

Supporting Information for

Original article

ADAR1 regulates vascular remodeling in hypoxic pulmonary hypertension through N1-methyladenosine modification of circCDK17

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Supplementary Figures

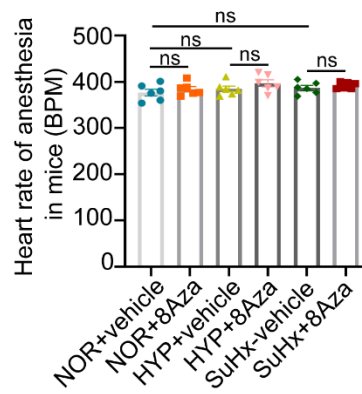


Figure S1. Effect of 8Aza on heart rate in different PH mouse models. n=6. NOR, normoxia; HYP, hypoxia; NC, negative control; 8Aza, 8-Azaadenosine; ns, no significance; Statistical analysis was performed with two-way ANOVA followed by Dunnett's test; All values are presented as mean \pm SEM.

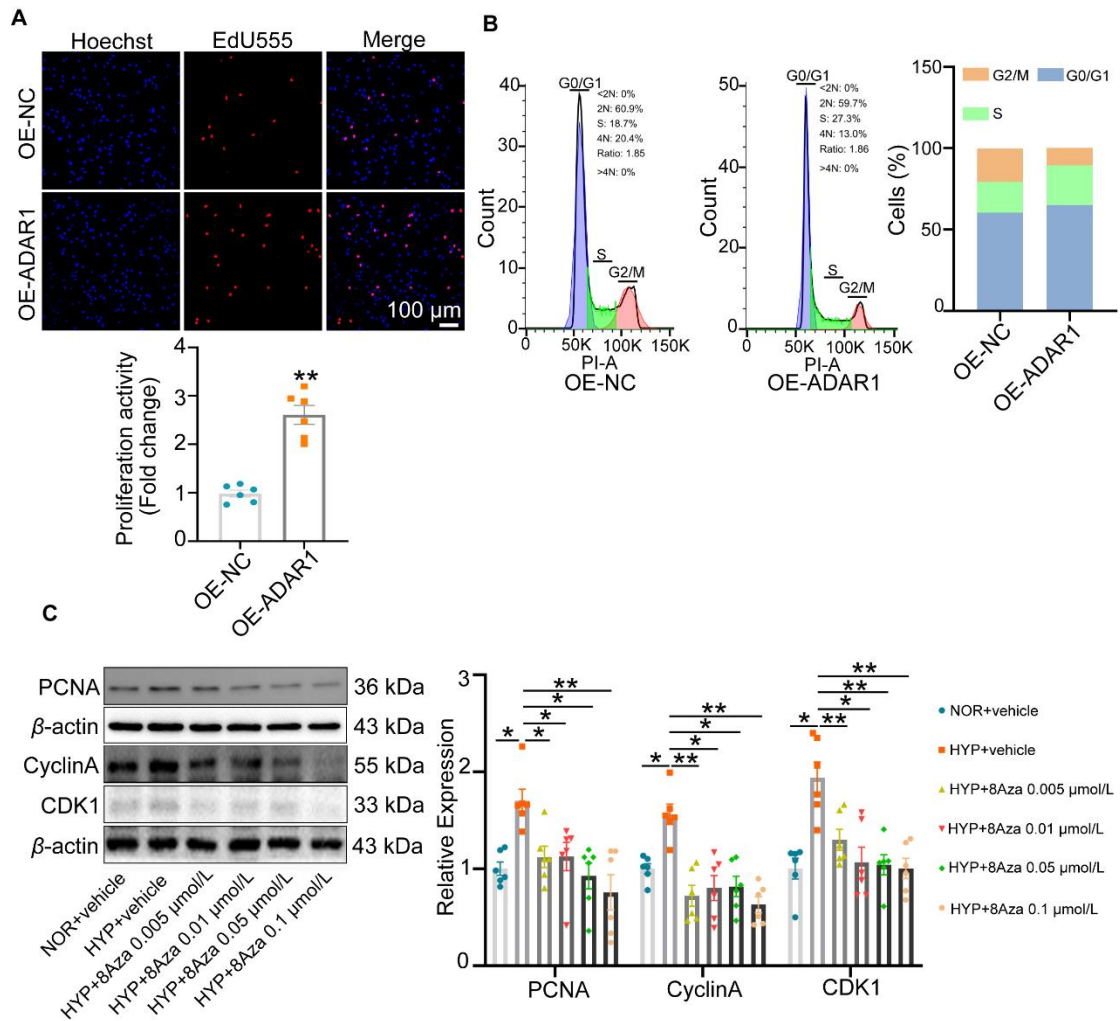


Figure S2. Effect of ADAR1 on cell proliferation in PSMCs. (A) EdU assay showing that overexpression of ADAR1 (OE-ADAR1) increased cell proliferation in PSMCs. n=6. **(B)** Flow cytometry showing that the effect of OE-ADAR1 on cell cycle progression. n=6. **(C)** Representative Western blots and group data showing the concentration-dependent effect of 8Aza on protein expression of PCNA, cyclin A and CDK1 in the hypoxia situation. n=6. NOR, normoxia; HYP, hypoxia; NC, negative control; OE, ADAR1 overexpression plasmid; Statistical analysis was performed with two-way ANOVA followed by Dunnett's test or the Student's t-test; All values are presented as mean \pm SEM. *P<0.05, **P<0.01, ***P<0.001.



Figure S3. Functional enrichment and pathway enrichment analysis of circRNAs by bioinformatics analysis. (A) Gene ontology (GO) classification. (B) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway classification.

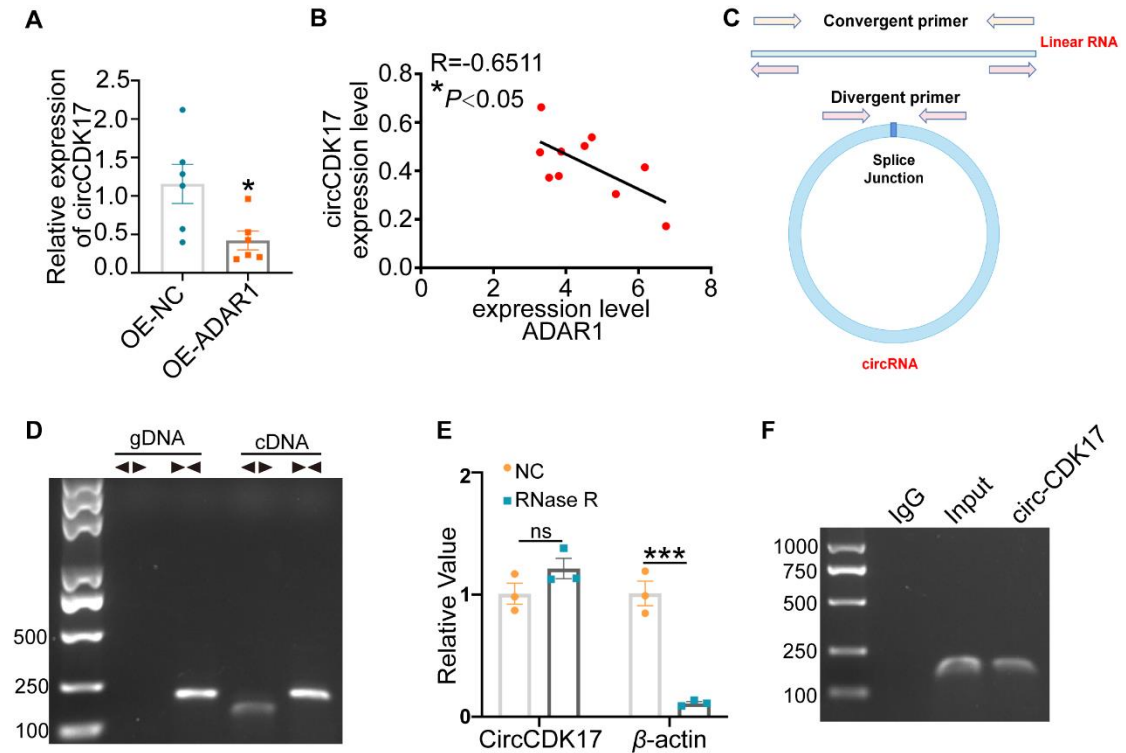


Figure S4. Verification of the structure of circCDK17 and its association with ADAR1. (A) qPCR analysis showing the expression of circCDK17 upon overexpression of ADAR1 (OE-ADAR1). n=6. (B) Correlation analysis between ADAR1 and circCDK17 *in vivo*. n=10. (C) Schematic illustration showing convergent and divergent primer design. (D) Agarose gel electrophoresis showing qPCR products of the opposite and back primers of circCDK17 cycling site. (E) RNase R experiment was performed to verify the ring structure of circCDK17. n=3. (F) RIP-qPCR experiment was performed to verify that ADAR1 binds to circCDK17. n=3. NC, negative control; OE, ADAR1 overexpression plasmid; ns, no significance; Statistical analysis was performed with the Student's t-test; All values are presented as mean \pm SEM. *P<0.05, **P<0.01, ***P<0.001.

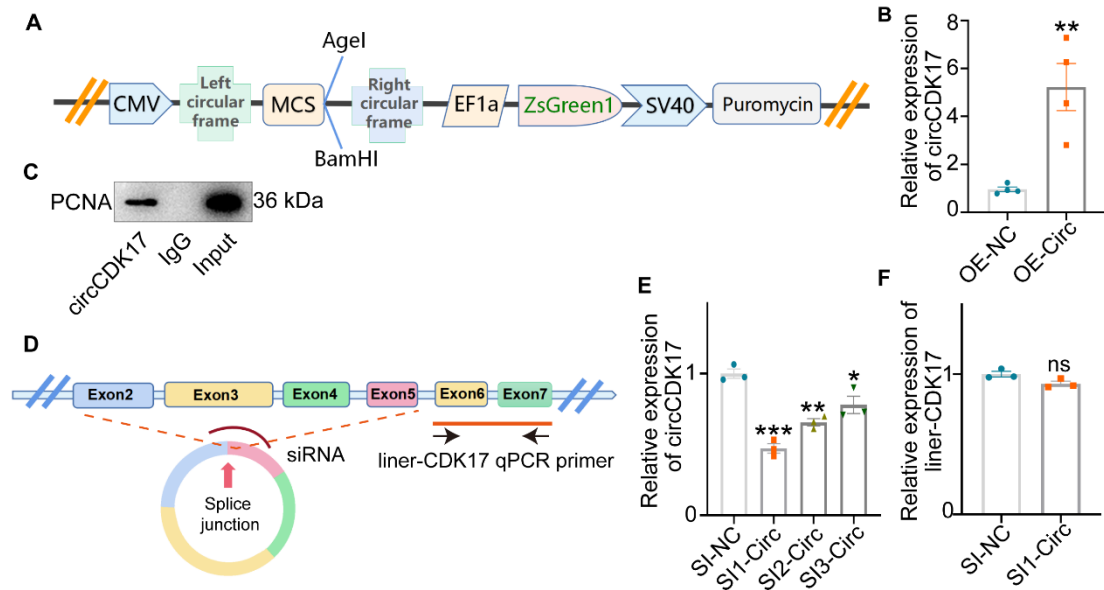


Figure S5. Molecular modulation of circCDK17 expression with overexpression (OE) plasmid or siRNA. (A-B) Schematic illustration showing the construction of circCDK17 plasmid (A) and qPCR analysis (B) showing the efficiency of circCDK17 overexpressed plasmid. n=4. **(C)** RNA immunoprecipitation showing that circCDK17 was directly bound to PCNA. n=3. **(D-E)** Schematic illustration showing the construction of circCDK17 siRNA and primer of liner CDK17 (D) and qPCR analysis showing the efficiency of circCDK17 siRNA (E). n=3. **(F)** qPCR analysis showing the effect of circCDK17 siRNA on linear CDK17. n=3. NC, negative control; SI, siRNA of circCDK17; OE, circCDK17 overexpression plasmid; ns, no significance; Statistical analysis was performed with one-way ANOVA followed by Dunnett's test or the Student's t-test; All values are presented as mean \pm SEM. *P<0.05, **P<0.01, ***P<0.001.

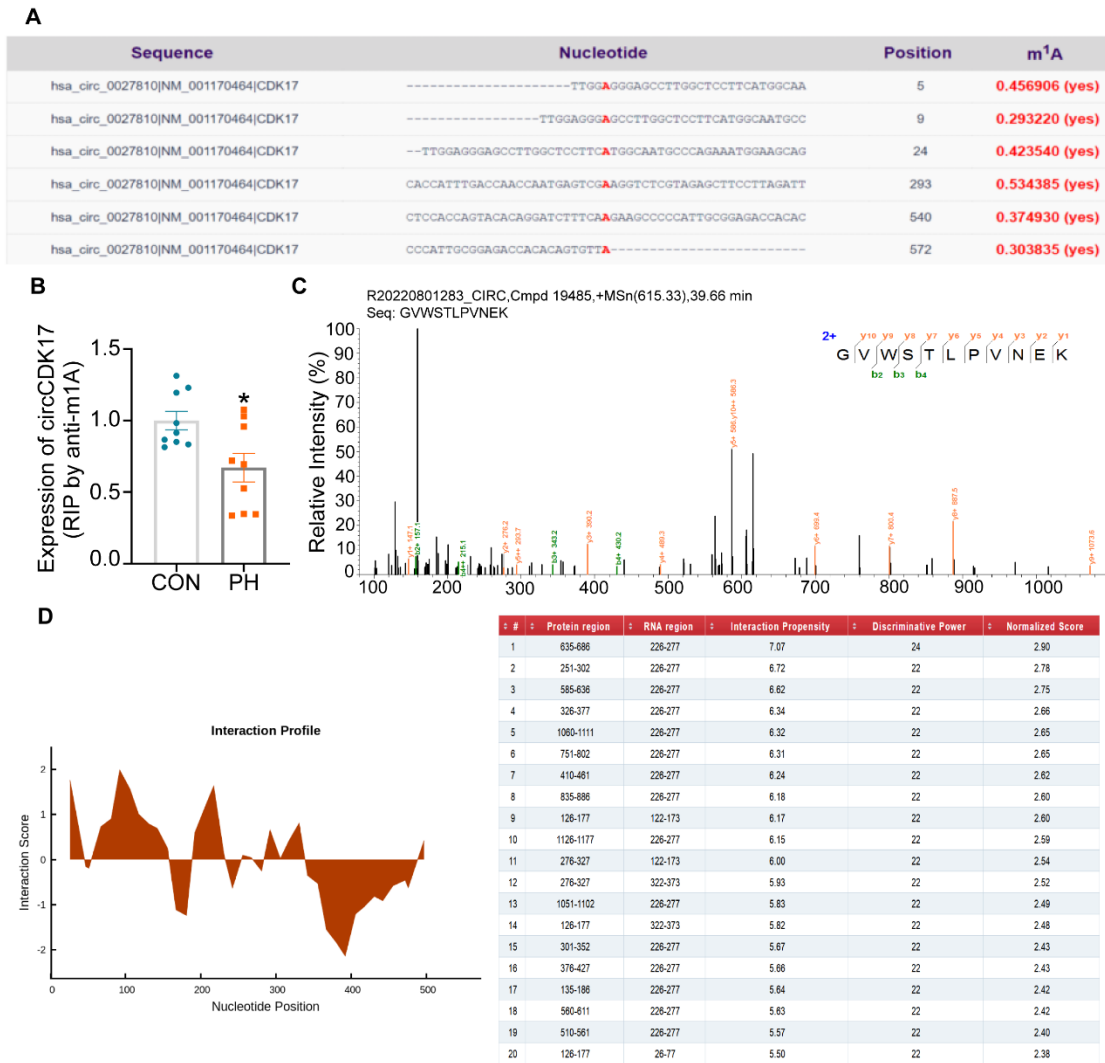


Figure S6. Software analysis showing the m¹A methylation modification sites in circCDK17 and its interaction with ADAR1. (A) Deepromise (<https://deepromise.erc.monash.edu/>) online software analysis of m¹A methylation modification sites. **(B)** RNA immunoprecipitation showing the m¹A modification level of circCDK17 in lung tissue of PH patients. n=9. **(C)** RNA pull-down combined mass spectrometry showing the presence of YTHDC1 specific peptide. **(D)** CatRAPID software analysis showing the interaction sites between ADAR1 and circCDK17 (http://service.tartaglialab.com/page/catrapid_group).

Abbreviations

PH Pulmonary hypertension

ADAR1 RNA adenosine deaminase 1

PCNA Proliferating cell nuclear antigen

PASMCs Pulmonary artery smooth muscle cells

PAECs Pulmonary artery endothelial cells

CircRNA Circular RNA

AAV9 serotype 9 adenovirus-associated virus

HE Hematoxylin and eosin

SiRNA Small interfering RNA

OE Overexpression

RIP RNA Binding Protein Immunoprecipitation

FISH Fluorescent in situ hybridization

DAPI 4',6-Diamidino-2-phenylindole

PBS Phosphate-buffered saline

HBSS Hank's Balanced Salt Solution

SMA Smooth muscle actin

Table S1 Related primer sequences.

qPCR primer	Forward	Reverse
ADAR1	CATCCATTTCAAGGCTATGAGC	GTACTGGAGGCAAGTAGTACTG
β -actin	CTCGCCTTTGCCGATCC	TCTCCATGTCGTCCCAGTTG
circCDK17	AGCTTCCTTAGATTTTTCAAGCCAC	AGGCCTGCCATTCTTCACAA
circCDC14B	CGGGCCTTGAAAAGCAGAAG	CCATTGCCAGATTGAGTGGT
circTBCD	CAGTGACAAGGCCCGAGATG	AACAGGCTCTACATCGGCAA
circAPBB2	ATGCAGCCACTGTTAACCCG	GGCGGCAAATCTGGATCAGT
CDK17	ATTTTGGACTAGCCCGAGCC	TCCGAGGAACCAAGAAGCAC

Name	Sequences
circCDK17 biotin probe	5' -3' TGTGGC+TTGAAAAA+TCTAAGGAAGC+TVTACG
circCDK17 fish probe	5' -3' TGAATGTGGCT+TGAAAATC+TAAGGAAGC+TCTACGAGA
SiRNA NC	sense (5' -3') UUCUCCGAACGUGUCACGUTT antisense (5' -3') ACGUGACACGUUCGGAGAATT
ADAR1 siRNA 1	sense (5' -3') CGGAUACUACACCCAUCCAUUTT antisense (5' -3') AAUGGAUGGGUGUAGUAUCCGTT
ADAR1 siRNA 2	sense (5' -3') GCAGGGUAUGUUGACUUUGAATT antisense (5' -3') UUCAAGUCAACAUACCCUGCTT
ADAR1 siRNA 3	sense (5' -3') GCAUGGGUUUCACAGAGGUAATT antisense (5' -3') UUACCUCUGUGAAACCAUGCTT
YTHDC1 siRNA	sense (5' -3') UGGAUUUGCAGGCGUGAAUUATT antisense (5' -3') UAAUUCACGCCUGCAAUCCATT
circCDK17 siRNA 1	sense (5' -3') GAGCUUCCUUAGAUUUUUAATT antisense (5' -3') UUGAAAAAUCUAAGGAAGCUCTT
circCDK17 siRNA 2	sense (5' -3') UUCUUAGAUUUUUAAGCCATT antisense (5' -3') UGGCUUGAAAAAUCUAAGGAATT
circCDK17 siRNA 3	sense (5' -3') CCUUAGAUUUUUAAGCCACATT antisense (5' -3') UGUGGCUUGAAAAAUCUAAGGTT

Table S2 Clinical data of PH patients and controls.

Patient ID	Assay	Age, y	Sex	Race/Ethnicity	Diagnosis/Cause of Death	mPAP (mmHg)
HPH-01	qPCR (Lung Plasma)	68	M	Asian	IPF associated PH	63
HPH-02	qPCR (Lung Plasma)	57	F	Asian	Pulmonary fibrosis associated PH	77
HPH-03	qPCR (Lung Plasma)	65	F	Asian	Pulmonary fibrosis associated PH	42
HPH-04	qPCR (Lung Plasma)	56	F	Asian	Pulmonary fibrosis associated PH	40
HPH-05	qPCR (Lung Plasma)	75	M	Asian	Pulmonary fibrosis associated PH	38
HPH-06	qPCR (Lung Plasma)	55	M	Asian	Pulmonary fibrosis associated PH	112
HPH-07	qPCR (Lung Plasma)	54	M	Asian	Pulmonary fibrosis associated PH	55
HPH-08	qPCR (Lung Plasma)	58	M	Asian	Pulmonary fibrosis associated PH	54
HPH-09	qPCR (Lung Plasma)	48	M	Asian	Pulmonary fibrosis associated PH	50
HPH-10	qPCR (Lung Plasma)	58	M	Asian	Pulmonary fibrosis associated PH	44
HPH-11	IF, IHC, WB, qPCR (Lung tissue)	53	M	Asian	Bronchiectasis PH	71
HPH-12	IF, IHC, WB, qPCR (Lung tissue)	43	M	Asian	Silicosis PH	97
HPH-13	IF, IHC, WB, qPCR (Lung tissue)	60	F	Asian	Pulmonary fibrosis associated PH	43
HPH-14	IF, IHC, WB, qPCR (Lung tissue)	13	F	Asian	Castleman's disease associated PH	59
HPH-15	IF, IHC, WB, qPCR (Lung tissue)	32	F	Asian	PVOD associated PH	112
HPH-16	IF, IHC, WB, qPCR (Lung tissue)	74	M	Asian	Pulmonary fibrosis associated PH	69
Control-01	qPCR (Lung Plasma)	54	F	Asian	Minimally invasive adenocarcinoma	N/A
Control-02	qPCR (Lung Plasma)	74	F	Asian	Minimally invasive adenocarcinoma	N/A
Control-03	qPCR (Lung Plasma)	55	M	Asian	Minimally invasive adenocarcinoma	N/A
Control-04	qPCR (Lung Plasma)	75	M	Asian	Minimally invasive adenocarcinoma	N/A
Control-05	qPCR (Lung Plasma)	64	F	Asian	N/A	N/A

Control-06	qPCR (Lung Plasma)	66	F	Asian	Benign biopsy	N/A
Control-07	qPCR (Lung Plasma)	70	F	Asian	N/A	N/A
Control-08	qPCR (Lung Plasma)	35	F	Asian	N/A	N/A
Control-09	qPCR (Lung Plasma)	41	F	Asian	Anoxia of brain	N/A
Control-10	qPCR (Lung Plasma)	18	F	Asian	Pneumatocele	N/A
Control-11	qPCR (Lung Plasma)	52	M	Asian	Pulmonary inflammatory pseudotumor	N/A
Control-12	qPCR (Lung Plasma)	44	F	Asian	Cystic teratoma	N/A
Control-13	qPCR (Lung Plasma)	66	F	Asian	Sclerosing hemangioma	N/A
Control-14	qPCR (Lung Plasma)	34	F	Asian	Pulmonary Atypical Adenomatous Hyperplasia	N/A
Control-15	qPCR (Lung Plasma)	50	F	Asian	N/A	N/A
Control-16	IF, IHC, WB, qPCR (Lung tissue)	M	64	Asian	Anoxia of brain	N/A
Control-17	IF, IHC, WB, qPCR (Lung tissue)	F	56	Asian	Adjacent normal tissues	N/A
Control-18	IF, IHC, WB, qPCR (Lung tissue)	F	47	Asian	Adjacent normal tissues	N/A
Control-19	IF, IHC, WB, qPCR (Lung tissue)	M	42	Asian	Anoxia of brain	N/A
Control-20	IF, IHC, WB, qPCR (Lung tissue)	F	71	Asian	Anoxia of brain	N/A
Control-21	IF, IHC, WB, qPCR (Lung tissue)	F	56	Asian	Adjacent normal tissues	N/A

Definition of abbreviations: HPH, hypoxic pulmonary hypertension; F, female; M, male; PH, pulmonary hypertension; IPF, idiopathic pulmonary fibrosis; PVOD, pulmonary venoocclusive disease; mPAP, mean pulmonary artery pressure (mmHg); N/A, data not available.