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Supplemental Information

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

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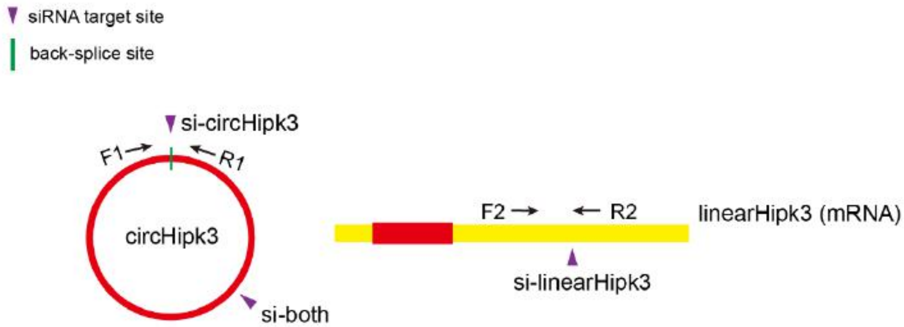
Supplemental Figure 1



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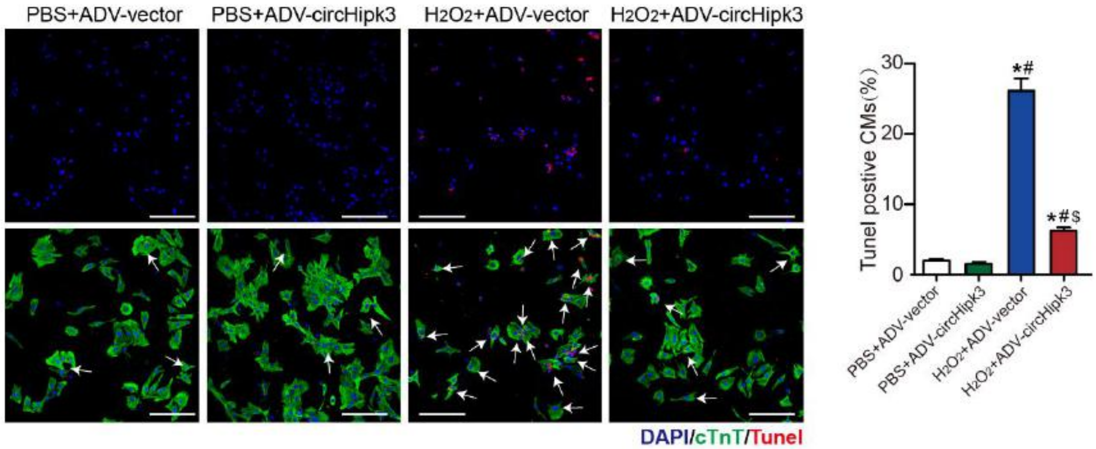

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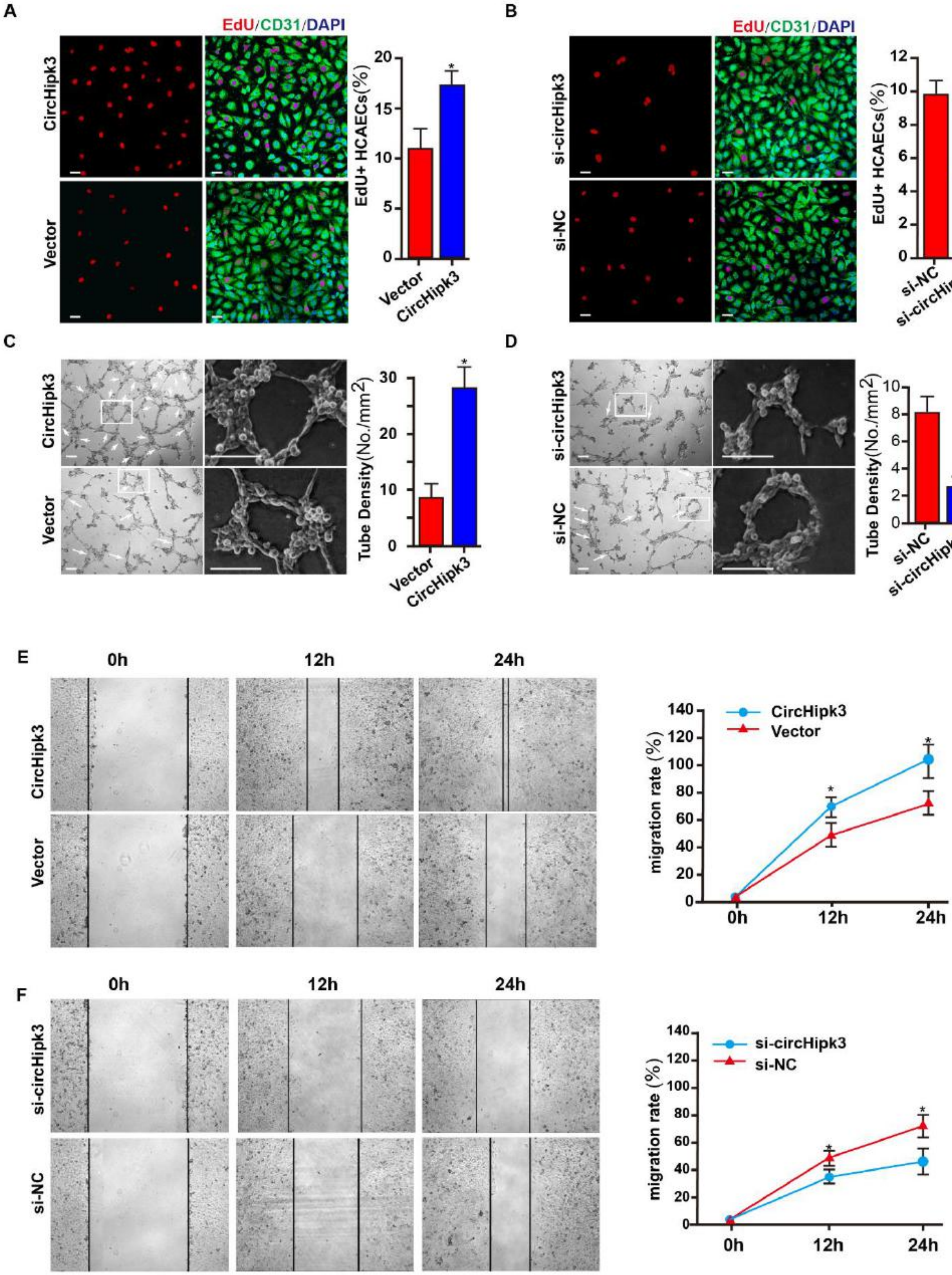

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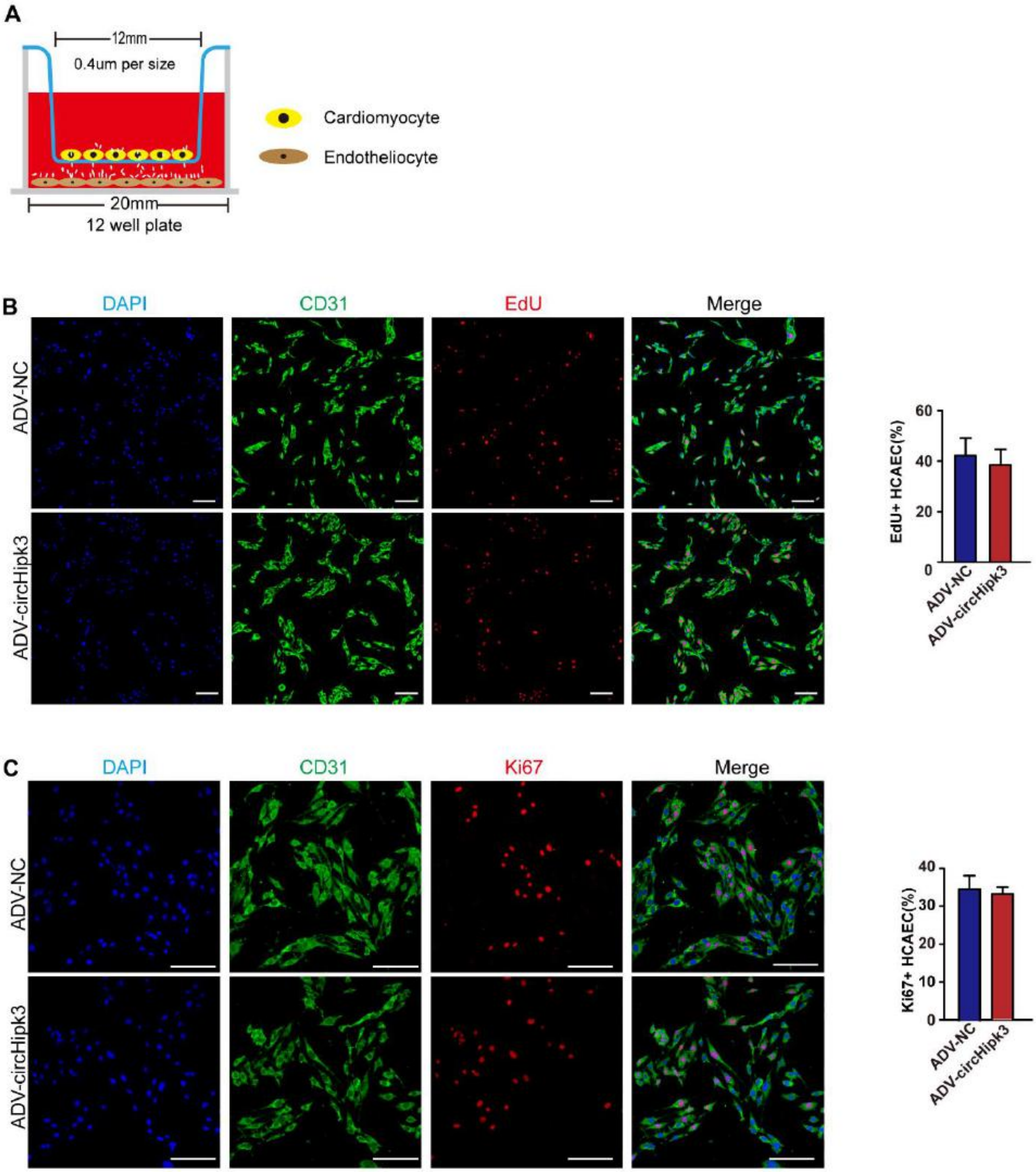

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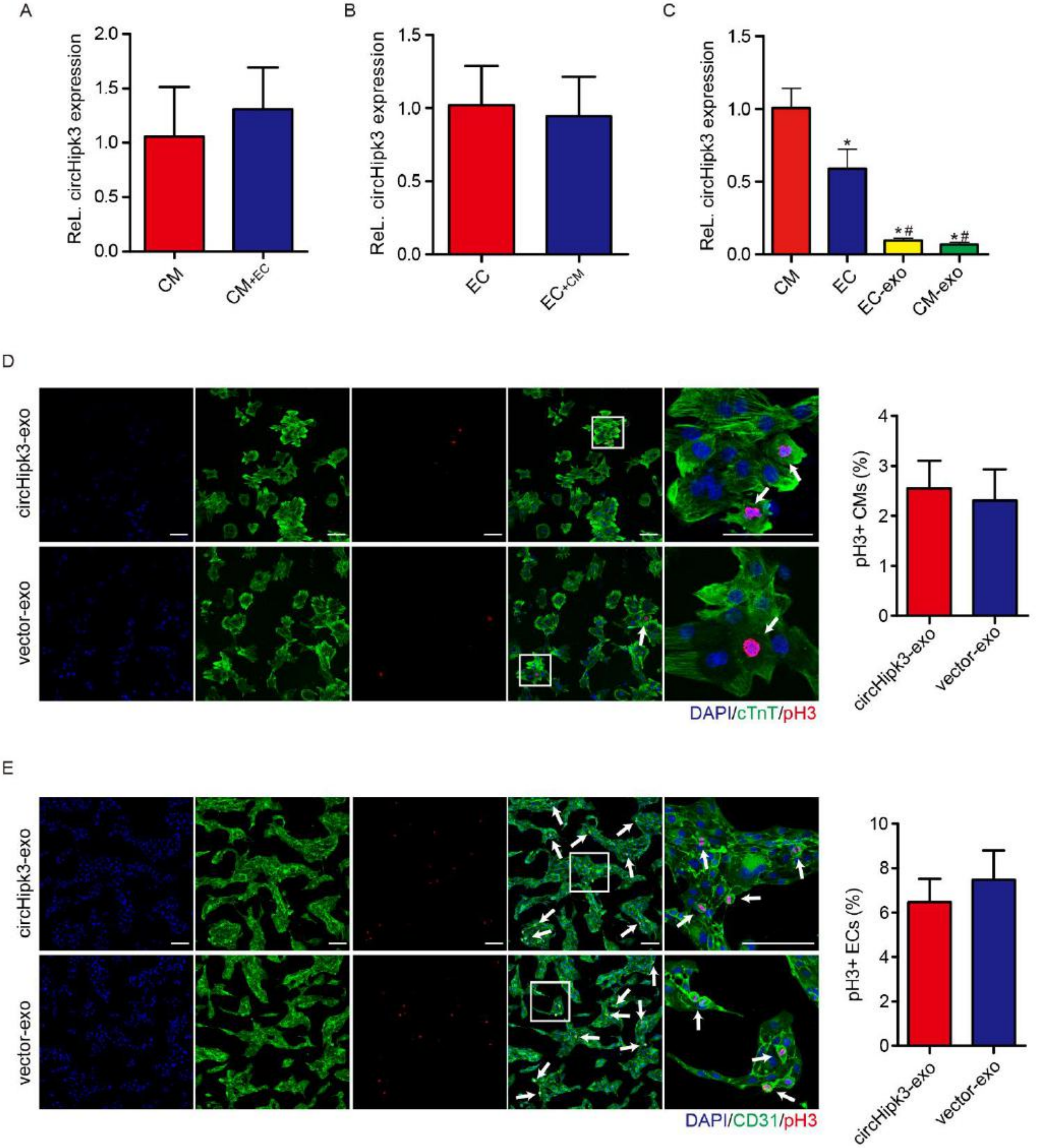

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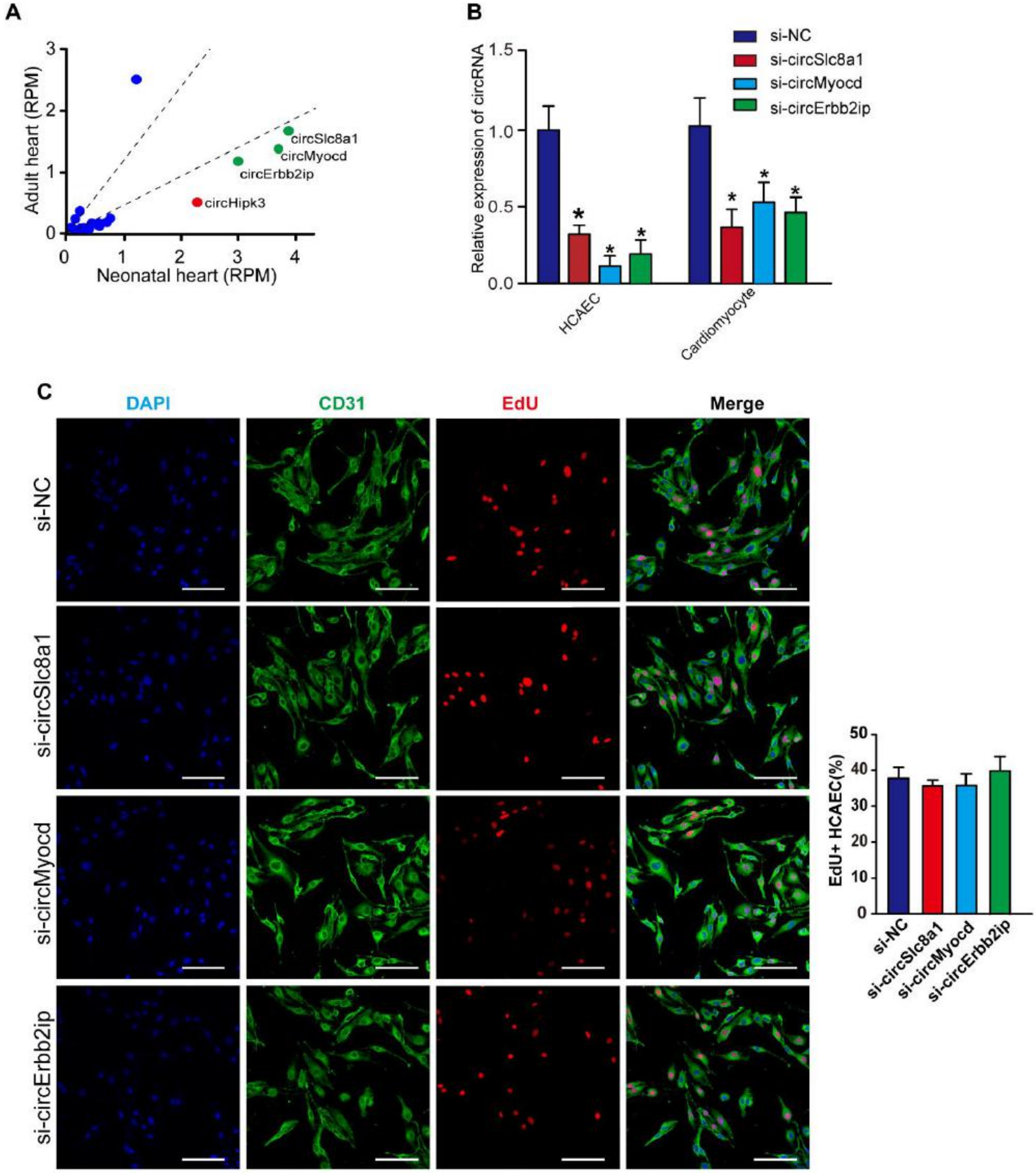

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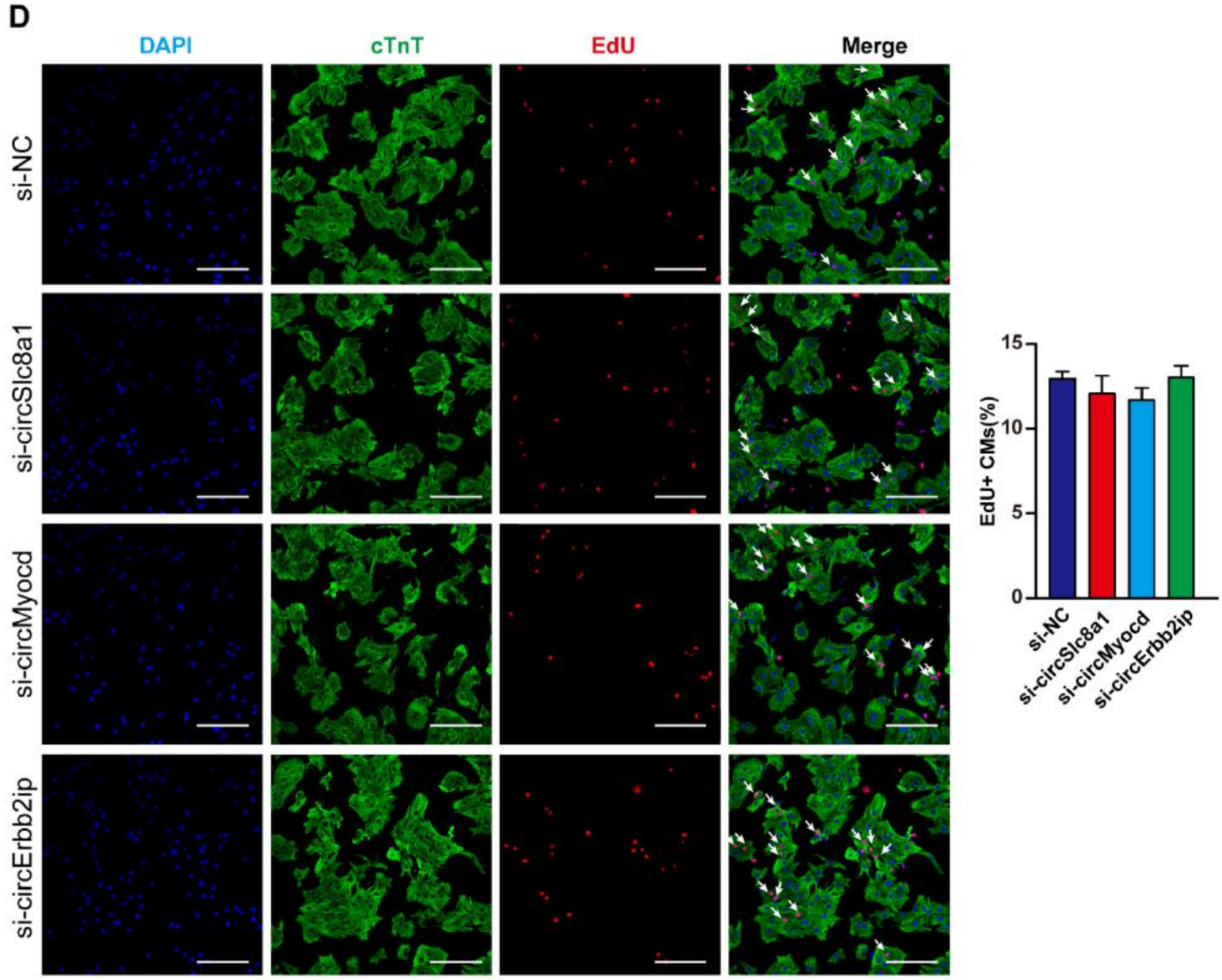

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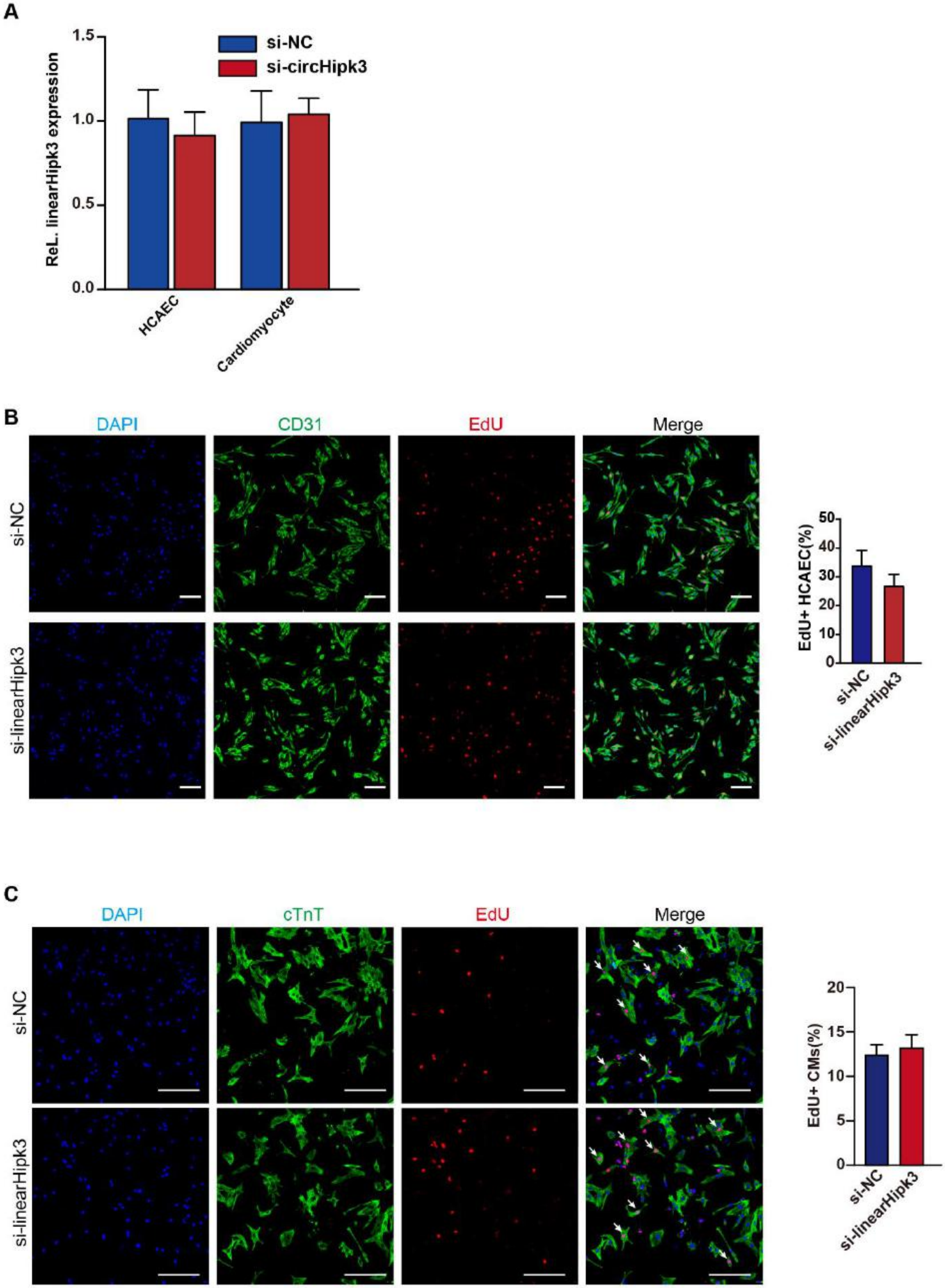

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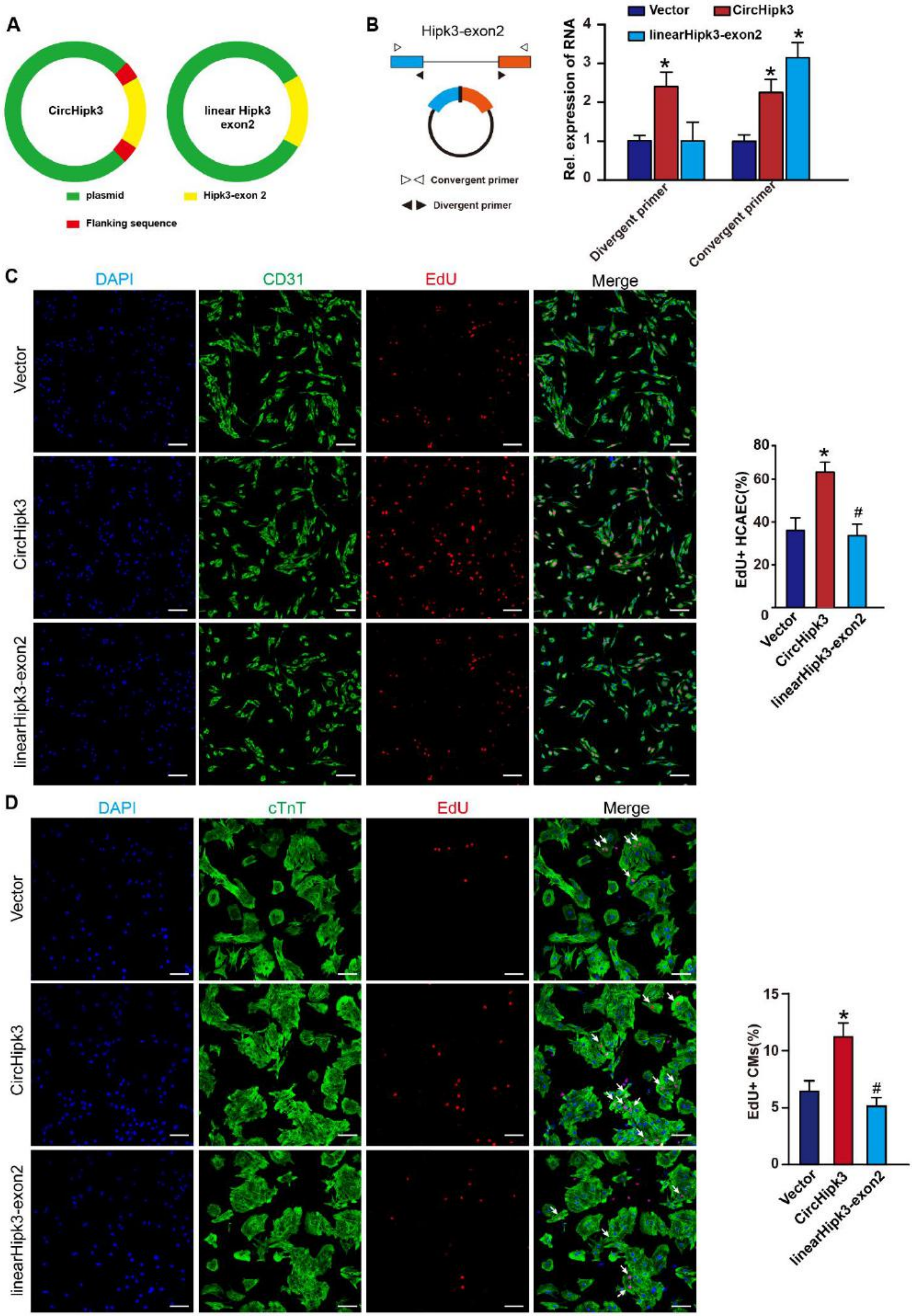

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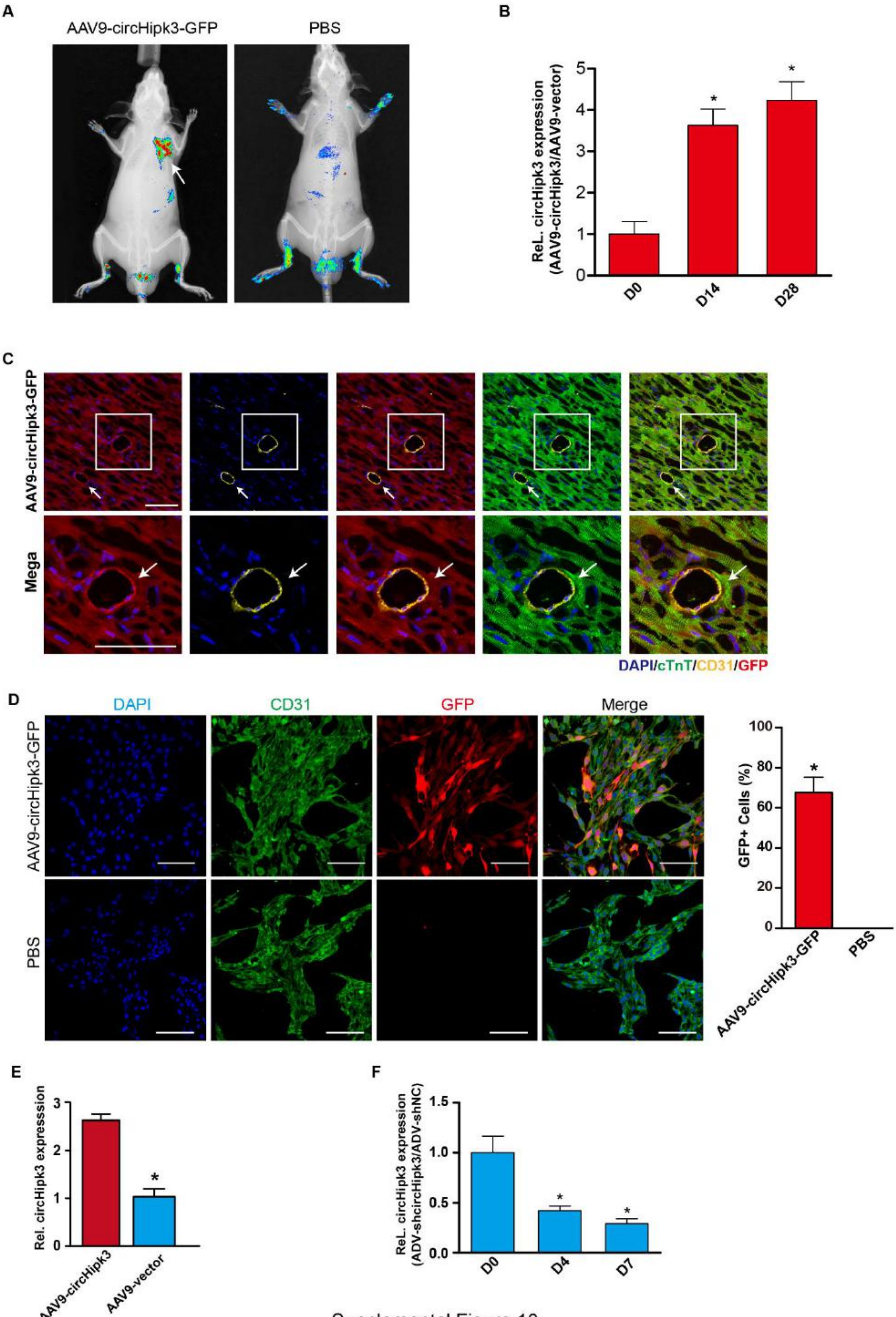

Supplemental Figure 10

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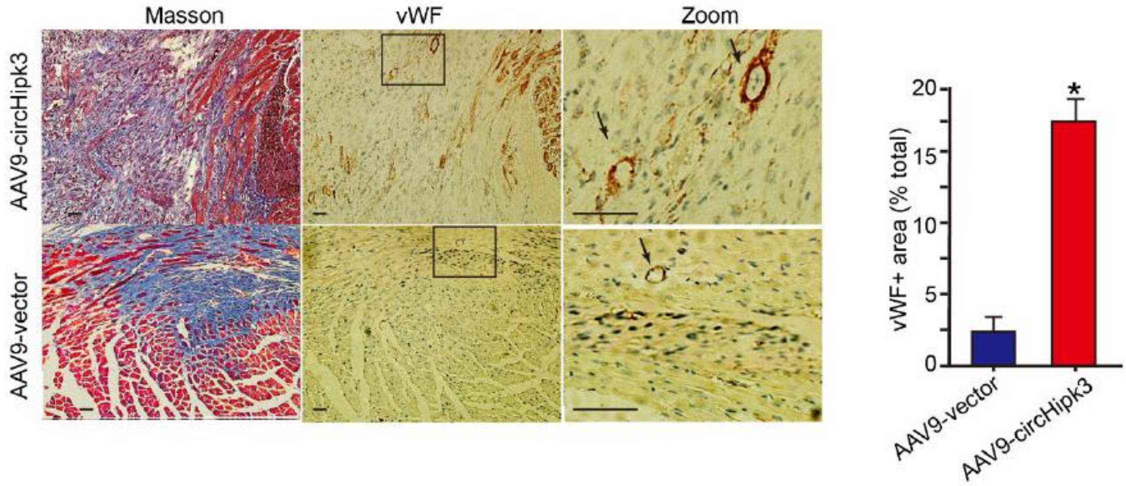

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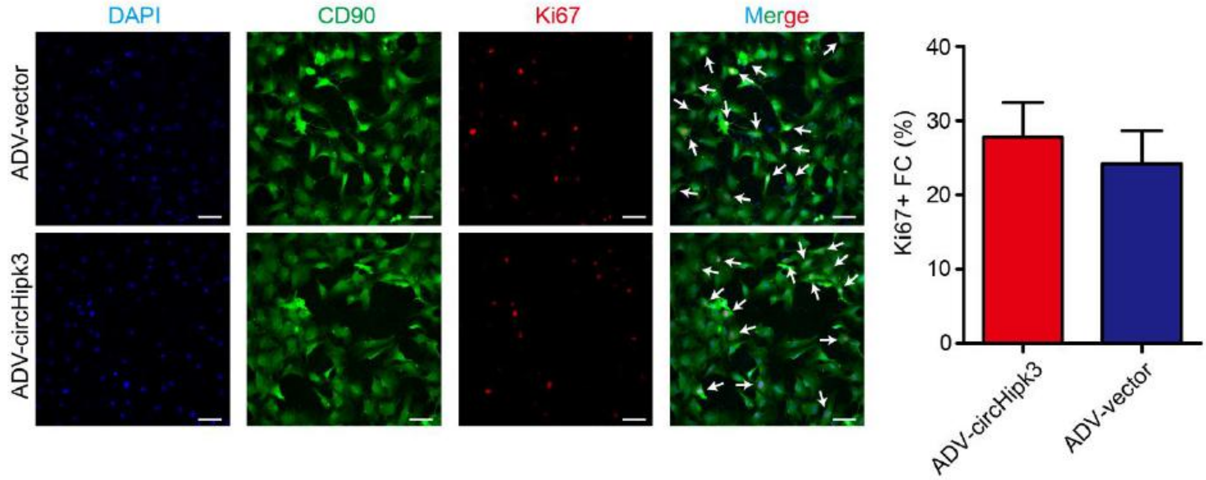

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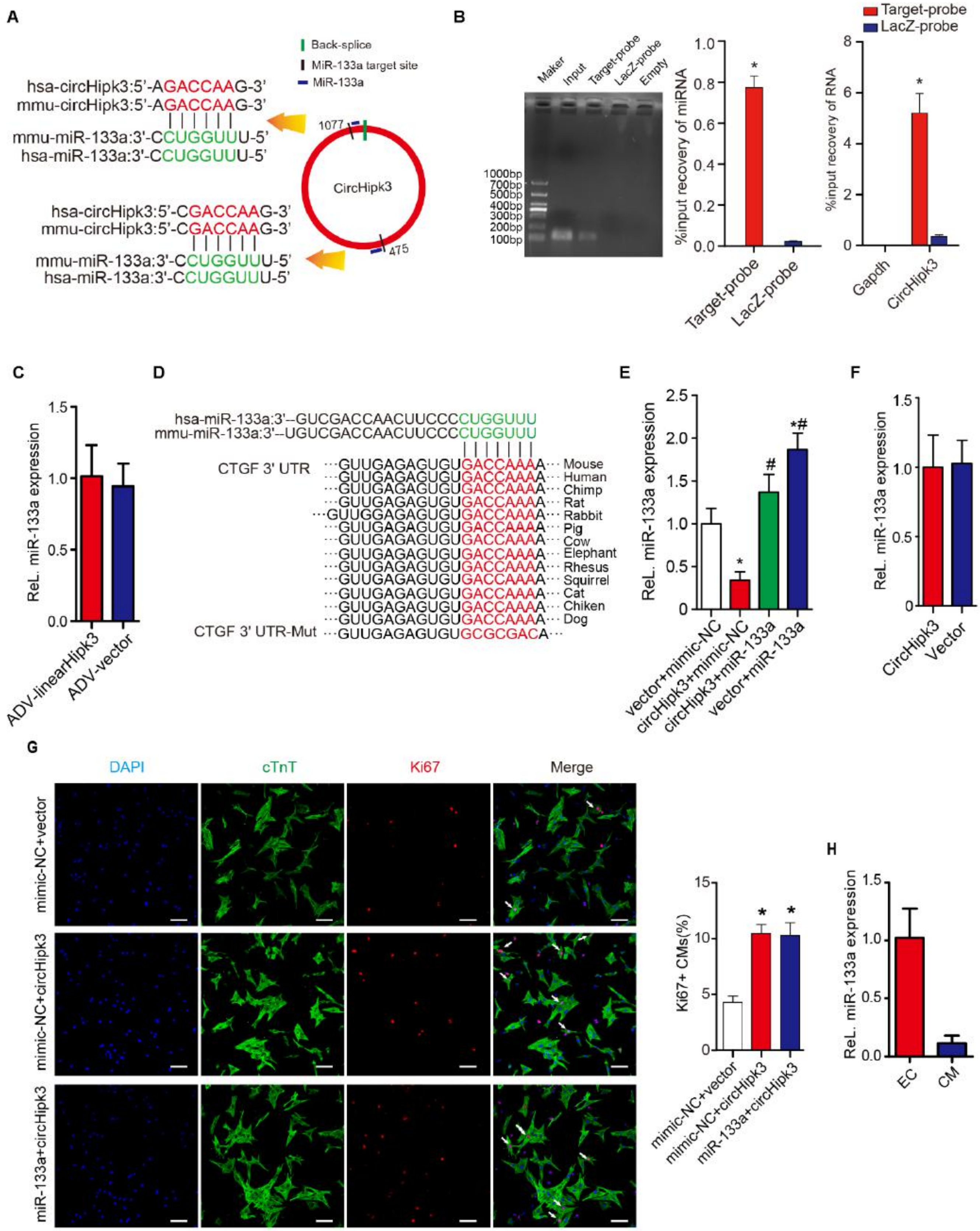

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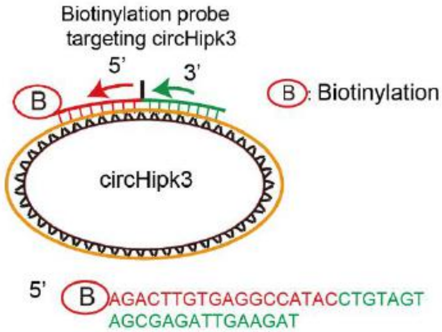
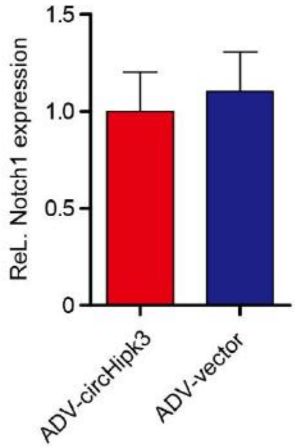


B

RNA	Protein	Prediction using SVM classifier	Prediction using RF classifier
mmu-circHipk3	Notch1	0.97	0.90
hsa-circHipk3	Notch1	0.99	0.65

C



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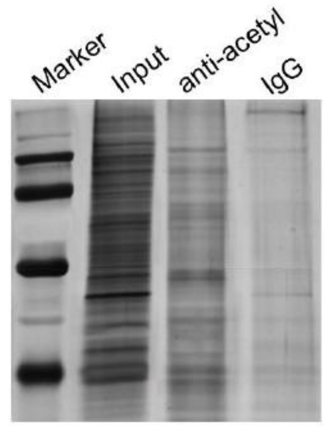

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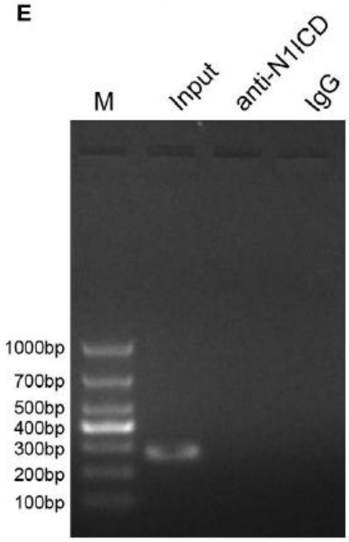

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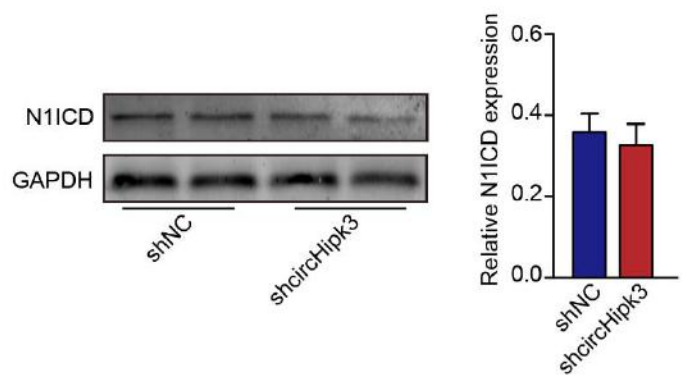

Supplemental Figure 14

Supplemental figure legends

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

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

Supplement Fig. 3 Detection of TUNEL+ CMs after ADV-circHipk3/ADV-vector transfection and H_2O_2 treatment, or control treatment. Neonatal mouse CMs were treated with 50umol H_2O_2 or PBS after transfection with ADV-circHipk3/ADV-vector for 48h. TUNEL+ CMs are indicated by arrows; n=500 CMs, *$P<0.05$ vs. PBS+ADV-vector group, #$P<0.05$ vs. PBS+ADV-vector group, \$ $P<0.05$ vs. H_2O_2+ADV-vector group, scale bar=100 μm.

Supplemental Fig. 4 CircHipk3 regulates coronary endothelial cell proliferation,

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

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

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

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

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

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

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

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

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

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

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

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

Supplemental Fig. 13 The relationship between circHipk3, miR-133a and CTGF. (A) The predicted binding of miR-133a/b to circHipk3. (B) RNA pulldown assays using the probe targeting circHipk3 back-splice site. The abundance of miR-133a, circHipk3 and Gapdh in bound fractions was evaluated by qRT-PCR analysis, **P*<0.05, n=3. The expression of miR-133a was also detected by gel electrophoresis. (C) Changes in miR-133a expression after linearHipk3 overexpression in HCAECs. *P<0.05, n=3. (D) The predicted binding of miR-133a to the 3'UTR of CTGF. (E) Co-injection with AAV9–miR-133a reversed circHipk3-induced miR-133a downregulation in adult hearts. **P*<0.05 vs. AAV9-vector, #*P*<0.05 vs. AAV9-circHipk3, n=6. (F) CircHipk3 has no effect on miR-133a expression in CMs, n=6. (G) MiR-133a mimics has no effect on circHipk3-induced CM proliferation, **P*<0.05 vs. vector+mimic-NC, n=400 CMs, scale bar=50 μm. (H) MiR-133a expression in neonatal cardiac EC and CM; **P*<0.05, n=3.

Supplemental Fig. 14 The relationship between circHipk3 and N1ICD. (A) The biotinylated probe targeting the back-splice junction of circHipk3. (B) The predicted interaction between circHipk3 and N1ICD using RNA-Protein interaction Prediction (RPISeq) programme. Predictions with probabilities > 0.5 indicated that the corresponding RNA and protein are likely to interact. (C) CircHipk3 overexpression has no effect on Notch1 mRNA expression in P7 CMs, n=4. (D) Protein immunoprecipitated by an acetylation-specific antibody and IgG antibody. The arrow indicated the position of N1ICD protein. (E) RIP assays were performed to investigate the interaction between circHipk3 and N1ICD in HCAECs. (F) Detection of N1ICD expression in HCAECs using western blotting assays after circHipk3 knockdown, n=4.

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

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

Primers name		Sequence (5’-3’)
circHipk3	+	GGGCAGATAGAAGTGAGC
	-	TGATAAACATACGGTGGG
MiR-133a	+	GCGTTTGGTCCCCTTCAAC
	-	AGTGCAGGGTCCGAGGTATT
	R	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCA CTGGATACGACCAGCTG
Notch1	+	GATGGCCTCAATGGGTACAAG
	-	TCGTTGTTGTTGATGTCACAGT
U6	+	GCGCGTCGTGAAGCGTTC
	-	GTGCAG GGTCCGAGGT
Gapdh	+	TGACCTCAACTACATGGTCTACA
	-	CTTCCCATTCTCGGCCTTG
circErbb2ip	+	CAACATTACCAGCCTCCAT
	-	GTCTCCTCTTCTCCTCGTA
circMyocd	+	ATTCTGCCGATGGATTCTT
	-	GGTATTAAGCCTTGGTTAGC
circSlc8a1	+	TCTGGAGCTCGAGGAAATGT
	-	TTGGGTGGGAGACTTAATCG
linearHipk3 mRNA	+	ACCTCACCCAGACCTT
	-	AGCATCCTCCACAAAA
mmu-miR-107-3p	+	GCGAGCAGCATTGTACAGGG
	-	AGTGCAGGGTCCGAGGTATT
	R	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA CGACTGATAG
mmu-miR-105	+	CGCCAAGTGCTCAGATGCT
	-	AGTGCAGGGTCCGAGGTATT
	R	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACG ACACCACA
mmu-miR-217-5p	+	CGCGTACTGCATCAGGAACTG
	-	AGTGCAGGGTCCGAGGTATT
	R	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACG ACTCCAGT
mmu-miR-292a-5p	+	GCGACTCAAACTGGGGGCT
	-	AGTGCAGGGTCCGAGGTATT
	R	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACG ACCAAAAG
mmu-miR-542-5p	+	CGCTCGGGGATCATCATG
	-	AGTGCAGGGTCCGAGGTATT
	R	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA

		CGACTCGTGA
mmu-miR-669a-5p	+	GCGAGTTGTGTGTGCATGTTC
	-	AGTGCAGGGTCCGAGGTATT
	R	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGACAT
mmu-miR-9-5p	+	GCGCGTCTTTGGTTATCTAGCT
	-	AGTGCAGGGTCCGAGGTATT
	R	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCATAC
mmu-miR-96-5p	+	GCGTTTGGCACTAGCACATT
	-	AGTGCAGGGTCCGAGGTATT
	R	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGCAAA
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	GGAACAACUCCUUCCACU
Si-circSLC8A1 (human)	GAAAUUGUGUUGUGACAGUUG
Si-circSlc8a1 (mouse)	AGAACGAUGAAAUAGUGUUGG
Si-circMYOCD (human)	AUAAAAGUUUUACAGUUAAGA
Si-circMyocd (mouse)	CGUCUUACAGUUACGGCUUCA
Si-circERBB2IP (human)	GGAUGUCAGCAAGAAUGAAUG
Si-circErbb2ip (mouse)	GGAUGUCAGCAAAAAUGAAUG

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