SUPPLEMENTAL MATERIAL

Cooperative binding of ETS2 and NFAT link Erk1/2 and calcineurin signaling in the pathogenesis of cardiac hypertrophy

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Supplemental Table I. Primers used in this study.

Genes	Species	Forward (5'-3')	Reverse (5'-3')	Test
ETS2	Mouse	CCTGTCGCCAACAGTTTTCG	TGGAGTGTCTGATCTTCACTGA	qPCR
ETS2	Human	AGAGACTGACGAGTGCGGTG	CGAAATCATTCATCCTGCCGCT	qPCR
ANP	Mouse	TCTTCCTCGTCTTGGCCTTT	CCAGGTGGTCTAGCAGGTTC	qPCR
BNP	Mouse	TGGGAGGTCACTCCTATCCT	GGCCATTTCCTCCGACTTT	qPCR
βMHC	Mouse	CGGACCTTGGAAGACCAGAT	GACAGCTCCCCATTCTCTGT	qPCR
18s	Mouse/Human	AAACGGCTACCACATCCAAG	TTGCCCTCCAATGGATCCT	qPCR
ETS2	Rat	TGGCATTCCCAAAAACCCCT	TTACACAGCATCTGGCCGTT	qPCR
ANP	Rat	GAAGATGCCGGTAGAAGATGAG	AGAGCCCTCAGTTTGCTTTTC	qPCR
BNP	Rat	GGTGCTGCCCCAGATGATT	CTGGAGACTGGCTAGGACTTC	qPCR
18s	Rat	CCGTTCTTAGTTGGTGGAGCGATT	TTGCTCAATCTCGGTGGCTGAAC	qPCR
Rcan1.4	Mouse/Rat	CCGTTGAAAAAGCAGAATGC	TCCTTGTCATACGTCCTGAAGAGGG	qPCR
MKP3	Rat	CCCCAATCTGCCCAATCTGT	TTGCCTCGGGCTTCATCTAT	qPCR
U6	Mouse/Rat	GCGCTCGTGAAGCGTTC	CTGGTGTCGTGGAGTCG	qPCR
miR-223	Mouse/Rat	GCCGTGTATTTGACAAGCT	CTGGTGTCGTGGAGTCG	qPCR
ANP	Rat	CTGTCGGCACCTCGGACACAAGTCG	GATTCATTGGCCTCGTGGGTGGACC	ChIP
BNP	Rat	CTGGAAGTGTTTTTGATGAGTCACC	GCTATAAACCGCAGGCCTTGTA	ChIP
βMHC	Rat	CCCTCCAAGAAAGTCAGGATACACC	CTGGGGAAAGGAGCAGCTGTTAC	ChIP
Rcan1.4	Rat	ACACTCCACCTTGCCTAGAGAAGGA	GCCCTAGAAGGCTGAGGATAGCA	ChIP
MKP3	Rat	GAGTAGCGAATAAGGGCTTTTGTGC	TCATTCACAAAAACGGAAAGGAATT	ChIP
miR-223	Rat	TATCAGGGATACAGTCATTA	TTTGTAAATAGTCTGTTCCT	ChIP
Rcan1.4	Human	ATTTTTATTGACCAAGACAGATTTC	ATCAACCTCTCTTGCAGCATAGT	ChIP
miR-223	Human	CCAGATTTCCGTTGGCTAAC	CACAAAGCAAATTAGTGGAAAGCTG	ChIP

Supplemental Table II. Clinical characteristics of patients with dilated cardiomyopathy.

Group	Subject	Age (years)	Gender	Body weight (kg)	Height (m)	BMI	Heart rate (BPM)	EF (%)	LVEDd (mm)	Medical treatment						
										β-Blocker	Digoxin	ACEI	Dobutamine	Diuretics	CCB	Valsartan
DCM	1	32	Male	75	1.71	25.6	89	30	61	No	No	No	Yes	No	No	No
DCM	2	55	Male	83	1.75	27.1	105	28	87	Yes	Yes	No	Yes	Yes	No	No
DCM	3	39	Male	80	1.74	26.4	107	32	69	Yes	Yes	No	Yes	Yes	No	Yes
DCM	4	31	Male	50	1.72	16.9	75	NA	NA	NA	NA	NA	NA	NA	NA	NA
DCM	5	55	Male	75.9	1.76	24.5	90	32	70	No	No	No	No	No	No	No
DCM	6	43	Male	79	1.89	22.1	84	21	67	No	No	No	No	No	No	No
DCM	7	45	Male	69	1.65	25.3	112	16	76	No	Yes	No	No	Yes	No	No
DCM	8	40	Female	51	1.6	19.9	74	42	48	Yes	No	No	Yes	Yes	No	No
DCM	9	34	Male	71.8	1.68	25.4	88	26	86	No	Yes	No	No	Yes	Yes	No
DCM	10	46	Female	80	1.6	31.3	92	26	80	No	Yes	Yes	Yes	Yes	No	No
DCM	11	46	Male	80	1.78	25.2	84	27	74	No	Yes	No	No	Yes	No	No
DCM	12	31	Male	50	1.72	16.9	75	NA	NA	NA	NA	NA	NA	NA	NA	NA
DCM	13	43	Male	78.9	1.89	22.1	82	21	67	No	No	Yes	No	Yes	No	No
DCM	14	43	Male	87	1.82	26.3	110	14	78	No	No	No	No	No	No	No
DCM	15	42	Male	73	1.7	25.3	125	22	81	No	Yes	No	No	Yes	No	No
DCM	16	67	Male	62	1.73	20.7	115	21	71	No	No	No	No	Yes	No	Yes
DCM	17	52	Female	50	1.55	20.8	118	18	60	No	No	No	Yes	Yes	No	Yes
DCM	18	30	Male	67.5	1.7	23.4	91	13	78	Yes	Yes	No	No	Yes	No	No
DCM	19	53	Male	NA	NA	NA	NA	21	77	No	Yes	No	No	Yes	No	No

DCM, dilated cardiomyopathy; BMI, body mass index; EF, ejection fraction; LVEDd, left ventricular end-diastolic diameter; β-Blocker, β-adrenergic blocker; ACEI, angiotensin converting enzyme inhibitor; CCB, calcium channel blocker; NA, not available.

Supplemental Table III. Echocardiography analyses of cardiac function in Cre, ETS2-floxed (F/F) and ETS2 knockout (KO) mice after 3 weeks of sTAC or sham.

	Sham			sTAC		
	Cre	**F/F**	**KO**	**Cre**	**F/F**	**KO**
N	6	6	7	9	8	11
HR (bpm)	672±8	681±13	699±15	701±7	682±14	698±8
EF (%)	92.12±1.46	92.19±1.63	91.24±1.15	68.41±3.61***	78.77±2.17**	88.85±1.52$^{\dagger\dagger\dagger, \ddagger}$
FS (%)	62.84±2.96	66.06±1.96	63.23±1.04	38.09±2.98***	47.16±2.44***	57.53±1.92$^{\dagger\dagger, \ddagger}$
LVID,d (mm)	2.44±0.05	2.52±0.06	2.44±0.06	3.16±0.08***	3.01±0.14**	2.59±0.08$^{\dagger\dagger\dagger, \ddagger\ddagger}$
LVID,s (mm)	0.91±0.08	0.86±0.06	0.9±0.04	1.97±0.14***	1.60±0.13***	1.11±0.08$^{\dagger\dagger\dagger, \ddagger\ddagger}$
LVPW,d (mm)	1.09±0.05	1.09±0.03	1.06±0.06	1.41±0.05***	1.42±0.06***	1.23±0.04$^{\dagger, \ddagger}$
LVPW,s (mm)	1.71±0.04	1.75±0.05	1.73±0.04	1.91±0.07*	1.98±0.08*	1.80±0.06
IVS,d (mm)	1.13±0.05	1.11±0.04	1.10±0.04	1.44±0.05***	1.43±0.04***	1.28±0.03$^{\dagger\dagger, \ddagger}$
LV mass (mg)	109.89±3.98	109.20±5.87	110.42±3.95	164.20±10.26***	174.31±9.97***	126.83±4.70$^{\dagger, \ddagger}$

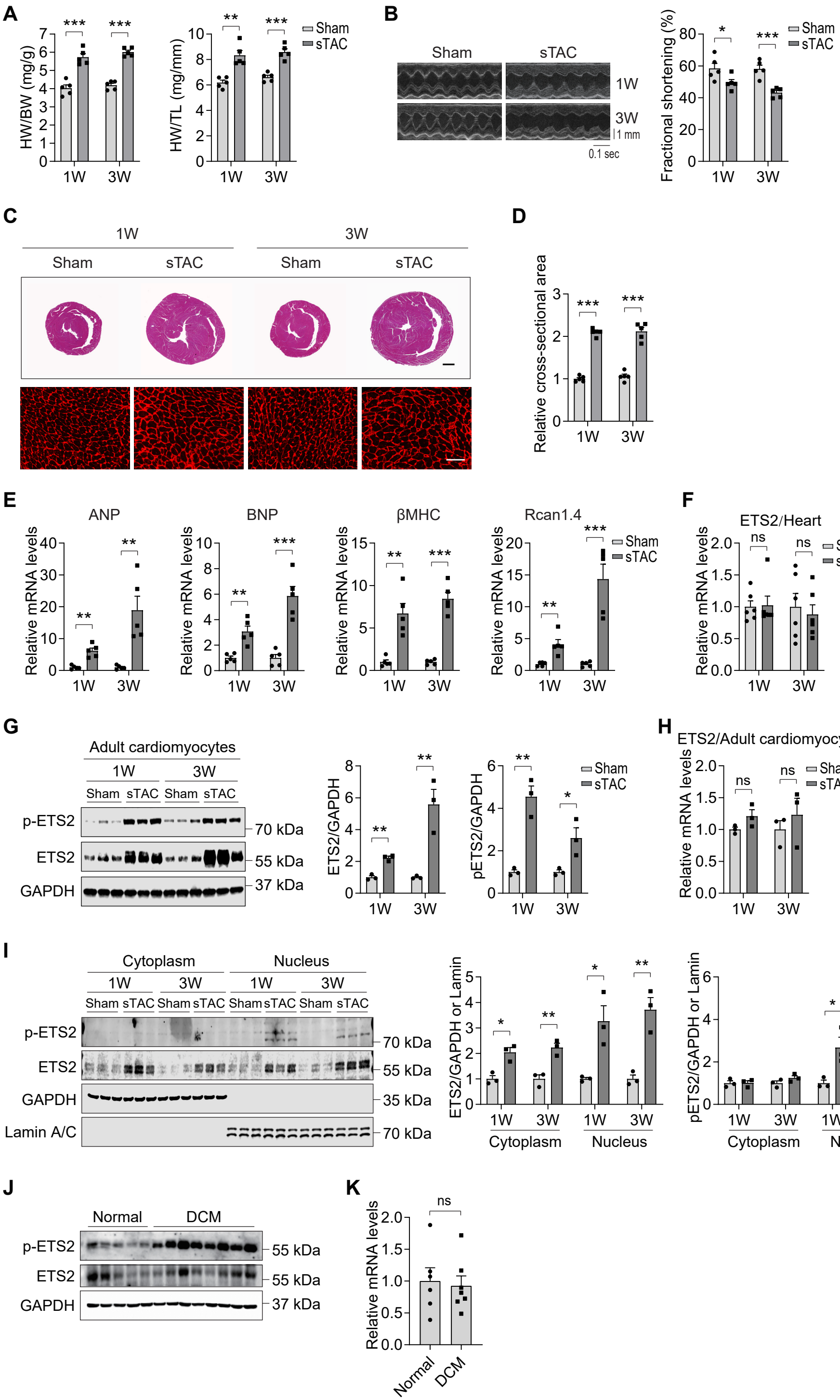
All values are presented as the mean ± SEM, *p<0.05, **p<0.01, ***p<0.001 vs. Sham; †p<0.05, ††p<0.01, †††p<0.001 vs. Cre-sTAC; ‡p<0.05, ‡‡p<0.01 vs. F/F-sTAC. HR: heart rate; EF: ejection fraction; FS: fractional shortening; LVID,d: left ventricular internal diastolic diameter; LVID,s: left ventricular internal systolic diameter; LVPW,d: left ventricular end diastolic posterior wall dimension; LVPW,s: left ventricular end systolic posterior wall dimension; IVS,d: end-diastolic interventricular septal wall thickness; LV mass: left ventricular mass.

Supplemental Table IV. Echocardiography analyses of cardiac function in ETS2-floxed (F/F) and ETS2 knockout (KO) mice intercrossed with cardiac specific calcineurin transgenic mice (CnA).

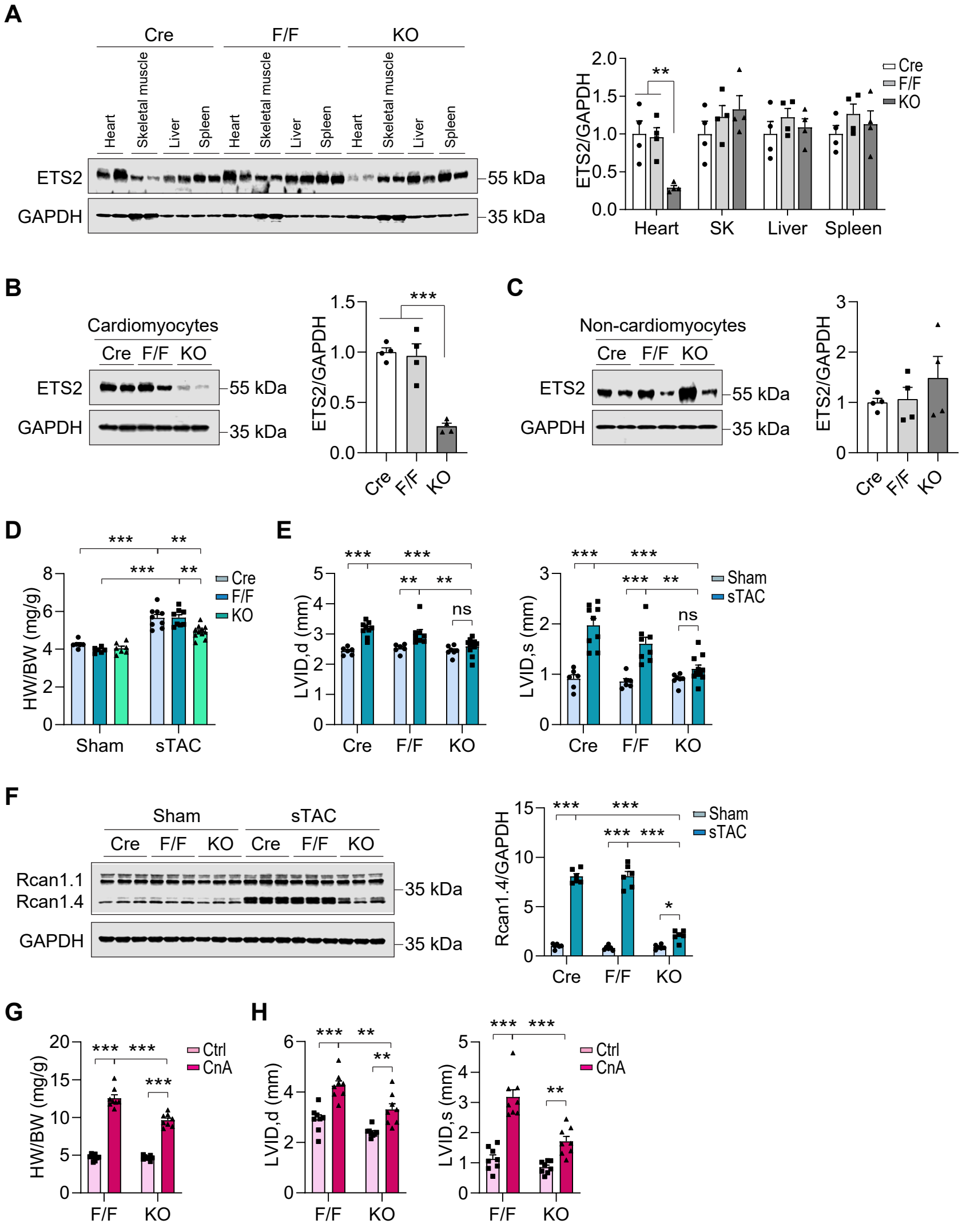
	Ctrl		CnA	
	F/F	**KO**	**F/F**	**KO**
N	8	8	8	8
HR (bpm)	636±23	629±23	513±25**	524±26**
EF (%)	91.14±1.73	92.73±1.40	50.12±5.19***	80.84±2.43*, †††
FS (%)	62.28±2.74	64.43±2.75	25.71±3.15***	48.60±2.70***, ††
LVID,d (mm)	2.92±0.17	2.40±0.07	4.26±0.19***	3.31±0.22**, ††
LVID,s (mm)	1.13±0.14	0.85±0.07	3.18±0.24***	1.71±0.16**, †††
LVPW,d (mm)	0.94±0.07	0.91±0.06	1.36±0.08**	1.05±0.05†
LVPW,s (mm)	1.38±0.03	1.35±0.06	2.09±0.08***	1.66±0.06**, †††
IVS,d (mm)	0.98±0.05	0.95±0.03	1.28±0.06**	1.19±0.04**
LV mass (mg)	90.43±4.78	85.32±6.14	216.73±14.37***	168.28±14.55*, †††

All values are presented as the mean ± SEM, *p<0.05, **p<0.01, ***p<0.001 vs. Ctrl; †p<0.05, ††p<0.01, †††p<0.001 vs. F/F-CnA. HR: heart rate; EF: ejection fraction; FS: fractional shortening; LVID,d: left ventricular internal diastolic diameter; LVID,s: left ventricular internal systolic diameter; LVPW,d: left ventricular end diastolic posterior wall dimension; LVPW,s: left ventricular end systolic posterior wall dimension; IVS,d: end-diastolic interventricular septal wall thickness; LV mass, left ventricular mass.

Supplemental Figure I

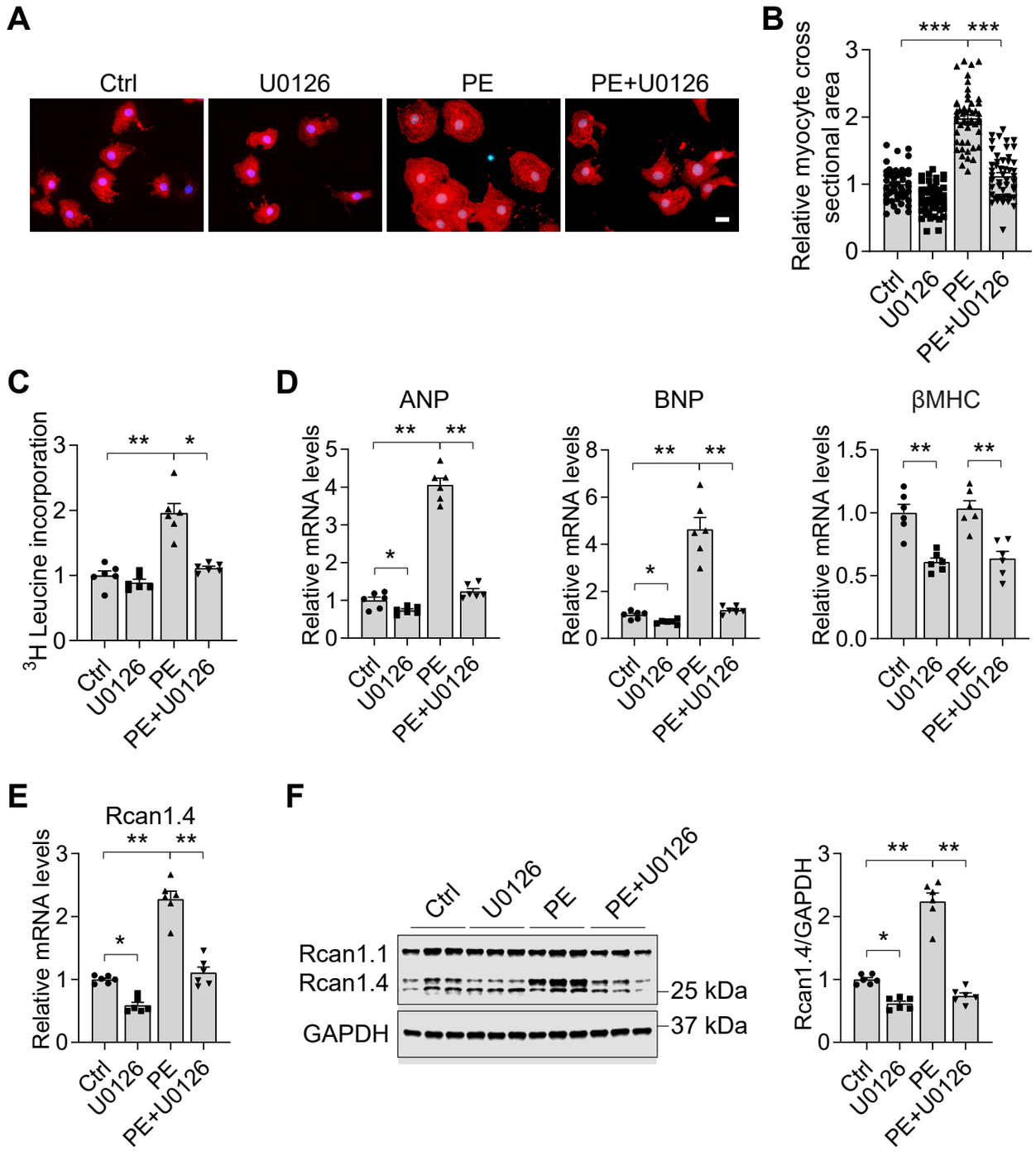


Supplemental Figure II



Supplemental Figure III

Supplemental Figure IV



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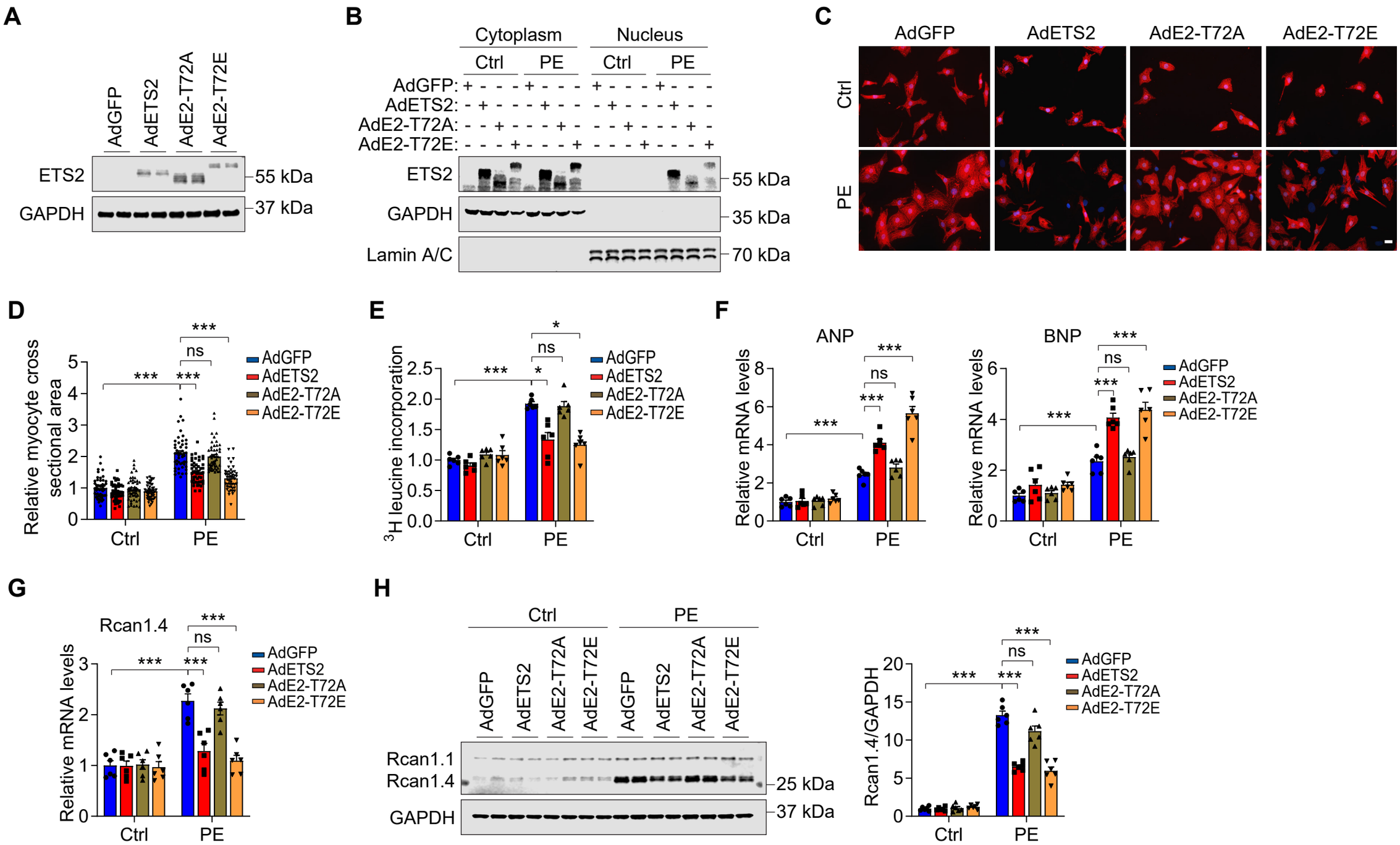
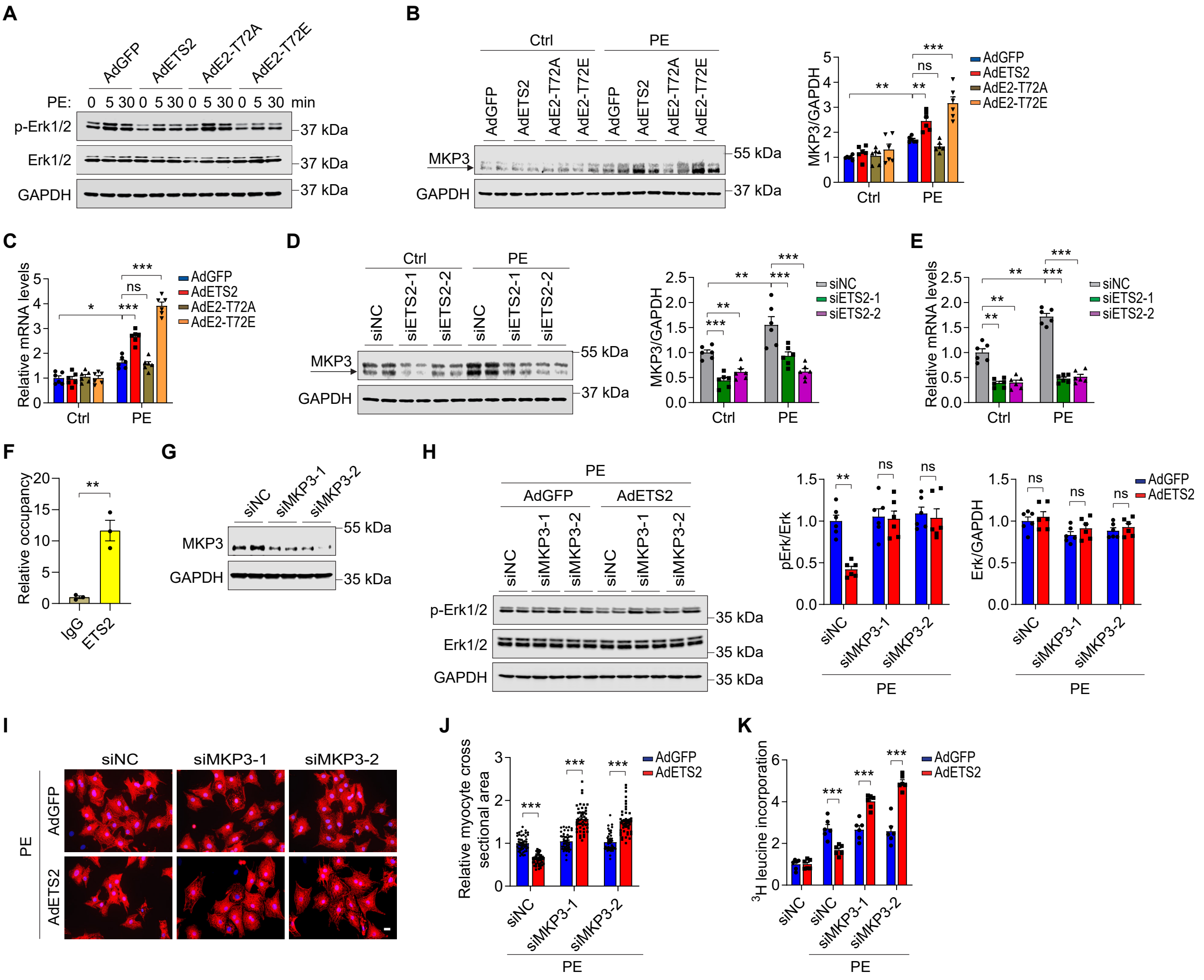
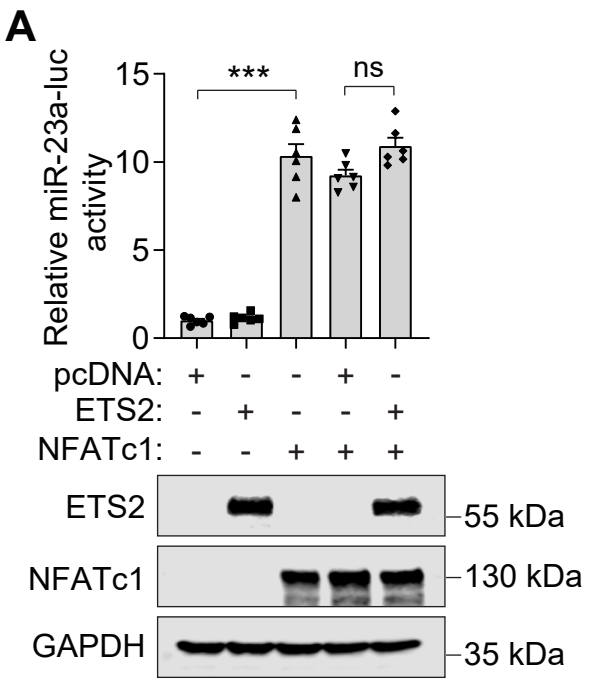
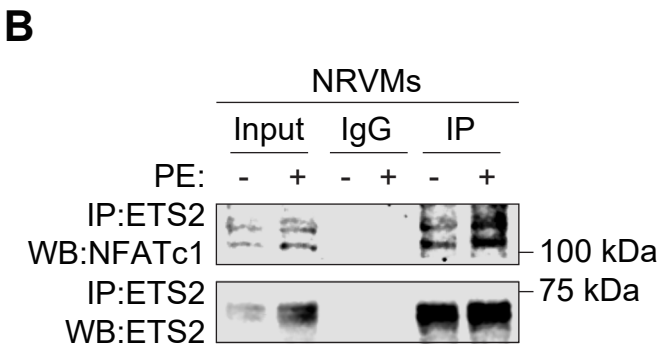


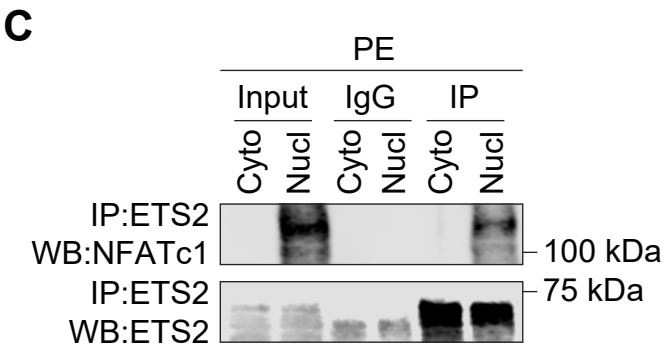
Supplemental Figure VI

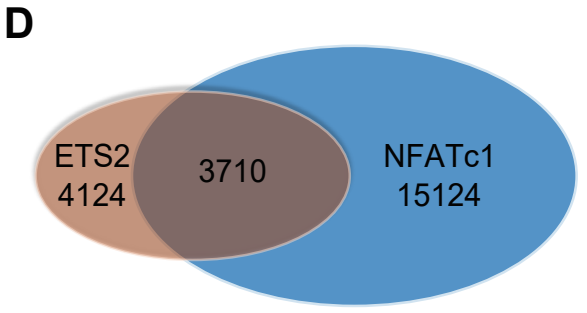


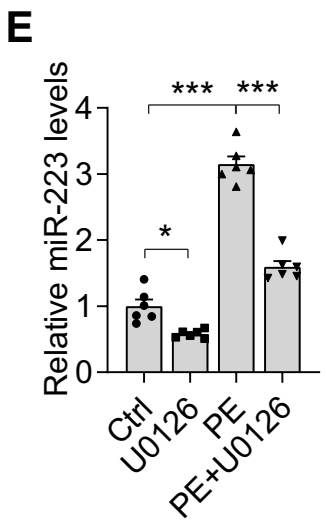
Supplemental Figure VII

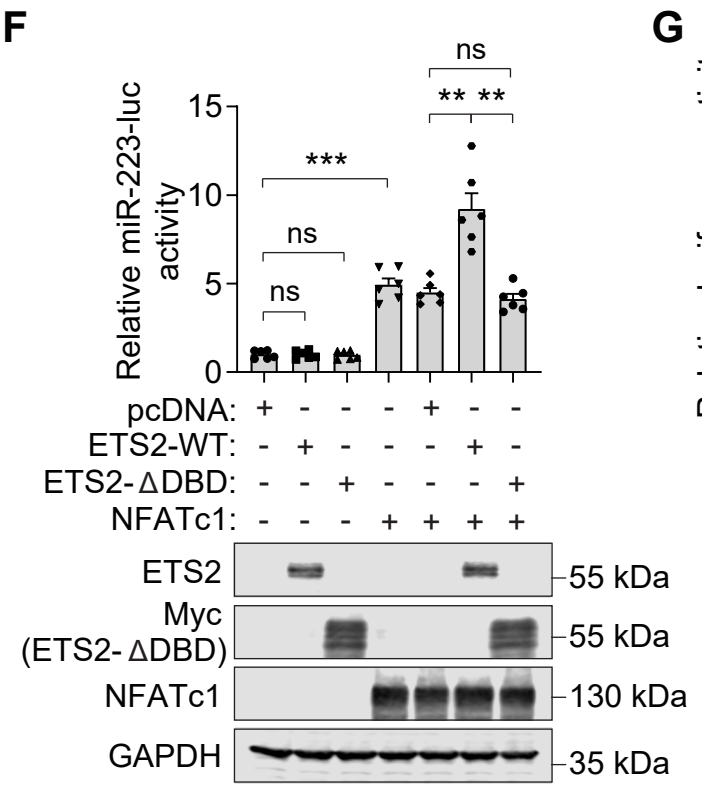


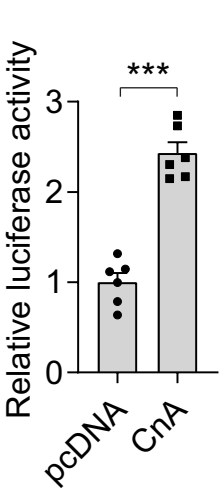


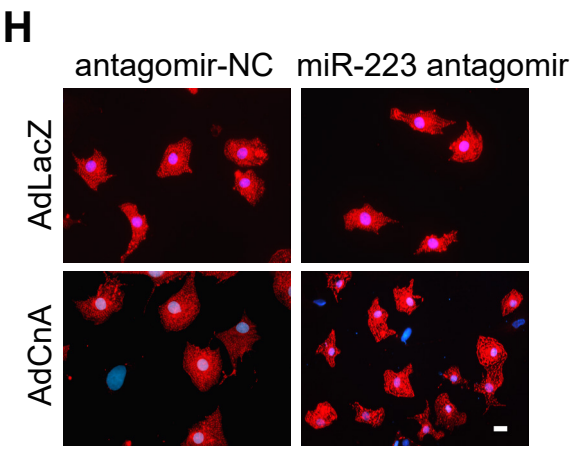


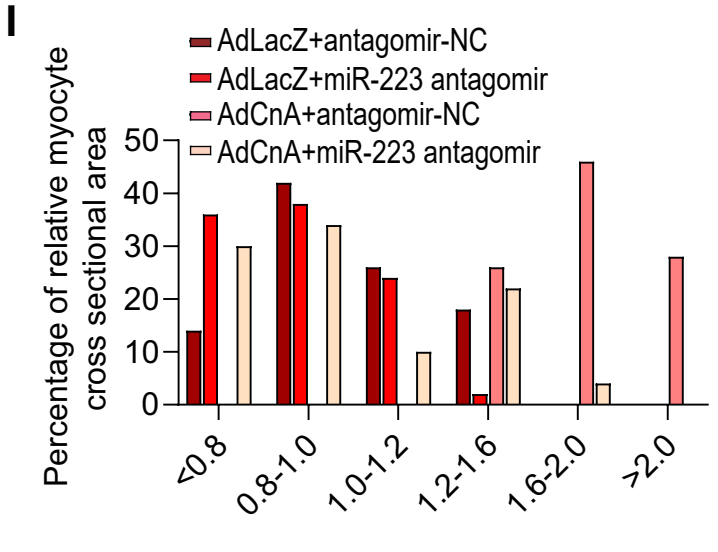


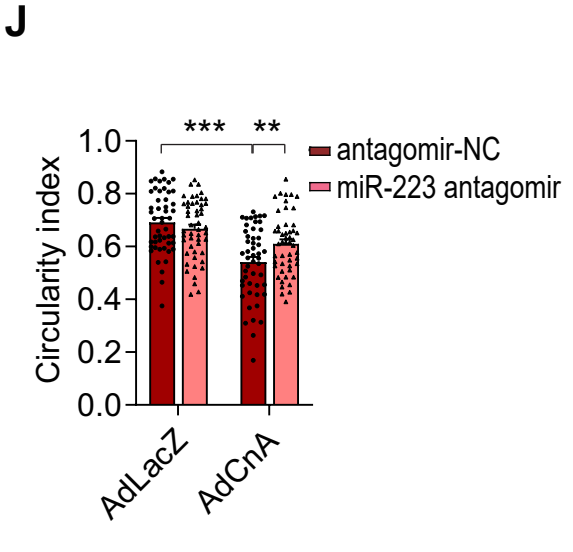


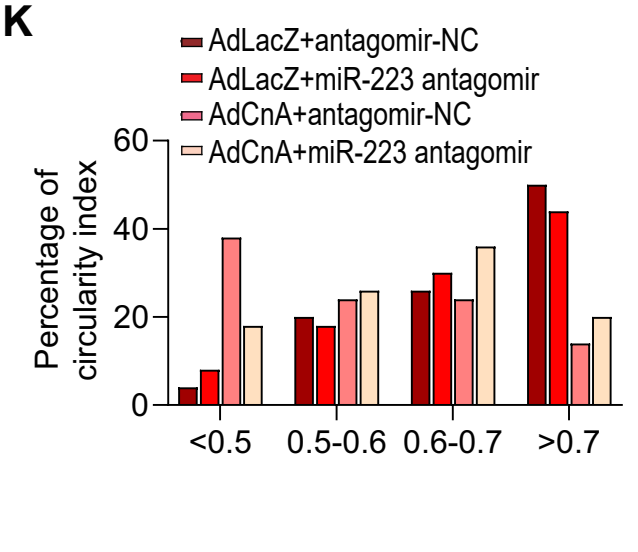












SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure I. Cardiac hypertrophy is induced by severe thoracic aortic constriction (sTAC).

(A) Ratios of heart weight/body weight (HW/BW) and heart weight/tibia length (HW/TL) in wild type mice after 1 or 3 weeks of sham operation or severe thoracic aortic constriction (sTAC) (n = 5).

(B) Contractile function is decreased after sTAC. Left, M-mode echocardiographic images. Right, fractional shortening (n = 5).

(C) Histological analyses of heart sections from mice subjected to sham or sTAC for 1 or 3 weeks. Heart sections were stained with hematoxylin-eosin (top row; scale bar, 1 mm) or wheat germ agglutinin (bottom row; scale bar, 20 mm).

(D) Quantification of the relative cross-sectional area of the indicated groups (n = 5).

(E) mRNA levels of hypertrophic marker genes in indicated groups (n = 5).

(F) ETS2 mRNA levels in mouse hearts at 1 or 3 weeks after sham operation or sTAC (n = 6).

(G) ETS2 phosphorylation and protein levels in adult cardiomyocytes isolated from sham-operated controls and sTAC-subjected mice at 1 or 3 weeks (n = 3).

(H) ETS2 mRNA levels in adult cardiomyocytes isolated from sham-operated controls and sTAC-subjected mice at 1 or 3 weeks (n = 3).

(I) Western blot analyses and quantification of ETS2 phosphorylation and protein levels in cytoplasmic and nuclear fractions of adult cardiomyocytes isolated from sham-operated controls and sTAC-subjected mice at 1 or 3 weeks (n = 3).

(J) ETS2 phosphorylation and protein levels in human heart samples from normal control donors and patients with dilated cardiomyopathy (DCM).

(K) ETS2 mRNA levels in human heart samples from normal control donors and patients with DCM (n = 6 for normal controls, n = 7 for DCM). ns, not significant; *p<0.05; **p<0.01; ***p<0.001.

Supplemental Figure II. Characteristics of ETS2 knockout mice.

(A) Western blot analyses and quantification of ETS2 protein levels in heart, skeletal muscle (SK), liver, and spleen of αMHC-Cre, ETS2-floxed (F/F) and ETS2 knockout (KO) mice (n = 4).

(B and C) ETS2 protein levels in adult cardiomyocytes (B) and non-cardiomyocytes (C) isolated from Cre, F/F, and KO mice (n = 4).

(D) Ratio of heart weight/body weight (HW/BW) in αMHC-Cre, $ETS2^{F/F}$ and KO mice after 3 weeks of sham or sTAC (n = 6-11).

(E) Left ventricular inner diameter (LVID) at both diastole (left) and systole (right) in αMHC-Cre, $ETS2^{F/F}$ and KO mice after 3 weeks of sham or sTAC (n = 6-11).

(F) Western blot analyses and quantification of Rcan1.4 protein levels in indicated groups (n = 6).

(G) HW/BW ratio of $ETS2^{F/F}$ and KO mice crossed with calcineurin transgenic mice (CnA) (n = 8).

(H) LVID in both diastole (left) and systole (right) in $ETS2^{F/F}$ and KO mice crossed with calcineurin transgenic mice (n = 8). ns, not significant; *p<0.05; **p<0.01; ***p<0.001.

Supplemental Figure III. ETS2 is required for calcineurin-induced hypertrophy *in vitro.*
(A) ETS2 mRNA levels in isolated neonatal rat ventricular myocytes (NRVMs) exposed to phenylephrine (PE) for 6 or 24 hours (n = 6).
(B) ETS2 phosphorylation in NRVMs treated with JNK inhibitor SP600125 or p38 inhibitor SB203580 for 24 hours (n = 6).
(C) ETS2 mRNA levels in NRVMs treated with PE and/or MEK inhibitor U0126 for 24 hours (n = 6).
(D) Western blot analyses and quantification of ETS2 phosphorylation and protein levels in NRVMs treated with PE and/or MEK inhibitor (PD0325901) for 24 hours (n = 6).
(E) ETS2 mRNA levels in NRVMs treated as in (D) (n = 6).
(F and G) Western blot analyses (F) and quantification (G) of ETS2 phosphorylation and expression in NRVMs infected with an adenovirus expressing constitutively activated calcineurin (AdCnA) or a control adenovirus (AdLacZ) (n = 6).
(H) ETS2 mRNA levels in calcineurin-over-expressing NRVMs (n = 6).
(I) Representative immunofluorescence images of α-actinin staining in NRVMs transfected with siRNA control (siNC) or siRNA targeting ETS2 (siETS2) and then infected with AdCnA for 48 hours. Scale bar, 20 μm.
(J) Quantification of the relative cardiomyocyte cross-sectional area (n = 50).
(K) Protein synthesis in NRVMs treated as in (I) (n = 6).
(L) mRNA levels of hypertrophic marker genes in NRVMs treated as in (I) (n = 6).
(M) Western blot analyses and quantification of Rcan1.4 protein levels in NRVMs treated as in (I) (n = 6).
(N) Western blot analyses and quantification of Rcan1.4 protein levels in PE-treated NRVMs (n = 6). ns, not significant; *p<0.05; **p<0.01; ***p<0.001.

Supplemental Figure IV. Inhibition of Erk1/2 represses PE-induced hypertrophy.
(A) Representative immunofluorescence images of α-actinin staining in NRVMs treated with PE and/or U0126 for 48 hours. Scale bar, 20 μm.
(B) Quantification of the relative cardiomyocyte cross-sectional area (n = 50).
(C) Protein synthesis in NRVMs treated with PE and/or U0126 (n = 6).
(D) mRNA levels of ANP, BNP and βMHC in NRVMs treated with PE and/or U0126 (n = 6).
(E and F) Rcan1.4 mRNA (E) and protein levels (F) in indicated NRVMs (n = 6). *p<0.05; **p<0.01; ***p<0.001.

Supplemental Figure V. ETS2 over-expression inhibits PE-induced hypertrophy in NRVMs.
(A) ETS2 expression in NRVMs infected with adenovirus expressing wild-type ETS2 (AdETS2) or mutated ETS2. AdE2-T72A: adenovirus expressing phospho-null mutant of ETS2 (mutated

threonine 72 to alanine); AdE2-T72E: adenovirus expressing phosphomimetic ETS2 (mutated threonine 72 to glutamic acid).
(B) Localization of wild-type ETS2 and mutated ETS2 in control NRVMs and PE-treated NRVMs.
(C) Representative immunofluorescence images of α-actinin staining in NRVMs infected with indicated adenovirus, and then treated with PE for 48 hours. Scale bar, 20 μm.
(D) Quantification of relative cardiomyocyte cross-sectional area (n = 50).
(E) Protein synthesis in NRVMs treated as in (C) (n = 6).
(F and G) mRNA levels of ANP, BNP (F) and Rcan1.4 (G) in NRVMs infected with indicated adenovirus and then treated with PE for 24 hours (n = 6).
(H) Western blot analyses and quantification of Rcan1.4 protein levels in NRVMs treated as in (G) (n = 6). ns, not significant; *p<0.05; ***p<0.001.

Supplemental Figure VI. ETS2 promotes PE-induced hypertrophy in MKP3-silenced NRVMs.
(A) ETS2 over-expression inhibits Erk1/2 signaling in response to PE. NRVMs were infected with indicated adenovirus for 48 hours and then treated with PE for 5 or 30 minutes.
(B and C) ETS2 over-expression up-regulates MKP3 protein (B) and mRNA (C) levels in PE-treated NRVMs (n = 6).
(D and E) MKP3 protein (D) and mRNA (E) levels in NRVMs transfected with siNC or siETS2, and then treated with PE for 24 hours (n = 6).
(F) ChIP analysis using an ETS2-specific antibody to detect ETS2 binding to MKP3 promoter in NRVMs (n = 3).
(G) MKP3 was silenced in NRVMs by siRNA transfection.
(H) MKP3 knockdown blocks the inhibitory effect of ETS2 on Erk1/2 signaling. NRVMs were transfected with siNC or siMKP3 and then infected with adenovirus expressing ETS2. Cells were treated with PE for 5 minutes before harvest (n = 6).
(I) Representative immunofluorescence images of α-actinin staining in MKP3-silenced NRVMs. MKP3 was silenced in ETS2-overexpressed NRVMs, and α-actinin staining was performed in cardiomyocytes after 48 hours of PE treatment. Scale bar, 20 μm.
(J) Quantification of the relative cardiomyocyte surface area (n = 50).
(K) Protein synthesis in NRVMs treated as in (I) (n = 6). ns, not significant; *p<0.05; **p<0.01; ***p<0.001.

Supplemental Figure VII. miR-223 is required for calcineurin-induced hypertrophy.
(A) Luciferase activity in HEK-293 cells transfected with a miR-23a luciferase reporter plasmid, along with expression plasmids for ETS2, and NFATc1 (n = 6).
(B) ETS2 interacts with NFATc1 in NRVMs. Endogenous IP was performed in control NRVMs and NRVMs treated with PE for 6 hours using an ETS2 specific antibody.
(C) ETS2 interacts with NFATc1 in the nucleus in PE-treated NRVMs. Cytoplasmic and nuclear fractions were isolated from PE-treated NRVMs, then an ETS2-specific antibody was used for IP analysis. Cyto: cytoplasm; nucl: nucleus.
(D) Venn diagram of genes carry ETS2 and/or NFATc1 binding sites.

(E) miR-223 levels in NRVMs treated with PE and/or MEK inhibitor U0126 (n = 6).
(F) Luciferase activity in HEK-293 cells transfected with a luciferase reporter plasmid containing a short miR-223 promoter without ETS2 binding sites, along with expression plasmids of ETS2, ETS2 lacking DNA binding domain (ETS-△DBD) and NFATc1 (n = 6).
(G) miR-223 luciferase activity in constitutively activated calcineurin (CnA) over-expressing HEK-293 cells. (n = 6).
(H) Representative immunofluorescence images of α-actinin staining in NRVMs transfected with control antagomir (antagomir-NC) or miR-223 antagomir, and then infected with AdCnA for 48 hours. Scale bar, 20 μm.
(I) Histograms of myocyte cross-sectional area.
(J) Circularity index of NRVMs treated as in (H). (n = 50).
(K) Histograms of circularity index in (J). ns, not significant; *p<0.05; **p<0.01; ***p<0.001.

SUPPLEMENTAL SPREADSHEETS

Supplemental Spreadsheet I. Genes harboring ETS2 and NFATc1 binding sites.

Supplemental Spreadsheet II. Target genes that harbor ETS2 and NFATc1 binding motifs in close proximity.