

Supplementary Materials for

Circular RNAs from *BOULE* play conserved roles in protection against stress-induced fertility decline

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Published 11 November 2020, *Sci. Adv.* **6**, eabb7426 (2020)
DOI: 10.1126/sciadv.abb7426

The PDF file includes:

Figs. S1 to S6

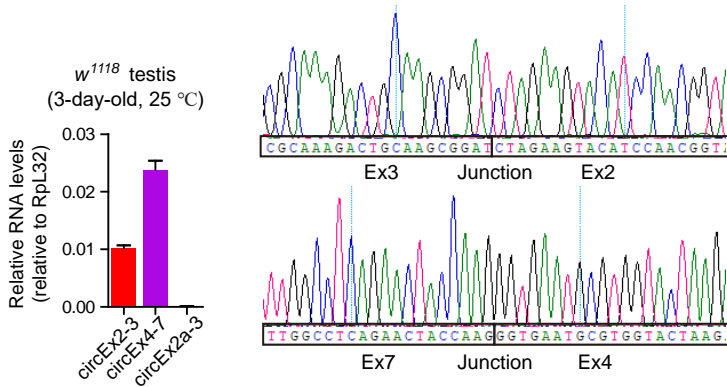
Other Supplementary Material for this manuscript includes the following:

(available at advances.sciencemag.org/cgi/content/full/6/46/eabb7426/DC1)

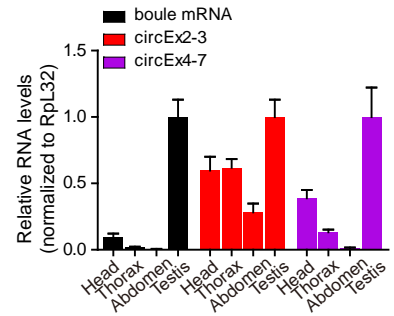
Tables S1 and S2

Fig. S1

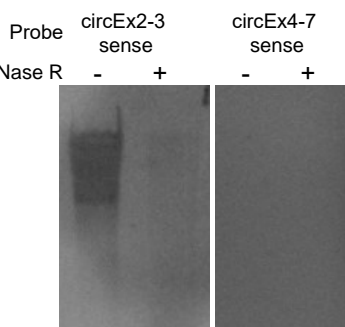
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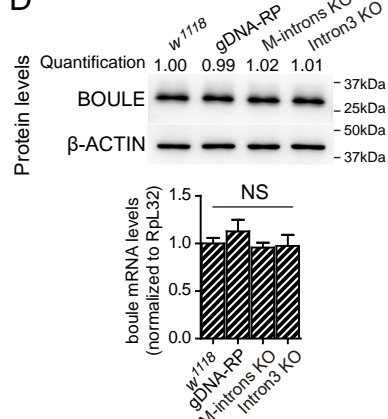
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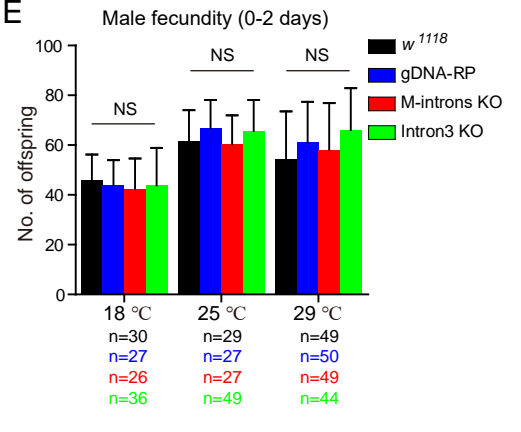
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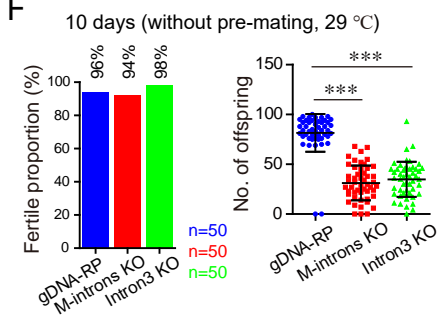
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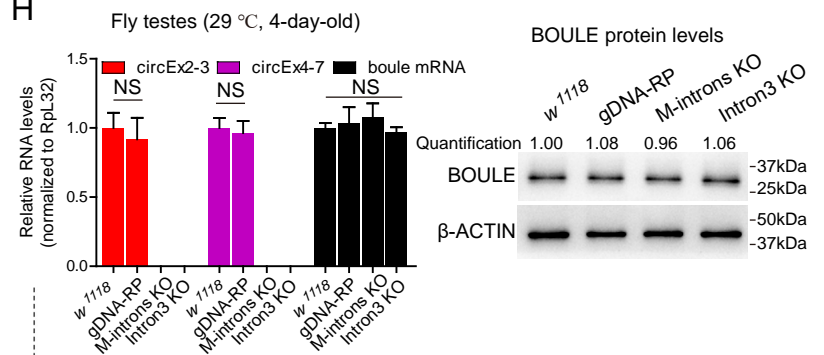
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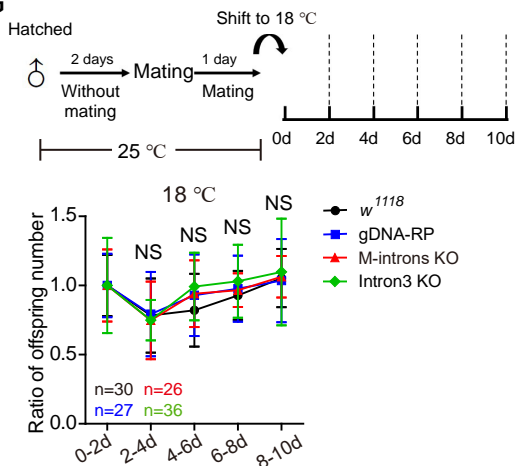
F



H



G



I

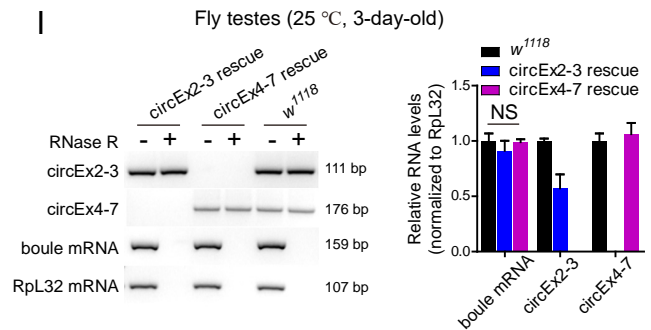


Fig. S1. CircBoule RNAs in fly.

(A) RNA levels of three circBoule RNAs were examined with w^{1118} testes via RT-qPCR, and sanger sequencing shown junction of circEx2-3 and circEx4-7.

(B) RNA levels of *boule* mRNA, circEx2-3 and circEx4-7 were detected in different tissues of w^{1118} male flies via RT-qPCR.

(C) Northern blotting of circEx2-3 and circEx4-7. Sense probes of circEx2-3 and circEx4-7 junctions (negative controls) were used to blot RNAs from w^{1118} fly testes.

(D) *boule* mRNA and BOULE protein were examined with testes of 2-day-old w^{1118} , gDNA-RP, M-introns KO and Intron3 KO flies (raised at 25 °C) via RT-qPCR and Western blot, respectively.

(E) Male fecundity of w^{1118} , gDNA-RP, M-introns KO and Intron3 KO flies showed no significant difference when shifted to 18 °C, 25 °C or 29 °C in the first 2 days. n = number of male flies tested.

(F) Males of gDNA-RP, M-introns KO and Intron3 KO flies reared at 29 °C for 10 days (without mating) can mate, although M-introns KO and Intron3 KO males had less progeny. n = number of male flies tested.

(G) Ratio of offspring numbers (normalized to 0-2 days progeny) of w^{1118} , gDNA-RP, M-introns KO and Intron3 KO male flies mating at 18 °C. Scheme of experimental setup was shown. There was no significant difference in fertility among w^{1118} , gDNA-RP, M-introns KO and Intron3 KO male flies at 18 °C. n = number of male flies tested.

(H) Left, RNA levels of *boule* mRNA, circEx2-3, and circEx4-7 were examined with testes of w^{1118} , gDNA-RP, M-introns KO and Intron3 KO flies reared at 29 °C for 4 days via RT-qPCR. Right, western blotting of BOULE protein in testes of w^{1118} , gDNA-RP, M-introns KO and Intron3 KO flies reared at 29 °C for 4 days.

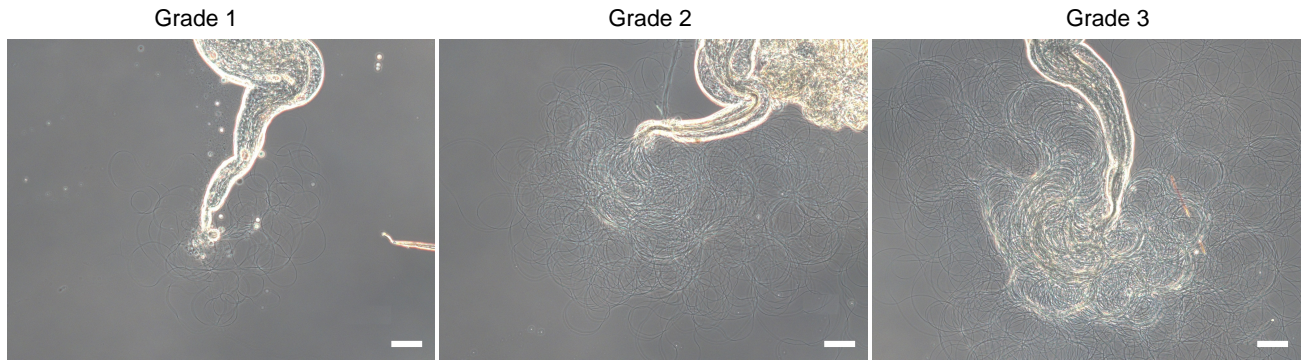
(I) Examination of successful expression and relative levels of circEx2-3 and circEx4-7 in testes of circEx2-3 rescue and circEx4-7 rescue flies.

Data are shown as means \pm SD; *p*-values from unpaired Student's *t*-test; NS, not significant; *** $p < 0.001$

Fig. S2

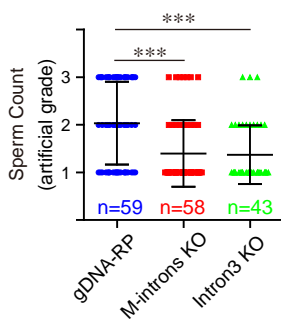
A

Sperm count (artificial grade) representative pictures



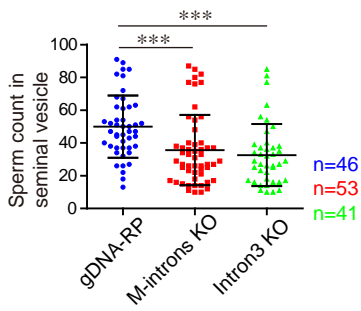
B

Continuous mating for 10 days (29 °C)



C

Continuous mating for 10 days (29 °C)



Without mating for another 2 days after a 10-day-mating (29 °C)

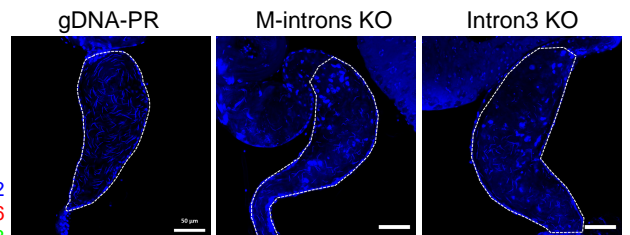
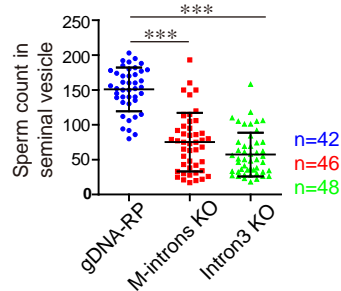


Fig. S2. Sperm count of gDNA-RP, M-introns KO and Intron3 KO males.

(A) Representative images of sperm counts were shown, dividing three levels: Grade 1 (low), Grade 2 (medium) and Grade 3 (high).

(B) Quantification of sperm counts in gDNA-RP, M-introns KO and Intron3 KO males continuously mating for 10 days at 29 °C. Representative images were shown.

(C) Sperm counts in seminal vesicles of gDNA-RP, M-introns KO and Intron3 KO males continuously mating for 10 days or without mating for 2 days after continuously mating for 10 days at 29 °C. Representative images of seminal vesicles of gDNA-RP, M-introns KO and Intron3 KO males without mating for 2 days after continuously mating for 10 days at 29 °C were shown.

Scale bar, 50 μm in (A to C). n= fly testis number. Data are shown as means \pm SD; *p*-values from unpaired Student's *t*-test; *** *p* < 0.001.

Fig. S3

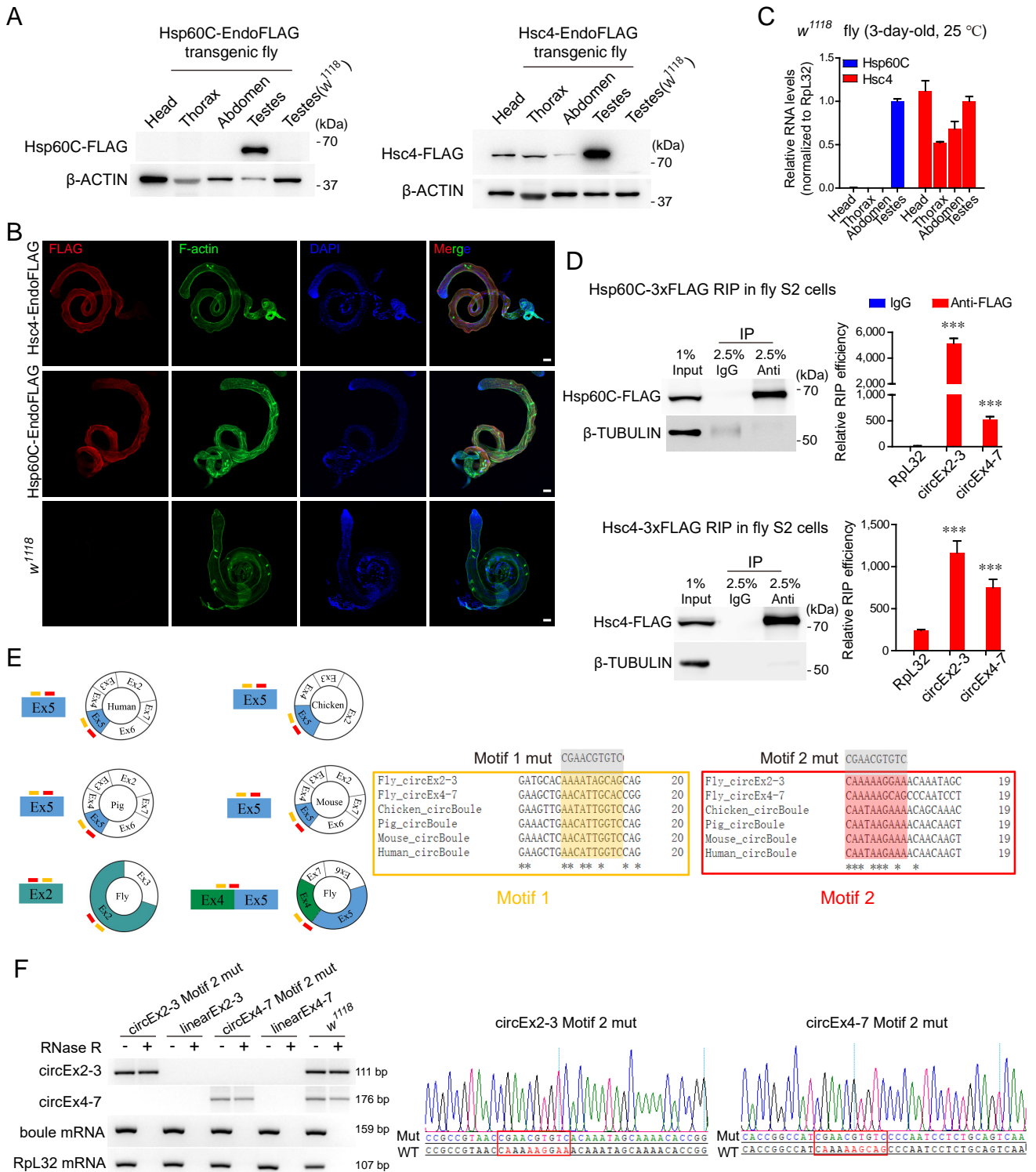


Fig. S3. Fly circBoule RNAs interact with Hsp60C and Hsc4 proteins.

(A) Western blotting of Hsp60C and Hsc4 proteins in different tissues from Hsp60C-EndoFLAG and Hsc4-EndoFLAG transgenic flies.

(B) Immunofluorescence staining of Hsp60C and Hsc4 proteins in whole testes of *w¹¹¹⁸*, Hsp60C-EndoFLAG and Hsc4-EndoFLAG transgenic flies using FLAG antibody. Scale bar, 50 μ m.

(C) mRNA levels of *Hsp60C* and *Hsc4* were examined in different tissues of *w¹¹¹⁸* male flies via RT-qPCR.

(D) Fly S2 cells transfected with circBoule RNAs, Hsp60C-3xFlag or Hsc4-3xFlag vectors were subjected to RIP with FLAG antibody. Precipitated RNAs were analyzed by RT-qPCR, and the enrichment was normalized to the IgG control. Successful pull-down of FLAGed proteins was examined with Western blot.

(E) Positions of conserved motifs (yellow, Motif 1; red, Motif 2), and sequence alignment of the motifs among species were shown. The sequences of mutated Motif 1 and Motif 2 were shown. Mut, mutation.

(F) Successful expression of circEx2-3 Motif 2 mut and circEx4-7 Motif 2 mut in fly testes. Sanger sequencing showed mutation of Motif 2 sequences (labelled by red box) in circEx2-3 or circEx4-7.

Data are shown as means \pm SD; *p*-values from unpaired Student's *t*-test; *** *p* < 0.001.

Fig. S4

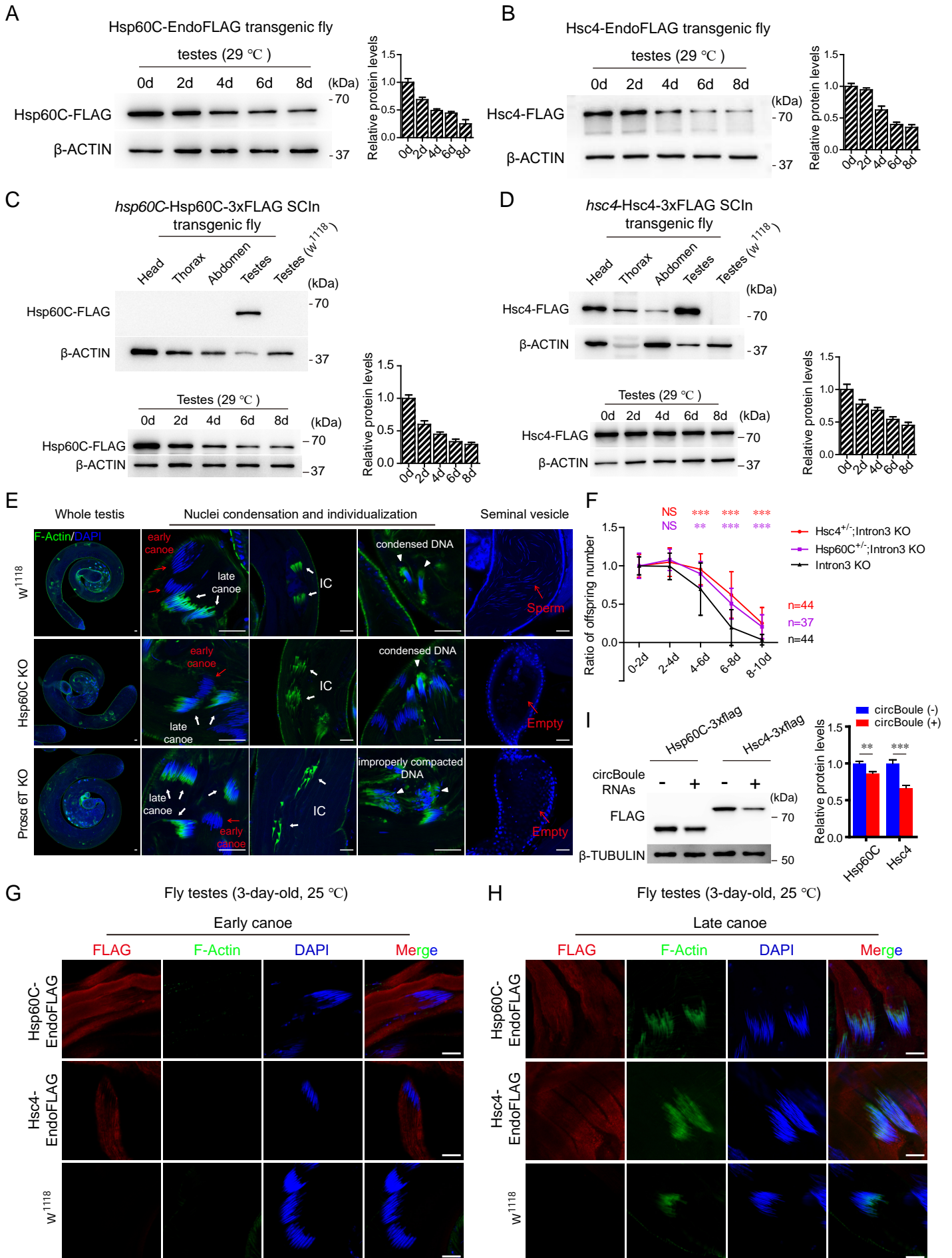


Fig. S4. Fly circBoule RNAs down-regulate Hsp60C and Hsc4 protein levels.

(A and B) The protein levels of Hsp60C (A) and Hsc4 (B) in testes of Hsp60C-EndoFLAG and Hsc4-EndoFLAG transgenic flies at different time points at 29 °C. Quantification of protein levels (relative to β -ACTIN) was shown in bar graph.

(C and D) Upper, western blotting of Hsp60C (C) and Hsc4 (D) proteins in different tissues from 2-day-old *hsp60C*-Hsp60C-3xFLAG SCIn and *hsc4*-Hsc4-3xFLAG SCIn male flies at 25 °C, respectively. Lower, the protein levels of Hsp60C (C) and Hsc4 (D) protein in testes of *hsp60C*-Hsp60C-3xFLAG SCIn and *hsc4*-Hsc4-3xFLAG SCIn flies at different time points at 29 °C. Quantification of protein levels (relative to β -ACTIN) was shown in bar graph.

(E) Immunofluorescence staining of *w¹¹¹⁸* (control), Hsp60C KO, and Prosa6T KO testes with phalloidin (green) and DAPI (blue). Mature sperm was absent in seminal vesicles of adult Hsp60C KO or Prosa6T KO testes. Groups of 64 elongating spermatid nuclei and IC (individualization complex) showed no obvious difference in *w¹¹¹⁸*, Hsp60C KO, and Prosa6T KO testes until the late canoe stage, before individualization stage. Following the late canoe stage, IC moves progressively through the cyst (from head to tail) in synchronous way (*w¹¹¹⁸*); in contrast, IC was asynchronous in both Hsp60C KO and Prosa6T KO testes. Hsp60C KO seems to have condensed bundles of nuclei DNA, but bundles of nuclei DNA appears uncondensed in Prosa6T KO testes. Scale bar, 20 μ m.

(F) Ratio of offspring numbers (relative to progeny of 0-2 days) of *Intron3 KO*, *Hsp60C^{+/-};Intron3 KO*, and *Hsc4^{+/-};Intron3 KO* males at 29 °C. n= males tested.

(G and H) Immunofluorescence staining of Hsp60C and Hsc4 protein in early canoe stage (G) and late canoe stage (H) sperm bundles using anti-FLAG antibody. Testes for staining were from 3-day-old flies reared at 25 °C. Early canoe stage had features of less condensed sperm nuclei and no F-Actin staining; while late canoe stage had condensed sperm nuclei and F-Actin staining. Scale bar, 10 μ m.

(I) CircBoule RNAs reduced the protein levels of Hsp60C and Hsc4 in fly S2 cells. CircBoule RNAs and heat shock proteins were expressed with plasmids in S2 cells. Quantification of protein levels (relative to β -TUBULIN) was shown in bar graph.

Data are shown as means \pm SD; *p*-values from unpaired Student's *t*-test; ** *p* < 0.01; *** *p* < 0.001.

Fig. S5

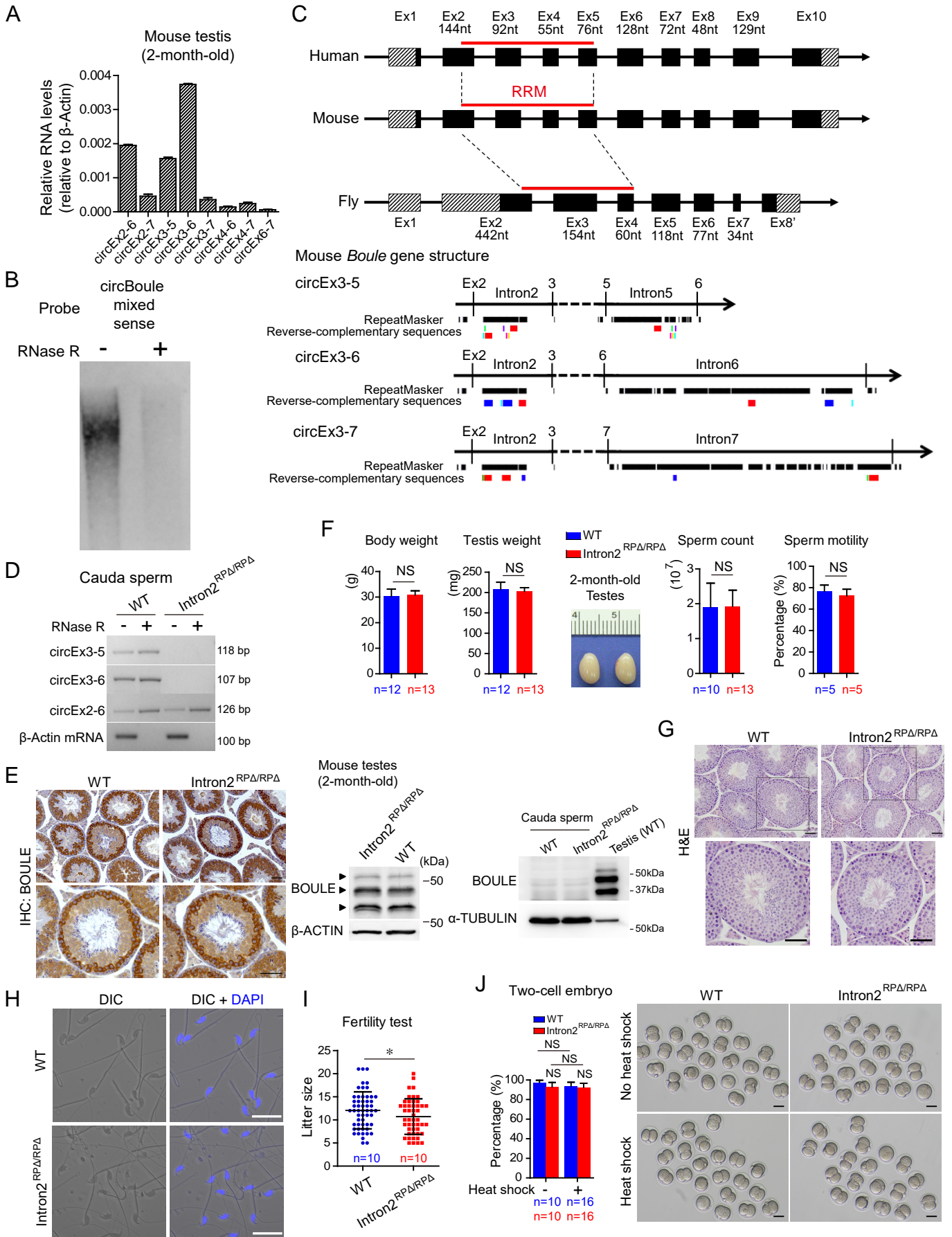


Fig. S5. Phenotypes of mice with or without circBoule RNAs.

(A) Multiple circRNAs were generated from mouse *Boule* gene locus, and the levels of circBoule RNAs were examined with 2-month-old mouse testis via RT-qPCR.

(B) Northern blotting of murine circBoule RNAs. Mix sense probes of circEx3-5, circEx3-6 and circEx3-7 junctions (as negative controls) were used to blot RNAs from wildtype mouse testes.

(C) Upper, gene structure homology among fly, mouse, and human *Boule*, and conserved RRM (RNA Recognition Motif) were marked by red line. Lower, schematics showed repeats (labelled by black rectangles) and reverse-complementary repeats (labelled by rectangles with the same color) in flanking introns of mouse *Boule* circEx3-5, circEx3-6 and circEx3-7.

(D) circEx3-5 and circEx3-6 were present in cauda sperm from wildtype mice but not detected in *Intron2^{RPΔ/RPΔ}* mice.

(E) Immunofluorescence staining and western blotting of BOULE protein in testis of 2-month-old wildtype and *Intron2^{RPΔ/RPΔ}* mice (black triangles indicated three BOULE protein isoforms). BOULE protein was not detected in cauda sperm of wildtype and *Intron2^{RPΔ/RPΔ}* mice.

(F) Body weight, testis weight, testis size, *sperm counts in epididymis* and sperm motility of 2-month-old wildtype and *Intron2^{RPΔ/RPΔ}* mice.

(G) H&E staining of 2-month-old wildtype and *Intron2^{RPΔ/RPΔ}* mice testes.

(H) Representative DIC and DAPI (blue) staining images of mature sperm in 2-month-old wildtype and *Intron2^{RPΔ/RPΔ}* mice.

(I) Litter size of adult wildtype and *Intron2^{RPΔ/RPΔ}* males continuously examined over four months.

(J) When the fertilized pronuclear eggs were examined, wildtype and *Intron2^{RPΔ/RPΔ}* showed no significant difference in developing into two-cell embryos, with or without heat shock.

n= number of male mice tested in (F, I, and J). Scale bar, 50 μm in (E, G, and J), 20 μm in (H). Data are shown as means ± SD; *p*-values from unpaired Student's *t*-test; NS, not significant; * *p* < 0.05.

Fig. S6

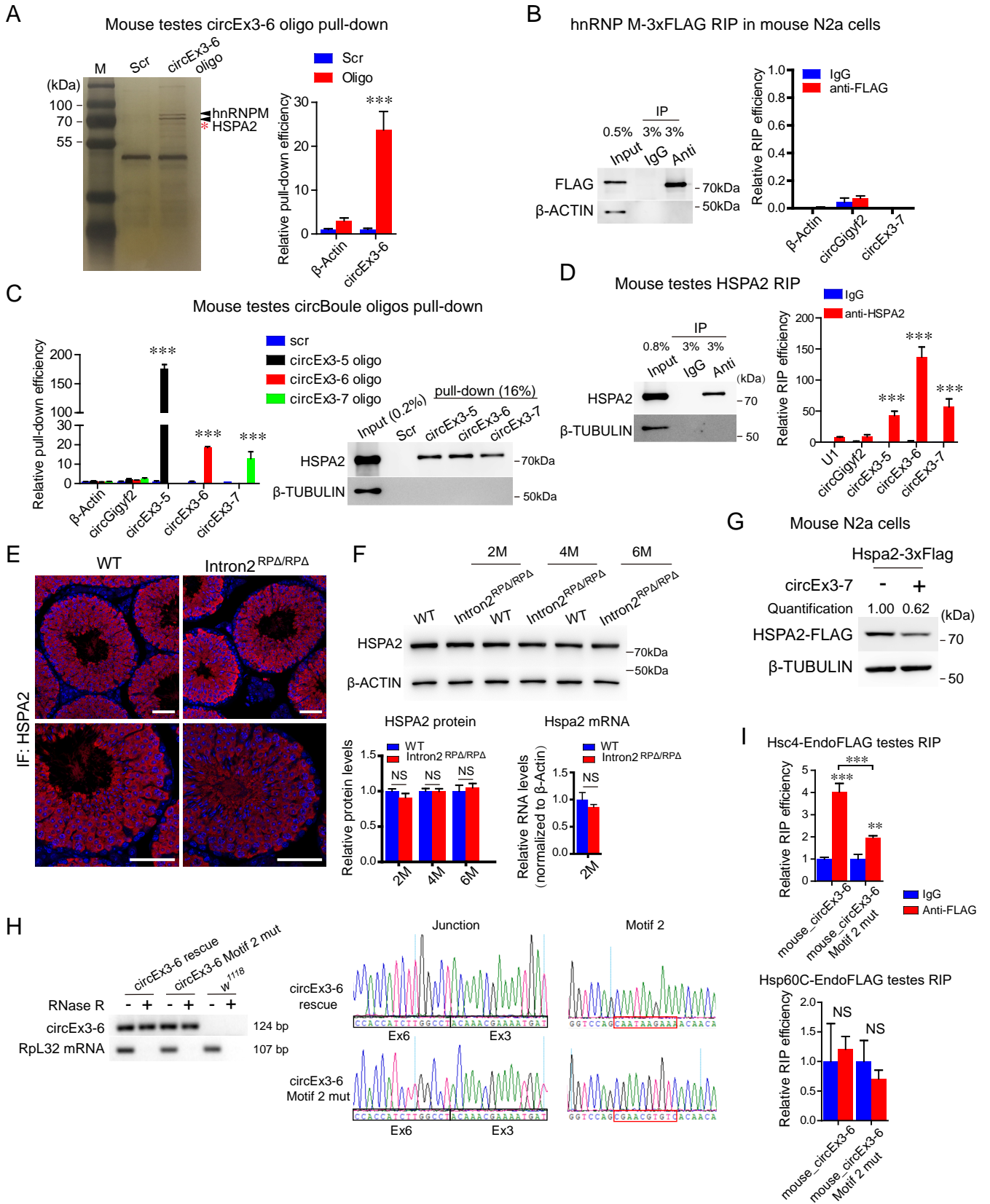


Fig. S6. Mouse circBoule RNAs interact with mouse HSPA2 and fly Hsc4 (when mouse circBoule RNA is artificially expressed in fly).

(A) Pull-down of circEx3-6 co-pulled down HSPA2 in mouse testis using biotin-labelled antisense oligos against the junction of circEx3-6. Proteins pulled down were analyzed by silver staining, and the bands indicated by triangles and red asterisk were identified to be hnRNP M and HSPA2 through mass spectrometry. The pull-down efficiency of circEx3-6 was showed by bar graph, and the enrichment was normalized to Scr control. Scr, negative control oligo with scrambled sequences.

(B) IP of hnRNP M-3xFLAG protein could not co-pulldown mouse circEx3-7 in cytoplasm of N2a cells. N2a cells transfected with circEx3-7 and hnRNP M-3xFlag plasmids were subjected to RIP with FLAG antibody. Precipitated RNAs were analyzed by RT-qPCR, and the enrichment was normalized to the IgG control. Successful pull-down of FLAGed protein was examined with Western blot.

(C) Pull-down of circBoule RNAs (circEx3-5, circEx3-6 and circEx3-7) co-pulled down HSPA2 in mouse testes. RNA pull-down using biotin-labeled antisense oligos against the junction of circEx3-5, circEx3-6 and circEx3-7, respectively. The pull-down efficiency of circBoule RNAs was showed by bar graph, and the enrichment was normalized to Scr control. Western blot of the HSPA2 co-pulled down was also shown. Scr, negative control oligo with scrambled sequences.

(D) IP of HSPA2 in mouse testes co-pulled down circBoule RNAs (circEx3-5, circEx3-6, and circEx3-7). The RIP enrichment of circBoule RNAs was showed by bar graph and normalized to IgG control. Western blot analysis of HSPA2 was shown.

(E) Immunofluorescence staining of HSPA2 protein in testes of 2-month-old wildtype and *Intron2^{RPA/RPA}* mice. Representative images were shown. Scale bar, 50 μ m

(F) Western blotting of HSPA2 protein in 2, 4 and 6-month-old testes of wildtype and *Intron2^{RPA/RPA}* mice. Quantification of HSPA2 protein levels (relative to β -ACTIN) was shown in bar graph. HSPA2 mRNA levels were examined with testes of 2-months-old wildtype and *Intron2^{RPA/RPA}* mice via RT-qPCR.

(G) CircEx3-7 reduced the protein level of HSPA2 in mouse N2a cells. CircEx3-7 and HSPA2-3xFlag were expressed with plasmids in N2a cells. Quantification of protein levels (relative to β -TUBULIN) was shown in bar graph.

(H) Successful expression of mouse_circEx3-6 and mouse_circEx3-6 Motif 2 mut in fly testes. Sanger sequencing of RT-PCR products shown junction and Motif 2 sequences (labelled by red box) of circEx3-6.

(I) IP of Hsc4 but not Hsp60C co-pulled down circEx3-6 in fly testes with expression of either mouse_circEx3-6 or mouse_circEx3-6 Motif 2 mut. The RIP enrichment of circEx3-6 was showed by bar graph and normalized to IgG control.

For A to D, F, G and I data are from three independent experiments, and shown as means \pm SD; *p*-values from unpaired Student's *t*-test. NS, not significant; ** *p* < 0.01, *** *p* < 0.001.