOVA, $P=0.012$) and PF (one-way ANOVA, $P=0.005$), we found the same rank of
282differentiation in miRNA expression levels: ≥ 18 BPF-enriched > PF-enriched > non-
283developmentally enriched miRNAs (figure 2B). *Post hoc* tests underscored the
284difference between ≥ 18 BPF- and non-developmentally enriched miRNAs (Tukey-
285Kramer HSD; ≥ 18 BPF, $P=0.019$; PF, $P=0.003$). Collectively, these results suggest
286that the two developmental stages sampled might be subject to different
287evolutionary dynamics, which affect the pace at which expression changes
288accumulate over evolutionary time and the global levels of differentiation of
289particular groups of miRNAs.

290**Evolutionary mode and developmental expression pattern.** We determined
291the mode of evolution (*i.e.*, stabilizing selection, genetic drift, or directional
292selection) of miRNA expression levels at ≥ 18 BPF and PF. We used a two-step
293ANOVA-based approach in which differences in expression levels were tested first
294within and then between species (supplementary text, Supplementary Material

295online). ~75% (39 out of 48) and ~80% (47 out of 55) miRNAs showed a mode of
296evolution consistent with the action of stabilizing selection at ≥ 18 BPF and PF
297respectively (figure 3A), a result reminiscent of previous observations for mRNA
298levels . Our results suggest that directional selection has acted on the expression
299level of a few miRNAs: *miR-34-5p* and *miR-956-3p* at ≥ 18 BPF, and *miR-34-5p*, *miR-*
300*312-3p*, and *miR-995-3p* at PF. The remaining miRNAs exhibited patterns of
301variation within and between species compatible with genetic drift alone or with
302other evolutionary scenarios such as relaxation of constraints in a lineage-
303dependent manner or combinations of genetic drift and some form lineage-
304dependent selection (figure 3B).

305If miRNAs with different developmental expression patterns differ in their degree of
306variation in expression levels (fig. 2B), they should also show a different propensity
307to evolve under stabilizing selection. We confirmed this non-random association
308especially at PF (supplementary figure S14, Supplementary Material online). At this
309developmental stage, non-developmentally enriched and PF-enriched miRNAs
310largely evolve under stabilizing selection (~83% in both cases) while only 50% of
311the ≥ 18 BPF-enriched miRNAs follow this mode of evolution. The difference in the
312proportion of miRNAs evolving under stabilizing selection is statistically significant
313between ≥ 18 BPF- and non-developmentally enriched miRNAs (two-tailed Fisher's
314exact test, FET; ≥ 18 BPF, $P=0.040$; PF, $P=0.011$). Why the expression level of
315 ≥ 18 BPF-enriched miRNAs is more variable overall than that of non-developmentally
316enriched miRNAs is unclear at this time. Nevertheless, this pattern reinforces the
317notion that miRNAs with different developmental expression patterns are exposed
318to different evolutionary pressures.

319 **Evolutionary mode and miRNA evolutionary age.** We examined whether the
320 evolutionary mode in miRNA expression is related to evolutionary age, *i.e.* the
321 moment at which we can parsimoniously date the emergence of a miRNA.
322 Presumably, more ancient miRNAs should be more stably integrated into regulatory
323 networks than younger miRNAs and thus should more likely evolve under stabilizing
324 selection ([Chen and Rajewsky 2007](#)). We dated the emergence of miRNAs
325 assuming a maximum parsimony framework (supplementary text and dataset S3,
326 Supplementary Material online) distinguishing between two main age classes. The
327 first class included miRNAs inferred to have emerged during the evolution of the
328 subgenus *Sophophora* after the split with the subgenus *Drosophila* -and therefore
329 less likely to evolve under stabilizing selection-, and the second class included
330 miRNAs inferred to have been present in the common ancestor of both subgenera -
331 and therefore more likely to be already stably integrated into the regulatory
332 network-. Subsequently, we examined whether these two age classes differ in the
333 extent to which they evolve under stabilizing selection versus other evolutionary
334 modes. MiRNAs inferred to have emerged during the evolution of the subgenus
335 *Sophophora* (6 expressed at ≥ 18 BPF and 8 at PF) do evolve less often under
336 stabilizing selection than more ancient miRNAs (46 expressed at ≥ 18 BPF and 51 at
337 PF), which is confirmed at PF but not at ≥ 18 BPF (FET; ≥ 18 BPF, $P=0.157$; PF,
338 $P=0.046$). Although this result should be taken cautiously due to the limited count
339 of the *Sophophora* specific miRNAs present in the analysis, it suggests that the
340 expression levels of more recently evolved miRNAs have not had enough time to be
341 shaped by natural selection to the extent the levels of expression of more ancient
342 miRNAs have been.

343 **Determinants of miRNA expression polymorphism in *D. melanogaster***
344 **males.** The regulatory role of recently emerged miRNAs is more likely to be still
345 evolving compared to that of miRNAs found in many phyla. Due to the potentially
346 detrimental effects on organismal fitness of recently evolved miRNAs if expressed to
347 a high level or across multiple tissues/organs, these miRNAs should exhibit lower
348 expression levels and narrower spatiotemporal expression profiles than ancient
349 miRNAs. Consistent with this notion, recently evolved miRNAs have been shown to
350 be expressed at a low level in primates and *Drosophila* species. Further, the
351 introduction of the relatively young *mir-310* family from *D. pseudoobscura* into the
352 *D. melanogaster* genome resulted in misexpression of numerous genes and in lower
353 organismal fitness. Based on these premises, miRNAs that are either present
354 across most metazoans, exhibit a detrimental phenotype if misexpressed, or are
355 more ubiquitously expressed across tissues and/or developmental stages should
356 possess lower expression polymorphism.

357 We tested for a negative relationship between the intraspecific $\log_{10}CV$ in
358 expression of 62 miRNAs expressed at least in one of two studied developmental
359 stages in *D. melanogaster* and evolutionary age, having associated a gain-of-
360 function phenotype, and expression breadth across developmental stages and
361 tissues. We did so for each developmental stage separately. For the evolutionary
362 age, we distinguished broadly between *Drosophila* evolved miRNAs versus miRNAs
363 inferred to have been present in the ancestor to the *Drosophila* genus and
364 *Anopheles gambiae* (age classes *young* and *ancient* in figure 4A), finding evidence
365 of a significant negative association with the level of expression polymorphism
366 (one-way ANOVA; ≥ 18 BPF, $P=0.086$; PF, $P=0.008$). When the number of age
367 classes considered was further divided into four based on additional phylogenetic

368partitions (supplementary text, Supplementary Material online), the trend was
369similar (one-way ANOVA; $\geq 18\text{BPF}$, $P=0.069$; PF, $P=0.034$). In this case, pairwise
370*post hoc* tests revealed, at least for PF, that the diametrically opposed relationship
371between the expression polymorphism of miRNAs originated during the evolution of
372the subgenus *Sophophora* versus that of the most ancient miRNAs is the main
373factor contributing to the pattern found (supplementary figure S15A and table S8,
374Supplementary Material online). Considering age as a continuous variable did not
375alter this observation (supplementary figure S15B, Supplementary Material online).

376Phenotypic effects upon inducing miRNA misexpression or being ubiquitously
377expressed were associated similarly with miRNA expression polymorphism in *D.*
378*melanogaster*. MiRNAs displaying gain-of-function phenotypes were found to harbor
379significantly lower CVs in expression than miRNAs with no phenotype especially at
380PF (one-way ANOVA; $\geq 18\text{BPF}$, $P=0.089$; PF, $P=0.017$) (figure 4B). Furthermore, for
381the expression breadth, we found a significant negative correlation between
382expression ubiquity and $\log_{10}\text{CV}$, a pattern confined to the PF stage ($r^2=0.228$,
383 $P=0.004$; $\geq 18\text{BPF}$, $r^2=0.021$, $P=0.426$) (figure 4C). These results confirm that
384miRNAs that are more necessary for obtaining a wild type phenotype and miRNAs
385with more ubiquitous expression profiles, which are likely to be exposed to
386conflicting functional requirements across tissues, are more constrained in their
387capability to accommodate segregating expression variance during population
388differentiation. Importantly, these functional constraints are more apparent at PF
389than at $\geq 18\text{BPF}$.

390The negative correlations observed between miRNA expression polymorphism and
391evolutionary age, gain-of-function phenotypes, and expression ubiquity are unlikely
392to be independent from one another. This would be the case if more recently

393originated miRNAs occupy less relevant positions in the regulatory network and
394have narrower spatiotemporal expression profiles, which can result in better
395accommodating higher levels of expression polymorphism. We did find statistical
396evidence of the association among these variables pointing to this parsimonious
397view (supplementary text, Supplementary Material online), which is consistent with
398a higher association of ancient miRNAs with disease phenotypes and with broader
399expression profiles across tissues, compared to recently originated miRNAs,
400reported in humans and *Diptera* respectively .

401

402**Using intraspecific variation in expression levels to uncover the landscape** 403**of the miRNA-mRNA regulatory network**

404To uncover the interface between miRNAs and mRNAs at the expression level at the
405onset of metamorphosis, we leveraged the intraspecific variation in expression
406levels for both molecules. We identified developmentally regulated miRNAs upon
407pooling the miRNA expression data from the males of the four strains of *D.*
408*melanogaster* ($P_{\text{adj}} < 0.05$; supplementary text, Supplementary Material online).
409Then, we assayed levels of mRNA abundance from the same biological samples and
410used a mixed-effects linear model to estimate the expression association between
411developmentally regulated miRNAs and the transcripts for which the microarray
412platform had distinctive probesets, *i.e.* the so-called mRNA exemplars (Material and
413Methods supplementary; figure S3 and text, Supplementary Material online). An
414mRNA exemplar might correspond to one or more transcripts. Permutation tests
415helped determine whether the observed miRNA-mRNA association values were
416higher than expected by chance alone ($P_{\text{adj}} < 0.05$; supplementary text,
417Supplementary Material online).

418 We identified 617 mRNA exemplars, representing 545 genes, as significantly
419 associated with 43 developmentally regulated miRNAs (supplementary datasets S4-
420 S5). The number of significant associations with mRNA exemplars varied
421 remarkably among miRNAs (average \pm SD / median; $\sim 217 \pm \sim 133$ / 212;
422 supplementary figure S16), with *miR-34-3p* displaying the highest number – 612.
423 These statistically significant associations between expression levels of miRNAs and
424 mRNAs may reflect concurrent co-regulation by a common upstream factor in the
425 transcriptional hierarchy or a *bona fide* causal regulatory relationship, which can
426 result from either direct or indirect targeting, the latter as it might occur between a
427 miRNA regulating a transcription factor and the battery of genes under the
428 transcription factor's control. We investigated the interplay between expression
429 associations and their potential causal regulatory nature by considering the sign of
430 the association, *i.e.* positive or negative, the link with miRNA developmental
431 expression patterns, and the associated biological coherent patterns.

432 We distinguished between positive and negative associations in expression for each
433 miRNA-mRNA exemplar pair. Importantly, when a miRNA covaried in expression
434 with an mRNA exemplar, it was more likely to show a negative than a positive
435 association (supplementary figure S16, Supplementary Material online). As
436 reported in other organisms such as primates, positive expression associations
437 were also abundant, which highlights the ambiguous nature of the interplay
438 between expression levels of miRNAs and mRNAs. Intriguingly, developmentally
439 regulated miRNAs showed a bimodal distribution for the proportion of negative
440 significant associations over the total exhibited by each miRNA (figure 5A). We
441 investigated whether this bimodal distribution was related to miRNA developmental
442 expression patterns in a non-random manner. Specifically, we calculated the

443 correlation between the proportion of negative significant associations and the
444 propensity of a miRNA in showing a particular type of developmental expression
445 pattern across the four strains assayed (figure 5B). We found that the higher is the
446 number of strains showing miRNA expression enrichment at PF, the higher is the
447 proportion of negative miRNA-mRNA expression associations (Spearman's
448 $Rho=0.687$, $P<0.0001$). Accordingly, ≥ 18 BPF-enriched miRNAs across *D.*
449 *melanogaster* strains were more likely to exhibit positive expression associations
450 with mRNAs while PF-enriched miRNAs were more likely to exhibit negative
451 associations.

452 These patterns could denote distinctive relationships with the covarying mRNAs
453 (figure 5C). Down-regulation of a miRNA alone does not result directly in an
454 increase of its targets' abundance unless that miRNA is involved in a feedback loop
455 to inhibit the transcription of its targets. Therefore, positive miRNA-mRNA
456 expression associations may often reflect the concurrent down-regulation of both
457 molecules at PF denoting no causal regulatory relationship (figure 5C, left panel).
458 Conversely, the up-regulation of a miRNA at PF may be important to facilitate the
459 degradation of truly regulated targets that are not necessary at this stage, resulting
460 in a decreasing abundance (figure 5C, right panel). This second pattern is
461 reminiscent of the degradation of maternally deposited mRNAs by a set of
462 zygotically expressed miRNAs in the *Drosophila* embryo . In consequence, negative
463 associations of PF-enriched miRNAs and mRNAs should more likely represent *bona*
464 *fide* causal regulations by miRNAs. To test this, we examined whether PF-enriched
465 miRNAs showing negative expression associations with predicted targets among the
466 617 mRNA exemplars were significantly overrepresented relative to ≥ 18 BPF-
467 enriched miRNAs displaying equivalent properties. Indeed, we found that there are

468more predicted targets among mRNAs negatively associated with PF-enriched
469miRNAs than among those with ≥ 18 BPF-enriched miRNAs, a pattern not shown in
470positive expression associations (Randomization test of goodness-of-fit; $P=0.013$
471and $P=0.246$ respectively; supplementary table S9, Supplementary Material online).
472This difference reinforces the possibility that positive expression associations are
473less likely than negative expression associations in denoting *bona fide* causal
474miRNA regulation at the onset of metamorphosis.

475We further examined the biological properties of the miRNA-mRNA exemplar
476associations by searching for biological coherent patterns in genes grouped by their
477patterns of expression association with the 43 developmentally regulated miRNAs.
478Briefly, ten clusters of mRNA exemplars were identified by hierarchical cluster
479analysis based on the sign of their expression associations (supplementary figure
480S17, Supplementary Material online). Subsequently, functional enrichment for
481functional rubrics in each cluster was tested with DAVID under several degrees of
482stringency (Materials and Methods). We found enrichment for biological processes
483and other functional rubrics unambiguously related to the onset of metamorphosis
484in nine of the ten clusters (supplementary table S10, Supplementary Material
485online). Nucleotide biosynthesis and energy production pathways, structural and
486regulatory genes related to muscle formation, and genes involved in molting
487formation were enriched among those down-regulated at PF in clusters 1, 3, 4, and
4888-10. On the other hand, histolysis upon tissue apoptosis and innate immune
489response pathway related genes were found to be overrepresented among those
490up-regulated at PF in clusters 5 and 7.

491A closer inspection of several clusters unveiled the complexity of the regulatory
492interactions that occur during the transition from larva to pupa. For instance, 66

493mRNA exemplars in Cluster 8 were negatively associated with up to 27 miRNAs and
494positively associated with up to 14 miRNAs. This cluster is enriched for genes
495involved in the functional rubric "molting cycle" and the cellular component rubric
496"muscle myosin complex" (supplementary table S11, Supplementary Material
497online). Among the seven genes in Cluster 8 annotated as part of the functional
498rubrics relevant to muscle development ("muscle myosin complex" or "muscle
499protein"), five were predicted to harbor binding sites in their 3'UTRs according to
500TargetScan. Three of these genes showed negative associations in expression with
501miRNAs while the other two showed positive associations. The remaining two genes
502in Cluster 8 (*Tm2* and *Mlc2*) did not have any predicted miRNA binding site. Similar
503patterns were found for the constituent genes of Cluster 10, which are annotated as
504part of the functional rubric "contractile fiber" (supplementary table S11,
505Supplementary Material online).

506An intriguing aspect is that none of the genes that are a part of functional rubrics
507related to muscle development in Clusters 8 and 10 possess binding sites for *let-7*
508complex miRNAs in their 3'UTRs or ORFs. Nevertheless, the expression levels of
509these genes are negatively associated with those of the *let-7* complex miRNAs. The
510*let-7* complex is required for the maturation of neuromuscular junction and
511deformation of abdominal neuromusculature, which at least in part is achieved by
512downregulating the BTB-zinc finger transcription factor Ab . Several non-mutually
513exclusive explanations may account for this observation. First, miRNAs other than
514*let-7* may contribute to the regulation of these genes during metamorphosis
515(supplementary table S11, Supplementary Material online). Second, the *let-7*
516complex may regulate these genes indirectly through the control of their upstream

517transcription factor(s) during metamorphosis. Third, the expression levels of these
518genes are not regulated post-transcriptionally by miRNAs denoting false positives.

519The innate immune response of *Drosophila* at the onset of metamorphosis is well
520represented in the significant expression associations found. This response is
521regulated by ecdysone and juvenile hormone and mainly consists of two
522components: localized melanization and antimicrobial peptides production . We
523found that Cluster 7 is enriched for genes involved in these two components. The
524genes *Dat*, *e*, and *ple* were found to participate in the functional rubric "dopamine
525metabolic process", which contributes to melanization among other biological
526functions . Three other genes encode antimicrobial peptides: *Drs*, *Drsl2*, and *Drsl5*.
527The expression levels of these six genes are significantly higher at PF as expected
528and this work). Interestingly, positive expression associations were mostly found
529between genes and miRNAs that are predicted to bind their ORFs while the only two
530negative associations involve genes (*Drsl2* and *ple*) presumably bound at their
5313'UTRs by miRNAs (supplementary table S12, Supplementary Material online).
532Further, the putative miRNAs regulating upstream genes of the innate immune
533pathway have been studied *in silico* . Some of these miRNAs were present in our
534miRNA-mRNA exemplars association list such as the *miR-2* family, *miR-9a* (both
535arms), *miR-125-5p*, *miR-279-3p*, and *miR-281-2-5p*. Thus, the expression levels of
536these innate immune response genes may be miRNA-regulated directly or through
537their upstream regulators in those same immune pathways.

538Our characterization of the landscape of miRNA-mRNA exemplars associations is
539limited in two ways. First, it is dependent on the differential miRNA expression
540between the developmental stages compared. Second, some miRNAs are
541expressed in tissue- or cell-type-specific manners and therefore expression

542associations may happen in multiple organs but in different directions resulting in a
543blurry association signal if any. It is remarkable therefore that we are still able to
544capture significant expression associations from our whole-body assays, which in
545some cases are suggestive of *bona fide* causal regulation.

546We have generated a portrait of the intra- and inter-specific differences in
547expression levels of sequence conserved miRNAs at the onset of *Drosophila*
548metamorphosis. In spite of the documented phylogenetic differentiation in
549expression levels, we find that the evolution of miRNA abundance is driven mainly
550by stabilizing selection. This agrees well with the stabilizing role that miRNAs play
551by repressing leaky expression or fine-tuning transcript levels. Notably, the type of
552developmental expression pattern of a miRNA appears to be an excellent predictor
553of the degree to which a miRNA can accommodate variation in expression level
554during the evolutionary process. The expression levels of some miRNAs are still
555evolving, which in a few cases seems compatible with the optimization of their
556functional role by directional selection. Whether this functional optimization goes
557beyond canalizing expression levels during metamorphosis contributing as well to
558the phenotypic diversification in the genus *Drosophila* remains to be established.
559Overall, we find distinct patterns of differentiation among miRNA expression levels
560between late third instar larva and white prepupa, which is also evidenced in how
561these evolved changes are linked to relevant proxies for the integration of miRNAs
562into the regulatory network. In addition, miRNAs with different developmental
563expression patterns exhibit marked differences in how they covariate with mRNAs in
564expression, which might reflect the type of regulatory relationship between both
565kind of molecules. Taken together, the profound differences in the biological

566 requirements of these two developmental stages seem to dictate the properties in
567 miRNA expression levels at the onset of *Drosophila* metamorphosis.

568

569

MATERIALS AND METHODS

570 **Fly stocks and husbandry.** Six strains representing three species of the *D.*
571 *melanogaster* species group were used (supplementary table S1, Supplementary
572 Material online). Flies were grown in standard corn meal medium, constant lighting
573 conditions, and at 25°C. Third instar larvae were identified as previously reported .
574 Briefly, larvae were raised under non-crowded conditions in medium with 0.05%
575 bromophenol blue. Wandering larvae exhibiting dark blue gut, which corresponds
576 to ~18 hours before puparium formation, were collected, rinsed with water, and
577 separated by sex. For the 0-1 hr white prepuparia, wandering larvae with light blue
578 to white gut were separated by sex, placed in a Petri dish on damped light-wipe
579 tissue until appropriate moment for collection . The presence of visible male
580 gonads was used for sex identification; trial collections were performed to assure
581 process accuracy. Samples were snap frozen in liquid nitrogen and stored at -80C
582 until RNA isolation.

583 **RNA extractions.** Two rounds of RNA collection were performed: the first, for
584 small RNA-seq; and the second, for the remaining expression profiling approaches
585 used (see below). For each collection, ~120 mg (60-80 individuals depending on
586 the strain) of frozen flies for each biological replicate were grinded using motorized
587 pestles and the total RNAs extracted and purified with *miRVana* miRNA isolation kit
588 (Ambion Inc.), which allows the optional separation of mRNAs from small RNAs. In
589 the first collection, the enriched small RNAs were isolated according to the

590manufacturer indications for subsequent small RNA library preparation. In the
591second collection, both total RNAs and small RNAs from four biological replicates for
592each strain/sex were isolated and used in all three expression profiling approaches.
593Concentration, quality, and integrity of the RNA samples were assessed using the
594NanoDrop 8000 Spectrophotometer and the RNA 6000 Nano and Small RNA kits
595(Agilent Technologies) in an Agilent 2100 Bioanalyzer. RNA extractions for different
596strains were performed independently to avoid cross-contamination.

597**Small RNA-seq.** Adaptor-ligated cDNAs were prepared according to Illumina small
598RNA preparation protocol (Preparing Samples for Analysis of Small RNA Using the
599Oligo Only Kit, <http://www.illumina.com>). Briefly, 16-28nt small RNAs were size
600selected from a denaturing polyacrylamide gels, 5' adaptors with four nucleotide
601indexes and 3' adaptors were ligated using T4 RNA ligase (Ambion 2140) and the
602resulting molecules were purified by size selection from denaturing polyacrylamide
603gels after each ligation reaction. Four samples with different indexes were pooled
604for 3' adaptor ligation and further preparation steps. The ligated small RNAs were
605reversed transcribed using Superscript II reverse transcriptase (Invitrogen 18064),
606subsequently PCR amplified with a Phusion high fidelity PCR DNA polymerase
607(Finnzymes F-506) through 10 cycles, and purified from denaturing polyacrylamide
608gels. Library preparation for different strains was performed independently to avoid
609cross-contamination. The quality of the cDNAs was evaluated with the High
610Sensitivity DNA kit (Agilent Technologies) using an Agilent 2100 Bioanalyzer before
611high-throughput sequencing using a Genome Analyzer II (Illumina) at the Genome
612Center of the University of California, Davis. Twelve libraries, named as L5-L16 in
613supplementary table S2 (Supplementary Material online), were sequenced in three

614lanes. Reproducibility of sequencing results was evaluated at biological and
615technical levels.

616Reads were sorted by distinctive indexes before their 5' and 3' adaptors sequences
617were trimmed. Reads matching yeast rRNAs and *D. melanogaster* 2S rRNA were
618discarded. The remaining reads were aligned against the stem-loop sequences of
619known miRNAs according to miRBase release 15 using Bowtie 0.12.5 . Because of
620the potential errors in the reference genome of *D. simulans* and *D. yakuba*, we also
621used the *D. melanogaster* genome sequence as a reference in the characterization
622of the libraries of these two species. Alignments and read numbers were recorded.
623Four libraries sequenced by 454, named as L1-L4 in supplementary table S2
624(Supplementary Material online), were also included and J.M. Ranz and M.
625Ashburner, unpublished results). In-house Perl scripts were used for sequence
626processing.

627**miRNA microarray profiling.** The miRNAarthropoda_15_UC_100610 array from LC
628Sciences based on the annotations of miRBase release 15 was used. In addition,
629100 custom probes were added to the array (supplementary table S3,
630Supplementary Material online). Custom probes include previously dubbed
631passenger sequences of known *D. melanogaster* miRNAs, putative miRNAs found in
632deep-sequencing experiments by us and others , and control reporters for the 2S
633rRNA gene harboring nucleotide differences in number and position to evaluate the
634impact of mismatches on hybridization kinetics. Probes for annotated and predicted
635miRNAs were present in triplicates. Internal quality controls included 43 spikes and
636six additional positive controls replicated either 4 or 16 times on the array.

637Four biological replicates per developmental stage per strain were used in
638competitive hybridizations. Labeling, hybridization, and image acquisition were

639 performed by LC Sciences. For a given array, the Cy3 and Cy5 fluorescent intensity
 640 values of each array were first adjusted by subtracting local background and then
 641 normalized according to a locally-weighted regression approach . The adjusted
 642 values were further log₂-transformed and normalized across arrays using the
 643 quantile method implemented in JMP Genomics 5.0 . The expression values for the
 644 280 reporters relevant to the six strains of interest were obtained by averaging over
 645 the three technical replicates spotted on separate blocks of the array. A mean
 646 intensity value lower than 32 was not considered to be reflective of expression
 647 above the background. A given miRNA was not considered to be expressed if it
 648 lacked evidence of expression across all sex by strain by developmental stage
 649 combinations assayed.

650 A linear model was used to test for differences in expression levels between
 651 developmental stages in any given strain by sex combination and for differences in
 652 expression levels between the sexes in any given strain by developmental stage
 653 combination. For this purpose, a linear mixed-effects gene model that takes into
 654 account both array and dye-specific effects was considered -

$$655 y_{ijklm} = \mu + A_i + T_j + S_k + D_l + Z_m + S_k D_l + S_k Z_m + D_l Z_m + S_k D_l Z_m + \epsilon_{ijklm}$$

656 where y_{ijklm} denotes the miRNA expression for the i^{th} array, j^{th} dye, k^{th} strain, l^{th}
 657 developmental stage and m^{th} sex. Also, μ is the baseline expression, A_i is the effect
 658 of the i^{th} array, T_j is the effect of the j^{th} dye, S_k is the effect of the k^{th} strain, D_l is the
 659 effect of the l^{th} developmental stage, and Z_m is the effect of the m^{th} sex. A was
 660 implemented as a random effect while T , S , D , Z , and the interaction effects were
 661 implemented as fixed effects in JMP Genomics 5.0. Next, the appropriate contrasts
 662 were made to obtain the differences of interest. The differences of interest were
 663 considered to be statistically significant at a false discovery rate (FDR) of 0.01.

664 For a given strain, to test the null hypothesis that the difference in magnitude of
665 developmental change was the same in both sexes, a linear mixed-effects model
666 was developed as follows -

$$667 y_{ijlm} = \mu + A_i + T_j + D_l + Z_m + DZ_m + \varepsilon_{ijlm}$$

668 where y_{ijlm} denotes the miRNA expression for the i^{th} array, j^{th} dye, l^{th} developmental
669 stage and m^{th} sex. Also, μ is the baseline expression, A_i is the effect of the i^{th} array,
670 T_j is the effect of the j^{th} dye, D_l is the effect of the l^{th} developmental stage, and Z_m is
671 the effect of the m^{th} sex. A was implemented as a random effect while T , D , Z , and
672 the interaction effect were implemented as fixed effects. Since there were two
673 developmental stages and two sexes the interaction term corresponded to the
674 difference of interest. The difference in magnitude of developmental change in
675 expression across sexes was considered to be statistically significant at a FDR of
676 0.05.

677 **qRT-PCR.** Triplicate total RNA samples for each strain by developmental stage by
678 sex analyzed were polyadenylated and reverse-transcribed using oligo-dT as a
679 primer following manufacturer conditions (Exiqon Universal cDNA Synthesis Kit,
680 203300). The qRT-PCR step was performed in a CFX-96 real-time instrument
681 (BioRad) using Locked Nucleic Acid (LNA) primers (Exiqon; supplementary table S5,
682 Supplementary Material online) and SYBR Green chemistry (Exiqon SYBR Green
683 Master Mix, 203450). Expression levels of the miRNAs analyzed were estimated
684 relative to the reference genes *miR-1-3p* and *miR-995-3p*, which were chosen based
685 on two criteria: i) expression uniformity in microarray experiments across all strain
686 by developmental stage by sex combinations; and ii) because they cover two
687 differentiated levels of expression (*miR-1-3p* is substantially more expressed than
688 *miR-995-3p* according to the small-RNA sequencing experiments performed in this

689study). Estimates were calculated using the $-2^{\Delta\Delta Cq}$ method implemented in the Bio-
690Rad CFX manager software and statistically significant differences among samples
691were interrogated in JMP Genomics 5.0 (SAS Institute Inc.).

692**mRNA microarray profiling.** We assayed levels of mRNA abundance for *D.*
693*melanogaster* males at ≥ 18 BPF and PF (supplementary table S1, Supplementary
694Material online). Three out of four biological samples used for assaying miRNA
695levels of expression were randomly chosen for this purpose. Per sample, 10 μ g of
696total RNA were reverse transcribed into cDNA using the SuperScript® Double-
697Stranded cDNA Labeling Kit (Invitrogen). The quality of the cDNAs was evaluated
698with the DNA 12000 kit (Agilent Technologies) using an Agilent 2100 Bioanalyzer.
699Probe labeling, hybridization, array scanning, and data extraction were performed
700by Roche NimbleGen Service Group in Iceland. Single color hybridizations were
701performed onto species-specific 12x135k NimbleGen oligonucleotide arrays
702corresponding to the FlyBase release 5.7 for *D. melanogaster*.

703The raw mRNA expression data were pre-processed using NimbleGen's DEVA
704software suite, which includes background correction, quantile normalization, and
705summarization of probeset expression using the robust multi-array average (RMA)
706method. The random probes present on the NimbleGen arrays were removed prior
707to mRNA data analysis.

708A linear mixed-effects model was used to test for differences in expression levels
709between developmental stages per strain as follows -

$$710 y_{kl} = \mu + S_k + D_l + S_k D_l + \varepsilon_{kl}$$

711where y_{kl} denotes the mRNA expression for the k^{th} strain and l^{th} developmental
712stage. Also, μ is the baseline expression, S_k is the effect of the k^{th} strain, and D_l is

713the effect of the l^{th} developmental stage. S , D , and interaction terms were
714implemented as fixed effects in JMP Genomics 5.0 . The difference in expression
715levels was considered to be statistically significant at a FDR of 0.05.

716**Genomic datasets.** Putative target genes with conserved miRNA binding sites
717were retrieved from TargetScan 6.2. . Association with gain-of-function phenotypes
718following miRNA overexpression was determined based on positive results in at
719least one of three screens (eye, wing, and ubiquitous activation) performed in *D.*
720*melanogaster* . MiRNA expression ubiquity values, τ , were taken from . These
721values were calculated as reported based on expression values derived from 28
722libraries of small RNAs representing different developmental stages and tissues of
723*D. melanogaster*. $1-\tau$ values were used here, which range from 0 to 1; high $1-\tau$
724values correspond to more ubiquitously expressed miRNAs.

725**Functional enrichment analysis.** Enrichment for Gene Ontology terms
726(biological process, molecular function, and cellular localization), and KEGG
727pathways in sets of protein-coding genes was evaluated with DAVID 6.7 . The false
728discovery rate adjustment was used to account for multiple testing at $P_{adj}<0.05$; the
729stringency was set to "Highest". As a background list, all the *D. melanogaster*
730genes on the NimbleGen array were used. To increase the stringency of the
731analysis, the list of 543 genes that turned out to show significant associations in
732expression with miRNAs was also used as background list in a second analysis.

733**Expression distance among strains.** Statistically significant differences in
734miRNA abundance were recorded across strains in a pairwise fashion (one-way
735ANOVA, $P<0.01$). One hundred and twenty miRNA reporters conserved in sequence
736across *D. simulans*, *D. yakuba*, and *D. melanogaster* (including the strain
737Zimbabwe-109) were deemed suitable for the analysis.

738 **Accession numbers.** Small RNA sequencing output, LC Sciences expression data,
739 and NimbleGen expression data have been deposited in the NCBI GEO database
740 under accession numbers GSE57438, GSE55562, and GSE55398, respectively.
741 Sequence data for protein-coding and miRNA loci have been deposited in NCBI
742 GenBank under accession numbers KJ767237-KJ67254 and KJ774561-KJ774633.

743

744

SUPPLEMENTARY MATERIAL

745 Supplementary text, supplementary alignments, supplementary figures S1-S17,
746 supplementary tables S1-S12, and supplementary datasets S1-S5 are available
747 online.

748

749

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756

Table 1. List of miRNAs showing differential expression between ≥ 18 BPF and PF stages

MiRNA	Males						Females		
	CS	ORR	Sam	ZW	sim	yak	ORR	ZW	sim
<i>let-7-5p</i>	PF	PF	PF	PF	PF	PF	PF	PF	PF
<i>miR-2a-3p</i>	PF	PF	PF	PF	PF	PF	PF	PF	PF
<i>miR-125-5p</i>	PF	PF	PF	PF	PF	PF	PF	PF	PF
<i>miR-279-3p</i>	PF ≥ 18 BP	PF ≥ 18 BP	PF ≥ 18 BP	PF ≥ 18 BP	PF ≥ 18 BP	PF ≥ 18 BP	PF ≥ 18 BP	PF ≥ 18 BP	PF ≥ 18 B
<i>miR-8-5p</i>	F ≥ 18 BP	F ≥ 18 BP	F ≥ 18 BP	F ≥ 18 BP	F ≥ 18 BP	F ≥ 18 BP	F ≥ 18 BP	F ≥ 18 BP	PF ≥ 18 B
<i>miR-14-5p</i>	F ≥ 18 BP	F ≥ 18 BP	F ≥ 18 BP	F ≥ 18 BP	F ≥ 18 BP	F ≥ 18 BP	F ≥ 18 BP	F ≥ 18 BP	PF ≥ 18 B
<i>miR-34-5p</i>	F ≥ 18 BP	F ≥ 18 BP	F ≥ 18 BP	F ≥ 18 BP	F ≥ 18 BP	F ≥ 18 BP	F ≥ 18 BP	F ≥ 18 BP	PF ≥ 18 B
<i>miR-313-5p</i>	F ≥ 18 BP	F ≥ 18 BP	F ≥ 18 BP	F ≥ 18 BP	F ≥ 18 BP	F ≥ 18 BP	F ≥ 18 BP	F ≥ 18 BP	PF ≥ 18 B
<i>miR-958-3p</i>	F ≥ 18 BP	F ≥ 18 BP	F ≥ 18 BP	F ≥ 18 BP	F ≥ 18 BP	F ≥ 18 BP	F ≥ 18 BP	F ≥ 18 BP	PF ≥ 18 B
<i>miR-958-5p</i>	F	F	F	F	F	F	F	F	PF
<i>miR-2c-3p</i>	PF ≥ 18 BP	PF ≥ 18 BP	PF ≥ 18 BP	PF	PF ≥ 18 BP	ns ≥ 18 BP	PF ≥ 18 BP	PF ≥ 18 BP	PF ≥ 18 B
<i>miR-34-3p</i>	F ≥ 18 BP	F ≥ 18 BP	F ≥ 18 BP	ne	F ≥ 18 BP	F ≥ 18 BP	F ≥ 18 BP	F ≥ 18 BP	PF ≥ 18 B
<i>miR-281-2-5p</i>	F ≥ 18 BP	F ≥ 18 BP	F ≥ 18 BP	ns ≥ 18 BP	F ≥ 18 BP	F ≥ 18 BP	F ≥ 18 BP	F	PF ≥ 18 B
<i>miR-284-5p</i>	F ≥ 18 BP	F ≥ 18 BP	F ≥ 18 BP	F ≥ 18 BP	F ≥ 18 BP	F ≥ 18 BP	F ≥ 18 BP	ns ≥ 18 BP	PF
<i>miR-316-3p</i>	F	F	F	F	F	F	F	F	ns
<i>miR-100-5p</i>	ne	PF	PF	PF	PF	PF	PF	PF	PF
<i>miR-2b-3p</i>	PF	PF	PF	PF	ns	PF	PF	PF	ns
<i>miR-2b-2-5p</i>	PF	ns	PF	PF	PF	ns	PF	PF	PF
<i>miR-92a-3p</i>	PF	PF	PF	PF	PF	ns	PF	PF	ns
<i>miR-276b-3p</i>	PF ≥ 18 BP	PF ≥ 18 BP	PF ≥ 18 BP	PF	PF ≥ 18 BP	PF ≥ 18 BP	PF ≥ 18 BP	ns	ns ≥ 18 B
<i>miR-956-3p</i>	F ≥ 18 BP	F	F ≥ 18 BP	ns ≥ 18 BP	F ≥ 18 BP	F ≥ 18 BP	F ≥ 18 BP	ns ≥ 18 BP	PF
<i>miR-1000-3p</i>	F	ne	F	F	F	F	F	F	ne
<i>miR-92b-3p</i>	PF	PF	PF ≥ 18 BP	PF ≥ 18 BP	ns ≥ 18 BP	ns	PF	PF	ns ≥ 18 B
<i>miR-289-5p</i>	PF	ns	F	F	F	ns	PF	ns	PF
<i>miR-306-5p</i>	PF	ns	PF	PF	PF	ns	PF	PF	ns
<i>miR-2a-1-5p</i>	PF	ne	PF	PF	PF	ns	ns	PF	ns
<i>miR-7-5p</i>	PF	ns	PF	PF	ns	ns	ns	PF	PF

Table 1. List of miRNAs showing differential expression between ≥ 18 BPF and PF stages

MiRNA	Males						Females		
	CS	ORR	Sam	ZW	sim	yak	ORR	ZW	sim
<i>miR-9a-5p</i>	PF	ns	PF	PF	ns	ns	PF	PF	ns
<i>miR-305-5p</i>	ns	PF	PF	PF	ns	PF	PF	ns	ns
<i>miR-1012-3p</i>	PF	ns	PF	PF	N/A	ne	PF	ns	N/A
<i>miR-276a-3p</i>	ns	PF	PF	ns	ns	PF	PF	ns	ns
<i>miR-1000-5p</i>	ns	PF	PF	PF	PF	ns	ns	ns	ns
<i>miR-1010-3p</i>	ne	ne	ne	PF	N/A	PF	PF	PF	N/A
<i>miR-79-3p</i>	PF	ne	ns	ne	PF	ns	ns	PF	ns
<i>miR-9a-3p</i>	ns	ns	PF	PF ≥ 18 BP	ns	ns	ns ≥ 18 BP	PF ≥ 18 BP	ns
<i>miR-964-5p</i>	ns	ns	ns	F	ns	ns	F	F	ne
<i>miR-988-3p</i>	PF	ne	ne	ne	ne	N/A	ns	PF	ns
<i>miR-314-5p-</i>	≥ 18 BP						≥ 18 BP		
<i>dme</i> <i>miR-2493-5p-</i>	F ≥ 18 BP	ne	ne	ns ≥ 18 BP	N/A	N/A	F	ns	N/A
<i>dme</i>	F	ne	ns	F	N/A	N/A ≥ 18 BP	ns ≥ 18 BP	ns	N/A
<i>miR-277-3p</i>	ns	ns	ns	ns	ns	F	F	ns	ns
<i>miR-998-3p</i>	ns	ns	PF	PF	ns	ns	ns	ns	ns
<i>miR-314-5p-</i>					≥ 18 BP	≥ 18 BP			
<i>dsi</i>	N/A	N/A	N/A	N/A	F	F	N/A	N/A	ne
<i>miR-2a-2-5p</i>	PF	ne	ne	ne	ne	ns	ns	ne	ne
<i>bantam-5p</i>	ns	ns	ne	PF	ns ≥ 18 BP	ns	ns	ne	ns
<i>miR-10-3p</i>	ns	ns	ns	ns	F	ns	ns	ns	ns ≥ 18 B
<i>miR-10-5p</i>	ns	ns	ns	ns	ns	ns	ns	ns	PF
<i>miR-11-3p</i>	ns	ns	ns	PF	ns	ns	ns	ns	ns
<i>miR-31a-5p</i>	ns	ns	ns	PF	ns	ns	ns	ns	ns
<i>miR-31b-5p</i>	ns	ns	ns	PF	ns	ns	ns	ns	ns
<i>miR-252-5p</i>	ns	ns	ns	PF	ns	ns	ns	ns	ns
<i>miR-275-3p</i>	ns	ns	ns	ns	ns	ns	ns	PF	ns
<i>miR-996-5p</i>	ns	ns	ns	PF	ns	ns	ns	ns	ns
<i>miR-998-5p</i>	ns	ns	ns	PF	ns	ns	ne	ne	ns
<i>miR-9b-5p</i>	ne	ne	ne	ne	N/A	ne	ne	PF	N/A
<i>miR-993-3p</i>	ne	PF	ne	ne	ns	N/A	ne	ne	ns

757CS, Canton-S; ORR, Oregon-R; Sam, Samarkand; ZW, Zimbabwe-109; sim, *D. 758simulans*; yak, *D. yakuba*.

759Differences in expression between developmental stages at $P_{adj} < 0.01$ (Materials and 760Methods). ne, non-expressed; ns, non-developmentally enriched; PF, enriched at 761PF; ≥ 18 BPF, enriched at ≥ 18 BPF; N/A, the analysis is not applicable to this miRNA 762due to its absence or sequence differences.

765**Fig. 1.** Developmental changes in miRNA expression at the onset of
766metamorphosis across six *Drosophila* males. (A) Differences in expression levels
767between ≥ 18 BPF and PF. x-axis, difference in normalized \log_2 -transformed
768expression levels between ≥ 18 BPF and PF; y-axis, significance of the difference as -
769 $\log_{10}(P$ value). Statistically significant differences were determined using a one-way
770ANOVA. Red dotted line, $P_{\text{adj}}=0.01$. (B) Examples of variation in miRNA abundance
771in males. The average expression level and the standard error of the mean are
772shown. Left, interstrain difference affecting the developmental expression pattern.
773*miR-956-3p* is ≥ 18 BPF-enriched in all strains but in Zimbabwe-109. In this strain,
774there is no significant difference in expression level between stages due to an
775increase in the level of expression at PF ($P_{\text{adj}}=2 \times 10^{-8}$). Right, interstrain difference
776not associated with differences in developmental expression patterns. *miR-1012-5p*
777is non-developmentally enriched in all strains but statistical significant differences in
778expression level were confirmed at PF ($P_{\text{adj}}=7.1 \times 10^{-3}$). Canton-S, Oregon-R,
779Samarkand, and Zimbabwe-109 are strains of *D. melanogaster*.

780**Fig. 2.** Distinct patterns of functional divergence in miRNA expression levels at the
781onset of *Drosophila* metamorphosis. (A) Expression distance in miRNA abundance
782and divergence time are not related in the same way at ≥ 18 BPF and PF. Branch
783lengths from Neighbor-Joining trees were used as a surrogate for divergence time
784among strains. Unlike at PF, expression changes do not accumulate in a linear
785fashion over time at ≥ 18 BPF. This result holds when estimated divergence times
786among strains are used instead of branch lengths and when an alternative
787statistical approach is used (supplementary text and figure S13, Supplementary
788Material online). (B) Comparison of the variation in expression levels among

789 miRNAs sets with different developmental expression patterns. Only miRNAs with
790 consistent developmental expression patterns across *D. melanogaster* strains and
791 identical in sequence across species were considered.

792 **Fig. 3.** Evolutionary modes of miRNA expression at the onset of male
793 metamorphosis in the *D. melanogaster* species subgroup. A) Counts of miRNAs with
794 patterns of intra- and inter-specific variation compatible with particular evolutionary
795 modes. B) MiRNAs illustrating four main evolutionary modes. Box plots are used to
796 show the dispersion around the median level of expression for each strain by miRNA
797 combination. CS, Canton-S; ORR, Oregon-R; Sam, Samarkand; Zw, Zimbabwe-109;
798 and sim, *D. simulans*. Within and between species differences in expression levels
799 were tested respectively by one-way ANOVA. Whether evidence of significant intra-
800 and inter-specific variation was found is indicated on the x- and y-axis respectively.
801 See supplementary text (Supplementary Material online) for the rationale followed
802 to categorize each miRNA under a particular evolutionary mode. MiRNAs showing
803 non-significant and significant differences in expression between *D. melanogaster*
804 and *D. simulans* are shown on top and bottom, respectively. Depending on the
805 combination of significant intra- and inter-specific differences in expression levels,
806 miRNAs are categorized as evolving under stabilizing selection (top left), directional
807 selection (bottom left), genetic drift (bottom right), or associated with complex
808 scenarios (top right). y-axis, log₂-transformed expression values in an arbitrary
809 scale. Examples shown correspond to miRNAs expressed at the PF stage.

810 **Fig. 4.** Relationship between the level of miRNA expression polymorphism in four
811 males of *D. melanogaster* and evolutionary age, gain-of-function phenotype, and
812 expression breadth. (A) Young miRNAs exhibit higher levels of polymorphism in
813 gene expression than ancient miRNAs in *D. melanogaster* males. Left, ≥ 18 BPF;

814right, PF. Average \pm SEM values of expression polymorphism are indicated on top of
815the chart. *Ancient* class, miRNAs inferred to have been already present in the
816ancestor to all currently existing *Drosophila* species and other insects and/or
817metazoans. *Young* class, miRNAs inferred to have been present in the ancestor to
818all *Drosophila* species but not in that of other insects or metazoans, or to have
819evolved subsequently during the radiation of the genus *Drosophila*. (B) Gain-of-
820function phenotype upon miRNA overexpression. Showing a gain-of-function
821phenotype in at least one of the three screens performed did result in a
822significantly lower level of expression polymorphism at both developmental stages
823being specially acute at PF. (C) Expression breadth. As miRNA expression ubiquity
824increases, the level of expression polymorphism decreases. This pattern is
825observed at PF but not at ≥ 18 BPF. The use of a non-parametric correlation statistic
826made no difference (Spearman's ρ ; PF, $P=0.006$; ≥ 18 BPF, $P=0.154$). Expression
827ubiquity values, $1 - \tau$, are close to 1 if expression is ubiquitous and close to 0 if
828expression is rather specific. τ values were taken from .

829**Fig. 5.** Relationship between the percentage of miRNA-mRNA negative expression
830associations and miRNA developmental expression pattern. (A) Distribution of the
831number of miRNAs based on the percentage of negative significant associations
832with mRNA exemplars relative to the total number of statistically significant
833associations. Thirty-eight miRNAs exhibited a proportion of negative over positive
834associations deviating significantly from the 1:1 ratio while five miRNAs did not. (B)
835MiRNAs with higher percentage of negative expression associations to mRNAs are
836more likely to be PF-enriched than ≥ 18 BPF-enriched (Spearman's $Rho=0.6895$,
837 $P<0.0001$). x-axis, developmental expression index. This index is calculated by
838counting the number of *D. melanogaster* strains that exhibit ≥ 18 BPF- or PF-

839enrichment and adding -1 or +1, respectively. For example, an index value of -3
840indicates that the miRNA was categorized as ≥ 18 BPF-enriched in three strains and
841non-developmentally enriched in one strain. y-axis, percentage of negative
842expression associations shown by miRNAs. (C) Ideogram representing separately
843the expression associations between miRNAs and mRNAs across the two
844developmental timepoints assayed. Left panel, the positive association between
845miRNA (blue) and mRNA (red) expression levels identified in miRNAs with lower
846expression level at PF may often reflect the concurrently transcriptional down-
847regulation of both kind of molecules. Right panel, the up-regulation of a miRNA at
848PF may be important for dampening mRNAs from genes that are not to be
849expressed at this stage. This second pattern is more likely to reflect a causal
850regulatory relationship between the miRNA and the mRNA, which can be exerted
851directly through miRNA binding or indirectly throughout a third party.

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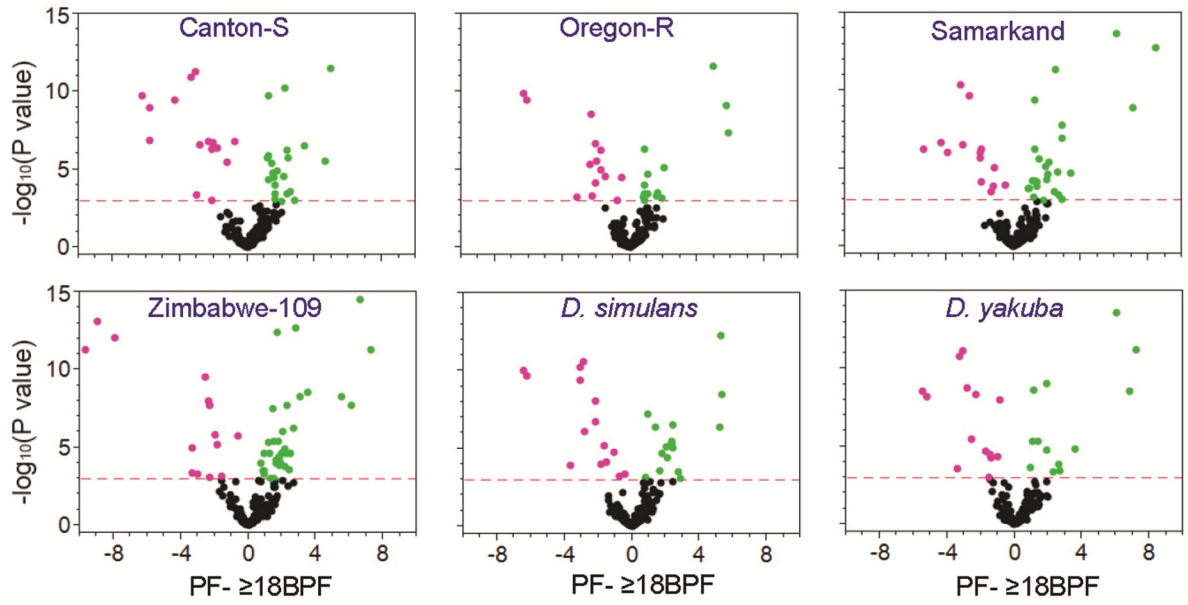
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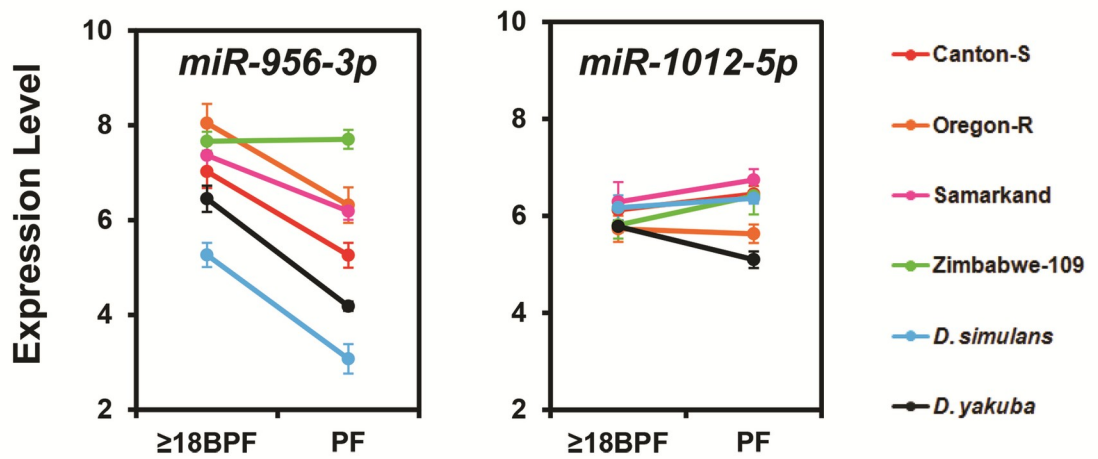
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A



B



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1093 **Fig. 1**

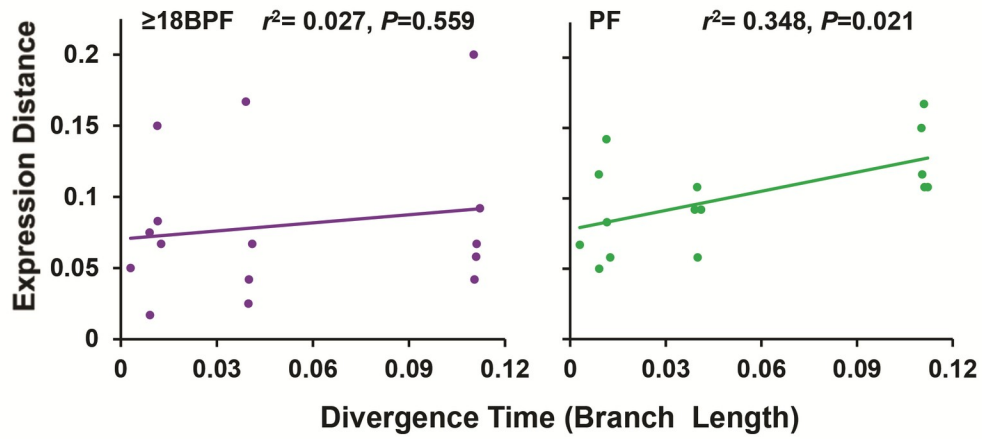
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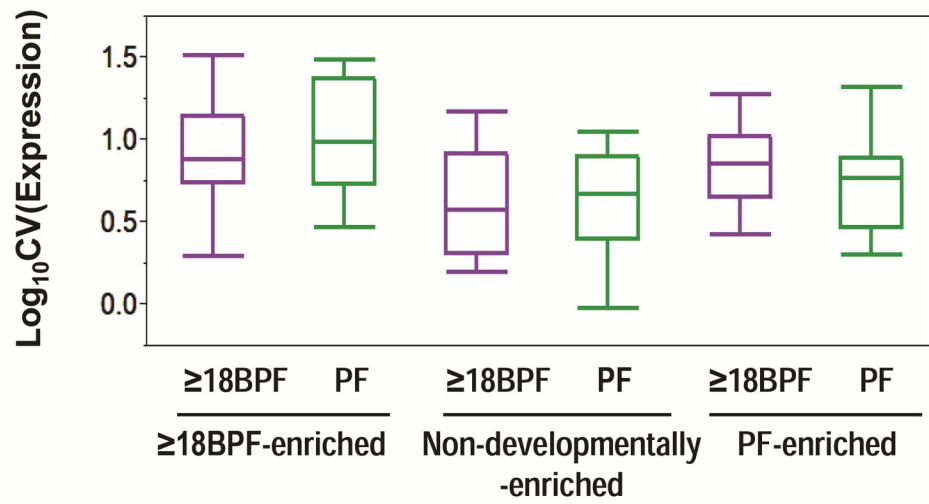
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A



B



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1099 **Fig. 2**

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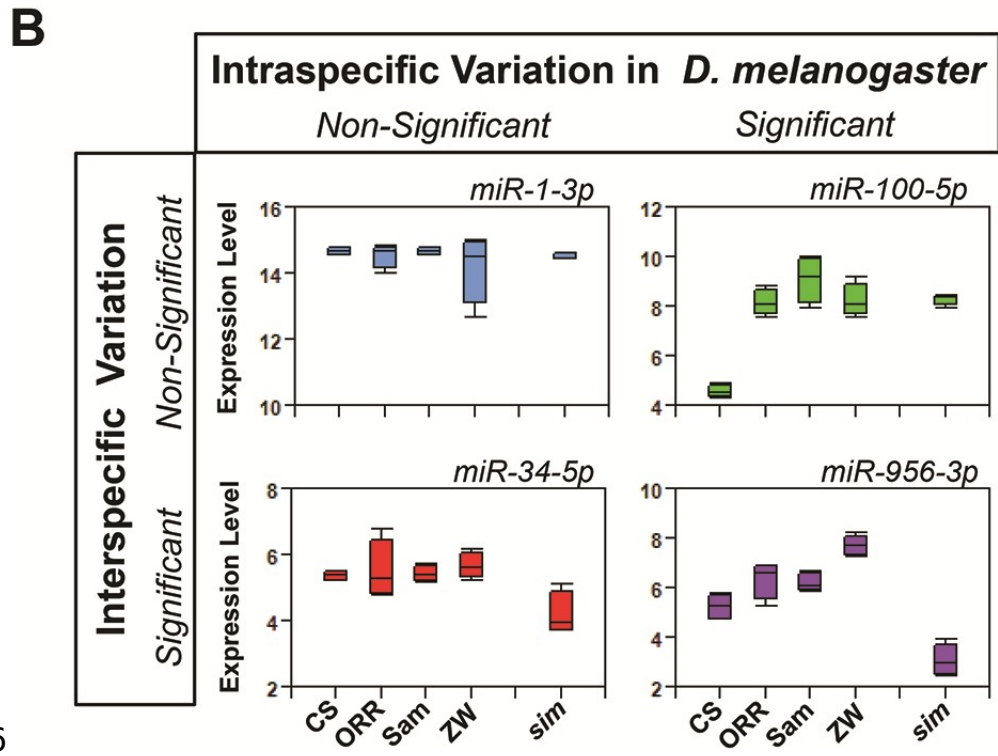
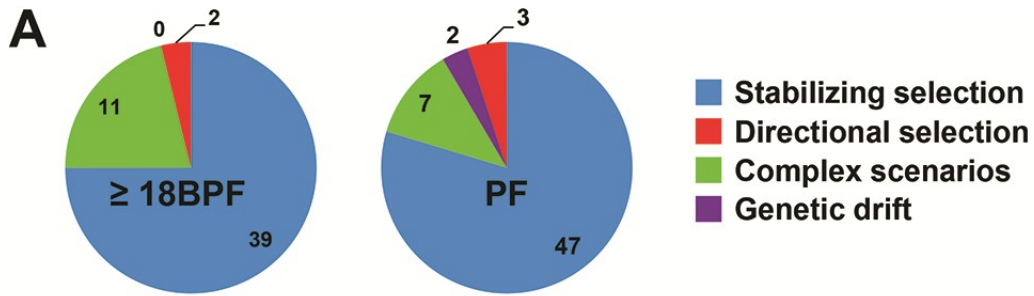
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1107 **Fig. 3**

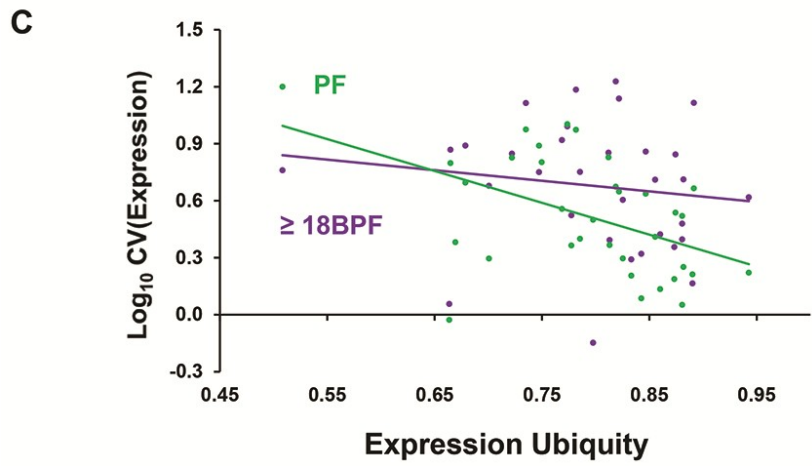
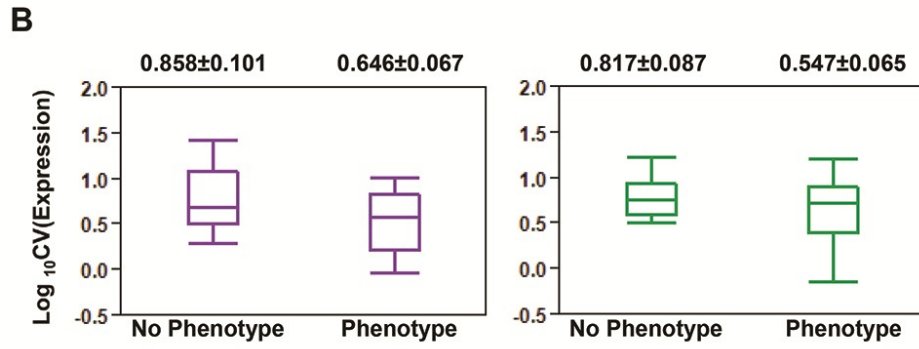
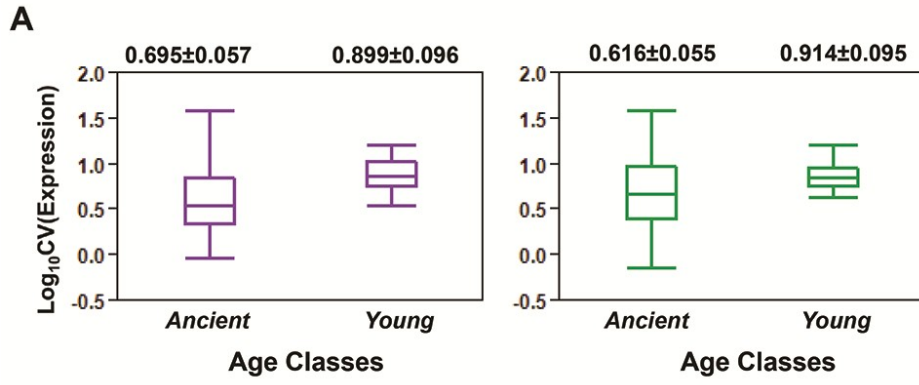
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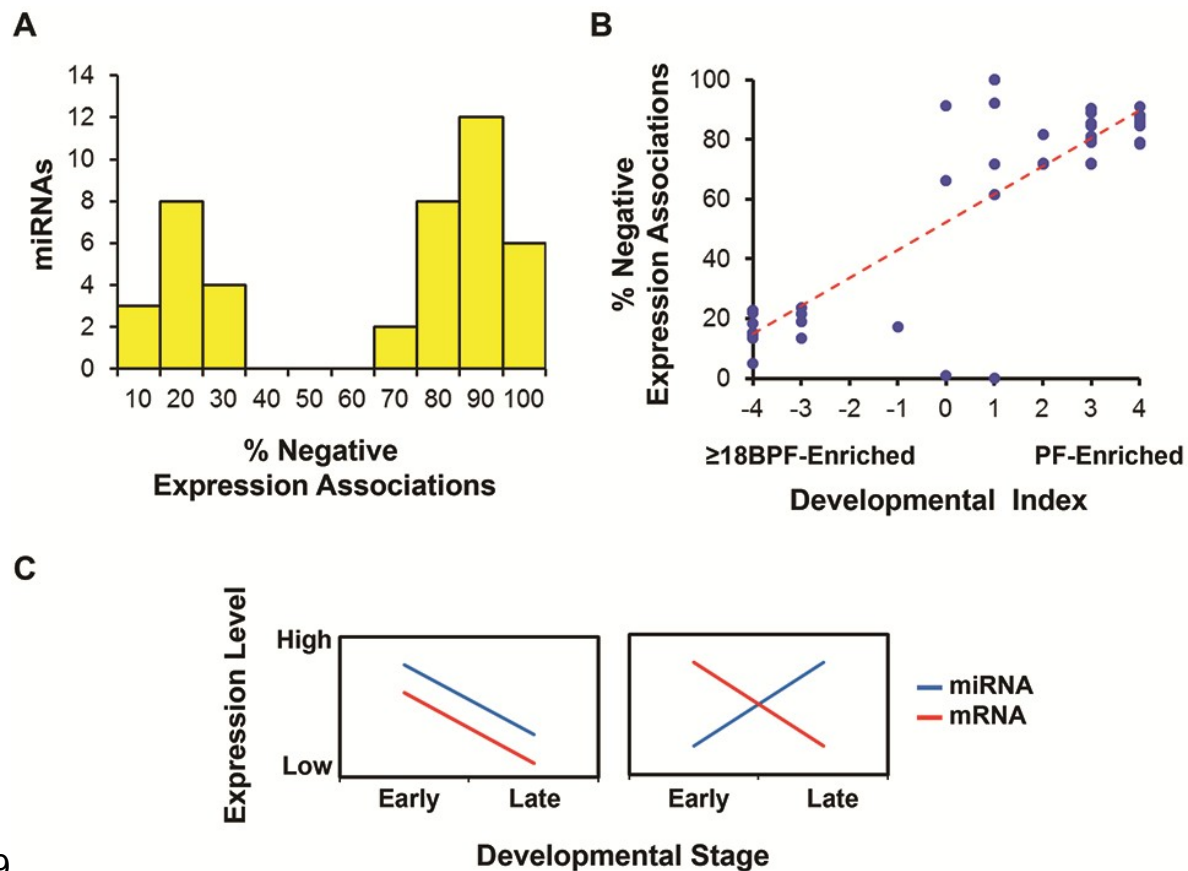
1114 **Fig. 4**

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1120 **Fig. 5**

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