

Supplementary Figures and Tables

Figure S1. Analysis of expression profile of the miR-959-964 cluster in small RNA libraries from different tissues. Heatmap represents Z-normalized frequencies of miRNAs abundance in total population of miRNAs whithin each tissue.

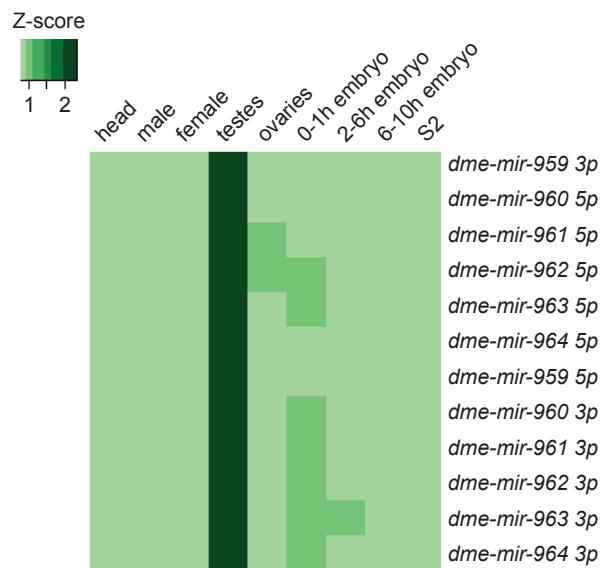


Figure S2. Deletion of the miR-959-964 results in male sterility.

(A) The scheme of the miR-959-964 deletion. (B) Fertility test of wild type and $\Delta miR-959-964$ males. (C) Fertility test of males with knockdown of CG31646 or CG18266 and carrying the mutation of CG31646. Control males of hp(CG31646) and hp(CG18266) strains carries transgenes with shRNA against genes while males of UAS/Dcr2+nos-GAL4 strain carries driver and helper Dcr2 transgenes; UAS/Dcr2+nos-Gal4+hp() carries both shRNA and driver. CG31646[MB09592] is males of 27790 strain from Bloomington Stock Center, carrying insertion of transgene in CG31646. (D) The effect of *MI03191* insertion within *miR-959-964* cluster on male fertility is dependent of the old age of males. Male fertility at (B), (C) and (D) refers to the average number of hatched progeny obtained from crossing of males of tested genotype with *Df(1)^{yw67c23(2)}* females.

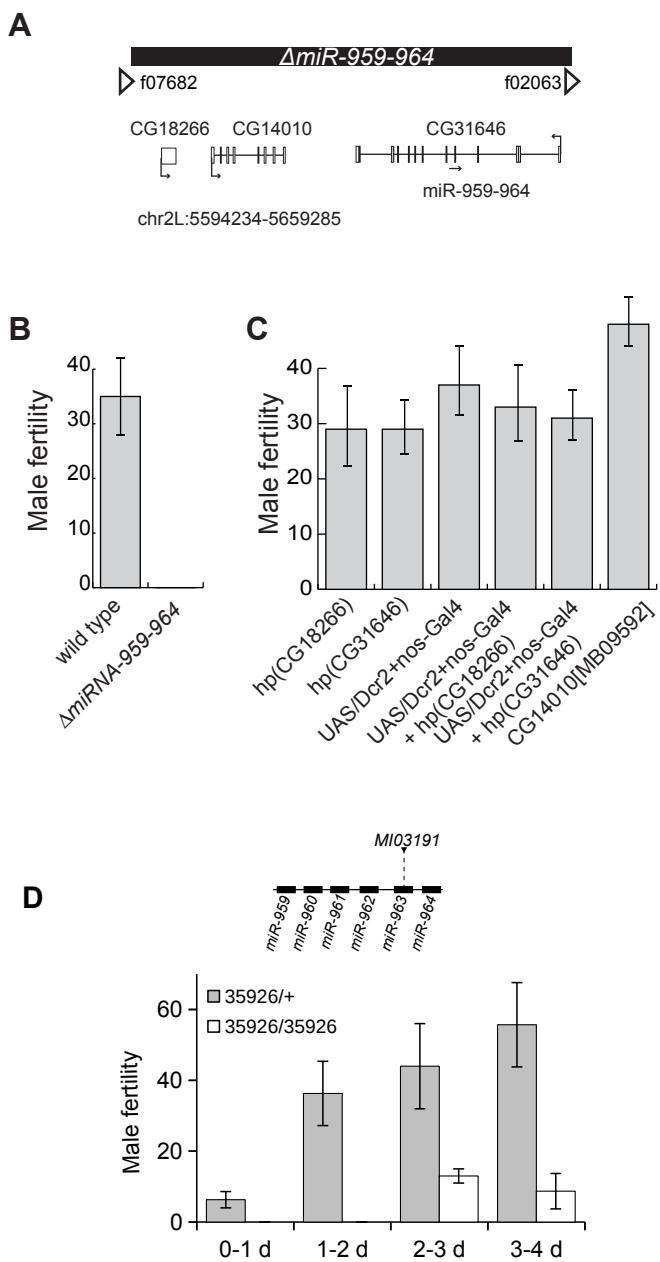


Figure S3. Phase-contrast images of wild-type and $\Delta miR-959-964$ testes.

(A) Post-mitotic mature primary spermatocytes with nuclei (arrow) and nucleoli (arrowhead).

(B) Post-meiotic round spermatids at the onion stage with nuclei (arrow) and Nebenkerns (arrowhead).

Scale bar, 10 μ m.

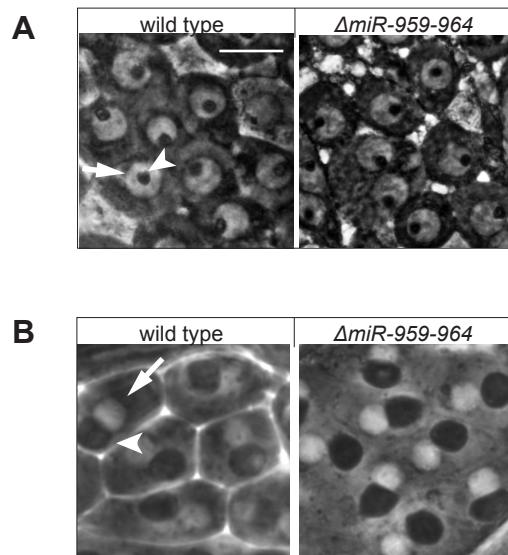


Figure S4. Testes of wild-type and $\Delta miR\text{-}959\text{-}964$ males.

(A,B) The typical testis of a 3-day old wild type male. (C,D,E) The typical testis of a 3-day old $\Delta miR\text{-}959\text{-}964$ male. (B,D and E) represent the magnification of the selected regions of testis as indicated at (A) and (C). Scale bar, 100 μm (A,C) and 25 μm (B,D,E). The spermatid nuclei are grouped in bundles in wild type testis while the spermatid nuclei of mutant testis demonstrate 'scattered' phenotype. Nuclei are stained by DAPI.

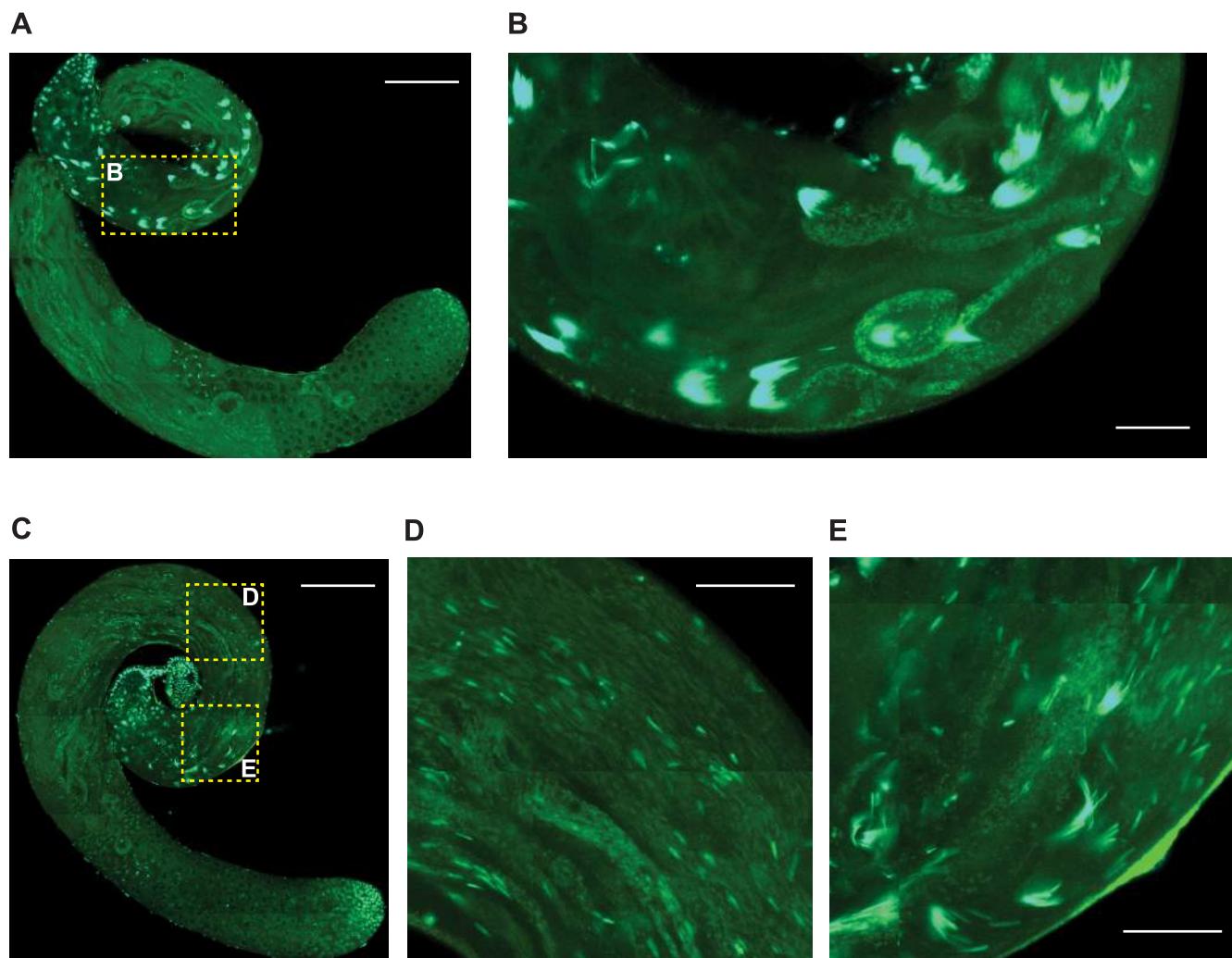


Figure S5. The novel putative miRNA in the miR-959-964 cluster.

(A) The location of novel putative pre-miRNA in miR-959-964. (B) The structure annotated alignment of novel pre-miRNA in different species evaluated by RNAalifold. The consensus secondary structure is present in dot-bracket notation. The minimum free energies (kcal/mol) and randfold *P*-values of pre-miRNAs are represented in the right panel. The significant *P*-values indicated the stable secondary structures are highlighted by gray color. (C) The MFE structure of novel putative pre-miRNA in *D. melanogaster* with unstable hairpin and *D. pseudoobscura* with stable hairpin drawing encode base-pair probabilities and evaluated by RNAfold.

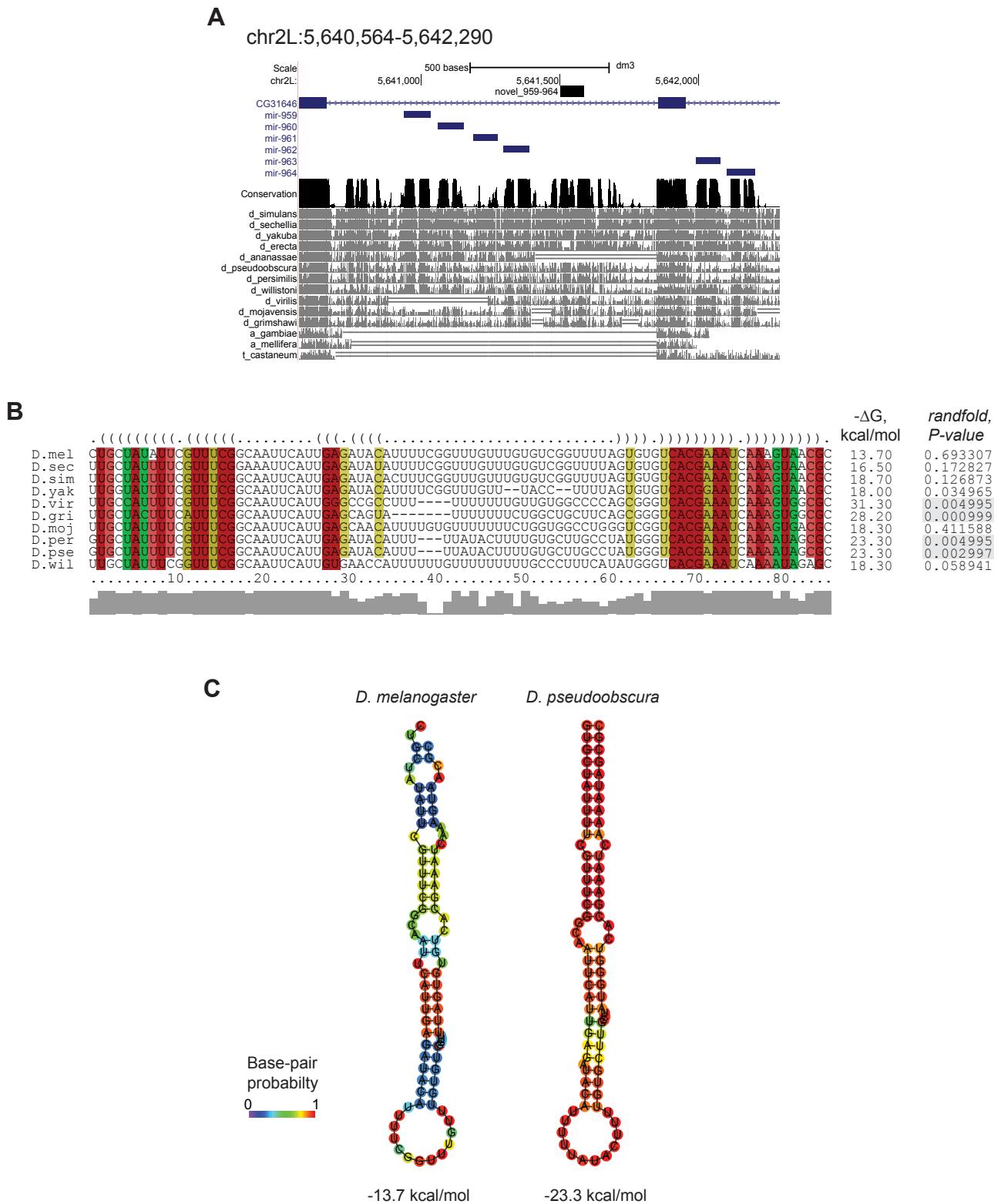


Table S1. Primers and probes used in the study.

Primers for amplification of <i>miRNA-959~964</i> cluster	
clu_d	5' - ATGTATGCTAGCAGCCACGCAATCTAGGAGAAC
clu_r	5' - TGGATTCTAGAGACCGATGCTCGATTTGTT

Primers for amplification of 3'UTR fragments of genes	
didum-s	5' - TGGATTGGATCCACCGTCGCATCTTAATCTGG
didum-as	5' - TGGATTGGATCCGTGGCTACCGGTATGGTGAC
CG10033-s	5' - TGGATTCTAGAAGCAATGCCAGCAGTATCT
CG10033-as	5' - TGGATTCTAGAATGTGCAACGCAATGAATT
CG10512-s	5' - TGGATTCTAGAGCATTGATGGACCAAAGC
CG10512-as	5' - TGGATTCTAGAGTTGCAATCGCAAGAAAAAA
CG1597-s	5' - TGGATTGGATCCATGCCACTTGACCACCTTCC
CG1597-as	5' - TGGATTGGATCCGGAGCAGTACGATGACACCA
CG33134-s	5' - TGGATTGGATCCCTAGATTCGCTTGGGATCG
CG33134-as	5' - TGGATTGGATCCTGCATAACACCCAGACTTCG
CG8824-s	5' - TGGATTCTAGACAATGCGTGCAAAAATGTCT
CG8824-as	5' - TGGATTCTAGATGCAGGTGACGTTGAACACT
CG8924-s	5' - TGGATTGGATCCTCAACGATCAGAAGCTGCAC
CG8924-as	5' - TGGATTGGATCCAGTACTGGGGCGACAAGAAA

Color legend:

- [ATGC]* - sequence added for improving the effectiveness of digestion
- [ATGC]* - restriction site of NheI
- [ATGC]* - restriction site of XbaI
- [ATGC]* - restriction site of BamH1

After amplification, PCR-products were purified, digested by the corresponding restriction endonucleases and cloned in the vector as described in the Material and Methods section.

Probes for Northern-blot	
anti-bantam	5' - ATCAGCTTCAAAATGATCTCA
anti-miR-960*	5' - C _t ATgCAaTCTGGaATaCTcA

*lowercase letters indicate LNA nucleotides

Primers for screening of deletion (see also Parks et al, 2009)		
FDD_l-f	5' - CCCACAAACAAACCTCCAGAAG	Left genome-transgene junction
WH3' pl-ri-f	5' - CCTCGATATAACAGACCGATAAAAC	
WH5' min r_f	5' - TCCAAGCGGCGACTGAGATG	Right genome-transgene junction
FDD_r_r	5' - AGCATACCCGGTCCTTGATG	

Primers for RT-qPCR	
didum-s	5' - TTTCGTGCCTGGTGCTTG
didum-as	5' - GATTCGCTGGTCTTGTGC
CG10512-s	5' - CTCAGGATCCCAGTGGTGAA
CG10512-as	5' - GAGTAGTGGCTCCGGACAT
fdl-s	5' - TCCCCAATCCCAGCCATTG
fdl-as	5' - TCGCGCTACTATCCAGAGTT
for-s	5' - GTTGCACATGGTTGGGAG
for-as	5' - ATGCATAACGTTGGGTGGC

Primers for rescue transgene cloning

Construct #1

clu_d5 5' -ATGTATCTCGAGAGCCACGCAATCTAGGAGAAC
clu_r2 5' -TGGATTCTAGAGACCGATGCTCGATTTGTT

Construct #2

CG31646_1 5' -GCCCTCACG_aATT_cCCATATCGGTGCTG
CG31646_2 5' -GCCCCC_GC_gC_gATTTTGCACTGC
CG31646_3 5' -TCCCGACTT_gCGG_cC_gCCGTTACGTTCTGCAG
CG31646_4 5' -GATTGGGATCACTTTAACGACCGAT_tCT_aGATTGTTG

Color legend:

- [ATGC]* - sequence added for improving the effectiveness of digestion
- [ATGC]* - restriction site of XhoI
- [ATGC]* - restriction site of XbaI
- [ATGC]* - restriction site of NotI

Small letters indicate the positions where genomic sequence was changed in the order to obtain the restriction site.

Table S2. Protein-coding genes with decreased level of expression in testis of $\Delta miRNA\text{-}959\text{--}964$. The adjusting of *P-value* for multiple hypothesis testing was performed with Benjamin-Hochberg algorithm.

Name	logFC	P-value	P _{adj} -value
CG31601	-2.66	3.93E-06	2.45E-03
<i>Der-1</i>	-2.58	5.70E-06	2.68E-03
CG12376	-2.33	1.86E-05	4.85E-03
CG11455	-2.19	3.64E-05	7.77E-03
CG4631	-2.11	3.70E-06	2.45E-03
CG1380	-1.99	1.02E-04	1.19E-02
CG18808	-1.91	1.48E-04	1.66E-02
<i>Mst77F</i>	-1.88	2.13E-06	2.45E-03
<i>Peb</i>	-1.86	4.18E-06	2.45E-03
CG15109	-1.82	1.64E-05	4.85E-03
CG31788	-1.75	3.45E-04	2.47E-03
<i>Kap-alpha1</i>	-1.75	3.54E-04	2.47E-03
CG4714	-1.73	3.85E-04	2.51E-03
CG31286	-1.69	4.79E-04	2.88E-03
<i>PebII</i>	-1.64	6.63E-05	1.03E-02
CG11023	-1.63	6.55E-04	3.21E-02
CG8565	-1.63	1.82E-05	4.85E-03
CG8565	-1.58	8.57E-04	3.69E-02
CR33318	-1.57	7.04E-05	1.03E-02
CG31210	-1.52	3.20E-05	7.51E-03
CG10919	-1.51	1.26E-03	4.90E-02
CG17005	-1.51	1.29E-03	4.90E-02
CG14183	-1.50	9.98E-05	1.19E-02
Cul-3	-1.49	4.94E-05	9.05E-03
CG32371	-1.46	9.16E-05	1.19E-02
CG4706	-1.45	5.01E-05	9.05E-03
CG32081	-1.40	1.81E-04	1.77E-02
<i>Ran</i>	-1.39	7.02E-05	1.03E-02
CG5045	-1.29	3.59E-04	2.47E-03
CG13243	-1.28	3.44E-04	2.47E-03
<i>Psa</i>	-1.27	2.08E-04	1.88E-02
CG9254	-1.27	1.75E-04	1.77E-02
<i>Pen</i>	-1.27	1.74E-04	1.77E-02
<i>Npl4</i>	-1.26	3.36E-04	2.47E-03
<i>f-cup</i>	-1.25	6.37E-04	3.21E-02
CG31948	-1.24	2.45E-04	2.06E-02
<i>skap</i>	-1.22	5.87E-04	3.19E-02
CG4983	-1.21	5.38E-04	3.01E-02
CR40459	-1.21	4.68E-04	2.88E-02
<i>ssp5</i>	-1.13	5.35E-04	3.01E-02
<i>endos</i>	-1.13	7.09E-04	3.40E-02
<i>Porin2</i>	-1.11	6.49E-04	3.21E-02
CG15523	-1.11	1.11E-03	4.44E-02
CG7742	-1.09	9.74E-04	4.08E-02
CG4021	-1.09	7.45E-04	3.43E-02
CG3927	-1.07	8.65E-04	3.69E-02
CG43342	-1.05	1.08E-03	4.43E-02
CG9389	-1.02	1.29E-03	4.90E-02

Table S3. Genes Ontology terms enrichment of down-regulated genes in testis of $\Delta miR-959-964$. Top ten enriched terms for each category are represented. *P-values* were evaluated by Fisher's exact test and adjusted for multiple hypothesis correction with Benjamin-Hochberg (P_{adj} -value ≤ 0.05).

Biological processes:

GO Term	<i>P-value</i>	P_{adj} -value	Number of genes
microtubule-based process [GO:0007017]	6.97E-06	3.89E-02	9
microtubule-based movement [GO:0007018]	2.29E-04		4
spermatid development [GO:0007286]	3.01E-04		4
sperm individualization [GO:0007291]	3.19E-04		3
spermatid differentiation [GO:0048515]	3.42E-04		4
spindle organization [GO:0007051]	5.83E-04		5
microtubule cytoskeleton organization [GO:0000226]	7.97E-04		6
protein complex subunit organization [GO:0071822]	2.10E-03		6
post-mating behavior [GO:0045297]	3.39E-03		2
spermatogenesis [GO:0007283]	3.42E-03		4

Cellular components:

GO Term	<i>P-value</i>	P_{adj} -value	Number of genes
cytoplasmic dynein complex [GO:0005868]	2.57E-07	2.33E-04	4
axonemal dynein complex [GO:0005858]	1.29E-07	2.74E-03	3
dynein complex [GO:0030286]	1.51E-05	2.93E-03	4
axoneme part [GO:0044447]	1.61E-05	2.99E-03	3
microtubule cytoskeleton [GO:0015630]	1.62E-05	3.66E-03	9
axoneme [GO:0005930]	1.98E-05	4.55E-03	3
microtubule associated complex [GO:0005875]	2.11E-05	5.86E-03	8
cytoskeleton [GO:0005856]	9.28E-05	1.05E-02	9
cytoskeletal part [GO:0044430]	2.97E-04	2.99E-02	8
cytoplasmic part [GO:0044444]	2.51E-03		15