Supplementary Materials for

Exosomal circEZH2_005, an intestinal injury biomarker, alleviates intestinal ischemia/reperfusion injury by mediating hnRNPA1/Gprc5a signaling

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Supplementary Figures

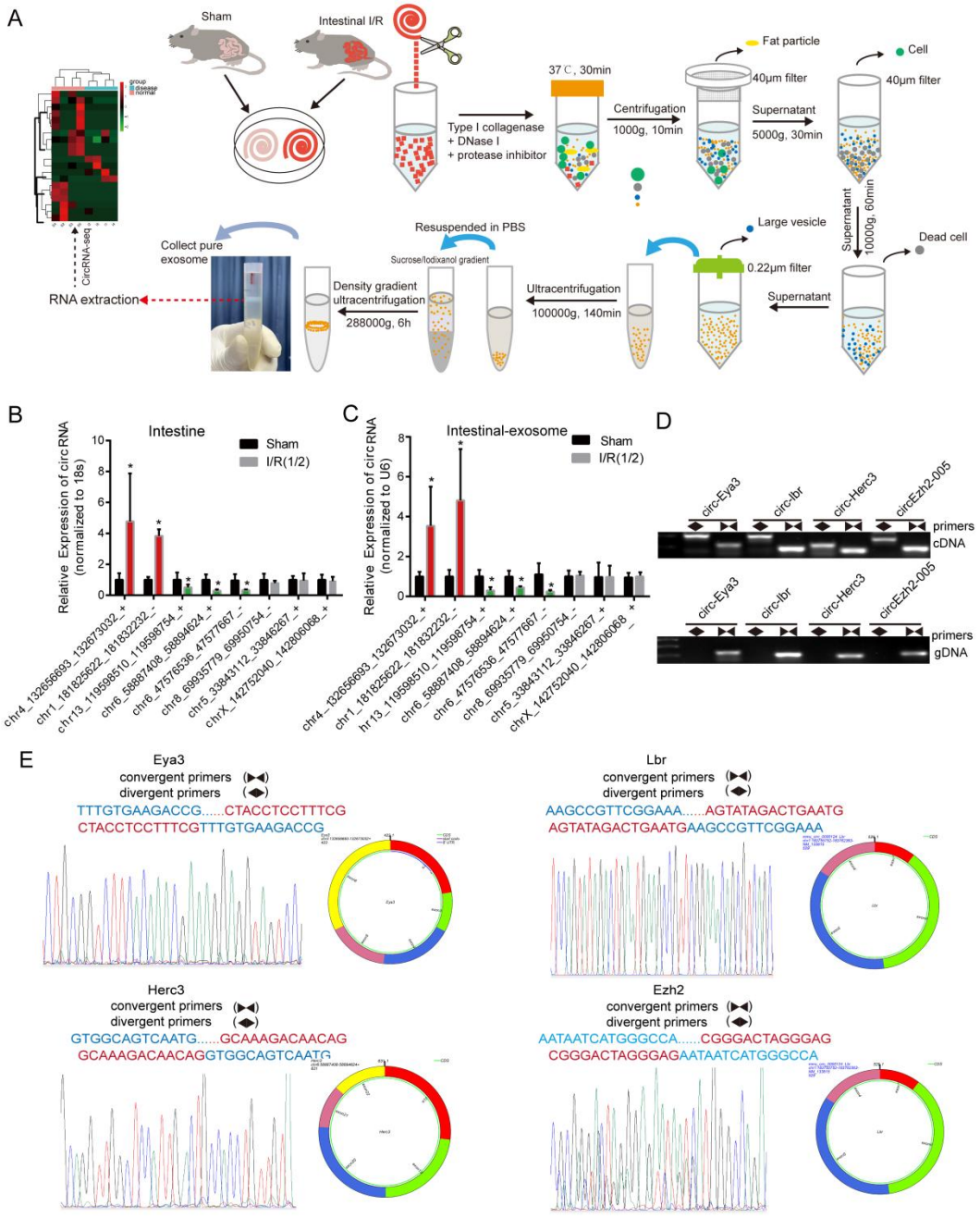

Fig. S1. Identification of the top four differentially expressed circRNAs. (**A**)The experimental scheme of exosome extraction from intestinal tissue. **(B, C)** RT-qPCR assay to illustrate the accuracy of circRNA-seq data from exosomes and confirmation of the eight highly expressed circRNAs. Data were presented as means ± SEM, n=5. (**D**)The amplification of circRNAs from cDNA or genomic DNA (gDNA) with divergent and convergent primers was shown by PCR. (**E**) Schematic illustration illustrating the generation of the top four putative target circRNAs from its host gene, then validated by Sanger sequencing.

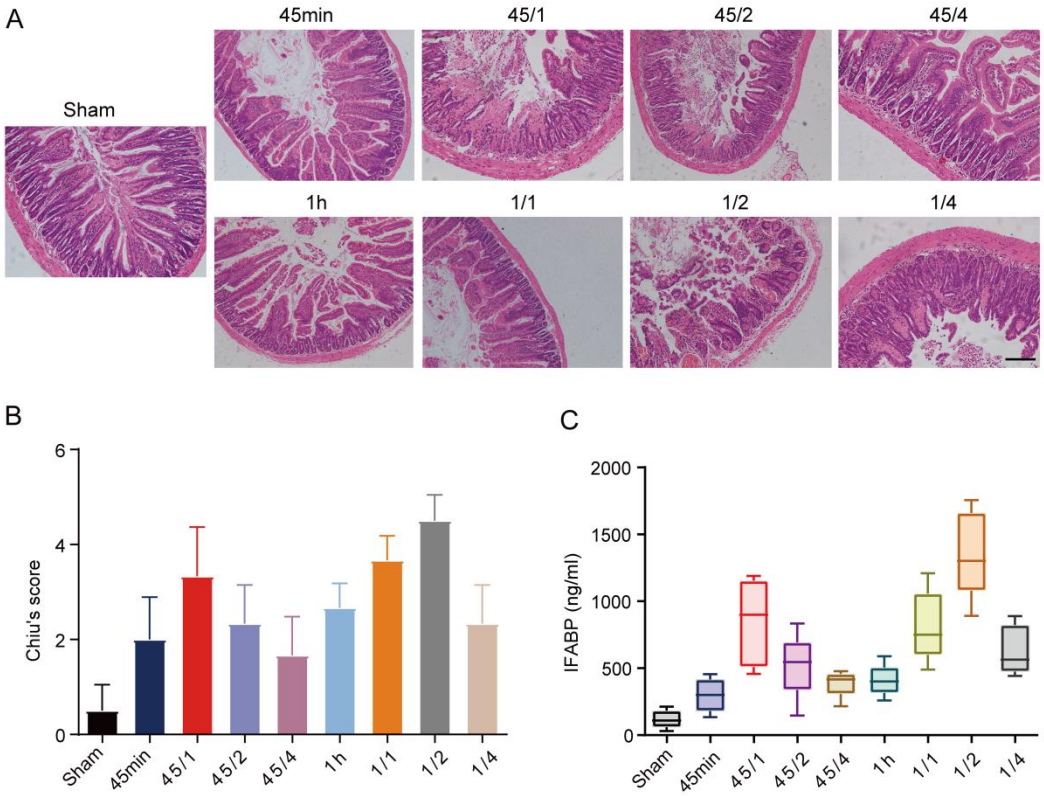

Fig. S2. Intestinal I/R model mice were established at different time points. (**A**) Mice were subjected to intestinal I/R mode and underwent different time points of ischemia (45 min and 1h of ischemia) and different time points of reperfusion (1h, 2h, and 4h of reperfusion), the histopathological was estimated by the H&E staining (n = 6). (**B**) Chui's score was used to assess the intestinal injury. (**C**) The ischemia-reperfusion-induced intestinal damage was evaluated by the serum I-FABP levels (n = 6). The data were analyzed by one-way ANOVA (Tukey's test) and presented as mean ± SEM. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

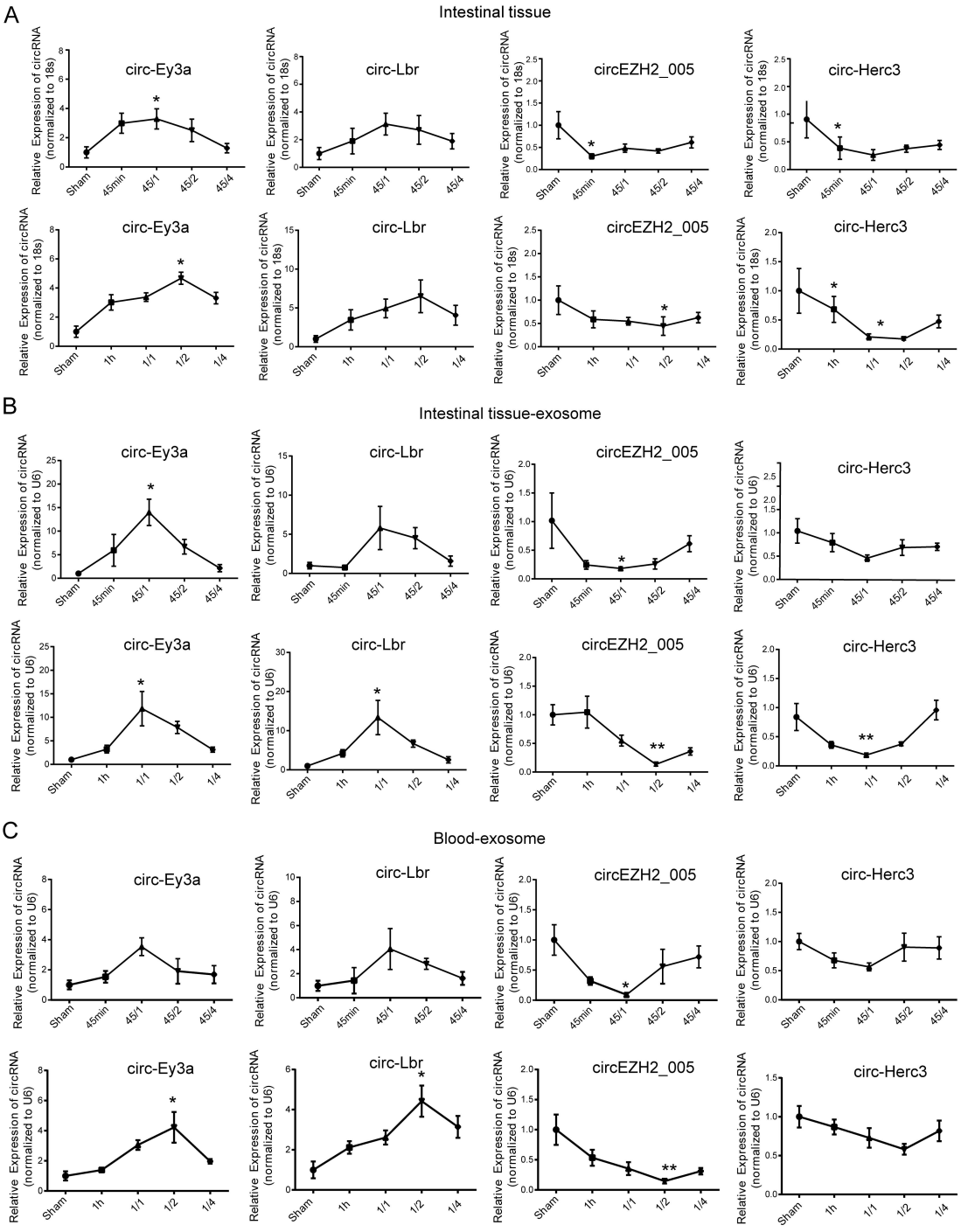

Fig. S3. The expression of the top four differentially expressed circRNAs. (A) RT-qPCR for the expression of the top four differentially expressed circRNAs in intestinal tissues at different time points of intestinal I/R mice mode. (**B**) Expression of the top four differentially expressed circRNAs in intestinal tissue exosomes at different time points of intestinal I/R mice mode. (**C**) Expression of the top four differentially expressed circRNAs in plasma exosomes at different time points of intestinal I/R mice mode. The data were analyzed by one-way ANOVA (Tukey's test) and presented as mean ± SEM, n=4. (* $p < 0.05$, ** $p < 0.01$).

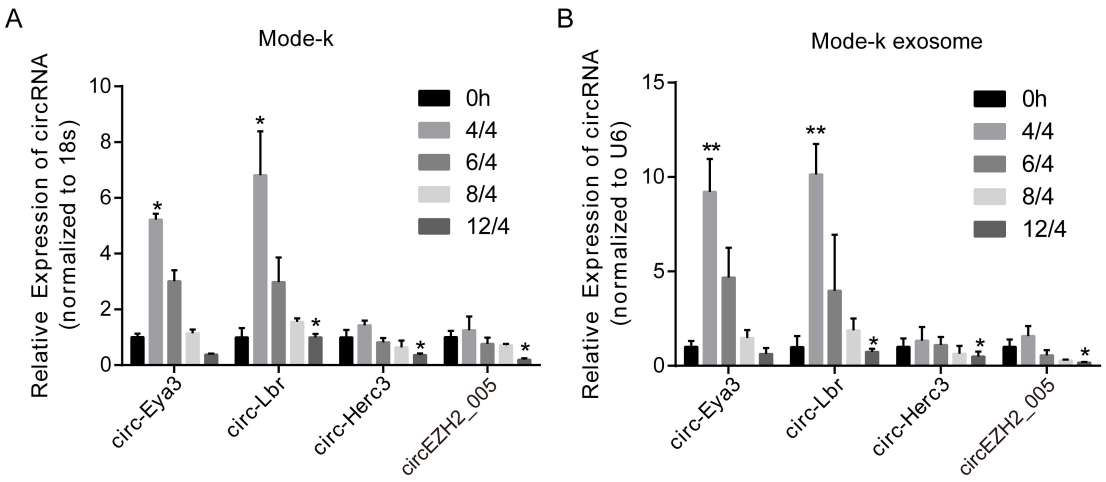

Fig. S4. The expression of the top four differentially expressed circRNAs in cells. RT-qPCR for the expression of the top four differentially expressed circRNAs in intestinal epithelial cell (Mode-K cell) and exosomes of cell supernatant subjected to different time points of hypoxia (4h, 6h, 8h, and 12h) and 4h of reoxygenation. The data were analyzed by one-way ANOVA (Tukey' s test) and presented as mean ± SEM, n=3. (*P < 0.05, **P < 0.01).

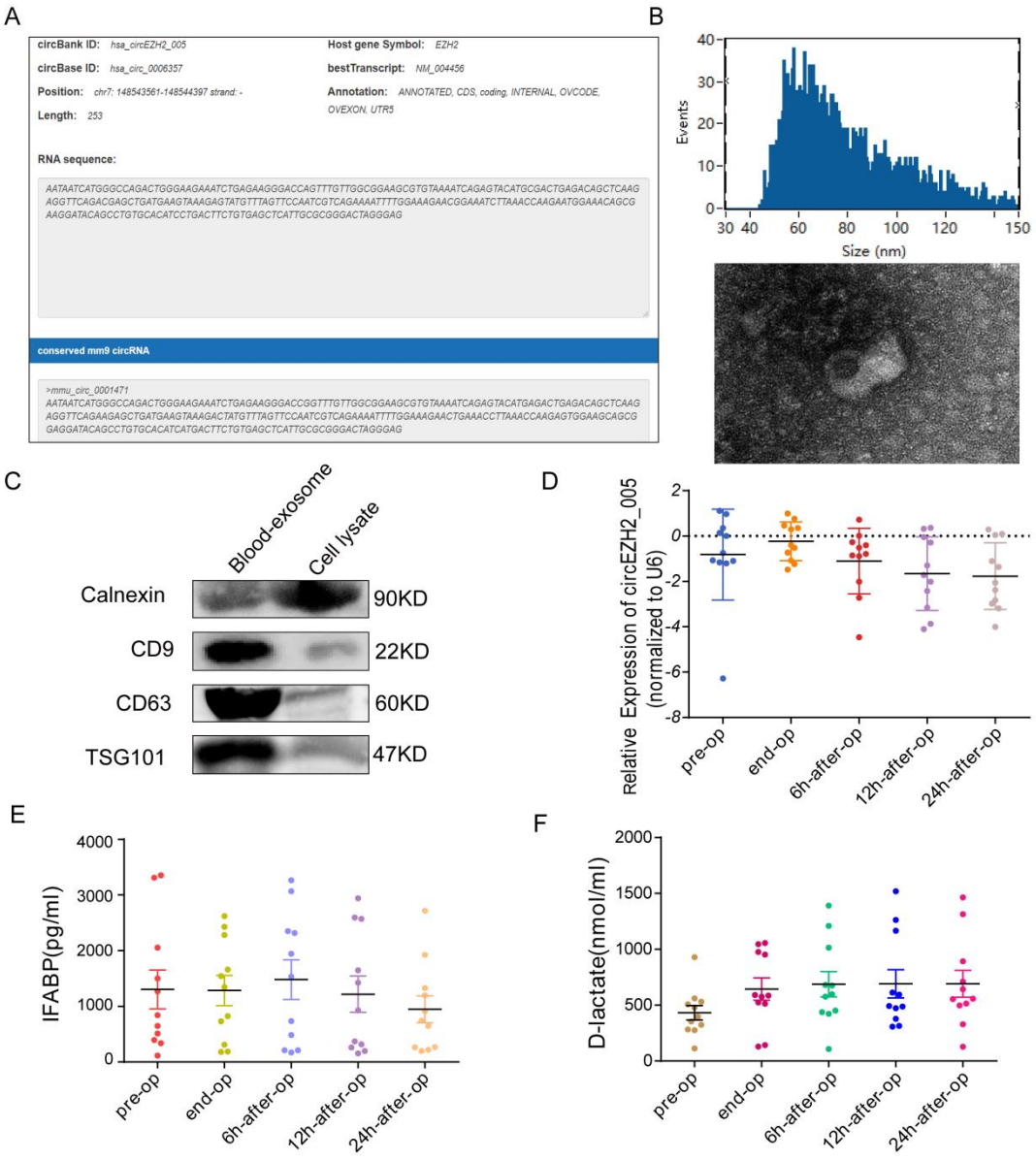

Fig. S5. Isolating and analyzing plasma exosomes from clinical patients. (**A**) Analysis of conservation of circEZH2_005 in humans and mice using circBank database. (**B**) The particle size and electron micrograph of plasma exosomes extracted by commercial kit. (**C**) The exosome-specific markers CD9, CD63, TSG101, and Calnexin were shown by western blot. (**D**) RT-qPCR analysis of exosomal circEZH2_005 expression in the plasma of patients without intestinal I/R injury in preoperative, end operative, 6 hours after operative, 12 hours after operative, and 24 hours after operative (n=11 in controls). (**E**, **F**) Plasma I-FABP and D-lactate levels were assessed by ELISA (n=11 in controls). The data were analyzed by one-way ANOVA (Tukey's test) and presented as mean ± SEM. (* p <0.05, ** p <0.01, *** p <0.001).

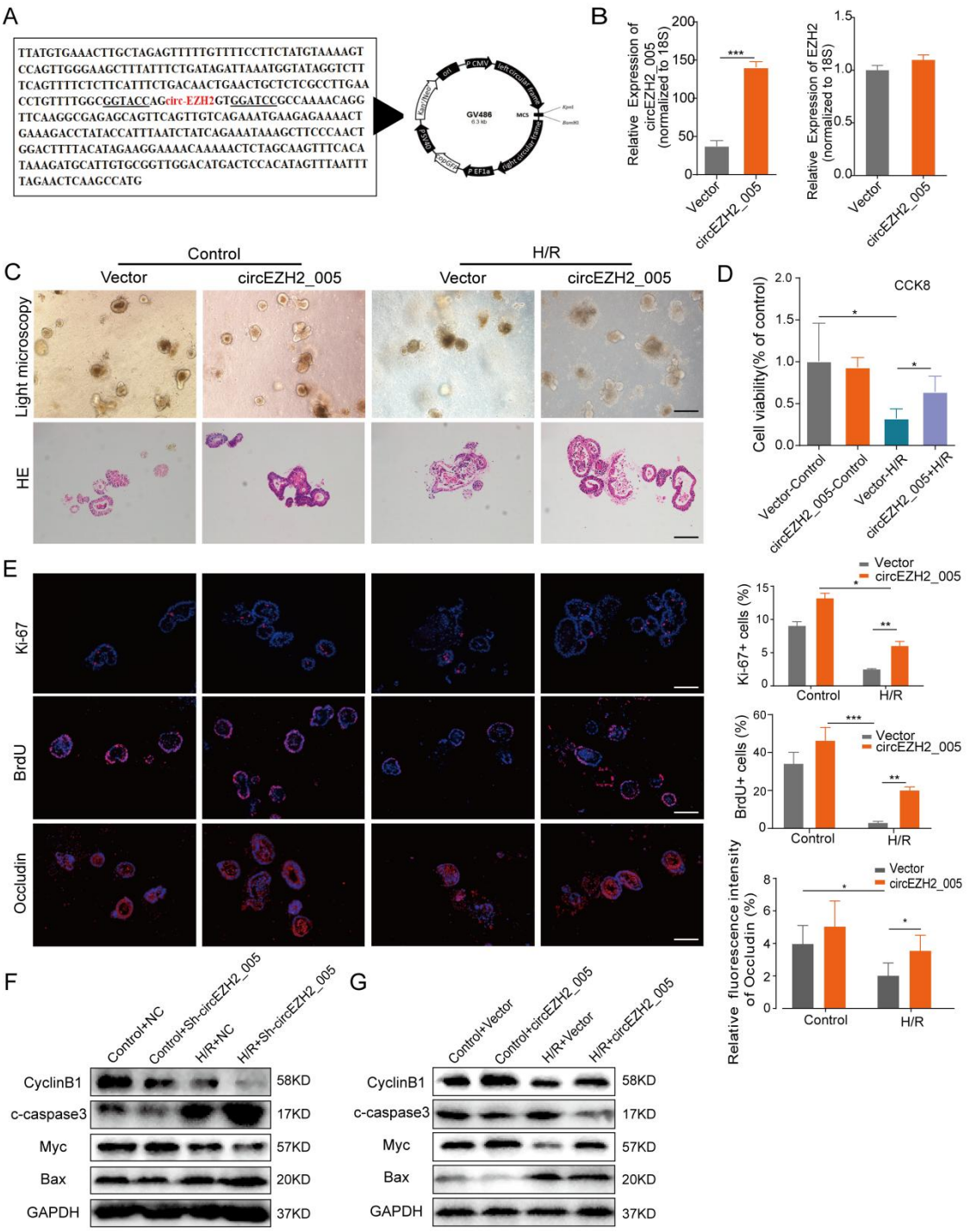

Fig. S6. CircEZH2_005 protects intestinal organoids from H/R injury in vitro. (A) The overexpression sequence of circEZH2_005 (the two end sequences of circEZH2_005 are partial concyclic sequences) was connected to the gv486 vector to construct circEZH2_005 overexpression plasmid, and then packaged on adenovirus vector. **(B)** RT-qPCR analysis of circEZH2_005 overexpression and EZH2 expression in circEZH2_005 -adenovirus transfected cells (n = 3). **(C)** Organoid morphology was observed by light microscopy and HE staining, scale bar is 100μm (n = 3). **(D)** The organoid viability was analyzed by CCK-8 (n = 5). **(E)**

Immunofluorescence staining for the Ki-67, BrdU, and occludin in the organoids for proliferation analysis, scale bar is 100μm (n = 3). **(F)** Western blot was conducted to analyze apoptotic and proliferation-related proteins induced by H/R and circEZH2_005 interference (n = 3). **(G)** Western blot was conducted to analyze apoptotic and proliferation-related proteins induced by H/R and circEZH2_005 overexpression (n = 3). The data were analyzed by the one-way ANOVA (Tukey's test) and presented as means ± SEM. (* p <0 .05, ** p < 0.01, *** p <0.001).

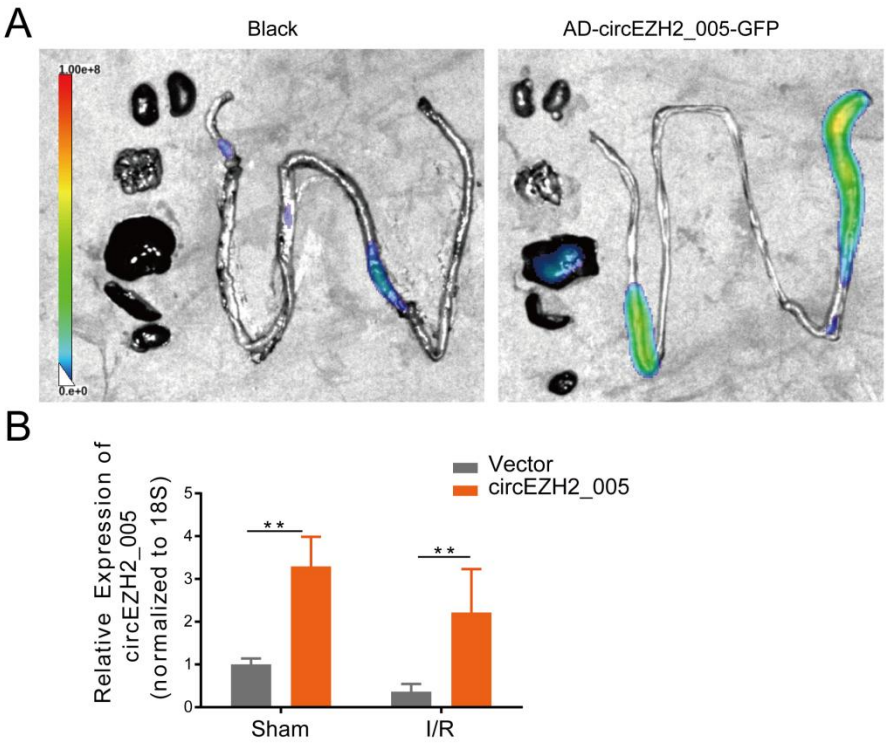

Fig. S7. Effect of circEZH2_005 overexpression in the mouse. (**A**) Fluorescence in vivo imaging was used to detect the overexpression position of circEZH2_005 adenovirus vector in mice after microinjecting with circEZH2_005 adenovirus. (**B**) RT-qPCR analysis of circEZH2_005 overexpression in intestinal tissues and intestinal crypt cell (n = 3). (* p <0 .05, ** p < 0.01, *** p <0.001).

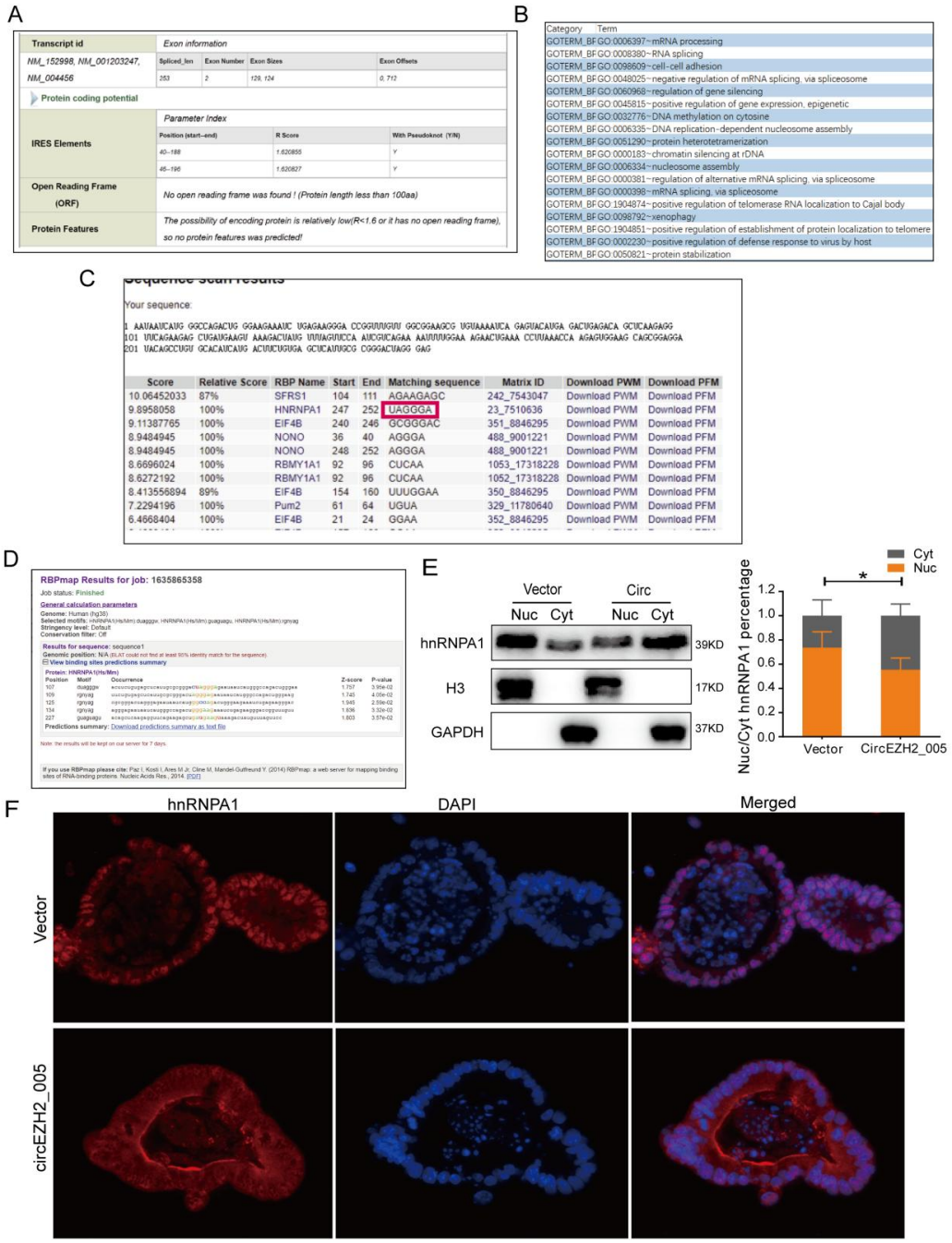

Fig. S8. Bioinformatics database analysis of circEZH2_005-bound proteins. (**A**) The CircRNADb website analyzed whether circEZH2_005 was able to translate the protein. (**B**) GO enrichment analysis was used to cluster and characterize the biological processes of circ-EZH2-binding proteins. (**C**) Proteins for circEZH2_005 binding are predicted by the RBPDB database. (**D**) Association between circEZH2_005 and hnRNPA1 was predicted by the RBPmap database. (**E**) Western blot was conducted to analyze the expression of cytoplasmic (Cyt) and nuclear (Nuc) hnRNPA1 in circEZH2_005-overexpressed intestinal crypt stem cells (n = 3). (**F**) Immunofluorescent of hnRNPA1 (red) in the organoids after overexpressing circEZH2_005. DAPI (blue) for nuclear staining, scale bar=50 μ m (n = 3).

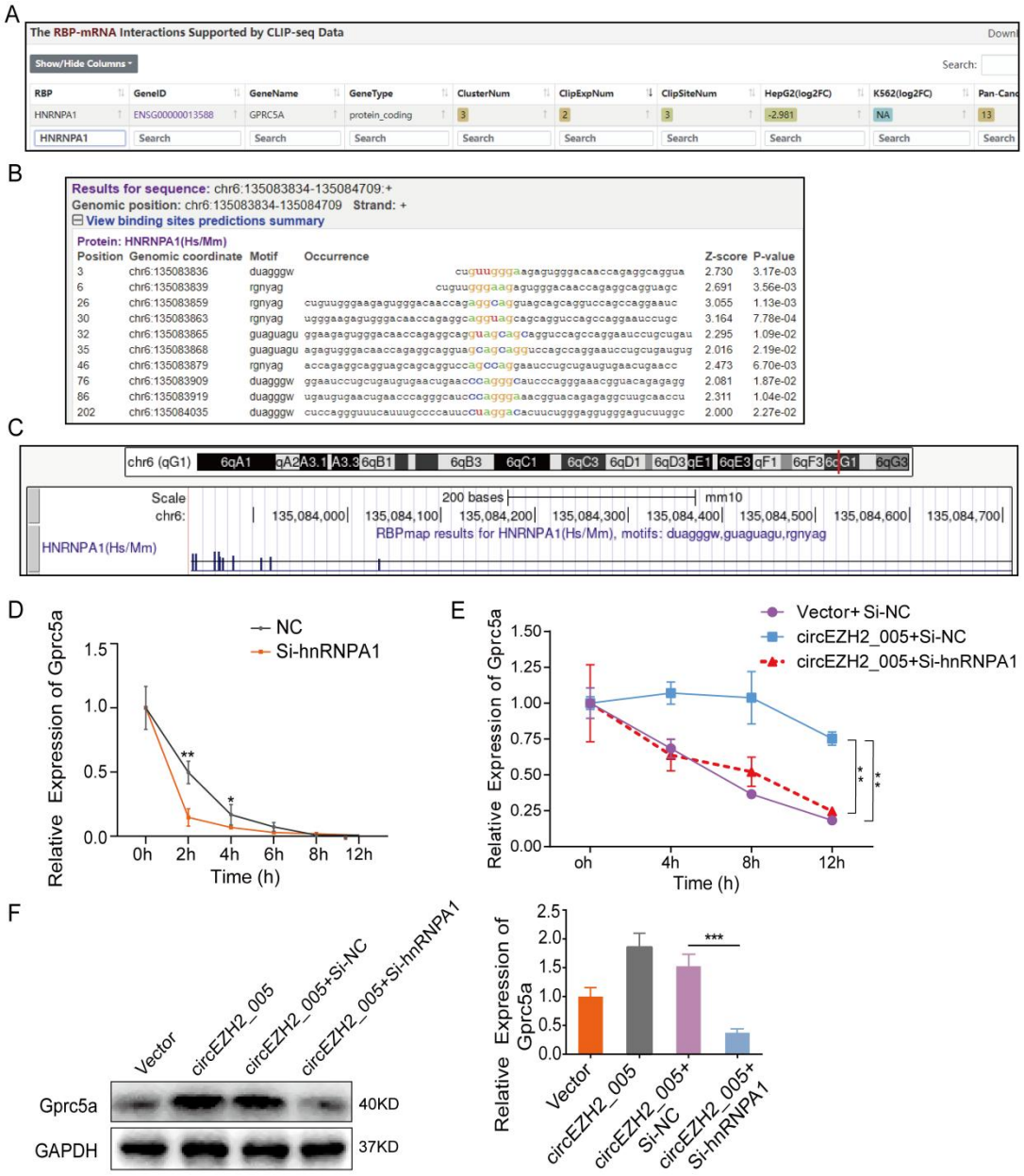

Fig. S9. CircEZH2_005 prevents intestinal I/R injury by stabilizing G*prc5a*. (**A**) The starBase data predicted that hnRNPA1 can bind to the mRNA of Gprc5a. (**B**) The hnRNPA1 binding sites in the Gprc5a 3'UTR region predicted by RBPmap. (**C**) The bottom blue vertical lines represented the hnRNPA1 binding sites in 3'UTR of Gprc5a mRNA. (**D**) The rate of degradation of the G*prc5a* was assessed after transfection with hnRNPA1 siRNA in intestinal crypt stem cells. (**E**) The degradation rate of Gprc5a mRNA in intestinal crypt stem cells was evaluated after circEZH2_005 overexpression and hnRNPA1 interference simultaneously. (**F**) Western blot assay examined whether interfering with hnRNPA1 could reverse the effect of circEZH2_005 on Gprc5a protein expression (n = 3).

Supplementary Tables

Supplementary Table S1. Baseline characteristics for AGI on the first operative day

	Total (n=50)	AGI < 2 (n=11)	AGI ≥2 (n=39)	p-Value
Age, median (IQR)	58.0 (53.0-65.5)	53.0 (39.0-56.0)	59.0 (55.0-70.0)	0.011
Sex, n (%) Male Female	 17 (34) 33 (66)	 3 (27) 8 (73)	 14 (36) 25 (64)	0.594
BMI (kg/m2), mean ± SD	23.8±0.5	22.2±1.2	24.2±0.6	0.095
ASA, n (%) III IV	 44 (88) 6 (12)	 10 (91) 1 (9)	 34 (87) 5 (13)	0.737
Duration of anesthesia (min), median (IQR)	329.0 (309.3-365.0)	318.0 (306.0-355.0)	330.0 (310.0-365.0)	0.482
Duration of surgery (min), median (IQR)	272.5 (244.5-302.0)	273.0 (242.0-291.0)	272.0 (245.0-302.0)	0.815
CPB total time (min), median (IQR)	135.0 (94.8-164.8)	106.0 (93.0-156.0)	142.0 (95.0-167.0)	0.426
Postoperative				
Duration of postoperative intubation (min), median (IQR)	1227.5 (1039.3-1665.0)	1077.0 (470.0-1270.0)	1245.0 (1053.0-2650.0)	0.015
Duration of postoperative fasting (min), median (IQR)	1622.5 (1393.8-2043.3)	1372.0 (1260.0-1580.0)	1732.0 (1478.0-2540.0)	0.006

Abbreviations: IQR, interquartile range; SD, standard deviation; AGI, acute gastrointestinal injury; BMI, body mass index; ASA: American Society of Anesthesiologists physical status; CPB: cardiopulmonary bypass.

Supplementary Table S2. The sequences of primers used for qRT-PCR

Gene name	Sequence (5'-3')
CircEZH2_005 (forward)	TTTAGTTCCAATCGTCAG
CircEZH2_005 (reverse)	CTCAGATTTCTTCCCAGT
Circ-Eya3 (forward)	ACCCAAACTTACGGACTA
Circ-Eya3 (reverse)	CTCAGGCTTCTCATCACT
Circ-Lbr2 (forward)	CTGCTCCACTTCCCTCCA
Circ-Lbr2 (reverse)	CACGCTGACGCTGTTTCC
Circ-Herc3 (forward)	ACAACAGGTGGCAGTCAA
Circ-Herc3 (reverse)	ACAAGGTGGTTCCTACGG
U6 (forward)	AACGCTTCACGAATTTGCGT
U6(reverse)	GCTTCGGCAGCACATATACTAA
Gprc5a (forward)	TACAACTGCCCCTAGCGGT
Gprc5a (reverse)	TGAGGAAAACGAGTGCAAACAT
hnRNPA1(forward)	GAAACAACCGACGAGAGTCTG
hnRNPA1 (reverse)	TGTGTGGTCTTGCATTCATGG
TNF-α (forward)	CCCTCACACTCAGATCATCTTCT
TNF-α (reverse)	GCTACGACGTGGGCTACAG
IL-6 (forward)	TAGTCCTTCCTACCCCAATTTCC
IL-6 (reverse)	TTGGTCCTTAGCCACTCCTTC
EZH2 (forward)	AGTGACTTGGATTTTCCAGCAC
EZH2 (reverse)	AATTCTGTTGTAAGGGCGACC
Hspa12a (forward)	TCGGGGACACAGGAATAACAC
Hspa12a (reverse)	GGTAAAGCTGTAGGCATAGCC
Cldn4 (forward)	GTCCTGGGAATCTCCTTGGC
Cldn4 (reverse)	TCTGTGCCGTGACGATGTTG
Eno3 (forward)	CACAGCCAAGGGTCGATTCC
Eno3 (reverse)	CCCAGGTATCGTGCTTTGTCT
Tmem146(forward)	ACACACACAAGCATCTACTTTGA

Tmem146 (reverse)	AGAGGACTGCACATTCACTGT
Jun (forward)	TGTGCCCCAAGAACGTGAC
Jun (reverse)	CCGGGTTGAAGTTGCTGAG
Gp1bb (forward)	TGACCGGCAACAACCTGAC
Gp1bb (reverse)	CAGCAGAGTAGACCGGGTG
Elmo1 (forward)	TGTAACCCACGATTTGCAGGA
Elmo1 (reverse)	TGCAGACATCCGTGAGTGTTC
Tlco2 (forward)	GCCTCTGGGATGGATGAGTCT
Tlco2 (reverse)	GCATGAGGATAAACGGGTAGGG
Lama3 (forward)	CTGTGACTACTGCAATTCTGAGG
Lama3 (reverse)	CAAGGTGAGGTTGACTTGATTGT
18S (forward)	ACACGGACAGGATTGACAGA
18S (reverse)	GGACATCTAAGGGCATCACA

Supplementary Table S3. Sequences of circEZH2_005 used for RNA pulldown analysis in the study

Gene name	Sequences (5'-3')
mmu_circ_0001471(3bio)_ChIRP Probe_1	GATTATTCTCCCTAGTCCCG
mmu_circ_0001471(3bio)_ChIRP Probe_2	ATGATTATTCTCCCTAGTCC
mmu_circ_0001471(3bio)_ChIRP Probe_3	CCATGATTATTCTCCCTAGT
mmu_circ_0001471(3bio)_Scramble ChIRP NC_1	CCTCGCAGTTGTTTACACCT

Supplementary Table S4. Si-RNAs were used in the study.

Gene name	Sequence
si-mmu_circ_0001471_001	CGGGACTAGGGAGAATAAT
si-mmu_circ_0001471_002	GGACTAGGGAGAATAATCA
si-mmu_circ_0001471_003	CTAGGGAGAATAATCATGG
si-hnRNPA1-1	GGAUGGAAGAGUUGUGGAATT
si-hnRNPA1-2	GAGGGCUGAGCUUCGAAACAATT
si-hnRNPA1-3	GAACAUCACCUACGAGAUUAUTT
si-Gprc5a-1	UCACCUUCGCCUUCAUCAUCATT
si-Gprc5a-2	CUGGAUCGUUCUGCUCCUGAUTT
si-Gprc5a-3	CAGGACCAACGUCAAUGUCUUTT