SUPPLEMENTAL MATERIAL

Expanded Methods

Animal

All experiments involving animals were approved by the Biomedical Research Ethics Committee of Peking University and are compliant with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All mice were housed in a specific pathogen-free environment under a 12-h light–dark cycle.

The HIP-55-knockout (HIP-55$^{-/-}$) mice with a C57BL/6J background were constructed by Cyagen Biosciences Inc. (Guangzhou, Guangdong, China). The genotypes were identified by PCR analysis of genomic DNA using primers: forward, 5′-AAGTGCTGGGATTAAAGGCGTGC-3′; reverse, 5′-GTCACTGTAGCTGAGCTGGAGGAAGA-3′.

The cardiac-specific expression of wild-type HIP-55 (HIP-55WT^{Tg}) and phosphorylation-deficient (HIP-55AA^{Tg}) mice were generated from C57BL/6J mice by Cyagen Biosciences. Genomic DNA was extracted for genotyping. The primers for HIP-55WT^{Tg} are: forward, 5′-GATCTCCTTTGACCCCGAGAACCTCA-3′; reverse, 5′- CTGGGTTTACTTGTCATCGTCGTCCT-3′. The primers for HIP-55AA^{Tg} are: forward, 5′- ATGACAGACAGATCCCTCCTATCTCC-3′; reverse, 5′-GCCTTCATAGGTAAAGAGAGCCCAGTCG-3′.

Heart failure model

Male mice (10-12 weeks) were used as experimental subjects. Mice heart failure

model was induced by continuous administration of the β-AR agonist isoproterenol (ISO, #I5627, Sigma-Aldrich) at a dose of 30mg/kg/day for 4 weeks by implanting a subcutaneous mini-osmotic pump. Control mice underwent the same procedure, but were administered saline. The same genotypic mice were randomly assigned to ISO or saline group.

Echocardiography

The Vevo 2100 ultrasound system (Visual Sonics Inc, Toronto, Canada) was used to evaluate anaesthetized mouse cardiac geometry and systolic function at 4 weeks after ISO administration. The echocardiographic investigator was unaware of treatment allocation. Averaged ejection fraction (EF%), fractional shortening (FS%), left ventricular end diastolic volume (LVEDV) and heart rates were evaluated. The mice which heart rate not reach 400 bp was excluded to evaluate cardiac function.

Histological and immunohistochemical analysis

Heart tissue was fixed with 4% paraformaldehyde and embedded in paraffin, and serially sectioned into 6-μm slices. For HIP-55 immunohistochemical (IHC) staining, slides were deparaffinized using xylene and graded ethyl alcohol. Heat-induced antigen epitope retrieval was performed by boiling the slides in 0.01 M citrate buffer in a pressure cooker, followed by 30-minute cooling at room temperature and washing with PBS. The slides were then blocked for 1 hour with PBS containing 10% goat serum and then incubated with the primary HIP-55 antibodies overnight in a humidified chamber at 4℃. For wild type mice cardiac HIP-55 staining, HIP-55

antibodies were diluted at 1:200; for cardiac-specific overexpression of HIP-55 transgenic staining, HIP-55 antibodies were diluted at 1:800. After recovering at room temperature for 30 minutes and washing with PBS, the slides were incubated with HRP-labeled secondary antibody (#PV-6001, ZSGB-BIO) for 30 minutes. After washing with PBS, the slides were stained with diaminobenzidine (#ZLI-9018, ZSGB-BIO). Stained slides were then counterstained with hematoxylin and cover-slipped. The negative control for IHC staining used PBS instead of HIP-55 antibodies during incubations, while the remaining steps were the same as the experimental group. Sirius red staining was performed according to the manufacturer's instructions (#G1472, Beijing Solarbio Science & Technology Co., Ltd.). HE staining was performed according to the manufacturer's instructions (#G1100, Beijing Solarbio Science & Technology Co., Ltd.).

Cell culture

Primary cardiomyocytes were isolated from neonatal C57BL/6 mice using a combination of mechanical dissociation (gentleMACS, Miltenyi Biotec, Bergisch Gladbach, Germany) and enzymatic degradation (neonatal heart dissection kit; #130-098-373, Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Primary cardiomyocytes were then cultured in Dulbecco's Modified Eagle Medium with 10% fetal bovine serum and antibiotics (50 mg/ml streptomycin, 50 U/ml penicillin) at 37 °C with 5% CO_2.

NIH-3T3 cells and HEK-293A were cultured in Dulbecco's Modified Eagle Medium

(DMEM) with 10% fetal bovine serum and antibiotics (50mg/ml streptomycin, 50U/ml penicillin) at 37°C with 5% CO_2.

Immunofluorescence

Cardiomyocytes were cultured in a confocal dish and then treated as indicated. Cardiomyocytes were fixed with 4% formaldehyde for 15 minutes, permeabilized with 0.1% Triton-X100 for 10 minutes, and washed with PBS. The cells were then blocked for 1 hour with PBS containing 10% goat serum and incubated with the primary HIP-55 antibodies overnight at 4℃. For endogenous HIP-55 staining, HIP-55 antibodies were diluted at 1:200; for exogenous HIP-55 staining, HIP-55 antibodies were diluted at 1:1000. Cardiomyocytes were then incubated with Alexa Fluor 488 conjugated goat anti-rabbit secondary antibody (Invitrogen, #A21206, diluted at 1:800) for 1 hour. A Zeiss 980 laser scanning confocal microscope (Carl Zeiss Canada Ltd., Toronto, Ontario, Canada) was used to capture the immunofluorescence images.

Antibodies and chemicals

Antibodies to GAPDH (WB, 1:5000, #5174), pan-AKT (WB, 1:3000, #9272), phospho-AKT substrate (WB, 1:5000, #9614), phospho-p38 (T180/Y182) (WB, 1:3000, #4613) and pan-P38 (WB, 1:3000, #9212), were from Cell Signaling Technology. In rabbits, we generated the following antibodies for western blots (diluted as indicated): anti-HIP-55 polyclonal antibodies against the full-length purified HIP-55 protein (1:3000), anti-phospho-HIP-55 (phosphorylated at S269) polyclonal antibodies against the synthesized phosphopeptide (KERAMpSTTS;

1:1000), and anti-phospho-HIP-55 (phosphorylated at T291) polyclonal antibodies against the synthesized phosphopeptide (FLQKQLpTQPE; 1:1000). MK 2206 (#HY-10358) was from MedChemExpress. AKT1/2-siRNA (#sc-43610) was from Santa Cruz Biotechnology.

Plasmid construction

For recombinant protein expression in E. coli, HIP-55WT and its variants, including the ADF-H domain, LCD, and SH3 domain modifications, HIP-55R/A, HIP-55DD, and HIP-55AA, respectively, were inserted into pET28a and fused with an N-terminal 6×His tag. DNA fragments from the ADF-H domain, LCD, and SH3 domain were generated from full-length HIP-55 by PCR amplification. The HIP-55R/A variant was synthesized by Genewiz Biotechnology. The pET28a-HIP-55DD and AA were generated by PCR using the pET28a-HIP-55WT as a template via site-directed mutagenesis.

For fluorescently labeled gene expression in mammalian cells, plasmids with HIP-55WT or one of its variants were constructed by subcloning the corresponding vector DNAs into the pEGFP-N1 or pmcherry-N1 vector using the EasyGeno Assembly Cloning Kit (TIANGEN Biotech).

Adenoviral infection and plasmid transfection

Adenovirus-mediated gene transfer was used for knockdown or overexpression of HIP-55 in primary cardiomyocytes. Adenoviral vector-mediated shRNA targeting mouse HIP-55 and scrambled control were commercially provided by Hanbio

Biotechnology (Shanghai, China). Adenoviral vector-mediated overexpression of Flag-tagged HIP-55WT and HIP-55AA were commercially provided by Hanbio biotechnology (Shanghai, China). The sequence of HIP-55WT and HIP-55AA were consistent with the corresponding plasmid vectors. After infection for 48 hours, the knockdown or overexpression efficiency of HIP-55 in cardiomyocytes was verified using western blot analysis for each experiment.

For gene transfer into NIH-3T3 or HEK-293A cells, cells were transfected with the corresponding plasmids using Lipofectamine 3000 (Invitrogen) following the manufacturer's instructions.

Pull-down assays

Cells were transfected with the corresponding plasmids, and the protein extracts were prepared using pull-down lysis buffer (1% NP-40, 150 mM NaCl, 100 mM Hepes, 5 mM Na4P2O7, 5 mM NaF, 2 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 10 mg/L aprotinin, 10 mg/L leupeptin, PMSF).

For GST pull-down assay, the lysates were incubated with Glutathione-Sepharose 4B beads (GE Healthcare) at 4°C 4 hours with rotation. After 3 washes with lysis buffer, the bound proteins were released in SDS loading buffer.

For Flag pull-down assay, the lysates were incubated with anti-Flag M2 affinity gel (Sigma) at 4°C overnight with rotation. After 3 washes with lysis buffer, the bead-conjugated proteins were then released with SDS loading buffer.

Recombinant protein expression and purification

HIP-55 and its variants were overexpressed in BL21 (DE3) E. coli at 25 °C for 13 hours after induction with 500 μM IPTG. Bacteria were harvested by centrifugation and lysed in a buffer containing 50 mM Tris-HCl (pH 7.5), 500 mM NaCl, 25 mM imidazole, 4 mM β-mercaptoethanol, and 2 mM PMSF. After centrifugation (16000 rpm, 4 °C, 0.5 hour), the supernatant was loaded onto a Ni column (GE Healthcare, USA). Proteins were eluted with buffer containing 50 mM Tris-HCl (pH 7.5), 500 mM NaCl, 500 mM imidazole, and 4 mM β-mercaptoethanol. Eluted proteins were fractionated via size exclusion chromatography in 50 mM Tris-HCl (pH 7.5), 500 mM NaCl, and 2 mM DTT.

Protein phosphorylation reaction

Purified HIP-55 were phosphorylated by AKT1 (Abcam, ab205801) *in vitro*. The reaction solution contained 40 μM HIP-55-WT or HIP-55-AA protein, 1 mM ATP (Sigma, A2383), 5 mM MgCl2, 1 mM EGTA, 0.2 mM EDTA, 2mM DTT, 160 mM NaCl, and 50 mM Tris-HCl (pH 7.5) with 1 μM AKT1. The reaction solution was incubated at 37 °C for 1 hour. An equal volume of AKT1 buffer replaced active AKT1 as the control. Phosphorylation of HIP-55 was confirmed by western blot. To determine the protein LLPS capacity, the final reaction solution was added to an equal volume of 20% (w/v) PEG 3,350.

Fluorescent labeling of purified proteins

Purified HIP-55 proteins were diluted with a reaction buffer containing 50 mM Tris-HCl (pH 7.5), 500 mM NaCl, and 4 mM Tris (2-carboxyethyl) phosphine (TCEP)

(Invitrogen, T2556) and desalted using a desalting column (GE Healthcare, USA) to remove the DTT from the original buffer. Proteins were then incubated with 5-fold AlexaFluor-647 C2-malemide (Invitrogen, A20346) at room temperature for 1 hour. Labeled proteins were then purified by Superdex 75 10/300 columns (GE Healthcare, USA) in buffer containing 50 mM Tris-HCl (pH 7.5), and 500 mM NaCl. Labeled protein was mixed with unlabeled protein at a molar ratio of 49:1 (labeled: unlabeled) for subsequent LLPS assays and confocal imaging.

DIC and fluorescent imaging of LLPS

Samples were prepared in LLPS buffers as indicated above. PEG 3,350 or the other polymers were added into the protein solution to induce protein LLPS at the final step. Induced LLPS samples were incubated in tubes for 5 minutes before imaging. Induced samples were then loaded onto glass slides with a coverslip. DIC or fluorescent images were acquired on a Leica TCS SP8 microscope with a 100×objective (oil immersion) at room temperature.

Turbidity measurement

Turbidity measurement was performed based on the optical absorption of an LLPS sample at 600 nm. OD_{600nm} were recorded on a Varioskan Flash spectral scanning multimode reader (Thermo Fisher) using a flat bottom and low volume 384-well plates (Corning, 10 μL per well) after a 5-minute incubation.

Fluorescence recovery after photobleaching (FRAP) assay

FRAP assays for HIP-55 condensates were performed using the FRAP module of the

confocal microscope. The region of interest within a fluorescent HIP-55 condensate was bleached using the laser beam. Images were captured over time as indicated after photo-bleaching. The fluorescent intensity of the bleached region was then acquired. Meanwhile, the fluorescent intensity of a nearby unbleached region was also recorded as a control. The fluorescent recovery (I_t) was calculated by normalizing the fluorescent intensity of the bleached region to the fluorescent intensity of the unbleached region at every time point (t). The relative fluorescent recovery for bleached intensity was then calculated. In brief, for each time point (t), relative fluorescent recovery fraction for bleached intensity was calculated with the formula: $(I_t - I_{min})/ (I_{t=0} - I_{min})$. I_{min} is the unbleached fraction after photobleaching. Images were analyzed with the Leica Application Suite X software and Fiji.

Fractionation of soluble and insoluble HIP-55 proteins

Cells were harvested in lysis buffer containing 1% NP-40, 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, and 1 mM EDTA with sonication. The cells were then lysed for 1 hour at 4°C and then centrifuged at 10,000g at 4°C for 10 minutes. The supernatant was collected as the NP-40 soluble fraction, while the pellet (NP-40 insoluble fraction) was resuspended in 8M urea at room temperature for 30 minutes.

Quantitative RT-PCR

In brief, total RNA was isolated from heart tissue with TRIzol reagent. Reverse transcription of RNA samples was carried out by using TransScript® II Two-Step RT-PCR SuperMix System (#AH401, Transgenbiotech) according to manufacturer's

instructions. Quantitative RT-PCR was performed with the GoTaq qPCR Master Mix (Promega) and specific primers in a Stratagene Mx3000P qPCR System.

The primers for ANP (mouse) were: forward, 5′- CTTCCAGGCCATATTGGAG -3′; reverse, 5′- GGGGGCATGACCTCATCTT -3′. The primers for BNP (mouse) were: forward, 5′- ACAAGATAGACCGGATCGGA-3′; reverse, 5′- AGCCAGGAGGTCTTCCTACA-3′. The primers for α-MHC (mouse) were: forward, 5′- CAGAGGAGAAAGCTGGCGTC -3′; reverse, 5′- TTGTCAGCATCTTCGGTGCC -3′. The primers for β-MHC (mouse) were: forward, 5′- AACCTGTCCAAGTTCCGCAAGGTG -3′; reverse, 5′- GAGCTGGGTAGCACAAGAGCTACT-3′. The primers for GAPDH (mouse) were: forward, 5′-ATGTTCCAGTATGACTCCACTCACG-3′; reverse, 5′- GAAGACACCAGTAGACTCCACGACA-3′.

Western blot

In brief, total protein lysates were extracted from heart tissue or cells, separated with SDS-PAGE, and transferred onto nitrocellulose filter (NC) membranes. Membranes were then incubated with indicated antibodies and visualized with enhanced chemiluminescent detection reagents (Pierce).

Table S1 - 2

Table S1. Major echocardiographic parameters in wild-type and HIP-55 KO mice

Mice	WT		HIP-55$^{-/-}$	
Group	Ctrl	ISO	Ctrl	ISO
n	7	9	6	8
HR (bpm)	497.0 ± 6.33	503.6 ± 4.75	497.5 ± 6.77	507.5 ± 6.12
LVID;d (mm)	3.747 ± 0.1186	4.268 ± 0.0540**	4.043 ± 0.0963	4.827 ± 0.0549††
LVID;s (mm)	2.327 ± 0.1080	3.348 ± 0.0662**	2.739 ± 0.0998	4.125 ± 0.1121††
LVPW;d (mm)	0.677 ± 0.0173	0.766 ± 0.0088**	0.727 ± 0.0251	0.833 ± 0.0296†
LVPW;s (mm)	1.111 ± 0.0562	0.953 ± 0.0411	1.152 ± 0.0705	0.919 ± 0.0557
LVAW;d (mm)	0.659 ± 0.0353	0.718 ± 0.0282	0.688 ± 0.0760	0.736 ± 0.0457
LVAW;s (mm)	1.155 ± 0.0807	0.959 ± 0.0456	1.165 ± 0.1129	0.874 ± 0.0667

Results are presented as the mean ± SEM and analyzed using 2-ANOVA followed by Tukey test. **P < 0.01, WT-ISO vs WT-Ctrl; †P<0.05, ††P<0.01, HIP-55$^{-/-}$-ISO vs WT-ISO.

WT: Wild type; HIP-55$^{-/-}$: HIP-55 knock mice; LVID;d: Left ventricular internal diameter end diastole and end diastole; LVID;s: Left ventricular internal diameter end diastole and end systole; LVPW;d: Left ventricular posterior wall end diastole; LVPW;s: Left ventricular posterior wall end systole; LVAW;d: Left ventricular anterior wall end diastole; LVAW;s: Left ventricular anterior wall end systole.

Table S2. Major echocardiographic parameters in wild-type and cardiac-specific overexpression of HIP-55 mice

Mice	WT		HIP-55WT^{Tg}		HIP-55AA^{Tg}	
Group	Ctrl	ISO	Ctrl	ISO	Ctrl	ISO
n	8	7	9	7	10	9
HR (bpm)	501.3 ± 5.04	497.4 ± 7.76	499.0 ± 4.19	502.1 ± 7.43	503.5 ± 4.24	502.8 ± 5.93
LVID;d (mm)	3.754 ± 0.1259	4.486 ± 0.0782**	3.813 ± 0.0641	4.060 ± 0.1516†	3.793 ± 0.1475	4.499 ± 0.0749‡
LVID;s (mm)	2.345 ± 0.1189	3.544 ± 0.1118**	2.320 ± 0.1101	2.997 ± 0.1331†	2.440 ± 0.1511	3.618 ± 0.0860‡‡
LVPW;d (mm)	0.678 ± 0.0065	0.750 ± 0.0163*	0.674 ± 0.0211	0.724 ± 0.0334	0.683 ± 0.0062	0.777 ± 0.0222
LVPW;s (mm)	1.171 ± 0.0161	0.871 ± 0.0165	1.160 ± 0.0579	1.071 ± 0.0282	1.197 ± 0.0297	0.904 ± 0.0319
LVAW;d (mm)	0.618 ± 0.0141	0.703 ± 0.0106	0.653 ± 0.0405	0.693 ± 0.0408	0.631 ± 0.0430	0.710 ± 0.0216
LVAW;s (mm)	1.095 ± 0.0225	0.913 ± 0.0371	1.126 ± 0.0383	0.991 ± 0.0409	1.058 ± 0.0354	0.926 ± 0.0558

Results are presented as the mean ± SEM and analyzed using 2-ANOVA followed by Tukey test. *$P < 0.05$, **$P < 0.01$, WT-ISO vs WT-Ctrl; †$P<0.05$, HIP-55WT^{Tg}-ISO vs WT-ISO; ‡ $P < 0.05$, ‡‡ $P < 0.01$, HIP-55WT^{Tg}-ISO vs HIP-55AA^{Tg}-ISO.

WT: Wild type; HIP-55WT^{Tg}: Cardiac-specific HIP-55WT overexpressing mice; IP-55AA^{Tg}: Cardiac-specific HIP-55AA overexpressing mice; LVID;d: Left ventricular internal diameter end diastole and end diastole; LVID;s: Left ventricular internal diameter end diastole and end systole; LVPW;d: Left ventricular posterior wall end diastole; LVPW;s: Left ventricular posterior wall end systole; LVAW;d: Left

ventricular anterior wall end diastole; LVAW;s: Left ventricular anterior wall end systole.