Supplementary Materials for

Mechanisms of gene death in the Red Queen race revealed by the analysis of *de novo* microRNAs

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This PDF file includes:

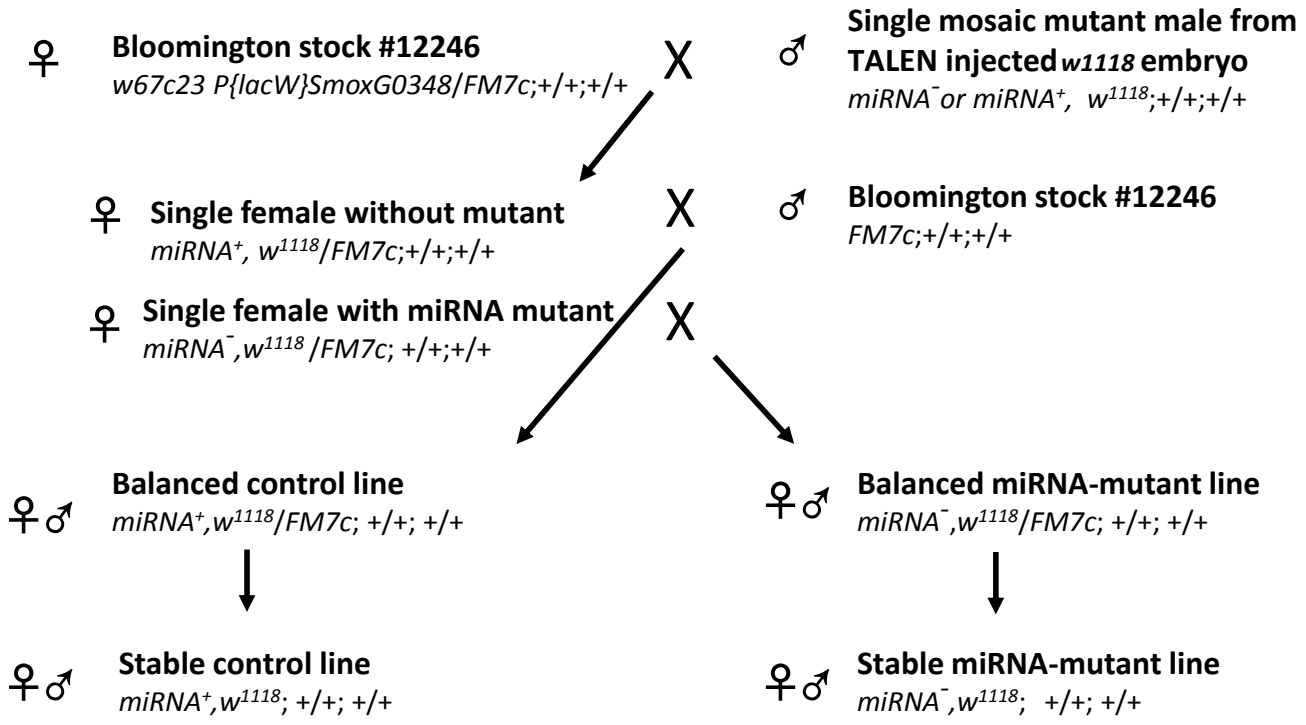


Fig. S1. Cross scheme for miRNA mutant generation.

Male fertility assay:

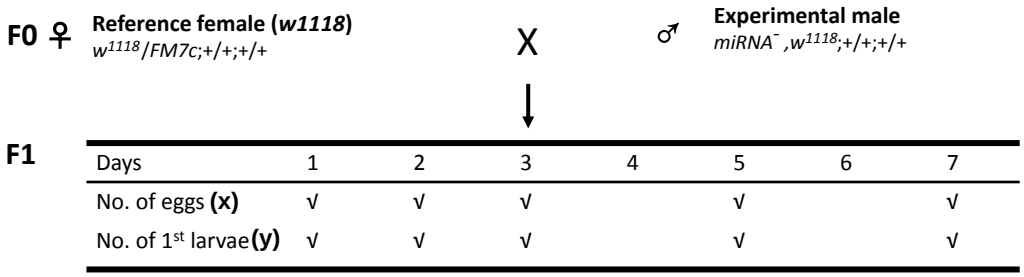


Fig. S2. Experimental procedure for the male fertility assays.

Similar crosses are set up for miRNA KO and control lines. For convenience, we use the miRNA KO flies to represent the experimental flies in the cross schemes.

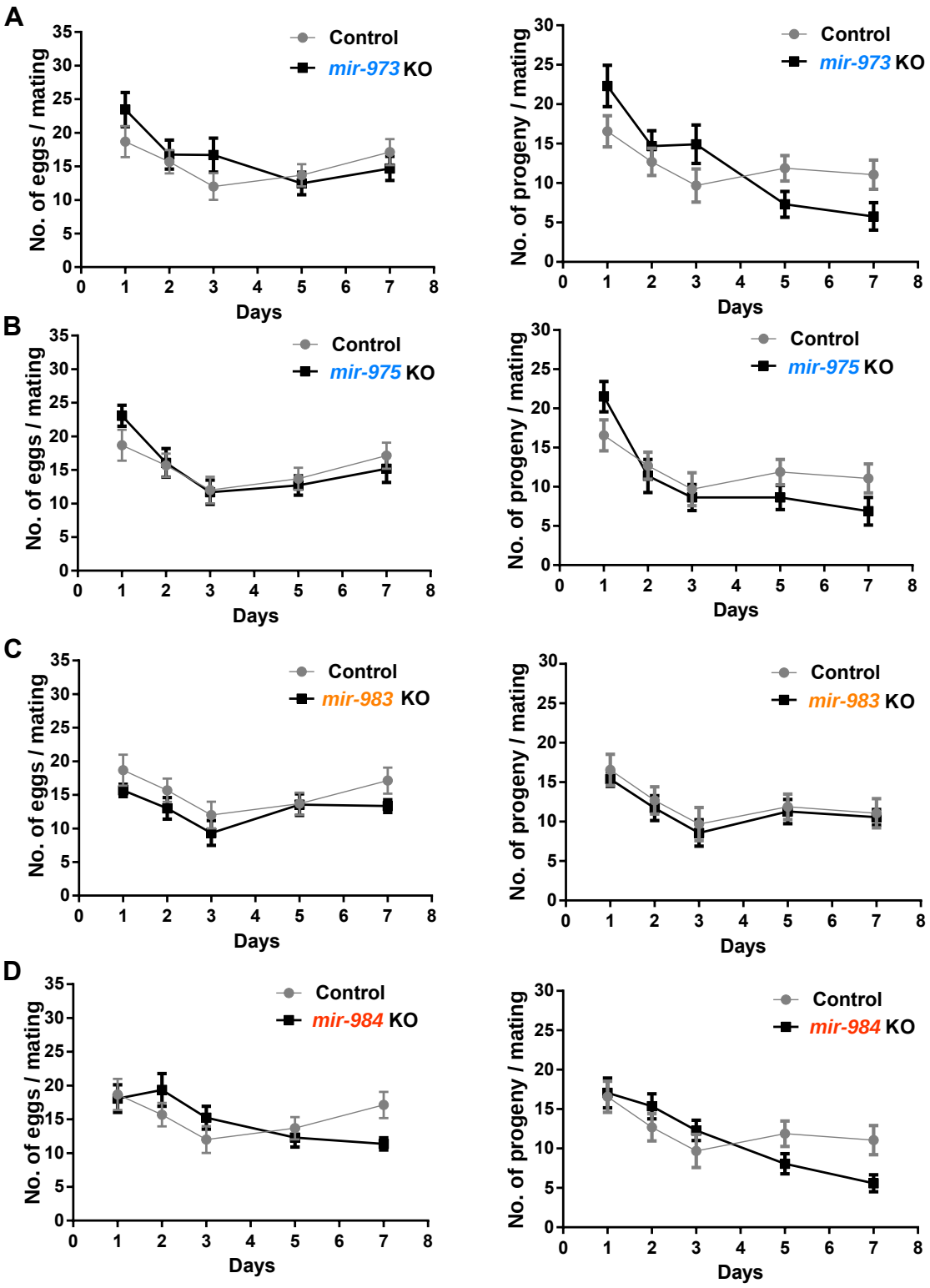


Fig. S3. Male fertility change in *de novo* miRNA mutants.

(A) to (D) depict results for *mir-973*, *mir-975*, *mir-983* and *mir-984* respectively. Data comes from single-pair mating of miRNA KO males or control males to control females from day one to day seven, n=~12. Each panel depicts mean ± SEM of the number of eggs laid by mated females on the left; mean ± SEM of the progeny number per day in the right.

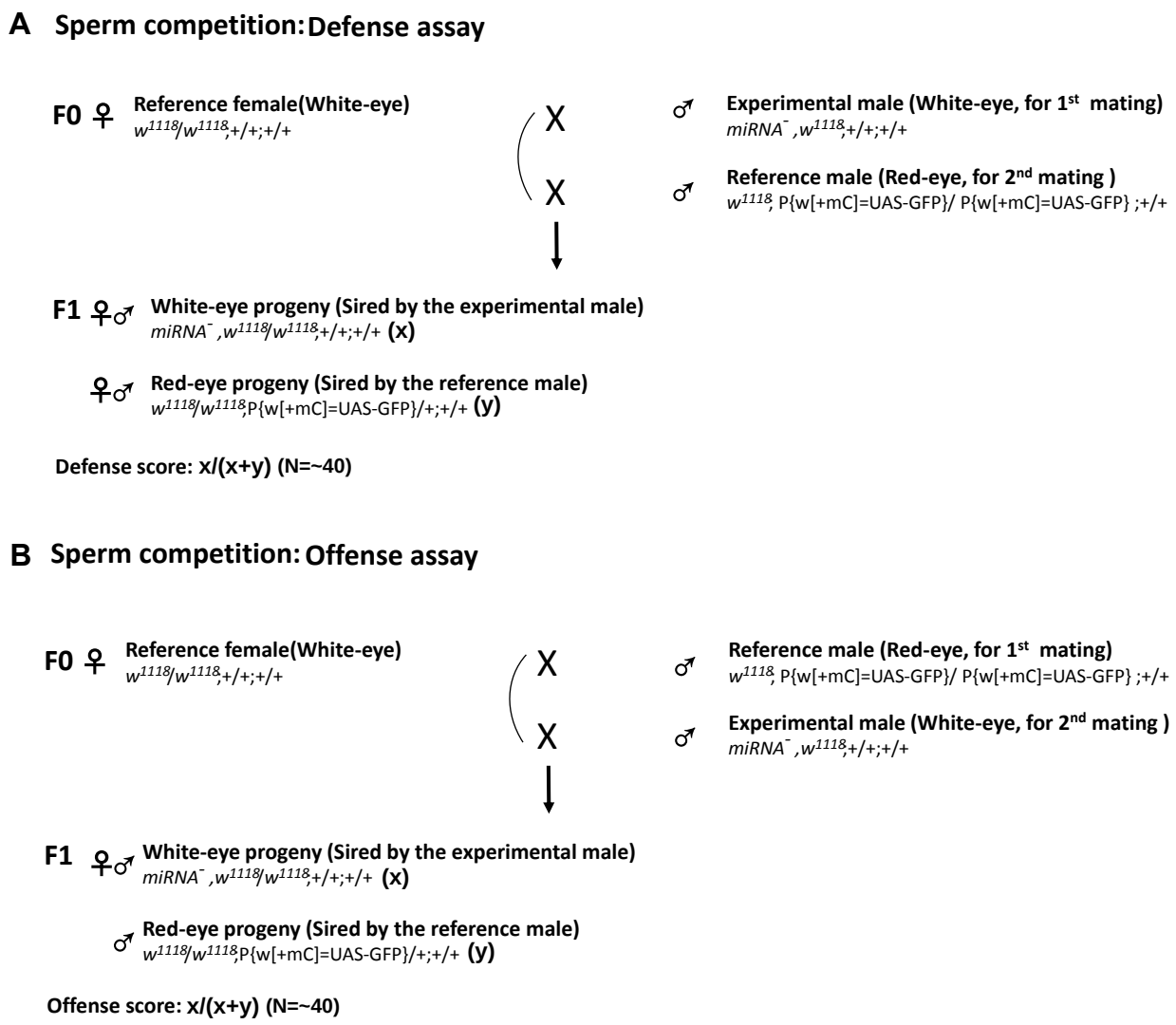
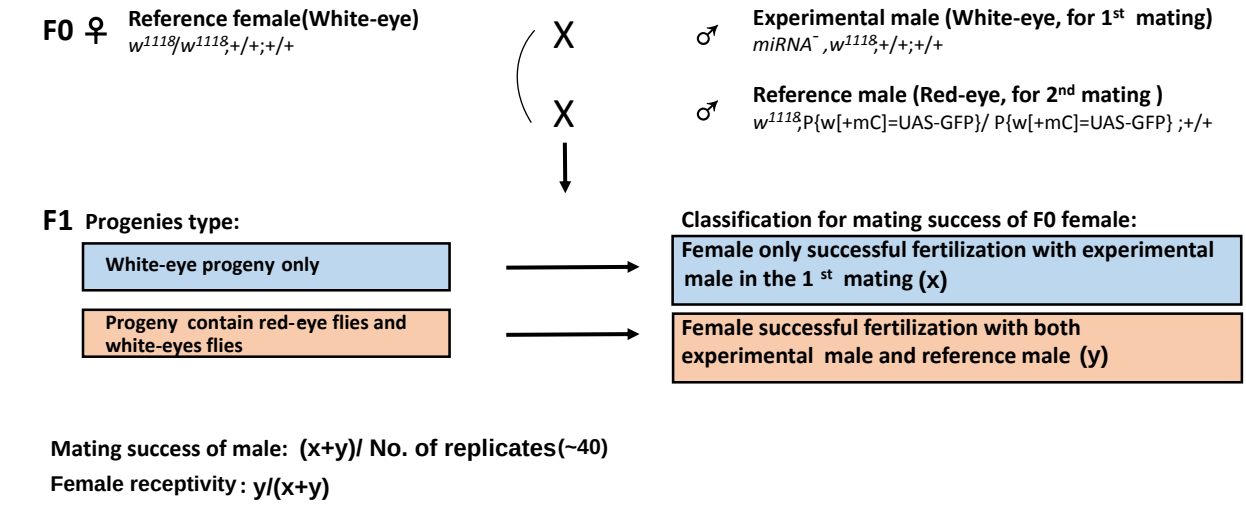


Fig. S4. Experimental procedure for the sperm competition assays.

Similar crosses are set up for miRNA KO and control lines. For convenience, we use the miRNA KO flies to represent the experimental flies in the cross schemes.

(A) Experimental procedures for the defense assays.

(B) Experimental procedures for the offense assays.



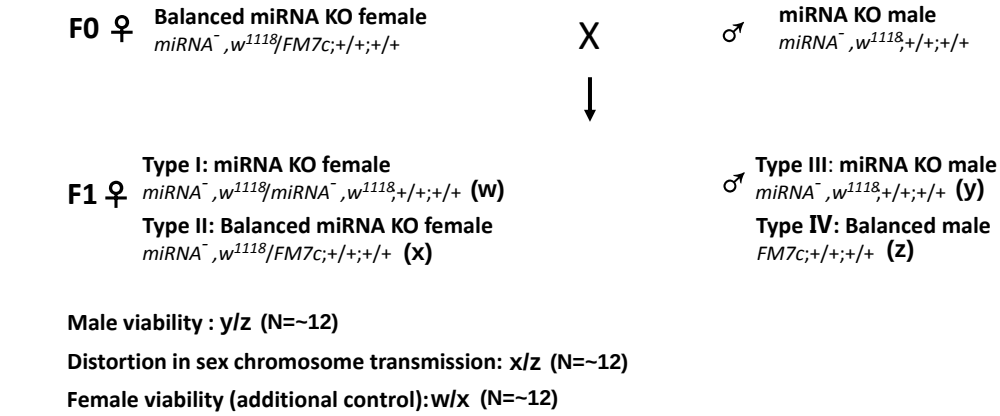


Fig. S5. Experimental procedure for other male fitness component assays.

Similar crosses are set up for miRNA KO and control lines. For convenience, we use the miRNA KO flies to represent the experimental flies in the cross schemes.

(A) Experimental procedures for measuring mating success and ability to repress female re-mating.

(B) Experimental procedures for assessing male viability and meiotic drive.

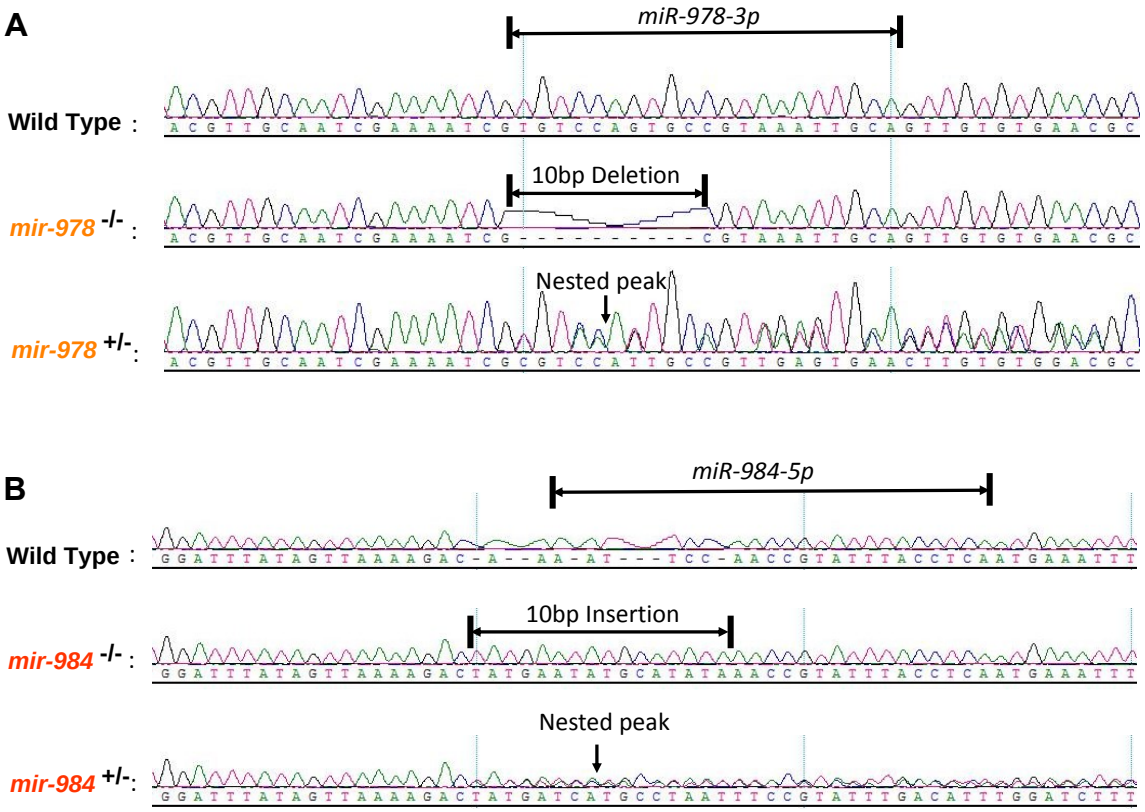


Fig. S6. Single-fly genotyping by PCR and Sanger sequencing.

Wild-type or miRNA KO can be distinguished by detecting a deletion in the mutant; heterozygotes can be detected by observing nested peaks.

(A) Genotyping of wild-type (upper panel), *mir-978* KO (middle panel), and heterozygote flies (bottom panel) in the *mir-978* population cage assay.

(B) Genotyping of wild-type (upper panel), *mir-984* KO (middle panel), and heterozygote flies (bottom panel) in the *mir-984* population cage assay.

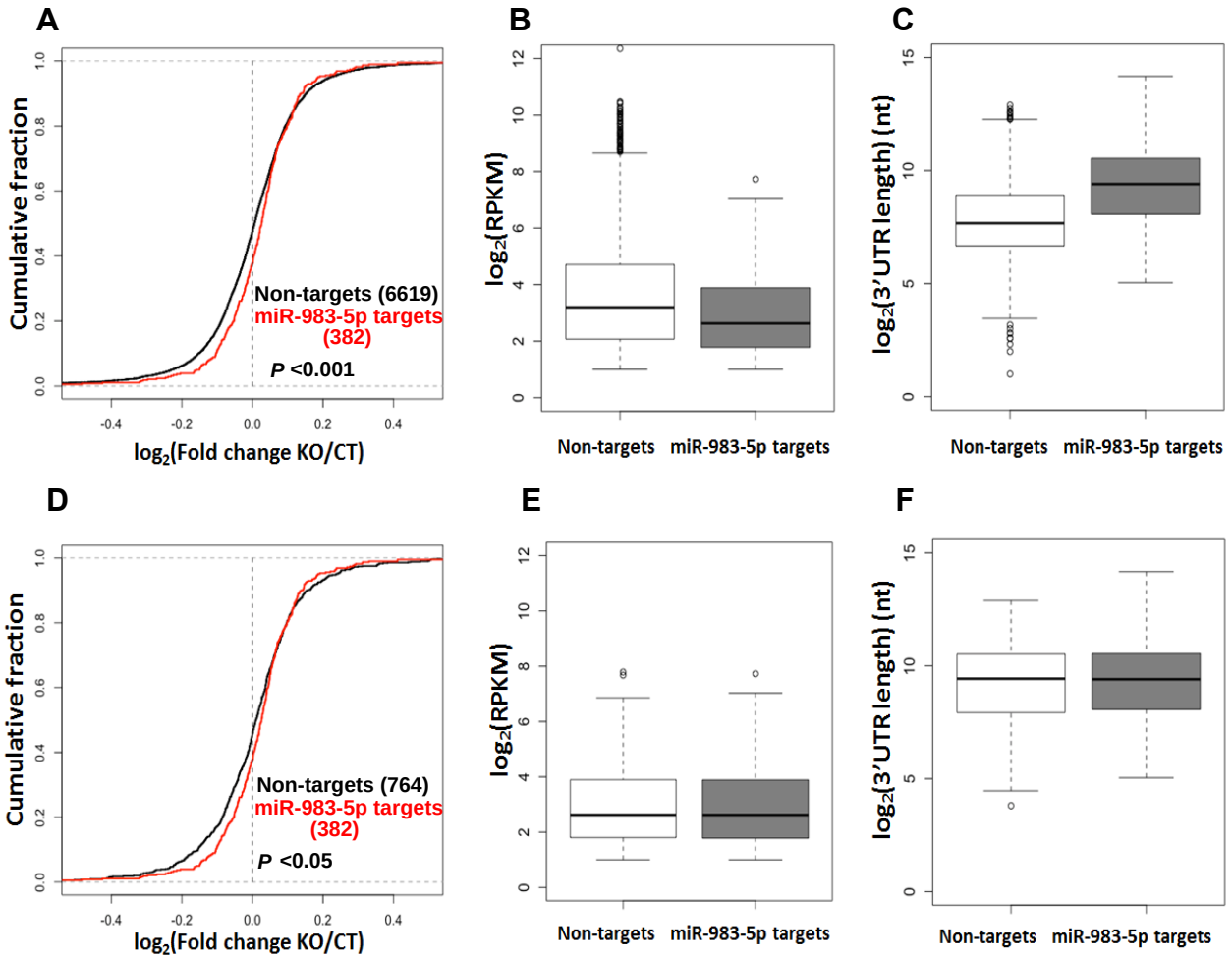


Fig S7. Re-analysis of *mir-983* target repression with refining background control.

(A-C) Original design, non-targets set is the gene pool containing all expressed genes without miR-983-5p targets. (D-F) Re-analysis of *mir-983* target repression, non-targets set is refined with the same expression and 3'UTR length distribution.

(A) *mir-983* target repression. Targets of *mir-983* (miR-983-5p) are significantly up-regulated compared to non-targets in the *mir-983* KO line (P<0.001, Kolmogorov-Smirnov test).

(B-C)Expression and 3’UTR length distribution for both non-targets and miR-983-5p targets.

(D) Re-analysis of mir-983 target repression. Targets of *mir-983* are significantly up-regulated compared to the refined non-targets in the *mir-983* KO line ($P<0.05$, Kolmogorov-Smirnov test).

(E-F)Expression and 3’UTR length distribution for both refined non-targets and miR-983-5p targets. No difference shown is significant (Kolmogorov-Smirnov test, overall $P>0.1$).

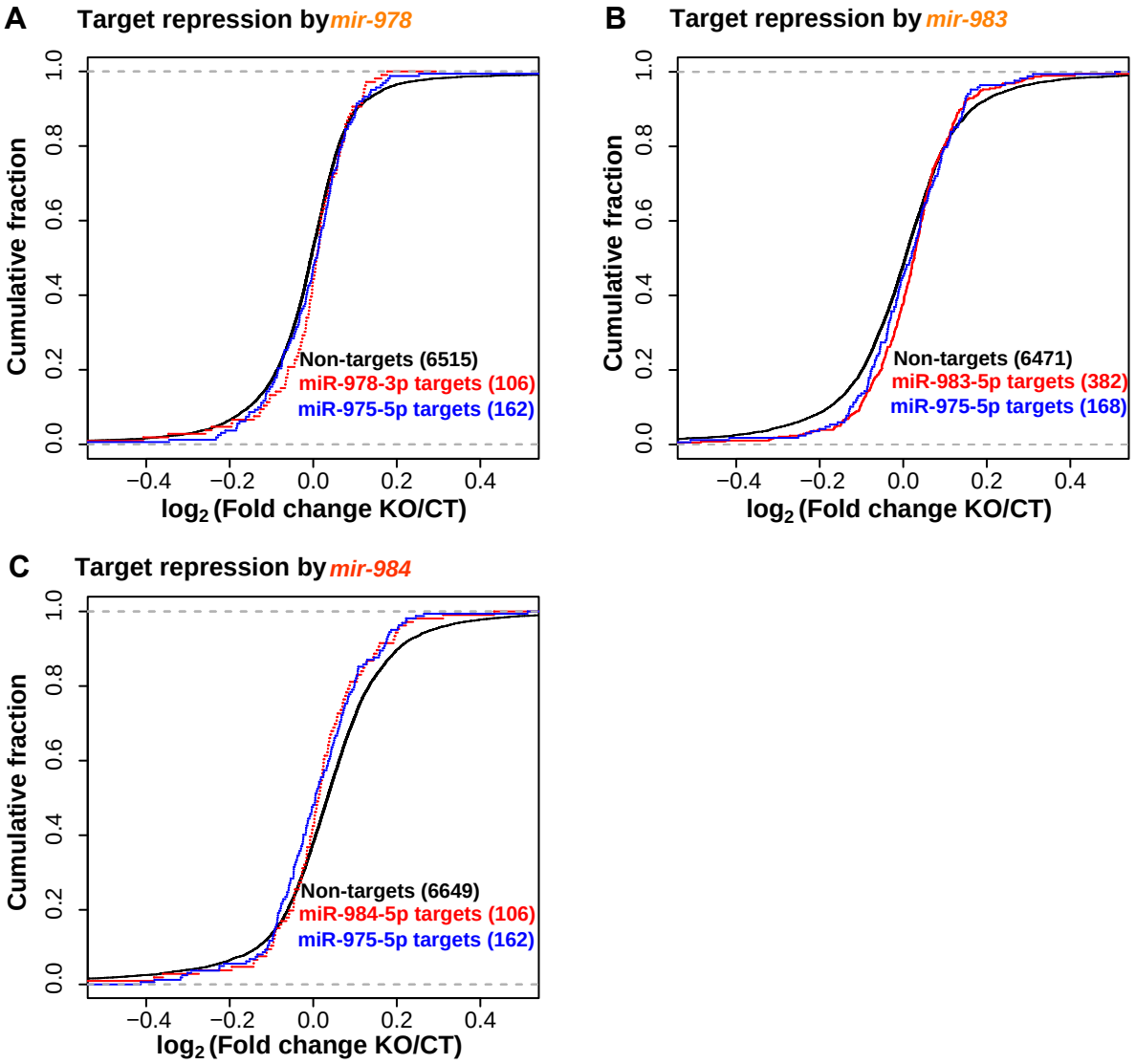


Fig. S8. Confirmatory analysis for the impact of *de novo* miRNA on target repression.

As an additional control, line highlighted in blue is the target pool of *mir-975*(miR-975-5p). (A-C)Target repression of *mir-978*, *mir-983* and *mir-984*. Note that in all cases, the targets of miR-975-5p do not have significant change compared to non-targets (Kolmogorov-Smirnov test, $P>0.05$).

Table S1. Expression of 6 *de novo* miRNAs in testis across 9 lines of *D. melanogaster.*

miRNA	Precursor	Normalized expression(RPM) in different Genetic Background									Total read count	Definition
		Oregon R-1	Oregon R-2	Oregon R-3	hs-Penelope	A1	yw67c23(2)	A2	Canton S	Z56		
dme-miR-973-3p	*dme-mir-973*	17.41	11.96	61.19	8.14	11.07	15.03	18.94	61.62	34.04	239.4	miR*
dme-miR-973-5p		672.6	358.49	71.83	696.07	411.05	945.55	389.57	36.54	29.7	3611.4	mature
dme-miR-975-3p	*dme-mir-975*	107.55	101.13	21.28	19.77	11.07	37.58	22.24	245.03	283.21	848.86	miR*
dme-miR-975-5p		66.24	67.85	5419.02	20.35	29.06	66.14	40.36	547.38	717.81	6974.21	mature
dme-miR-977-3p	*dme-mir-977*	7510.57	4871.74	20024.05	1141.51	1853.17	1649.83	1375.45	481.46	507.03	39414.81	mature
dme-miR-977-5p		80.92	63.95	436.29	25.59	261.58	75.16	259.44	68.78	39.11	1310.82	miR*
dme-miR-978-3p	*dme-mir-978*	647.33	535.27	20766.27	1346.78	1461.5	2178.23	1360.62	133.98	187.6	28617.58	mature
dme-miR-978-5p		123.94	106.59	396.38	71.53	127.33	157.09	157.31	5.02	5.79	1150.98	miR*
dme-miR-983-5p	*dme-mir-983*	868.92	649.91	803.41	172.13	770.89	489.31	500.76	3578.72	2741.58	10575.63	miR*
dme-miR-983-3p		2073.1	1322.96	16520.44	753.64	1125.19	1241.7	935.63	81.68	82.57	24136.91	mature
dme-miR-984-3p	*dme-mir-984*	1541.17	730.24	101.09	881.57	1008.93	1077.09	1056.71	56.6	25.35	6478.75	miR*
dme-miR-984-5p		14159.05	8872.6	5506.81	8969.26	2111.98	10800.95	2350.61	7038.51	8155.2	67964.97	mature

Note: Small RNA-seq data are retrieved from the GEO database, accession numbers: GSM909277 for Oregon R-1,GSM 909278 for Oregon R-2, GSM 280085 for Oregon R-3,GSM 548591 for hs-Penlope, GSM 548589 for A1,GSM 548584 for yw67c23(2) , GSM 548582 for A2, GSM2562978 for Canton S, GSM2562975 for Z56.

Table S2. TALEN binding sites for generating the miRNA mutants.

miRNA	TALEN binding sites design for miRNA	RVD sequences of binding site pairs
mir-973	T ACTTCACTTTACCGTCG TCGACTTTTCGTGG TTGGTGGTTGAACTTCG A	F: NI HD NG NG HD NI HD NG NG NG NI HD HD NN NG HD NN R: HD NN NI NI NN NG NG HD NI NI HD HD NI HD HD NI NI
mir-975	T TTTTGATTTTAAACAC TTCCTACATCCTGT ATGTGTTTTGCATCCG A	F: NG NG NG NG NN NI NG NG NG NG NI NI NI HD NI HD R: HD NN NN NI NG NN HD NI NI NI NI HD NI HD NI NG
mir-977	T TCAGTGTTCGAAATCTGATGAGATATTCACGTTGTCT AAATCATGTTTTGTACG A	F: HD NI NN NG NN NG NG HD NG NI NI NI NG HD NG NN NI NG R: HD NN NG NI HD NI NI NI NI HD NI NG NI NG NG NG
mir-978	T ACGTTGCAATCGAAAAT CGTGTCCAGTGCCGT AAATTGCAGTTGTGTGA A	F: NI HD NN NG NG NN HD NI NI NG HD NN NI NI NI NI NG R: NG HD NI HD NI HD NI NI HD NG NN HD NI NI NG NG NG
mir-983	T CAGTTCATTCATTAGGTAGTTACGCATTATCT AGTTGTTGTAAACATTC A	F: HD NI NN NG NG HD NI NG NG HD NI NG NG NI NN NN NG R: NN NI NI NG NN NG NG NG NI HD NI NI HD NI NI HD NG
mir-984	T TTCATTGAGGTAAATACGGTTGGAATTTT GTCTTTTAACTATAA A	F: NG NG HD NI NG NG NN NI NN NN NG NI NI NI NG R: NG NG NI NG NI NN NG NG NI NI NI NI NN NI HD

Note: For the TALEN binding sites design, mature miRNA sequences are in red, the forward and reverse TALEN binding sites are underlined.

Table S3. Primers used for this study.

A *Primers used for mutant detecting and expression analysis.*

Genes	Primer pair used for mutant detecting	Primer pair used for qRT-PCR (Universal reverse primer: **GTGCAGGGTCCGAGGT**)
miR-972-3p	N.A	RT:GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCA GAGCCAACGCCTAA F:GGCGUTGTACAATACGAATAT
miR-973-5p	F: TCTCATTCGTTTAGCGAGAGG R:GAAAAGCATGAGATCCTCGAC	RT:GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAG AGCCAACAAAATCG F:GGCGTGGTTGGTGGTTGAA
miR-975-5p	F:AATACGAAAGTGCGGACCAC R:TCTGATAGGGAAGGCACTCG	RT:GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAG AGCCAACATACAGG F: GGCGTAAACACTTCCTACAT
miR-977-5p	F:AGCGGAGAATACGAAAGTGC R:TCTGATAGGGAAGGCACTCG	RT: GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAG AGCCAACTTAGAC F: GGCGTGAGATATTCACGTT
miR-978-3p	F: GCAAACCCCGTAGCAGGA R: GATCAACAGCATGGCGAAGA	N.A
miR-983-5p	F:TGCGCCATTCAATGTCTATC R: TGAGTCGTCAACTCGGTGAG	RT:GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGA GCCAACTCATTA F:GGCGATAATACGTTTCGAAC
miR-984-5p	F:TGCGCCATTCAATGTCTATC R: TGAGTCGTCAACTCGGTGAG	RT:GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGA GCCAACAAATTCC F: GGCGTGAGGTAAATACGGTT
2s(Reference gene)	N.A	RT:GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGA GCCAACTACAAC F:GGCGTGCTTGGACTACATATGG

B *The sequence of TaqMan probes used for qRT-PCR.*

Genes	Target sequence
U14(Reference gene)	GCGGUUUCCACCAGAAAGCUUCGGCUUAAUGAUGGUCUAAGGCGUCUGACU
miR-975-5p (Positive control)	UAAACACUUCCUACAUCCUGUAU
miR-978-3p.1	UGUCCAGUGCCGUAAAUUGCAG
miR-978-3p.2	GUGUCCAGUGCCGUAAAUUGCAG

Note: For *mir-978* expression analysis, TaqMan probes are used to get higher specificity for detecting its two iso- miRNAs.

TableS4. Viability of miRNA KO lines.

Measurements in wild type males	Relative fitness of miRNA knock out males vs. wild type males						
	Wild type (Control)	*mir-973*	*mir-975*	*mir-977*	*mir-978*	*mir-983*	*mir-984*
Male viability							
1.07±0.04	1	1.04	1.07	1.10	1.29*	1.21*	1.20*
Female viability							
1.12±0.06	1	1.00	1.02	0.98	0.95	0.94	0.91

Note: Note: Male viability data is also presented in Table 1, relative fitness estimates are presented in materials and methods. (*)$P<0.05$.

Supplemental text S1 Testis-specific expressions of de novo miRNA genes

We note that the small RNA libraries of "imaginal discs" in (Lyu et al. 2014) and miRBase are pooled samples that also contain brain and gonad tissues, in addition to imaginal discs. In these data, the de novo mirs of this study are detected but the expression should be attributed to the inclusion of testes in the samples. In no other expression studies (published or unpublished) that use testis-free samples did we find these de novo miRNAs expressed. We would also like to mention that the fitness analyses carried out in this study do extend beyond the strict association with testicular functions. For example, the long-term population experiments include all fitness components in the life cycle of flies and no unexpected fitness contributions are detected.

Technical details: The pooled disc samples, generated by (Ruby et al. 2007) and used in miRBase, are described as 'mass isolated imaginal discs'(Berezikov et al. 2011). These samples are collected by the filtering method of Fristrom (Fristrom 1972). In its execution, the 'mass isolated imaginal discs' samples are commingled with similarly sized tissues including gonads and brains. Indeed, in the recent publications by the same authors of the miRBase data (Mohammed et al. 2014), these six miRNAs (*mir-973*, *mir-975*, *mir-977*,mir-*978* ,*mir-983* and *mir-984*) are characterized as testis-restricted. Apparently, the expression levels of these mirs in the imaginal disc data can be accounted for by the testicular contribution.

Finally, we have recently surveyed the expression patterns of these same miRNAs in various

male larval tissues by qRT-PCR. Indeed, these miRNAs are expressed only in the gonads (additional file 1 available by request to the corresponding author).

References:

Berezikov E, Robine N, Samsonova A, Westholm JO, Naqvi A, Hung JH, Okamura K, Dai Q, Bortolamiolbecet D, Martin R. 2011. Deep annotation of Drosophila melanogaster microRNAs yields insights into their processing, modification, and emergence. *Genome Research* **21**: 203.

Fristrom JW. 1972. *The Biochemistry of Imaginal Disk Development*. Springer Berlin Heidelberg.

Lyu Y, Shen Y, Li H, Chen Y, Guo L, Zhao Y, Hungate E, Shi S, Wu C-I, Tang T. 2014. New MicroRNAs in Drosophila—Birth, Death and Cycles of Adaptive Evolution. *Plos Genetics* **10**: 229-231.

Mohammed J, Bortolamiolbecet D, Flynt AS, Gronau I, Siepel A, Lai EC. 2014. Adaptive evolution of testis-specific, recently evolved, clustered miRNAs in Drosophila. *RNA* **20**: 1195-1209.

Ruby JG, Stark A, Johnston WK, Kellis M, Bartel DP, Lai EC. 2007. Evolution, biogenesis, expression, and target predictions of a substantially expanded set of Drosophila microRNAs. *Genome Research* **17**: 1850.

Supplemental Text S2 Technical issues of the off-target effects.

For the possible off-target effects in this study. First, we have considered this issue on the experimental design, off-targeting matches have been avoided in the design of TALEN binding sites (Supplemental Table S1). Second, generally, off-target effects are very unlikely to increase the fitness. In contrast, we observe these miRNA KO have advantageous effect in most fitness components, suggesting the fitness of these strains is in the miRNA-independent manner. In addition, to rule out the possibility of off-target effects, we use a couple of components of female fitness as a control in the viability assay. Since these miRNAs show testis restricted expression, one would expect their mutants should not have impact on female fitness. As showed in Supplemental Table S4, compared to control, *mir-978*,*mir-983* and *mir-984* KO have significantly advantages in male viability whereas no difference in female viability.