

OMTN, Volume 21

Supplemental Information

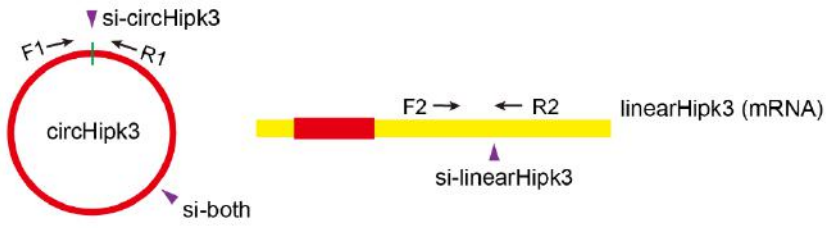
circRNA Hipk3 Induces Cardiac Regeneration after Myocardial Infarction in Mice by Binding to Notch1 and miR-133a

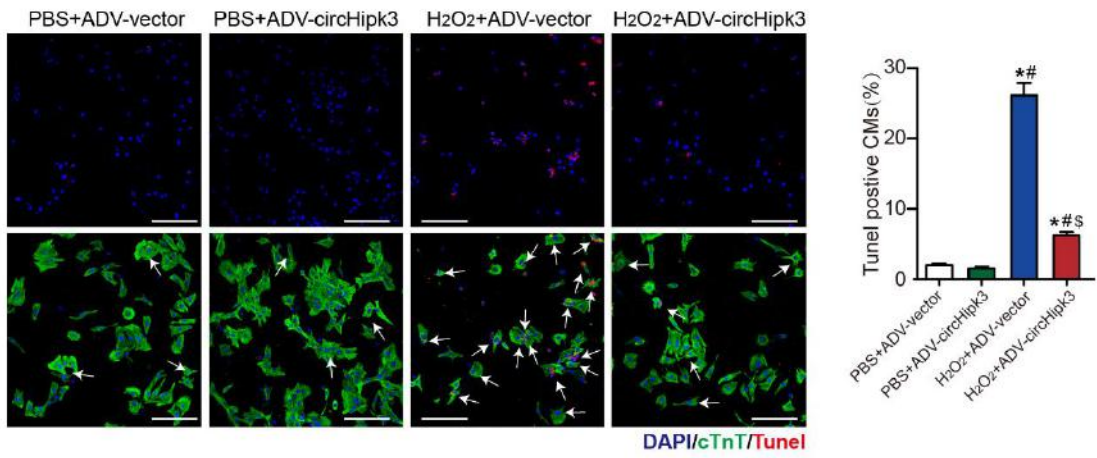
Xiaoyun Si, Hao Zheng, Guoquan Wei, Mengsha Li, Wei Li, Houmei Wang, Haijun Guo, Jie Sun, Chuling Li, Shenrong Zhong, Wangjun Liao, Yulin Liao, Senlin Huang, and Jianping Bin

hsa_circHipk3 mmu_circHipk3 rno_circHipk3	GTATGGCTCACAAAGTCTTGGTCTAACCACCATATGTTTATCAAACCTAGTCAAGTGCT GTATGGCTCACAAAGTCTTGGTCTAACCACCATATGTTTATCAAACCTAGTCAAGTGCT GTATGGCTCACAAAGTCTTGGTCTAACCACCATATGTTTATCAAACCTAGTCAAGTGCT *****	hsa_circHipk3 mmu_circHipk3 rno_circHipk3	ATGAAATGTAGCAATCAAAATTTTGAAGAATCACTCTTCTTATGCCCCTAAGGTCAA ATGAAATGTAGCAATCAAAATTTTGAAGAATCACTCTTCTTATGCCCCTAAGGTCAA ATGAAATGTAGCAATCAAAATTTTGAAGAATCACTCTTCTTATGCCCCTAAGGTCAA *****
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hsa_circHipk3 mmu_circHipk3 rno_circHipk3	TTGATTTTCTGGTGGAGGACGTTTGGCCAGGTAGTAAATGCTGAAAAGAGGGACAA TGGATTTTCTGGTGGAGGACGTTTGGCCAGGTAGTAAATGCTGAAAAGAGGGACAA TGGATTTTCTGGTGGAGGACGTTTGGCCAGGTAGTAAATGCTGAAAAGAGGGACAA *..*****		

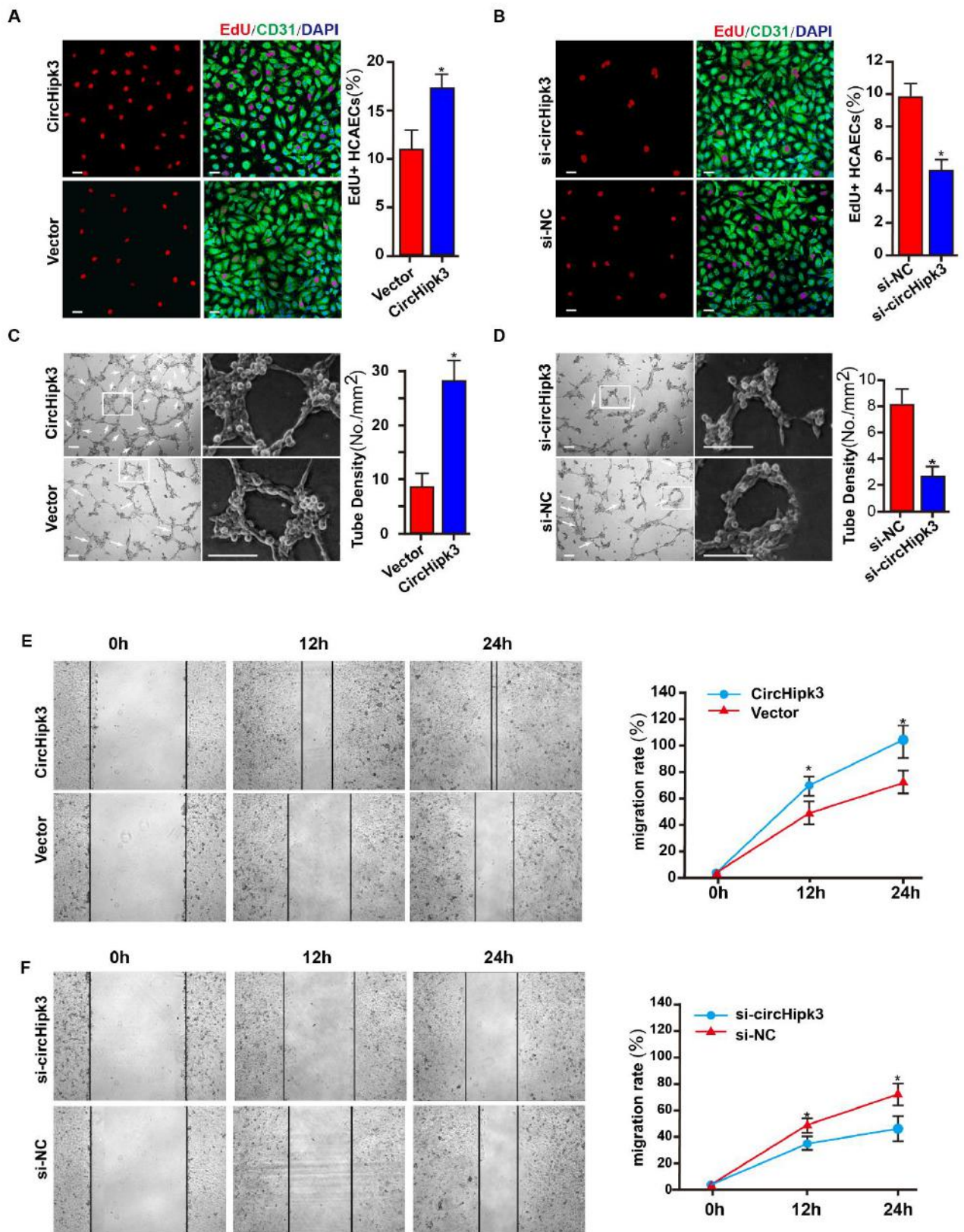
Supplemental Figure 1

▼ siRNA target site
| back-splice site

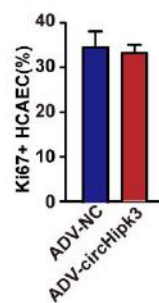
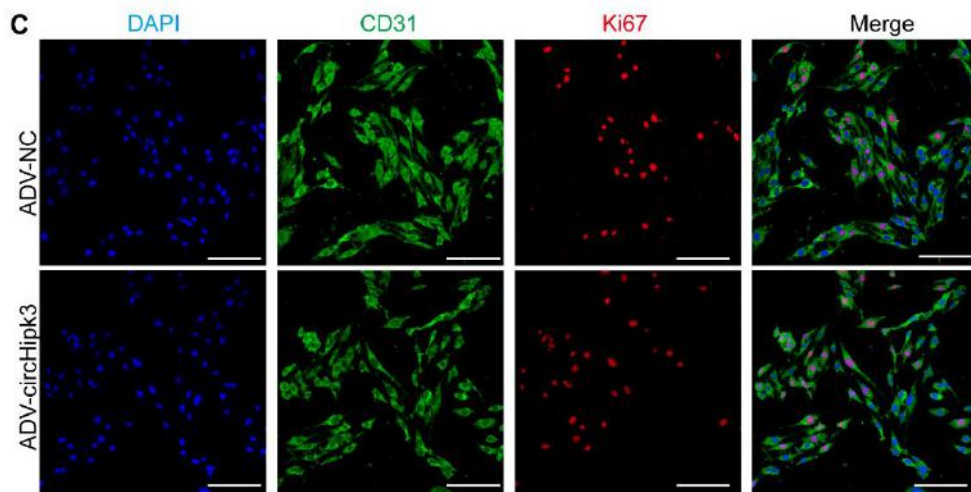
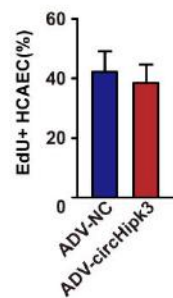
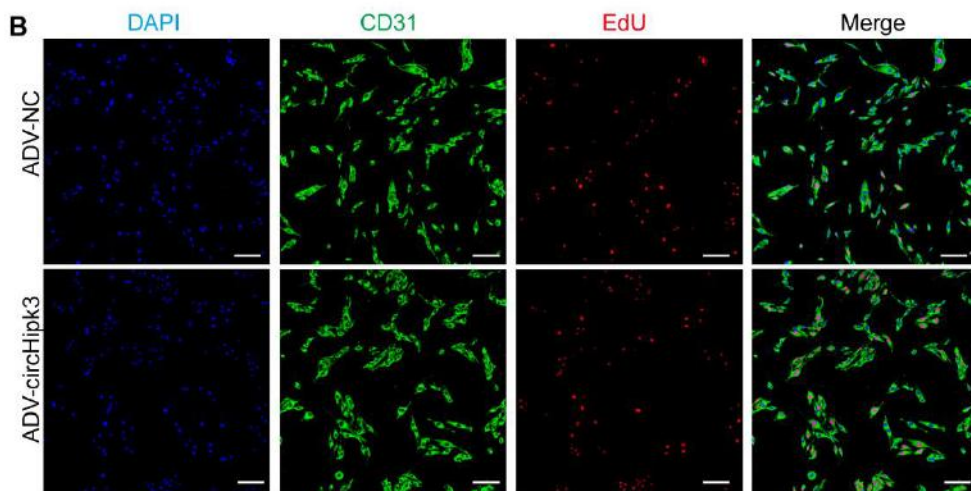
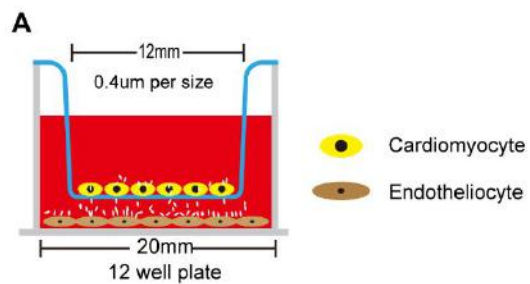


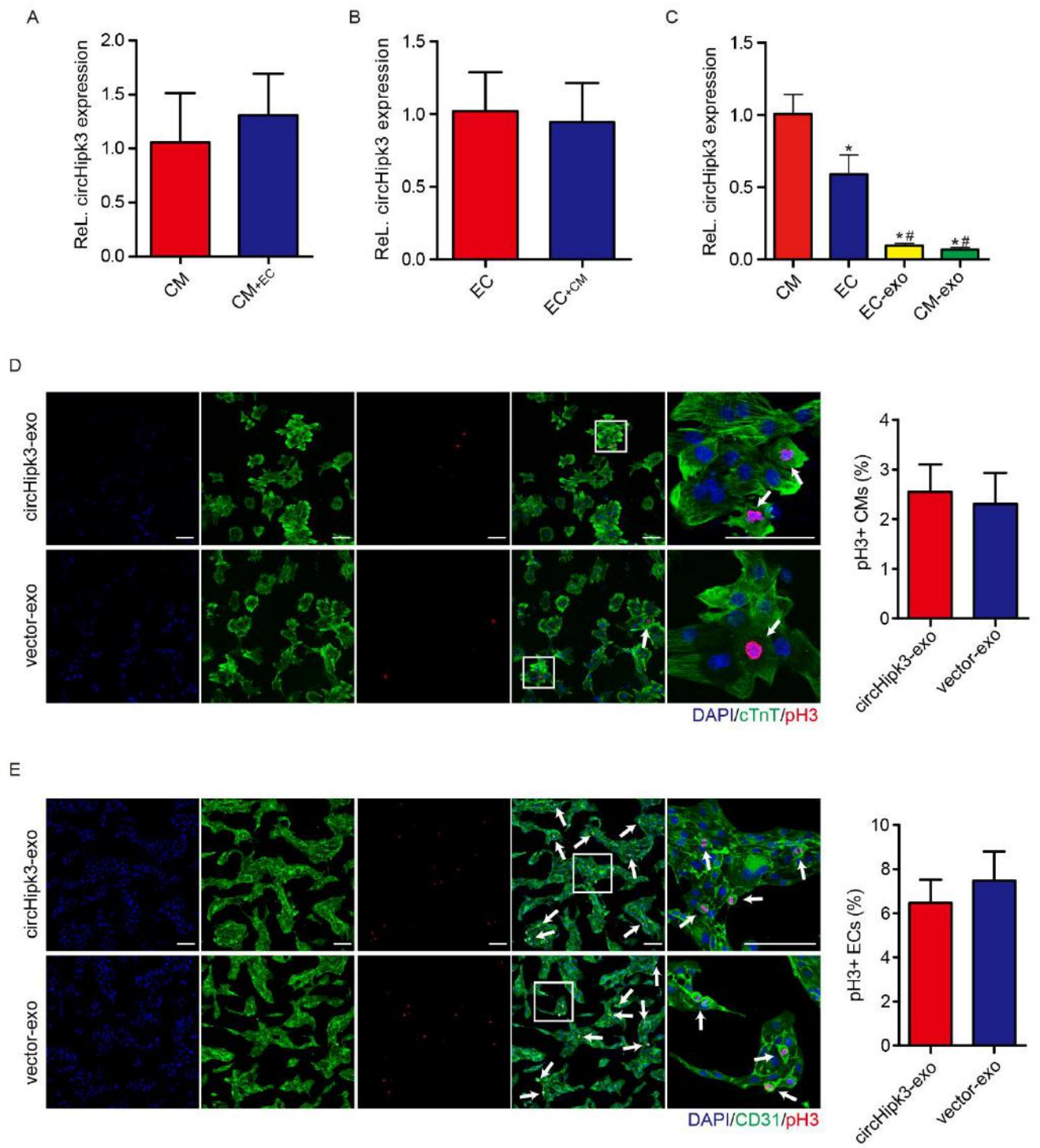


Supplemental Figure 3

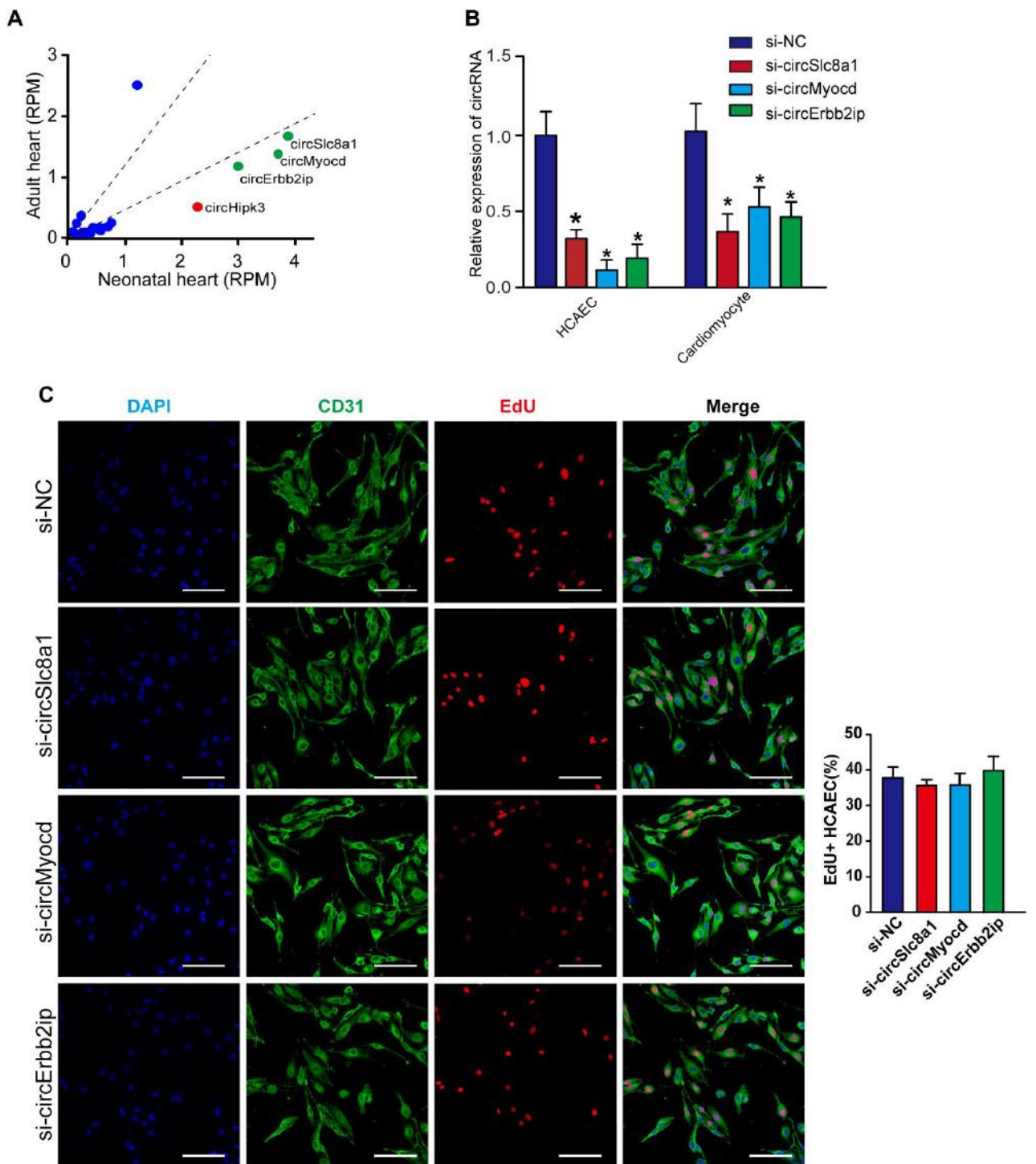


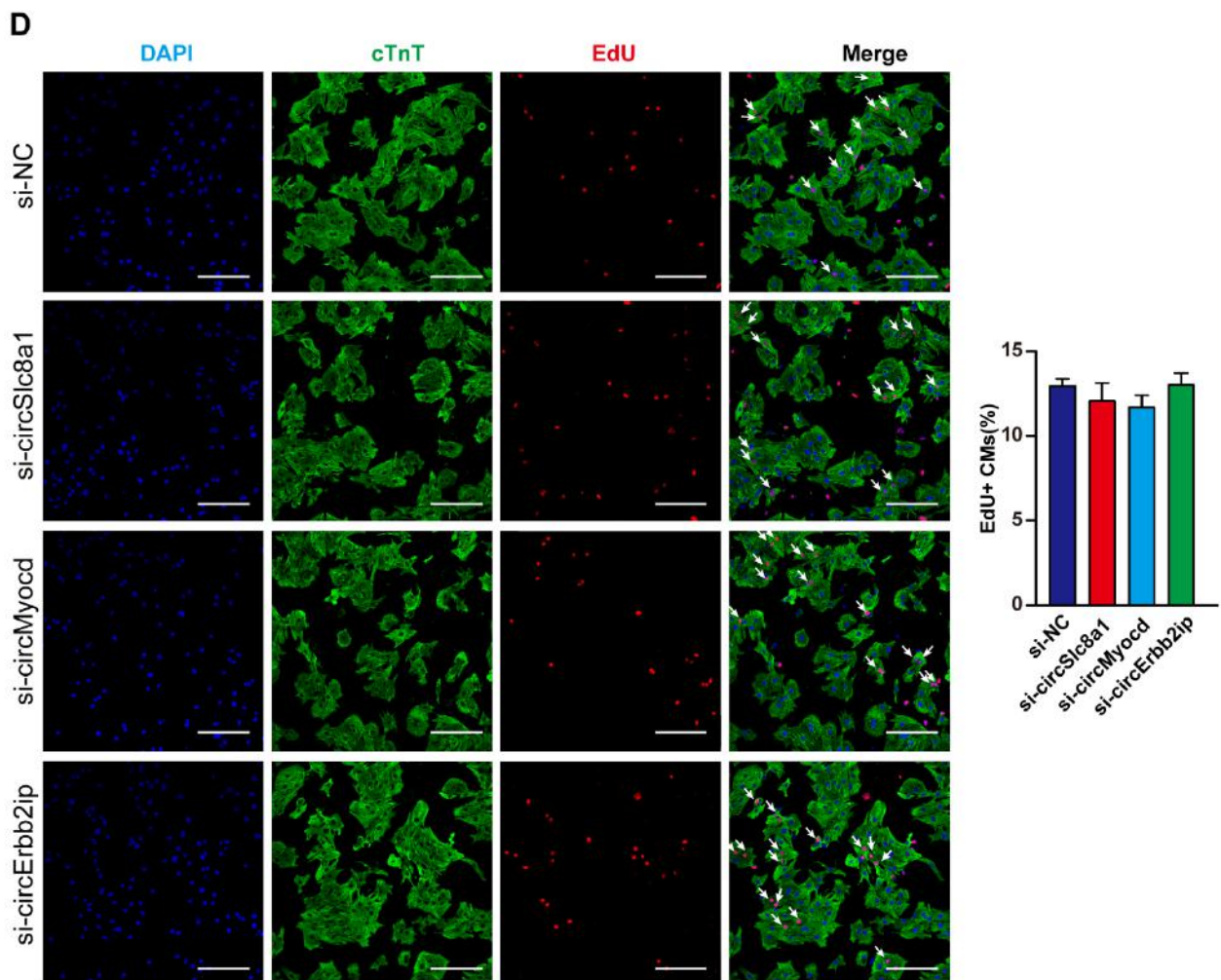
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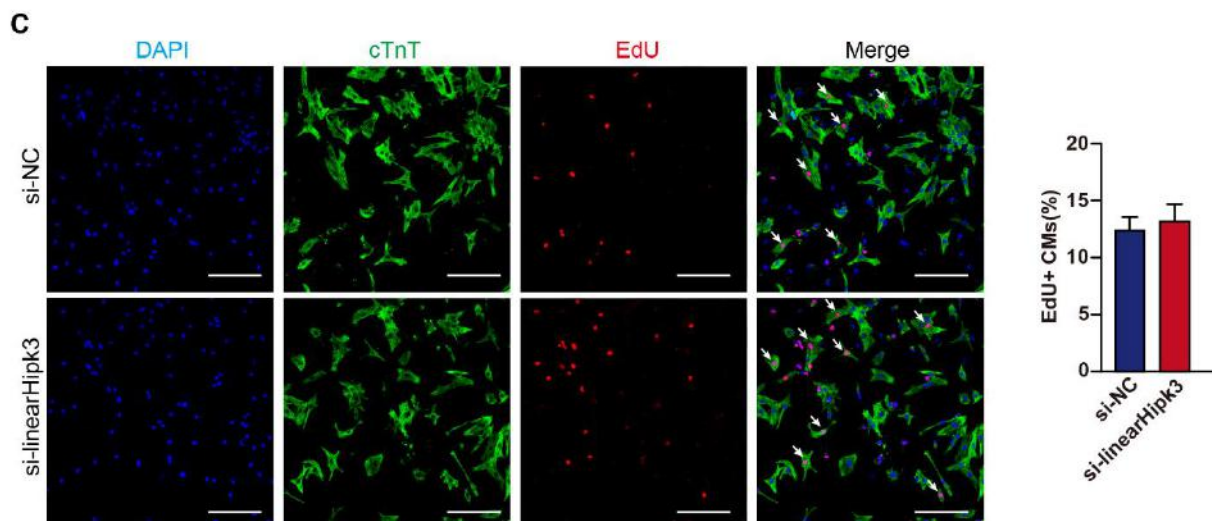
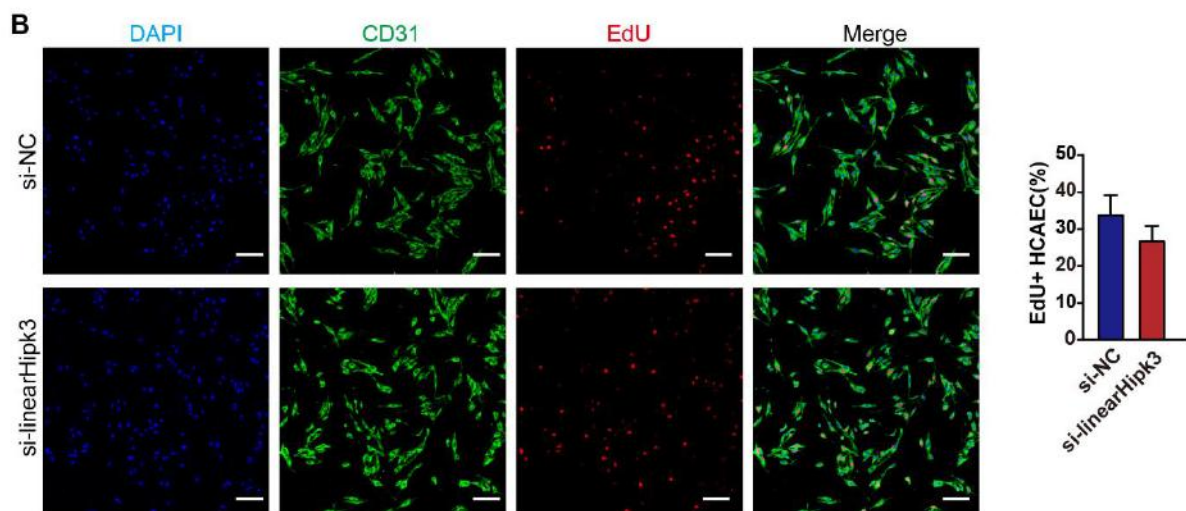
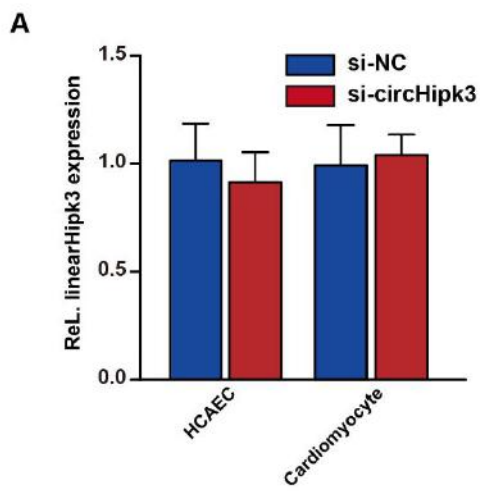


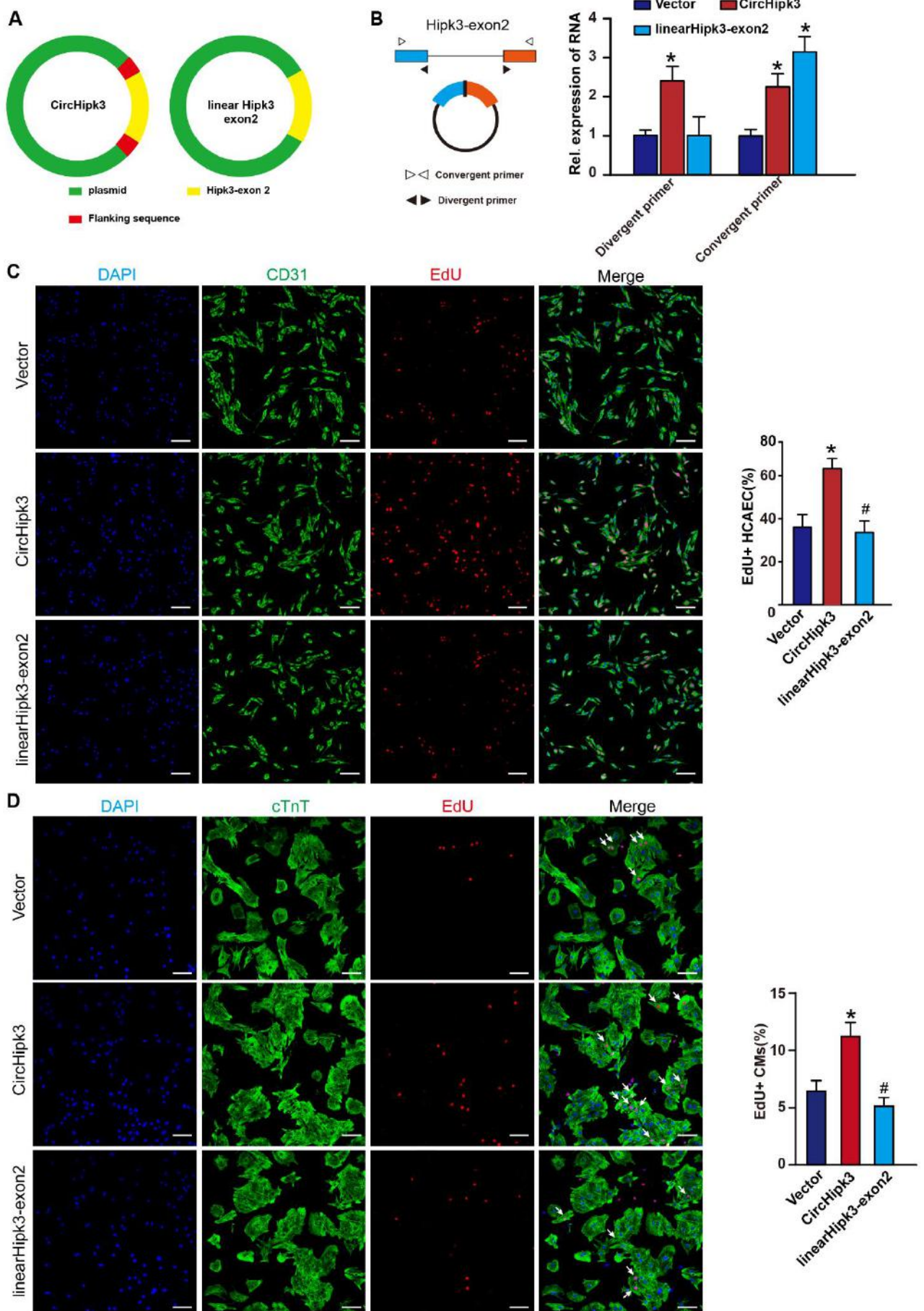


Supplemental Figure 6

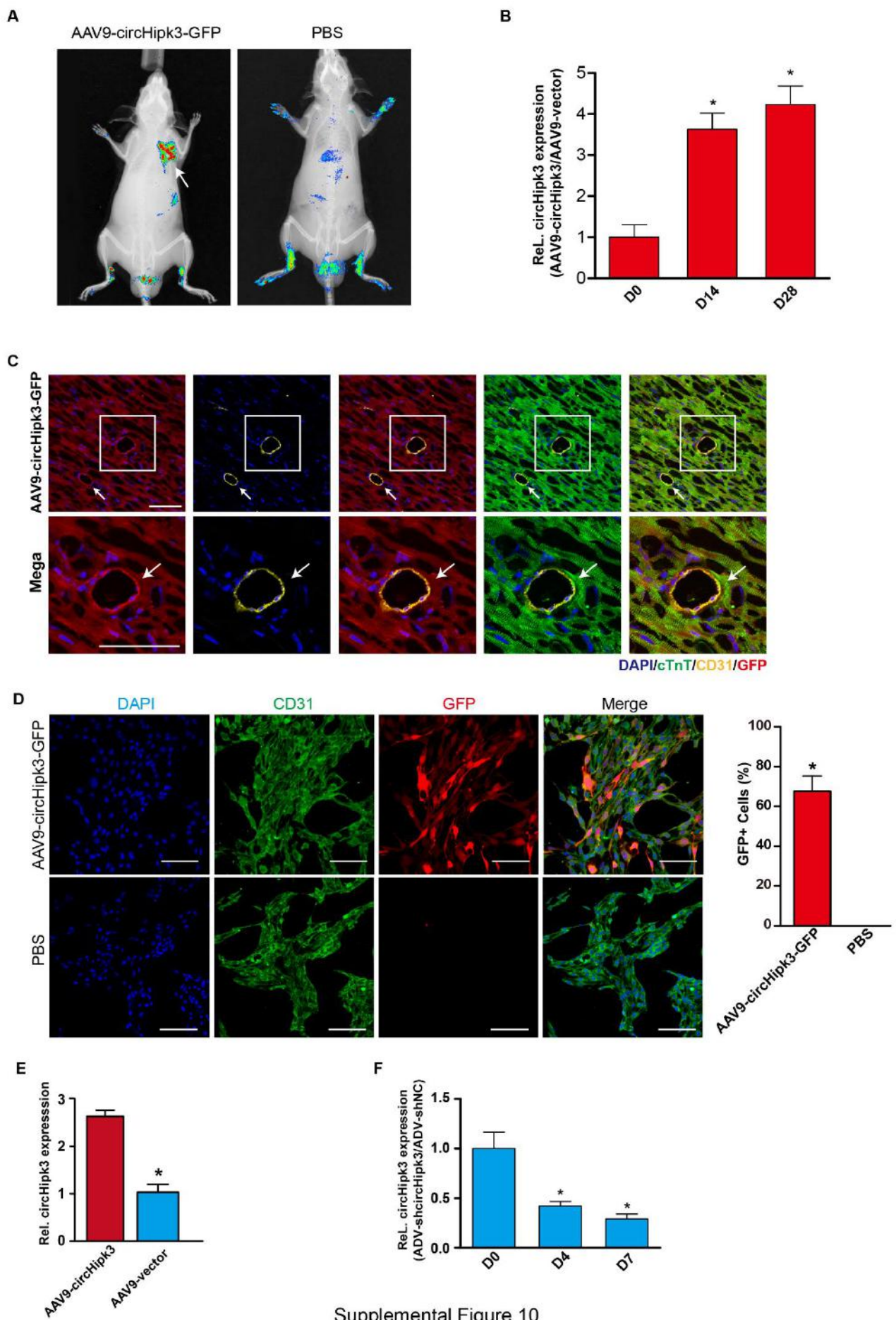






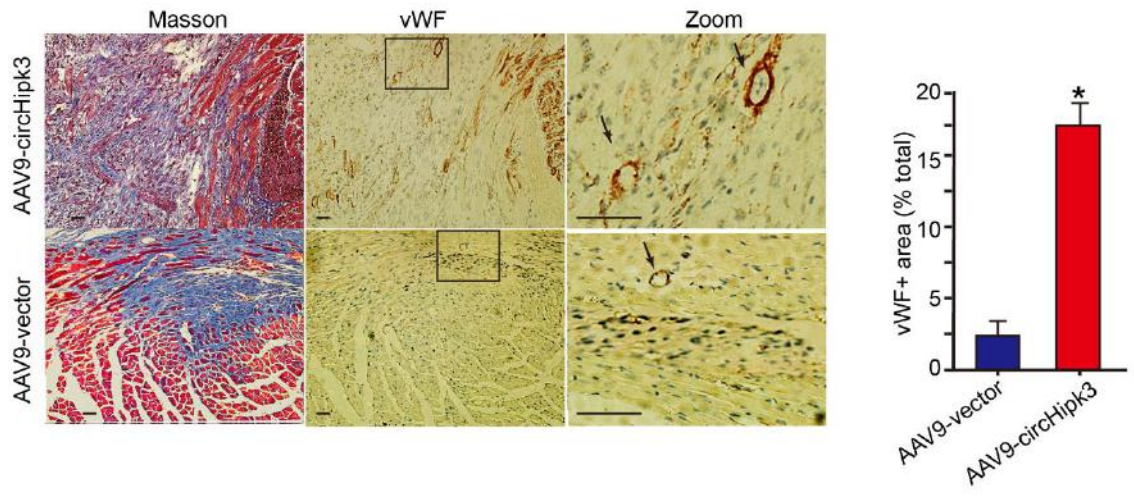


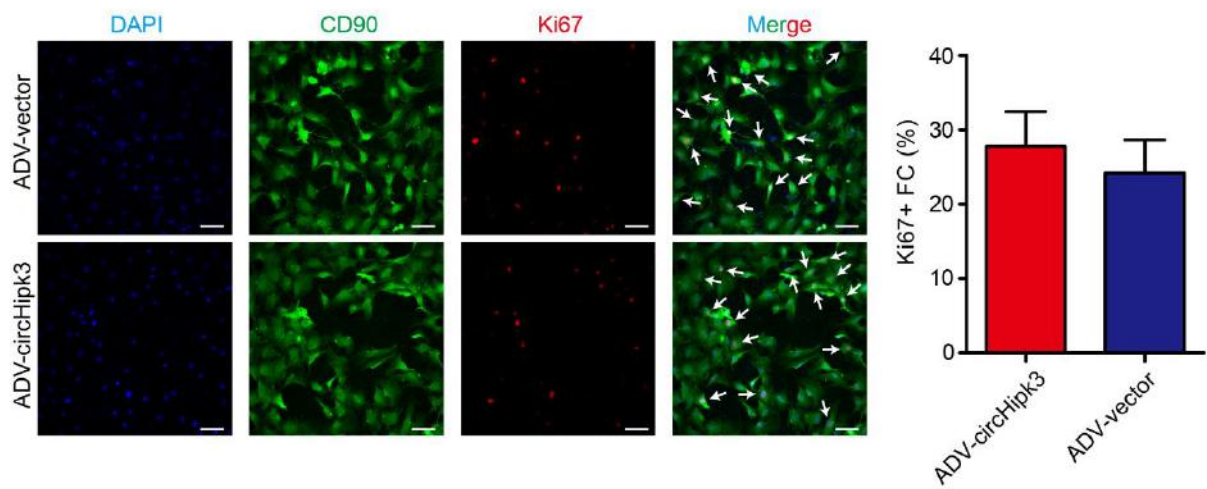
Supplemental Figure 9



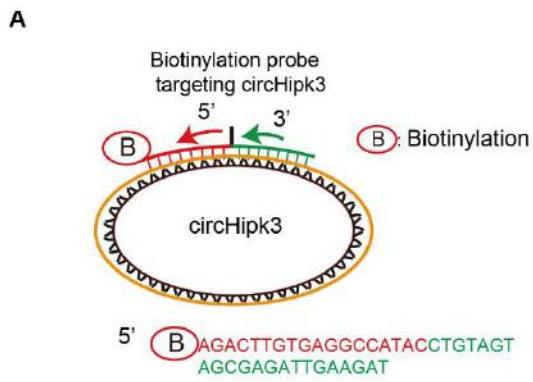
Supplemental Figure 10

A



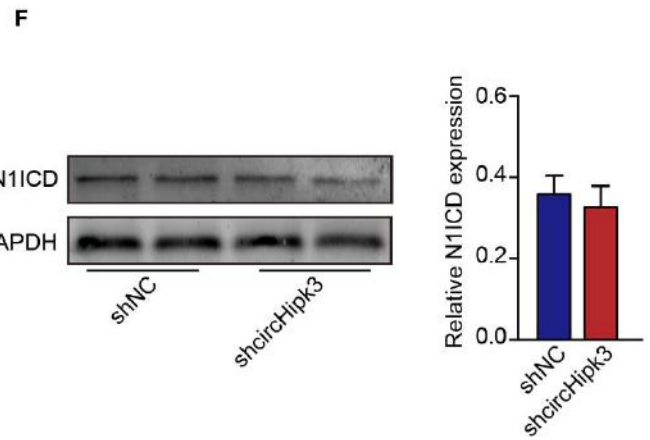
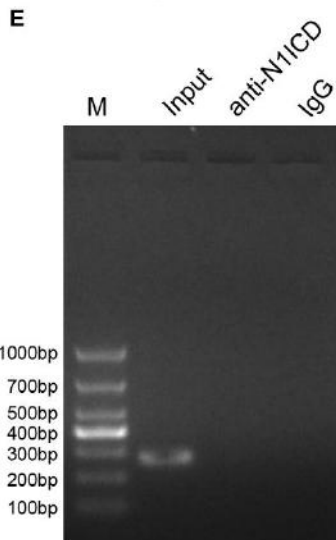
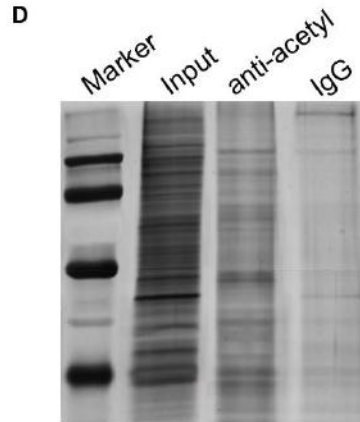
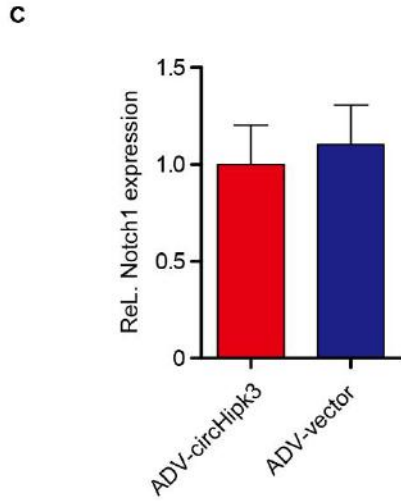


Supplemental Figure 12



B

RNA	Protein	Prediction using SVM classifier	Prediction using RF classifier
mmu-circHipp3	Notch1	0.97	0.90
hsa-circHipp3	Notch1	0.99	0.65



1 **Supplemental figure legends**

2 **Supplemental Fig. 1 CircHipk3 homology analysis of the mouse, human and rat**
3 **genome using multiple sequence alignment tool (MAFFT,**
4 **<https://www.ebi.ac.uk/Tools/msa/>)**

5

6 **Supplemental Fig. 2 Illustration of three targeted siRNAs.** siRNA-circHipk3
7 (si-circHipk3) targets the back-splice sequence of circHipk3, siRNA-linearHipk3
8 (si-linearHipk3) targets sequence only in the linear Hipk3 mRNA, and si-both targets
9 sequence in a circularized exon shared by both linear and circular species. The red
10 region of linearHipk3 mRNA harbors circHipk3 sequence. F1/R1 and F2/R2 indicates
11 the forward/reverse primers used to specifically detect circHipk3 and linearHipk3
12 expression, respectively. All siRNAs' sequence is provided in the Supplemental Table
13 2.

14

15 **Supplement Fig. 3 Detection of TUNEL+ CMs after ADV-circHipk3/ADV-vector**
16 **transfection and H₂O₂ treatment, or control treatment.** Neonatal mouse CMs were
17 treated with 50umol H₂O₂ or PBS after transfection with ADV-circHipk3/ADV-vector
18 for 48h. TUNEL+ CMs are indicated by arrows; n=500 CMs, * $P < 0.05$ vs.
19 PBS+ADV-vector group, # $P < 0.05$ vs. PBS+ADV-vector group, \$ $P < 0.05$ vs.
20 H₂O₂+ADV-vector group, scale bar=100 μ m.

21

22 **Supplemental Fig. 4 CircHipk3 regulates coronary endothelial cell proliferation,**

1 **migration and tube formation.** (A) CircHipk3 overexpression increased the ratio of
2 EdU+ HCAECs. * $P < 0.05$, $n = 800$ HCAECs, scale bar = 50 μm . (B) CircHipk3
3 knockdown decreased the ratio of EdU+ HCAECs. * $P < 0.05$, $n = 800$ HCAECs, scale
4 bar = 50 μm . (C) CircHipk3 overexpression promoted tube formation of HCAECs.
5 * $P < 0.05$, $n = 6$, scale bar = 50 μm . (D) CircHipk3 knockdown inhibited tube formation
6 of HCAECs. * $P < 0.05$, $n = 6$, scale bar = 50 μm . (E) CircHipk3 overexpression
7 promoted migration of HCAECs. * $P < 0.05$ vs. vector group at each time point, $n = 8$,
8 scale bar = 50 μm . (F) CircHipk3 knockdown inhibited migration of HCAECs.
9 * $P < 0.05$ vs. si-NC group at each time point, $n = 8$, scale bar = 50 μm .

10

11 **Supplemental Fig. 5 The effect of CM/HCAEC co-culture on HCAEC**
12 **proliferation.** (A) Diagrammatic representation of CMs and HCAECs co-culture
13 system. (B) Detection of EdU+ HCAECs after co-cultured with CMs; $n = 1000$
14 HCAECs, scale bar = 50 μm . (C) Detection of Ki67+ HCAECs after co-cultured with
15 CMs; $n = 1000$ HCAECs, scale bar = 50 μm .

16

17 **Supplement Figure 6 CircHipk3 is not involved in CM/EC crosstalk through**
18 **exosome secretion.** (A) Detection of circHipk3 expression level in CM after
19 co-culture with EC or not. CM+EC: neonatal mouse CM co-cultured with neonatal
20 mouse EC, $n = 4$. (B) Detection of circHipk3 expression level in EC after co-culture
21 with CM or not. EC+CM: neonatal mouse EC co-cultured with neonatal mouse CM,
22 $n = 4$. (C) Detection of circHipk3 expression level in CM/EC and their exosome.

1 Exosome was isolated from CM/EC, namely CM-exo/EC-exo, respectively. N=3,
2 * P <0.05 vs. CM, # P <0.05 vs. EC. (D) Neonatal mouse CMs were cultured with
3 exosome from ADV-circHipk3 or ADV-vector transfected ECs and examined for pH3
4 immunostaining. Exosome was isolated from ADV-circHipk3/ADV-vector transfected
5 ECs, namely circHipk3-exo/vector-exo, respectively. N=500 CMs, * P <0.05, scale
6 bar=50 μ m. (E) neonatal mouse ECs were cultured with exosome from
7 ADV-circHipk3 transfected CMs and examined for pH3 immunostaining. Exosome
8 was isolated from ADV-circHipk3/ADV-vector transfected CMs, namely
9 circHipk3-exo/vector-exo, respectively. N=700 ECs, * P <0.05, scale bar=50 μ m.

10

11 **Supplemental Fig. 7 Silencing circSlc8a1, circMyocd and circErbb2ip expression**
12 **has no effect on HCAEC or CM proliferation.** (A) CircSlc8a1, circMyocd and
13 circErbb2ip was differentially expressed in the adult and neonatal rodent heart. The
14 expression level of circRNA is shown in RPM. Dashed lines indicate the interval of
15 two-fold change; n=3. (B) QRT-PCR assays detecting circSlc8a1, circMyocd and
16 circErbb2ip expression levels in HCAECs and CMs after transfection with siRNAs
17 targeting their back-splicing sites; * P <0.05 vs. si-NC in each cell type, n=6. (C)
18 Detection of EdU+ HCAECs after transfection with siRNAs targeting circSlc8a1,
19 circMyocd and circErbb2ip; n=1000 HCAECs, scale bar=50 μ m. (D) Detection of
20 EdU+ CMs after transfection with siRNAs targeting circSlc8a1, circMyocd and
21 circErbb2ip. EdU+ CMs are indicated by arrows; n=500 CMs, scale bar=50 μ m.

22

1 **Supplemental Fig. 8 The association between Hipk3 linear transcript and cell**
2 **proliferation.** (A) QRT-PCR assays detecting Hipk3 expression level after circHipk3
3 knockdown. n=6. (B) Detection of EdU+ HCAECs after silencing of linearHipk3
4 mRNA; n=1000 HCAECs, scale bar=50 μ m. (C) Detection of EdU+ CMs after
5 silencing of linearHipk3 mRNA. EdU+ CMs are indicated by arrows; n=500 CMs,
6 scale bar=50 μ m.

7
8 **Supplemental Fig.9 The circular form is essential for the role of circHipk3 on**
9 **regulating cell proliferation.** (A) Vectors designed to overexpress circHipk3
10 sequence with and without circularization, namely circHipk3 and LinearHipk3-exon2,
11 respectively. (B) QRT-PCR assays detecting circular Hipk3 and non-circular Hipk3
12 expression level after transfection with vectors overexpressing circHipk3 sequence
13 with or without circularization. The negative control group was named vector group.
14 * P <0.05 vs. vector group, # P <0.05 vs. circHipk3 group, n=6. (C) Detection of EdU+
15 HCAECs after transfection with vectors overexpressing circHipk3 sequence with or
16 without circularization; n=1000 HCAECs, * P <0.05 vs. vector group, # P <0.05 vs.
17 circHipk3 group, scale bar=50 μ m. (D) Detection of EdU+ CMs after transfection
18 with vectors overexpressing circHipk3 sequence with or without circularization.
19 EdU+ CMs are indicated by arrows; n=500 CMs, * P <0.05 vs. vector group, # P <0.05
20 vs. circHipk3 group, scale bar=50 μ m.

21
22 **Supplemental Fig. 10 The transfection efficiency of AAV9-circHipk3 and**

1 **ADV-shcircHipk3.** (A) *In vivo* bioluminescence images captured on 2 weeks after
2 injection with AAV9-circHipk3-GFP virus. PBS was used as the control treatment.
3 The white arrow indicates the heart with GFP fluorescence. (B) CircHipk3 expression
4 in adult mouse hearts at 0, 14 and 28 days after transfection with AAV9-circHipk3
5 and AAV9-vector. The y axis shows circHipk3 expression in AAV9-circHipk3 group
6 versus that in AAV9-vector group. * $P < 0.05$ vs D0, n=4. (C) cTnT/CD31/GFP
7 co-staining in myocardium tissue of adult hearts 4 weeks after injection of
8 AAV9-circHipk3-GFP or PBS. Bar=100 μm . GFP+ vessels are indicated by arrows.
9 (D) CD31/GFP co-staining in cardiac endothelial cells isolated from adult hearts 4
10 weeks after injection of AAV9-circHipk3-GFP or PBS, N=500 cardiac endothelial
11 cells, * $P < 0.05$, bar=100 μm . (E) QRT-PCR assays detecting circHipk3 expression in
12 cardiac endothelial cells isolated from adult hearts 4 weeks after injection of
13 AAV9-circHipk3 and AAV9-NC, n=6, * $P < 0.05$. (F) CircHipk3 expression in P0
14 mouse hearts at 0, 4 and 7 days after transfection with ADV-shcircHipk3 and
15 ADV-shNC. The y axis shows circHipk3 expression in ADV-shcircHipk3 group
16 versus that in ADV-shNC group. * $P < 0.05$ vs. D0, n=4.

17

18 **Supplemental Fig. 11 Masson staining and vWF immunostaining in**
19 **peri-infarcted zone in the shcircNfix and shNC groups 14 days after MI.** * $P < 0.05$,
20 n=6, scale bar=50 μm .

21

22 **Supplement Fig. 12 Detection of Ki67+ FC after ADV-circHipk3 or ADV-vector**

1 **transfection.** Ki67+ FCs are indicated by arrows; n=600 FC, * P <0.05, scale bar=50
2 μ m. FC: fibrocytes.

3

4 **Supplemental Fig. 13 The relationship between circHipk3, miR-133a and CTGF.**

5 (A) The predicted binding of miR-133a/b to circHipk3. (B) RNA pulldown assays
6 using the probe targeting circHipk3 back-splice site. The abundance of miR-133a,
7 circHipk3 and Gapdh in bound fractions was evaluated by qRT-PCR analysis,
8 * P <0.05, n=3. The expression of miR-133a was also detected by gel electrophoresis.

9 (C) Changes in miR-133a expression after linearHipk3 overexpression in HCAECs.

10 * P <0.05, n=3. (D) The predicted binding of miR-133a to the 3'UTR of CTGF. (E)

11 Co-injection with AAV9-miR-133a reversed circHipk3-induced miR-133a
12 downregulation in adult hearts. * P <0.05 vs. AAV9-vector, # P <0.05 vs.

13 AAV9-circHipk3, n=6. (F) CircHipk3 has no effect on miR-133a expression in CMs,

14 n=6. (G) MiR-133a mimics has no effect on circHipk3-induced CM proliferation,

15 * P <0.05 vs. vector+mimic-NC, n=400 CMs, scale bar=50 μ m. (H) MiR-133a

16 expression in neonatal cardiac EC and CM; * P <0.05, n=3.

17

18 **Supplemental Fig. 14 The relationship between circHipk3 and N1ICD.** (A) The

19 biotinylated probe targeting the back-splice junction of circHipk3. (B) The predicted

20 interaction between circHipk3 and N1ICD using RNA-Protein interaction Prediction

21 (RPISeq) programme. Predictions with probabilities > 0.5 indicated that the

22 corresponding RNA and protein are likely to interact. (C) CircHipk3 overexpression

1 has no effect on Notch1 mRNA expression in P7 CMs, n=4. (D) Protein
2 immunoprecipitated by an acetylation-specific antibody and IgG antibody. The arrow
3 indicated the position of N1ICD protein. (E) RIP assays were performed to investigate
4 the interaction between circHipk3 and N1ICD in HCAECs. (F) Detection of N1ICD
5 expression in HCAECs using western blotting assays after circHipk3 knockdown,
6 n=4.

7

8 **Supplemental Movie. 1:** Time-lapse imaging of P7 CMs after transfection with
9 ADV-circHipk4, corresponding to Fig. 2H. Time-lapse imaging started 12hr
10 post-transfection (1/3 hr/Frame).

11

Supplementary Table 1: The primers used for qPCR in this study.

Primers name		Sequence (5'-3')
circHipk3	+	GGGCAGATAGAAGTGAGC
	-	TGATAAACATACGGTGGG
MiR-133a	+	GCGTTTGGTCCCCTTCAAC
	-	AGTGCAGGGTCCGAGGTATT
	R	GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGCA CTGGATACGACCAGCTG
Notch1	+	GATGGCCTCAATGGGTACAAG
	-	TCGTTGTTGTTGATGTCACAGT
U6	+	GCGCGTCGTGAAGCGTTC
	-	GTGCAG GGTCCGAGGT
Gapdh	+	TGACCTCAACTACATGGTCTACA
	-	CTTCCATTCTCGGCCTTG
circErbB2ip	+	CAACATTACCAGCCTCCAT
	-	GTCTCCTCTTCTCCTCGTA
circMyocd	+	ATTCTGCCGATGGATTCTT
	-	GGTATTAAGCCTTGGTTAGC
circSlc8a1	+	TCTGGAGCTCGAGGAAATGT
	-	TTGGGTGGGAGACTTAATCG
linearHipk3 mRNA	+	ACCTCACCCAGACCTT
	-	AGCATCCTCCACAAAA
mmu-miR-107-3p	+	GCGAGCAGCATTGTACAGGG
	-	AGTGCAGGGTCCGAGGTATT
	R	GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGCACTGGATA CGACTGATAG
mmu-miR-105	+	CGCCAAGTGCTCAGATGCT
	-	AGTGCAGGGTCCGAGGTATT
	R	GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGCACTGGATACG ACACCACA
mmu-miR-217-5p	+	GCGTACTGCATCAGGAACTG
	-	AGTGCAGGGTCCGAGGTATT
	R	GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGCACTGGATACG ACTCCAGT
mmu-miR-292a-5p	+	GCGACTCAAACCTGGGGGCT
	-	AGTGCAGGGTCCGAGGTATT
	R	GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGCACTGGATACG ACCAAAAAG
mmu-miR-542-5p	+	CGCTCGGGGATCATCATG
	-	AGTGCAGGGTCCGAGGTATT
	R	GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGCACTGGATA

		CGACTCGTGA
mmu-miR-669a-5p	+	GCGAGTTGTGTGTGCATGTTC
	-	AGTGCAGGGTCCGAGGTATT
	R	GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGCACTGGAT ACGACAGACAT
mmu-miR-9-5p	+	GCGCGTCTTTGGTTATCTAGCT
	-	AGTGCAGGGTCCGAGGTATT
	R	GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGCACTGGAT ACGACTCATAAC
mmu-miR-96-5p	+	GCGTTTGGCACTAGCACATT
	-	AGTGCAGGGTCCGAGGTATT
	R	GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGCACTGGATAC GACAGCAAAA
LinearHipk3-exon2	+	GGATCGGCCAGTCATGTATC
	-	ACCGCTTGGCTCTACTTTGA

Supplementary Table 2: The RNA interference used in this study

siRNA name	Sequence (5'-3')
ShcircHipk3/si-circHipk3 (mouse)	UCUCGCUACUACAGGUAUG
ShcircHIPK3/si-circHIPK3 (human)	CUACAGGUAUGGCCUCACA
Si-linearHipk3	GCAGUACUGCUACACUGAC
Si-both	CUUUGUACGAGCUUAUGAA
Si-Notch1	GGAACAACUCCUCCACU
Si-circSLC8A1 (human)	GAAAUUGUGUUGUGACAGUUG
Si-circSlc8a1 (mouse)	AGAACGAUGAAAUAGUGUUGG
Si-circMYOCD (human)	AUAAAAGUUUUACAGUUAAGA
Si-circMyocd (mouse)	CGUCUUACAGUUACGGCUUCA
Si-circERBB2IP (human)	GGAUGUCAGCAAGAAUGAAUG
Si-circErb2ip (mouse)	GGAUGUCAGCAAAAUGAAUG

Supplementary Table 4: The results of mass spectrometry analysis after RNA pulldown related to Fig. 8A.

Protein	Score	Mass	Matches	Sequences	emPAI
Bptf	24	294193	4 (1)	2 (1)	0.01
Usp9x	25	293509	3 (1)	3 (1)	0.01
Notch1	17	284624	2 (1)	1 (1)	0.01
Pcdh15	37	190454	1 (1)	1 (1)	0.02
Mag13	23	162655	4 (1)	3 (1)	0.02
Dsg1a	78	115551	5 (2)	4 (1)	0.06
Gpld1	18	93767	3 (1)	2 (1)	0.03
Pkp1	15	82270	1 (1)	1 (1)	0.04
Mroh6	28	78839	2 (1)	2 (1)	0.04
Tgm3	59	77717	2 (1)	1 (1)	0.04
Dyrk4	24	73028	5 (1)	5 (1)	0.04