Supplemental Figures and Tables

OTUD1 promotes pathological cardiac remodeling and heart failure by targeting STAT3 in cardiomyocytes

Short title: OTUD1 promotes cardiac remodeling

Supplemental (Online) File: 5 Tables, and 8 Figures

Table S1: Primer sequences for real-time qPCR assay and genotyping.

Gene	Species	Sequence
Actb	Rat	AAGTCCCTCACCCTCCCAAAAG AAGCAATGCTGTCACCTTCCC
Col1a1	Rat	GACATCCCTGAAGTCAGCTGC TCCCTTGGGTCCCTCGAC
Il1	Rat	CACCTCTCAAGCAGAGCACAG GGGTTCCATGGTGAAGTCAAC
Il6	Rat	GAGTTGTGCAATGGCAATTC ACTCCAGAAGACCAGAGCAG
Myh7	Rat	GAGGAGAGGGCGGACATT ACTCTTCATTCAGGCCCTTG
Tgfb1	Rat	GCAACAACGCAATCTATGAC CCTGTATTCCGTCTCCTT
Tnf	Rat	TACTCCCAGGTTCTCTTCAAGG GGAGGCTGACTTTCTCCTGGTA
Actb	Mouse	CCGTGAAAAGATGACCCAGA TACGACCAGAGGCATACAG
Col1a1	Mouse	TGGCCTTGGAGGAAACTTTG CTTGGAAACCTTGTGGACCAG
Il1	Mouse	TCGCAGCAGCACATCAACAAGAG AGGTCCACGGGAAAGACACAGG
Il6	Mouse	GAGGATACCACTCCCAACAGACC AAGTGCATCATCGTTGTTCATACA
Myh7	Mouse	CAAAGGCAAGGCAAAGAAAG TCACCCCTGGAGACTTTGTC
Tgfb1	Mouse	TGACGTCACTGGAGTTGTACGG GGTTCATGTCATGGATGGTGC
Tnf	Mouse	TGATCCGCGACGTGGAA ACCGCCTGGAGTTCTGGAA
Otud1 (KO genotyping)	Mouse	ATGCAGCTCTACAGCAGCGT GTGAGCCTCGGCTTCGGGAT
Otud1	Human	CCCACGGTGTCTACCATGATTCA CTTCGTCGCGTTTCCTTTGCA
Actb	Human	CCTGGCACCCAGCACAAT GCCGATCCACACGGAGTACT

Table S2: siRNA sequences for OTUD1 knockdown in neonatal rat ventricular myocytes

siRNA	Species	Sequence
OTUD1 #1	Rat	5'-CUGACGGUAACUGCCUUUATT-3' 5'-UAAAGGCAGUUACCGUCAGTT-3'
OTUD1 #2	Rat	5'-GGUGCAAGCAAACCCAAAUTT-3' 5'-AUUUGGGUUUGCUUGCACCTT-3'
OTUD1 #3	Rat	5'-GCCUAGUAUUUGGCUUAGUTT-3' 5'-ACUAAGCCAAAUACUAGGCTT-3'
Negative control (NC)	Rat	5'-UUCUCCGAACGUGUCACGUTT-3' 5'-ACGUGACACGUUCGGAGAATT-3'

Table S3: Biometric and echocardiographic parameters in Ang II-challenged mice.

Model 1 (Ang II model)	Sham		Continuous Ang II Pump Infusion	
	WT	OTUD$^{-/-}$	WT	OTUD$^{-/-}$
	n=6	n=6	n=6	n=6
EF, %	84.84±0.04	80.92±0.03	69.66±0.08**	80.10±0.03#
FS, %	47.85±0.05	43.50±0.03	33.87±0.05**	43.27±0.03##
HR, bpm	615.00±48.27	609.33±20.92	592.33±43.98	590.17±52.30
LVIDd, mm	2.63±0.40	2.73±0.51	2.93±0.30	2.51±0.30#
IVSD, mm	0.57±0.05	0.68±0.04	0.73±0.05**	0.67±0.05#
PWD, mm	0.58±0.04	0.68±0.04	0.7±0.06**	0.6±0.08
E wave, m/s	0.95±0.11	0.71±0.17	0.73±0.38	0.68±0.13
Tei Index	0.71±0.01	0.70±0.11	0.92±0.16*	0.76±0.12
IRT, ms	13.5±2.3	15.67±1.63	17.33±0.8**	16.33±0.82#
HW/BW, mg/g	5.63±0.09	5.86±0.55	6.51±0.66**	5,83±0.34#
HW/TL, mg/mm	7.30±0.42	7.33±0.54	7.75±0.19*	7.12±0.30##

Transthoracic echocardiography was performed on mice at the end of the animal study. Ang II = angiotensin II; BW = body weight; EF = ejection fraction; E wave = peak mitral E velocity; FS = fractional shortening; HR = heart rate; HW = heart weight; IVRT = isovolumic relaxation time; IVSD = diastole interventricular septal thickness; LVIDd = diastole left ventricle internal dimension; PWD = diastole posterior wall thickness; Tei index = a myocardial performance index. Data presented as Mean ± SEM; * = $p < 0.05$ and ** = $p < 0.01$ compared to WT-Sham; # = $p < .05$ and ## = $p < 0.01$ compared to WT + Ang II.

Table S4: Biometric and echocardiographic parameters in TAC-challenged mice.

Model 2 (TAC model)	Sham		TAC	
	WT	OTUD1$^{-/-}$	WT	OTUD1$^{-/-}$
	n=6	n=6	n=6	n=6
EF, %	81.41±0.03	80.69±0.02	73.27±0.05*	82.54±0.02##
FS, %	43.90±0.04	42.32±0.04	36.92±0.05*	44.82±0.02##
HR, bpm	538.50±32.67	523.67±27.87	532.67±37.13	530.50±28.46
LVIDd, mm	2.5±0.54	2.56±0.18	2.93±0.27	2.53±0.26#
IVSD, mm	0.63±0.05	0.70±0.09	0.90±0.09**	0.78±0.04#
PWD, mm	0.63±0.05	0.70±0.09	0.90±0.09**	0.72±0.04##
E wave, m/s	0.62±0.09	0.66±0.07	0.66±0.17	0.62±0.06
Tei Index	1.48±0.15	1.43±0.09	1.87±0.18**	1.44±0.11##
IRT, ms	17.00±1.26	16.67±1.51	20±2.10*	15.83±3.37#
HW/BW, mg/g	4.66±0.29	4.92±0.19	6.11±0.73**	4.90±0.30##
HW/TL, mg/mm	5.13±0.21	5.42±0.23	6.88±0.78**	5.89±0.32#

Transthoracic echocardiography was performed on mice at the end of the animal study. BW = body weight; EF = ejection fraction; E wave = peak mitral E velocity; FS = fractional shortening; HR = heart rate; HW = heart weight; IVRT = isovolumic relaxation time; IVSD = diastole interventricular septal thickness; LVIDd = diastole left ventricle internal dimension; PWD = diastole posterior wall thickness; Tei index = a myocardial performance index. Data presented as Mean ± SEM; * = $p < 0.05$ and ** = $p < 0.01$ compared to WT-Sham; # = $p < .05$ and ## = $p < 0.01$ compared to WT + TAC.

Table S5: Biometric and echocardiographic parameters in Ang II-challenged mice treated with OTUD1-AAV9.

Model 3 (Ang II model)	WT+AAV-NC	WT+AAV-OTUD1	Continuous Ang II Pump Infusion		
			WT + AAV-NC	WT + AAV9-OTUD1	WT+AAV9-OTUD1 +Static (10mg/kg)
	n=6	n=6	n=6	n=6	n=6
EF, %	84.5±0.02	87.2±0.02	73.7±0.07	64.9±0.06*	83.9±0.04##
FS, %	47.3±0.02	50.3±0.02	36.9±0.06	30.0±0.04*	47.7±0.05##
HR, bpm	534±25.91	534.2±27	548.8±36.22	527±37.45	547.5±38.5
LVIDd, mm	1.45±0.10	1.28±0.14	2.65±0.10**	2.76±0.10	2.00±0.35##
IVSD, mm	0.68±0.04	0.82±0.17	1.1±0.09	1.15±0.08ns	0.75±0.05##
PWD, mm	0.68±0.04	0.72±0.08	0.97±0.10	1.11±0.10*	0.72±0.04##
E wave, m/s	0.72±0.16	0.76±0.07	0.61±0.02	0.55±0.06*	0.73±0.06##
Tei Index	0.70±0.02	1.01±0.27	1.21±0.10	1.53±0.34*	1.14±0.19#
IRT, ms	16.33±1.03	18±0.63	22.83±3.60	26.33±1.03*	19.5±2.17##
HW/BW, mg/g	4.29±0.27	4.32±0.31	5.02±0.11	5.46±0.22**	4.59±0.29##
HW/TL, mg/mm	5.62±0.42	5.56±0.38	6.46±0.13	6.80±0.05**	5.78±0.41##

Transthoracic echocardiography was performed on mice at the end of the animal study. Ang II = angiotensin II; BW = body weight; EF = ejection fraction; E wave = peak mitral E velocity; FS = fractional shortening; HR = heart rate; HW = heart weight; IVRT = isovolumic relaxation time; IVSD = diastole interventricular septal thickness; LVIDd = diastole left ventricle internal dimension; PWD = diastole posterior wall thickness; Tei index = a myocardial performance index. Data presented as Mean ± SEM; * = $p < 0.05$ and ** = $p < 0.01$ compared to WT-Sham; # = $p < .05$ and ## = $p < 0.01$ compared to WT + Ang II.

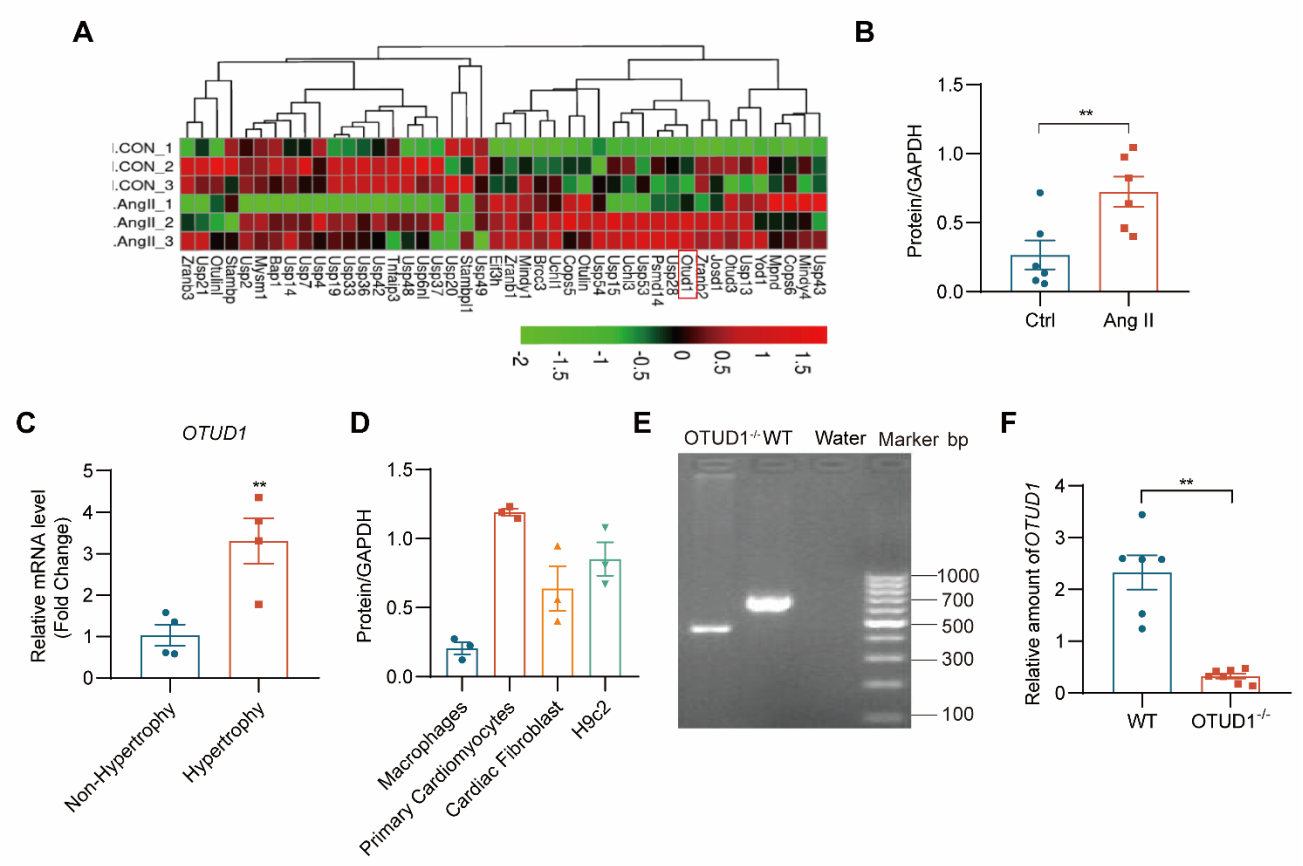

Figure S1: Heatmap showing deubiquitinating enzymes in heart tissues of Ang II-challenged mice.

(A) We performed RNA-sequencing analysis using the wildtype B6 mice (Ctrl) and mice challenged with Ang II for 4 weeks. Figure showing heatmap of the top 44 differentially expressed deubiquitinating enzymes. Cut-off was set as log2 > 1.5 ($p<0.05$). **(B)** Densitometric quantification of blots in Figure 1B. **(C)** Real-time qPCR analysis of the mRNA expression of OTUD1 in hypertrophic myocardial tissues of heart failure patients and normal human heart tissues. n = 4 per group. The mRNA level from each group was normalized to one value from the control/non-hypertrophy group, which was set to 1. Data were normalized to *Actb*. All quantitative data are presented as Mean ± SEM; ** = $p < 0.01$. **(D)** Densitometric quantification of blots in Figure 1D. **(E)** Genotyping of wildtype (WT) and OTUD1 knockout mice ($OTUD1^{-/-}$). Genotyping primers detected 690 bp product in WT mice and <500 bp product in OTUD1 knockout mice. **(F)** mRNA level of *OTUD1* in hearts of wild-type mice and $OTUD1^{-/-}$ mice determined by RT-qPCR. Transcripts were normalized to *Actb* (n=6 per group).

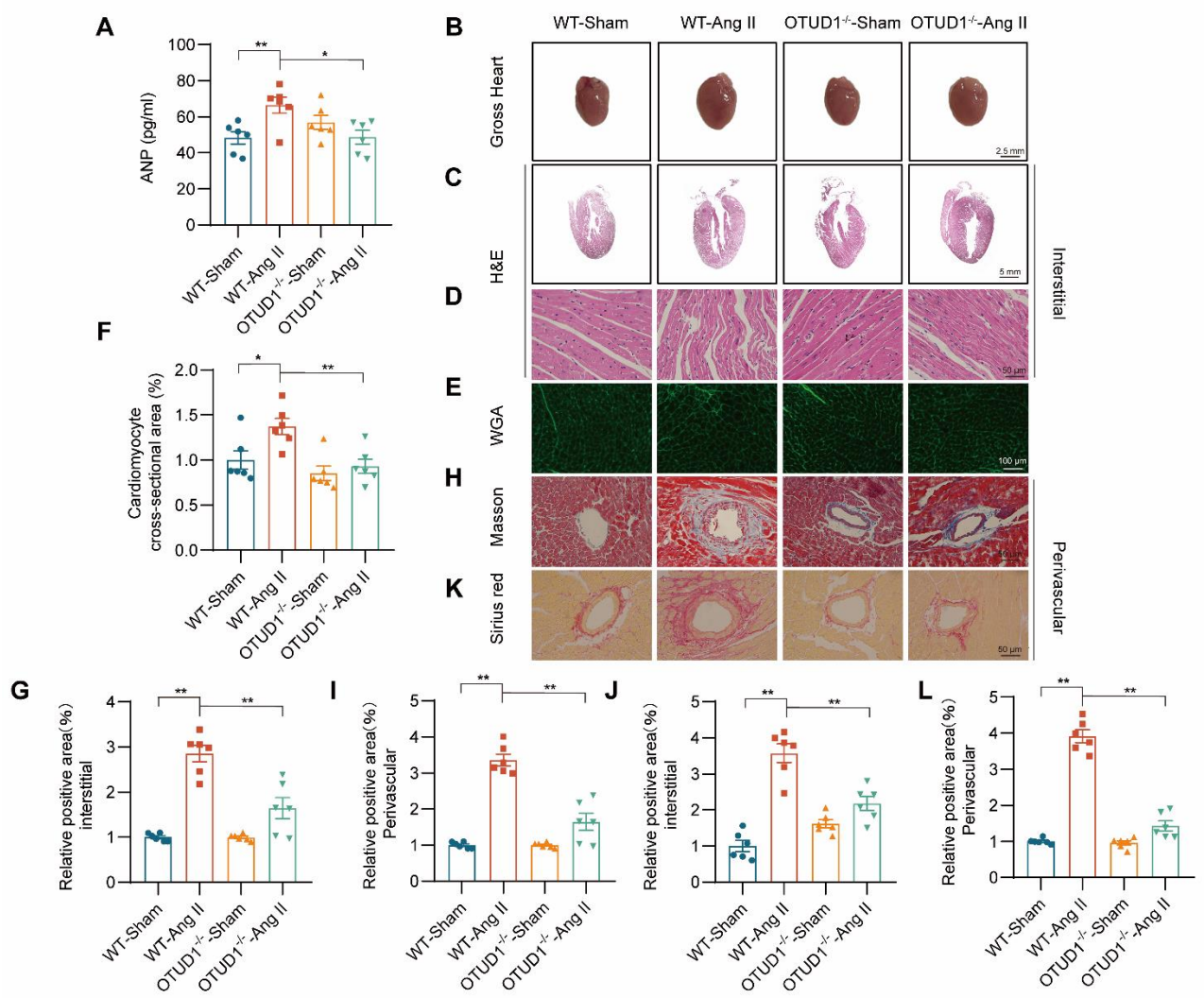

Figure S2: OTUD1 deficiency prevents Ang II-induced myocardial hypertrophy and fibrosis. **(A)** Atrial natriuretic peptide (ANP) levels in serum of wildtype (WT) and OTUD1 knockout mice, infused with saline (Sham) or Ang II for 4 weeks. **(B)** Representative whole heart images [scale bar = 2.5 mm]. **(C**, **D)** Hematoxylin and eosin-stained heart tissues [scale bar = 5 mm (C) and 50 μm (D)]. **(E)** Heart tissues were stained with wheat germ agglutinin (WGA) to assess cell size changes [scale bar = 100 μm]. **(F)** Quantification of cardiomyocyte size following WGA staining. **(G)** Interstitial fibrosis was quantified from Masson's Trichrome staining of heart tissues. Representative stained images are shown in Figure 2J. **(H**, **I)** Perivascular fibrosis was detected by staining heart tissues with Masson's trichrome (H) [scale bar = 50 μm]. Quantification is shown in panel I. **(J)** Quantification of Picro Sirius Red staining of heart tissues in Figure 2K. **(K, L)** Picro Sirius Red staining and quantification of interstitial fibrosis sin heart tissues of mice. Representative staining images are shown in Panel K [scale bar = 50 μm]. Quantification is shown in Panel L. All quantitative figures showing Mean ± SEM; n = 6; * = $p < 0.05$ and ** = $p < 0.01$ compared to WT-Sham.

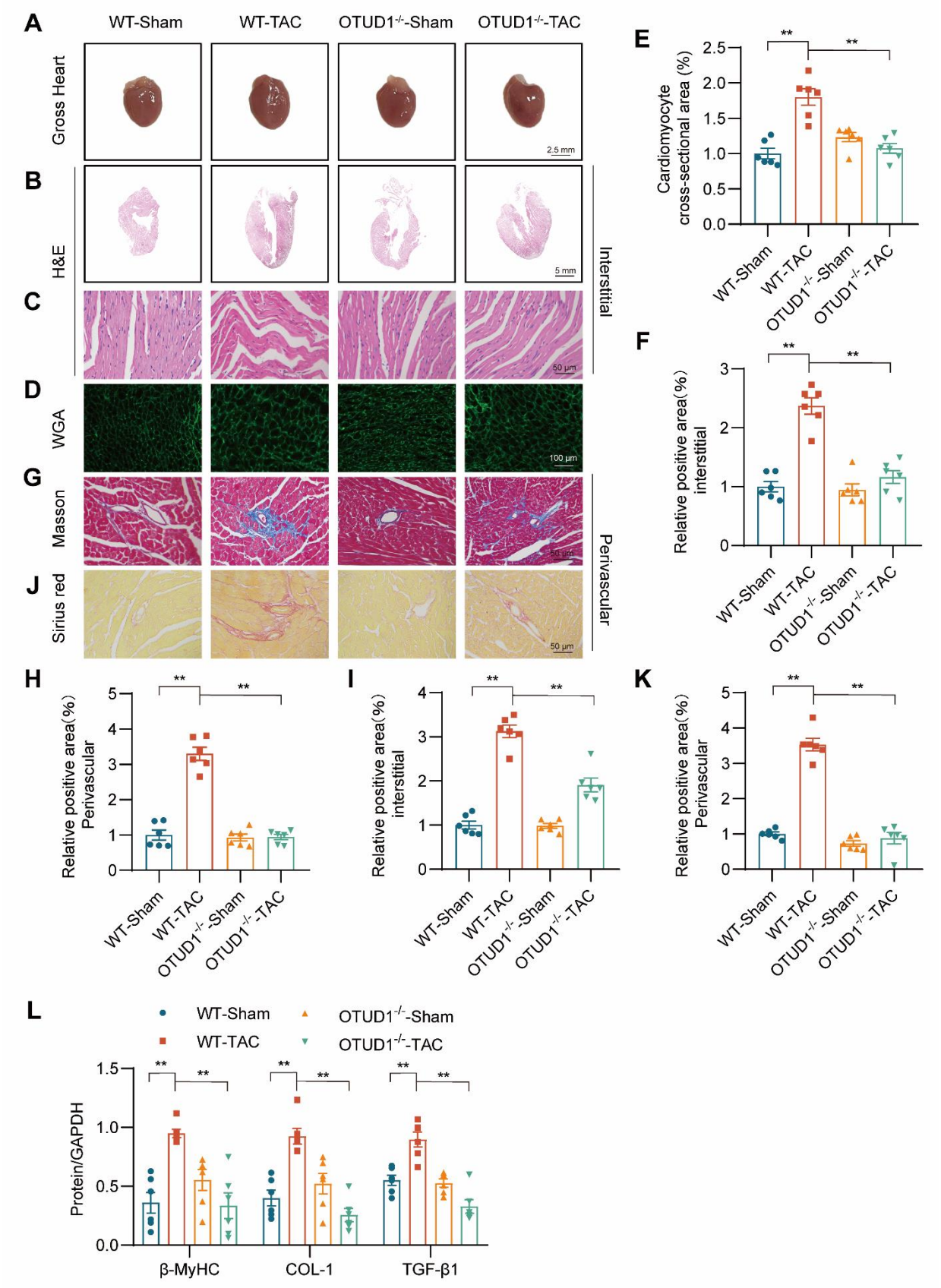
A
WT-Sham
WT-TAC
OTUD1-/--Sham
OTUD1-/--TAC
Gross Heart
2.5 mm
B
H&E
5 mm
Interstitial
C
50 μm
D
WGA
100 μm
G
Masson
50 μm
Perivascular
J
Sirius red
50 μm
E
Cardiomyocyte cross-sectional area (%)
F
Relative positive area(%) interstitial
H
Relative positive area(%) Perivascular
I
Relative positive area(%) interstitial
K
Relative positive area(%) Perivascular
L
WT-Sham
WT-TAC
OTUD1-/--Sham
OTUD1-/--TAC
Protein/GAPDH
β-MyHC
COL-1
TGF-β1

Figure S3: OTUD1 deficiency prevents TAC-induced myocardial hypertrophy and fibrosis. C57BL/6 wildtype (WT) and OTUD1 knockout mice were subjected to TAC. Tissues were harvested after 4 weeks and assessed. **(A)** Representative whole heart images [scale bar = 2.5 mm]. **(B**, **C)** Hematoxylin and eosin (H&E) staining of heart tissues from mice. Whole hearts (B) and high-power stained slices (C) are shown [scale bar = 5 mm (B) and 50 μm (C)]. **(D)** Cardiomyocyte size changes were detected by staining heart tissues with wheat germ agglutinin (WGA) [scale bar = 100 μm]. **(E)** Quantification of cardiomyocyte size, from WGA-stained heart tissues. **(F)** Quantification of interstitial fibrotic area (%) from Masson's trichrome-stained heart sections in Figure 3I. **(G**, **H)** Masson's Trichrome staining of heart tissues for perivascular fibrosis [scale bar = 50 μm]. Quantification of fibrotic area (%) is shown in Panel H. **(I)** Quantification of interstitial fibrotic area (%) from Picro Sirius Red staining in Figure 3J. **(J**, **K)** Interstitial fibrosis was detected in heart tissues by Picro Sirius Red staining (J) [scale bar = 50 μm]. Quantification of staining is shown in Panel K. (scale bar, 50 μm for Sirius Red staining). **(L)** Densitometric quantification of blots in Figure 3K. All quantitative figures showing Mean ± SEM; n = 6; * = $p < 0.05$ and ** = $p < 0.01$ compared to WT-sham.

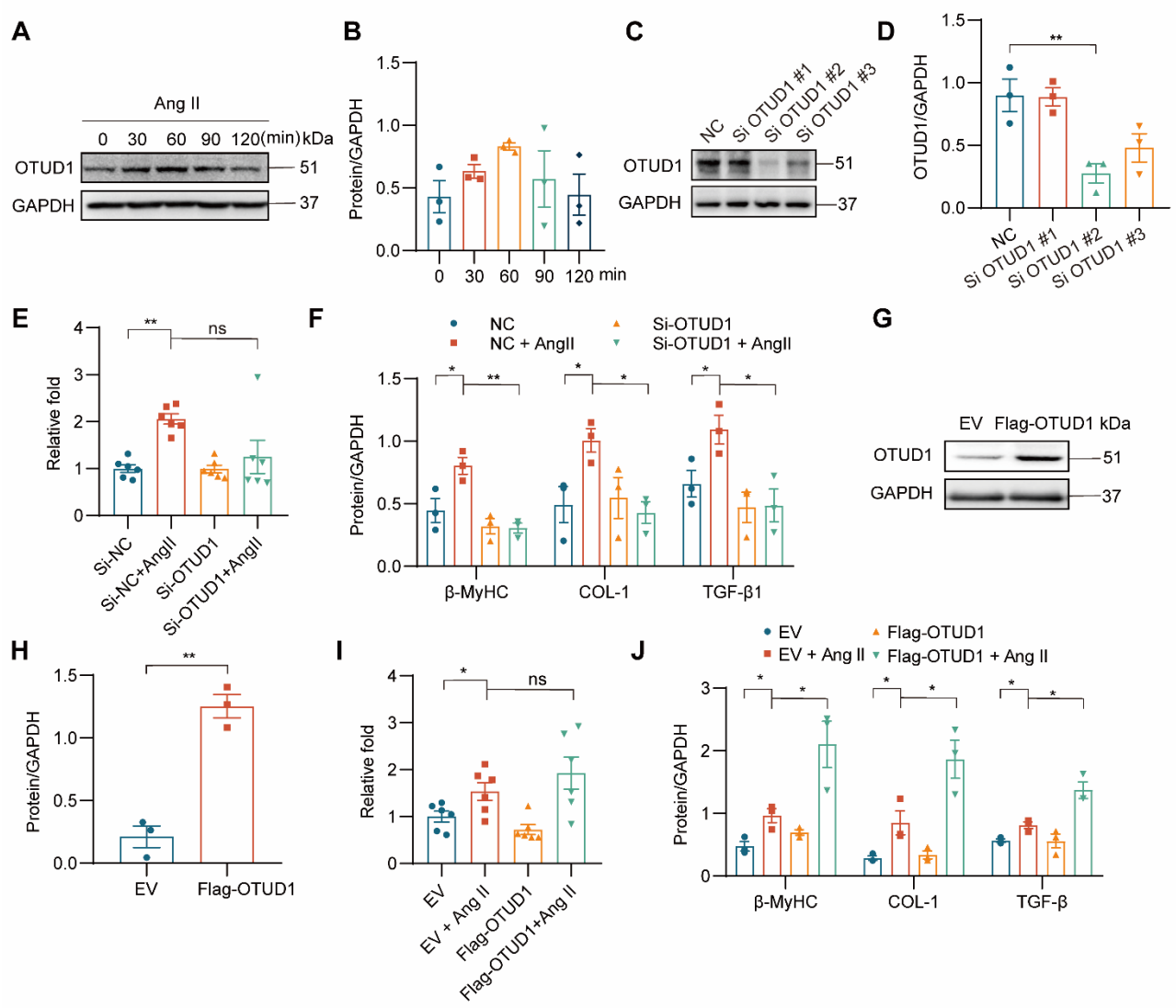

Figure S4: OTUD1 deficiency suppresses Ang II-induced cardiomyocyte hypertrophy and fibrotic responses *in vitro*.

(A) Representative western blots for OTUD1 protein in primary cardiomyocytes (neonatal rat ventricular cardiomyocytes; NRVMs) following Ang II exposure for different times. GAPDH was used as loading control. **(B)** Densitometric quantification of OTUD1 immunoblots in panel A. **(C, D)** OTUD1 gene silencing was performed in NRVMs by siRNA (si-OTUD1). Negative control (NC) included scrambled siRNA transfections. After 48 h, levels of OTUD1 proteins were detected by immunoblotting (C) and densitometric quantification (D). **(E)** Quantification of cell size changes in NRVMs in response to Ang II in Figure 4A. A minimum of 100 cells were measured from different visual fields of 3 samples per group. **(F)** Densitometric quantification of blots in Figure 4B. **(G, H)** OTUD1 was expressed in NVRMs using Flag-tagged OTUD1 vector. Control included empty vector (EV) transfections. After 48 h, levels of OTUD1 proteins were detected by immunoblotting (G) and densitometric quantification (H). **(I)** Quantification of cell size increase in NRVMs in response to Ang II in Figure 4E. A minimum of 100 cells were measured from different visual fields of 3 samples per group. **(J)** Densitometric quantification of blots in Figure 4F. All quantitative figures showing Mean ± SEM; n = 3 independent examinations; * = $p < 0.05$ and ** = $p < 0.01$.

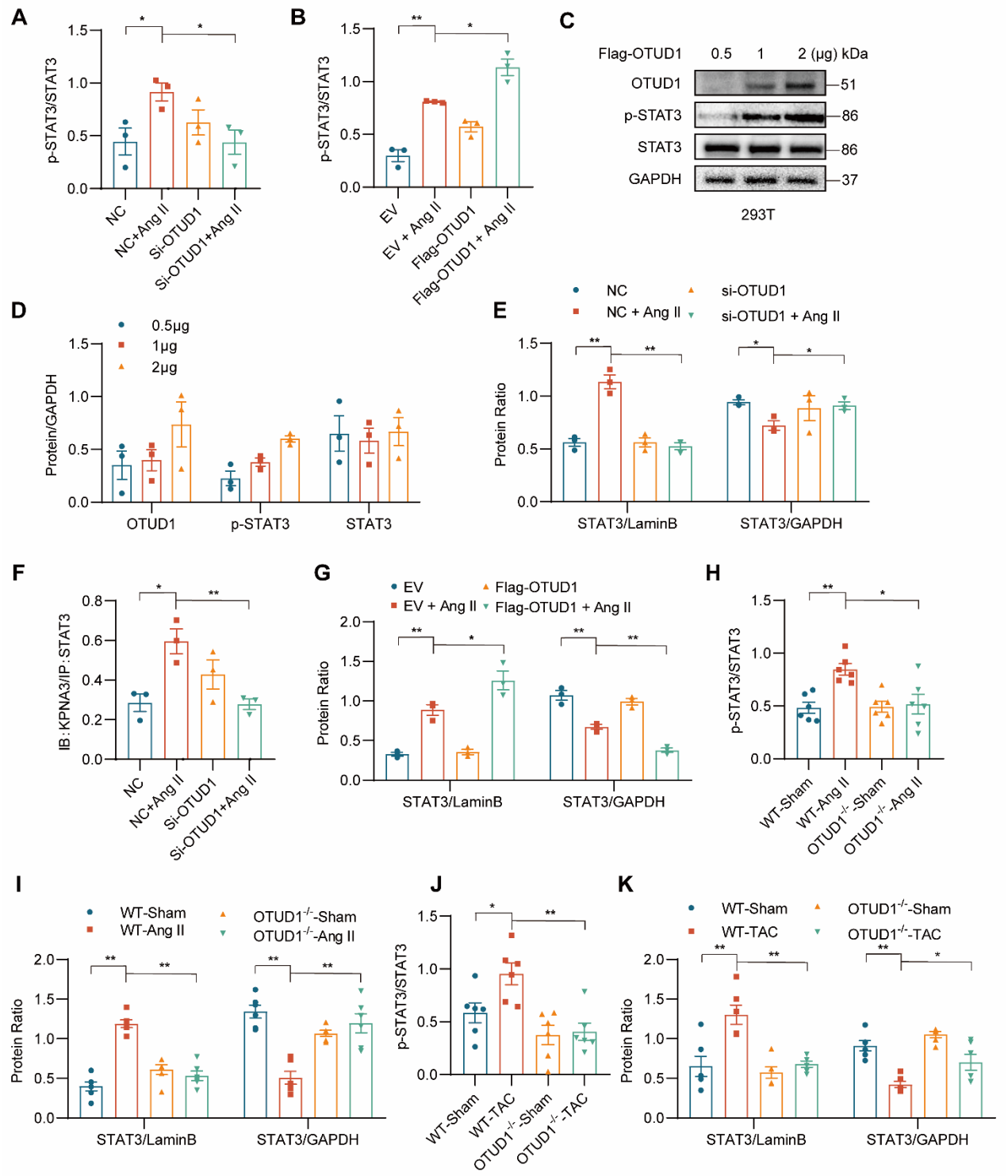

Figure S5: OTUD1 interacts directly with STAT3.

(A) Densitometric quantification of blots in Figure 5E. **(B)** Densitometric quantification of blots in Figure 5F. **(C, D)** HEK-293T were transfected with OTUD1 expressing vector. Levels of OTUD1 and (p-) STAT3 were detected by immunoblotting. Representative blots are shown in Panel C and densitometric quantitation is shown in D. **(E)** Densitometric quantification of blots in Figure 5G. **(F)** Densitometric quantification of blots in Figure 5H. **(G)** Densitometric

quantification of blots in Figure 5I. **(H-I)** Densitometric quantification of blots in Figures 5J and 5K. **(J**, **K)** Densitometric quantification of blots in Figures 5L and 5M. All quantitative figures showing Mean ± SEM; n = 3 independent examinations (Panels A-G) and n = 6 per group (Panels H-K); * = $p < 0.05$ and ** = $p < 0.01$.

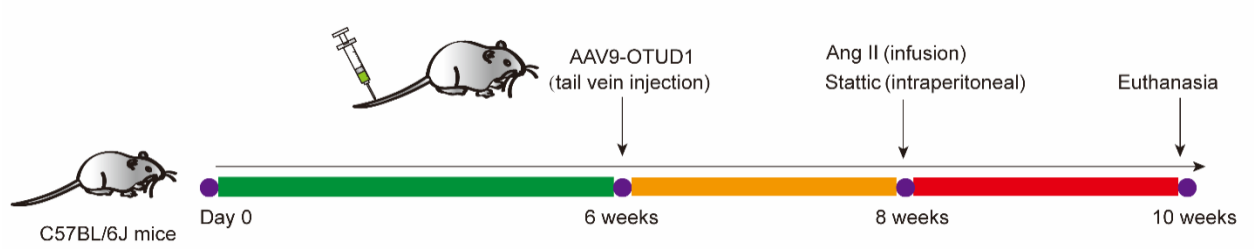

Figure S6: The flow chart of Ang II model with AAV9-OTUD1 injection and Stattic treatment.

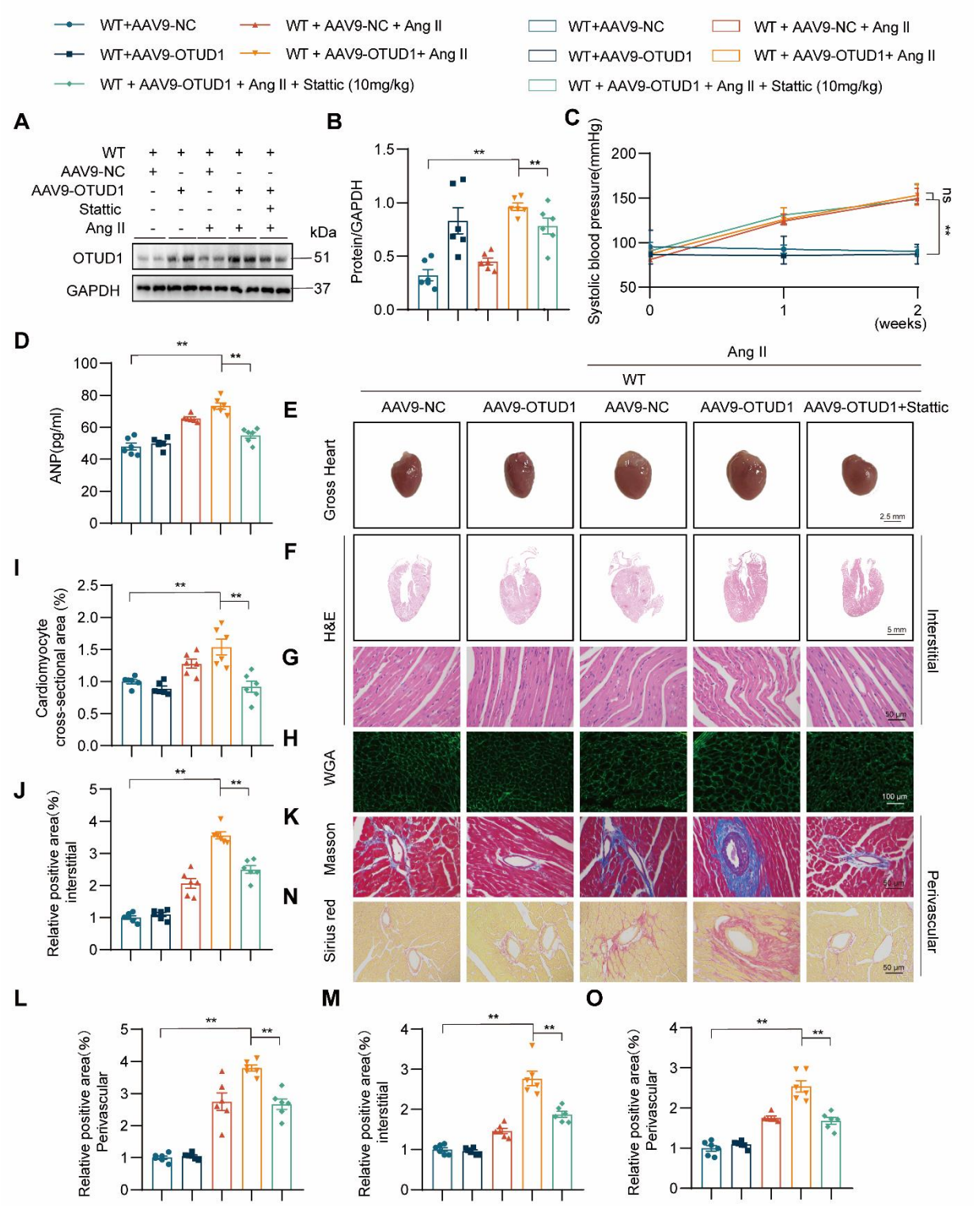

Figure S7: OTUD1 prevents Ang II-induced myocardial hypertrophy and fibrosis by regulating STAT3.

We infected the 6 weeks old wildtype C57BL/6 mice with adeno-associated virus serotype 9 (AAV9) that encodes OTUD1 (AAV9-OTUD1) and negative control (AAV9-NC). Mice were injected with AAV9 via tail vein (3×10^{11} viral particles/mouse) for 2 weeks. **(A**, **B)** OTUD1

protein levels in heart tissues were measured by immunoblotting. Representative blots are shown in Panel A and densitometric quantification is presented in Panel B. **(C)** Systolic blood pressure was determined by non-invasive tail-cuff on a weekly basis. **(D)** ANP protein levels were measured in serum samples obtained from mice after 2 weeks of Ang II infusion. **(E)** Representative whole heart images [scale bar = 2.5 mm]. **(F**, **G)** Hematoxylin and eosin (H&E) staining of heart tissues showing whole hearts (F) and high-power slices of tissues (G) [scale bar = 5 mm (F) and 50 μm (G)]. **(H**, **I)** Cardiomyocyte hypertrophy was measured by staining heart tissues with wheat germ agglutinin (WGA). Representative staining images are shown in Panel H [scale bar = 100 μm]. Cell size measurements are shown in Panel I. **(J)** Quantification of interstitial fibrotic area (%) from Masson's Trichrome staining of heart tissues in Figure 7I. **(K**, **L)** Masson's Trichrome staining was performed to assess fibrosis in heart tissues of mice. Representative staining images are shown in Panel K [scale bar = 50 μm]. Quantification of fibrotic area is shown in Panel L. **(M)** Quantification of interstitial fibrotic area (%) from Picro Sirius Red staining of heart tissues in Figure 7J. **(N**, **O)** Heart tissues were stained with Picro Sirius Red to detect interstitial fibrosis. Representative staining images are shown in Panel N [scale bar = 50 μm]. Quantification of staining is presented in Panel O. All quantitative figures showing Mean ± SEM; n = 6; ns = not significant; * = $p < 0.05$ and ** = $p < 0.01$.

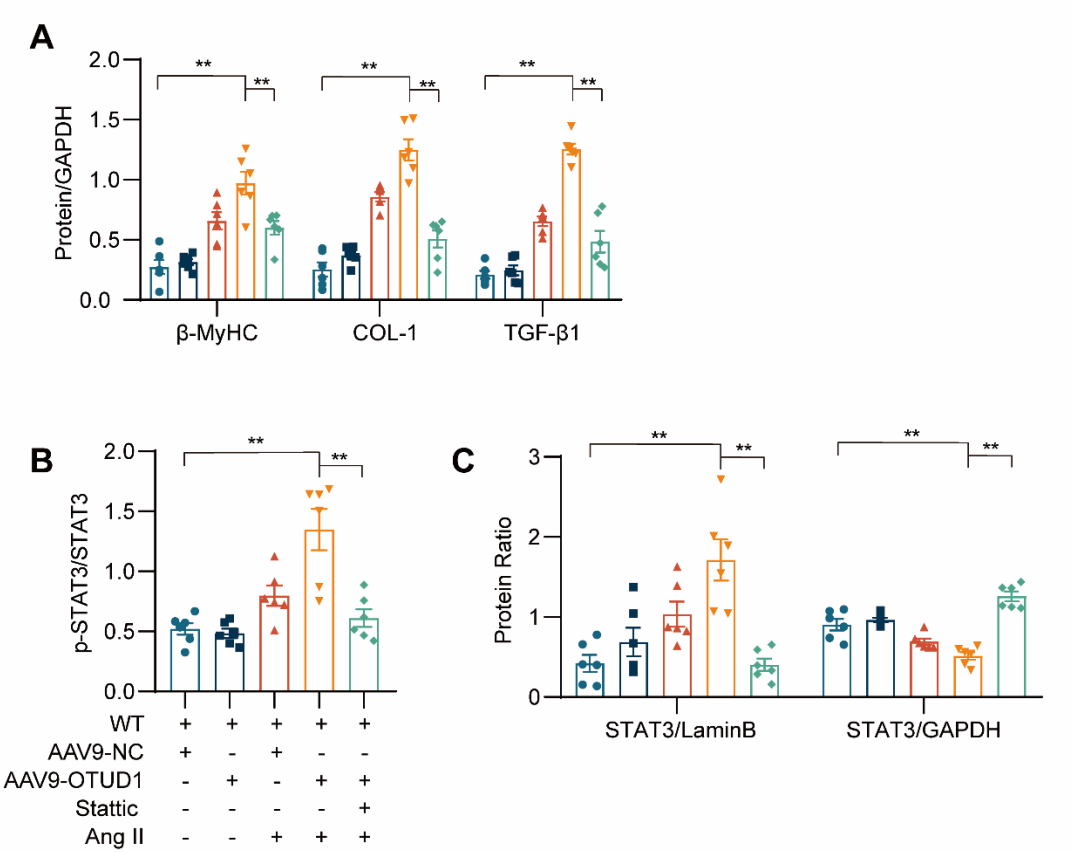

Figure S8: Fibrogenic factors and STAT3 activation in heart tissues of Ang II-challenged mice following OTUD1 expression.

(A) Densitometric quantification of blots in Figure 7K. **(B, C)** Densitometric quantification of blots in Figures 7N and 7O. All quantitative data is presented as Mean ± SEM; n = 6; * = $p < 0.05$ and ** = $p < 0.01$.