SUPPLEMENTAL MATERIAL

Supplemental Methods

Thoracic aortic banding (TAB) model and colchicine treatment

Male C57BL/6 mice (9-10 weeks) were subjected to sham or pressure overload by TAB surgery as described.[1]_Colchicine (Sigma, St. Louis, MO, USA) was injected intraperitoneally from 2 days after the Sham or TAB procedure once every other day. The injections began with 0.4 mg/kg and progressed to 1 mg/kg (0.4 mg/kg, 0.6 mg/kg, 0.8 mg/kg and 1 mg/kg) to allow the mice to adjust to the drug, as previously described.[2] Then 1 mg/kg colchicine continued until 5 weeks after TAB. Control mice were injected with saline. LV function was examined by echocardiography [3] at the end of 5 weeks after TAB. In situ confocal imaging of T-tubule structure from epicardial myocytes of intact hearts was performed on the next day after echocardiography.

Adult mouse ventricular myocytes isolation, cell culture and viral transfection

Adult ventricular cardiomyocytes were isolated from C57BL/6 mice or JP2 knockdown (JP2-KD) mice (2 ~ 3 months old) using standard enzymatic method described previously,[4] and ≥75% Ca^{2+} tolerant rod-shaped myocytes were used for experiments. After stabilizing in 1.0 mMol/L Ca^{2+}Tyrode's solution (in mmol/L: NaCl 137, KCl 5.4, HEPES 10, Glucose 10, $MgCl_2$ 1, NaH_2PO_4 0.33, pH adjusted to 7.4 with NaOH) for 10 minutes (min), cell pellet was suspended in Minimum Essential Medium (MEM) with 10% fetal bovine serum (FBS) and plated in 35 mm dishes with laminin (10 μg/ml)-precoated glass at the bottom. The myocytes were cultured in 5% CO2 incubator at 37 ^{0}C for 2 hours (hrs), then the medium was changed to FBS-free MEM medium and myocytes were cultured at a period of 24 hrs to 72 hrs. For adenoviral-mediated

gene expression, freshly isolated cardiomyocytes were infected with adenoviruses expressing Kif5b (i.e. Kinesin 1) wild type (WT) or a dominant negative (DN) mutant [5] for 48 hrs.

In some experiments, cardiomyocytes were isolated from JP2-KD mice. JP2-KD mice were generated by crossing JP2-shRNA mice (expressing a cardiac-specific, tamoxifen-inducible junctophilin-2 shRNA,[6] C57BL/6 background) with MerCreMer (MCM) mice (Jackson Laboratory). Two to three month old male MCM (used as control) and double transgenic JP2-KD mice (MCM x JP2-shRNA) mice were treated with daily tamoxifen (~40 mg/kg, Sigma-Aldrich Co., St. Louis, MO) by intraperitoneal injection for 10 days. The next day following the last injection, cardiomyocytes were isolated and cultured as above.

T-tubule imaging and Analysis

T-tubules of single cardiomyocytes was stained with Di-8-ANEPPS (10 μmol/L, AAT BioQuest, CA) in Ca^{2+} free Tyrode solution at room temperature for 30 mins. T-tubules from intact hearts were stained with MM 4-64 (2.5 μmol/L, AAT BioQuest, CA) in Ca^{2+} free Tyrode solution via Langendorff perfusion at room temperature for 30 min. And the structure of T-tubules was visualized with confocal microscope (LSM510, Carl Zeiss MicroImaging Inc., Germany) on 63× lens. Quantitative analysis of T-tubule integrity was processed with IDL image analysis program as previously reported.[7] The power value (TT_{power}) reflects the strength of the regularity of T-tubule organization.

Immunofluorescent staining of LV cryosections and cardiomyocytes

Hearts from sham and TAB mice were fixed by perfusion with 4% paraformaldehyde (PFA) for 15-20 min and postfixation of the longitudinally-cut heart with 4% PFA at 4°C for 24-48 hrs. Fixed heart tissue was then incubated in 10%, 20% and 30% sucrose sequentially at 4°C for cryoprotection before cryoembedding with optimal cutting temperature (OCT) compound. The heart tissues were longitudinally sectioned (10 μm) in cryostat at -24°C. Sections were

stored at -80°C before immunofluorescent staining. Isolated cardiomyocytes were fixed in 4% PFA at 37°C for 15 min. For immunofluorescent staining, samples were washed 3 times with PBS for 10 min, and followed by permeabilization with 0.3% Triton-X 100 in PBS for 30 min. Following 1% BSA blocking 30 min at room temperature, samples were incubated with anti-β-tubulin antibody (1:100, T4026, Sigma, St. Louis, MO, USA) / anti-α-tubulin antibody (1:300, ab18251, abcam, Cambridge, MA, USA), or anti-JP2 (1:50, sc-51313; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight followed by incubation with fluorescent-labeled secondary antibodies at room temperature for 2 hrs. Staining was visualized by confocal microscopy with a 63X lens. Microtubule density was quantitated using NIH Image J. JP2 organization ($JP2_{power}$) was processed with IDL image analysis program. Mean JP2 fluorescence around the cell periphery (within ~4 μm from cell edge) and mean JP2 fluorescence inside the cell were quantitated using a home-complied automatic program coded in MatLab.

Protein preparation

Single cardiomyocytes was suspended (Whole hearts or LV were homogenized) and sonicated in lysis buffer (in mmol/L: Tris-HCl 50 [pH 7.4]; NaCl 150; NaF 10; Na_3VO_4 1; EGTA 5; EDTA 5; 0.5% Triton X-100; 0.5% Na deoxycholate and 0.1% SDS), containing protease inhibitors (Sigma, P8340). Then the lysates were centrifuged at 13,000g 4 ^{0}C for 15 min. The supernatants were kept as whole cell proteins. Cytosol and membrane fraction was obtained from single cardiomyocytes by using modified method as previously reported.[8] Cardiomyocytes were suspended and sonicated in lysis buffer (in mmol/L: Tris-HCl 25; sucrose 250, EDTA 1, EGTA 1, Na_3VO_4 1, NaF 1 and protease inhibitor). The lysates were centrifuged at 13,000 g for 15 min, producing the supernatants designated as cytosol fraction. After being washed with lysis buffer 3 times, the pellets were re-suspended with the above lysis buffer with 0.5% Triton X-100,

0.5% sodium deoxycholate and 0.1% SDS, and then were centrifuged at 13,000 g for 15 min. The supernatants were saved as membrane fraction.

Free and polymerized tubulin were prepared by using modified method as previously reported.[9] Fresh LV were homogenized in microtubule stabilization buffer (MTSB) (in mmol/L: GTP 1, Na_2HPO_4 10, EGTA 0.5, $MgCl_2$ 0.5, 50% glycerol, 5% DMSO). Protease inhibitor cocktails were included in this buffer, and the lysate was centrifuged at 100,000 x *g* 25°C for 20 min. The supernatants were saved as free tubulin. After being washed once with MTSB, the pellets were re-suspended with depolymerizing buffer (in mmol/L: PIPES 80, MgCl 1, EGTA 1, $CaCl_2$ 5, 1% Triton X-100 and protease inhibitors) and kept on ice for 30 min. Then the suspensions were centrifuged at 20,000x *g* at 4°C for 10 min. The supernatants were saved as polymerized tubulin. The protein concentration was determined by BCA assay.

Western blotting

Whole cell proteins, cytosol and membrane fractions, free and polymerized tubulin were electrophoresed on 4-12% Bis-Tris gels. Proteins were transferred to PVDF and probed with primary antibodies recognizing JP_2 (1:1000), β-tubulin (1:000) and GAPDH (1:10,000, G8975, Sigma), respectively, overnight at 4°C. After being washed with PBS solution 3 times, the binding of the primary antibodies was detected by horseradish peroxidase (HRP)-conjugated second antibodies (1:5,000-1:10,000 dilution in PBS solution). The immunoreactions were visualized using an enhanced-chemiluminescent detection kit and the protein bands were quantified with Quantity One 1-D Analysis Software (Bio-Rad, USA).

Confocal Ca^{2+} Imaging

Cultured cardiomyocytes were loaded with 5 μmol/L Fluo-4 AM (Invitrogen, Grand Island, NY) in 1.8 mmol/L Ca^{2+}Tyrode solution for 30 min. Cells were then washed with dye-free Tyrode solution for 20 min for de-esterification before imaging. The Ca^{2+} images were acquired using

confocal microscope in line–scan mode along the longitudinal axis of myocytes. Steady-state Ca^{2+} transients were measured in Tyrode's solution containing 1.8 mmol/L Ca^{2+} under field stimulation of 1 Hz. Ca^{2+} sparks were recorded ~ 5 sec after halt of field stimulation.

Statistical Analysis

Data are expressed as mean ± SE. Bonferroni procedure followed a one-way ANOVA was applied to multiple group comparisons of in vivo animal experiments. Bonferroni procedure after a global test based on a linear mixed-effects model was performed to multiple group comparisons of in vitro cardiomyocyte experiments. A compound symmetry correlation structure was assumed for linear mixed-effects model tests. Student's *t*- test was used for two group comparison. Statistical analysis were carried out using SPSS V15.0 software. Values of $p < 0.05$ were considered statistically significant.

Supplemental References

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Supplemental Figures

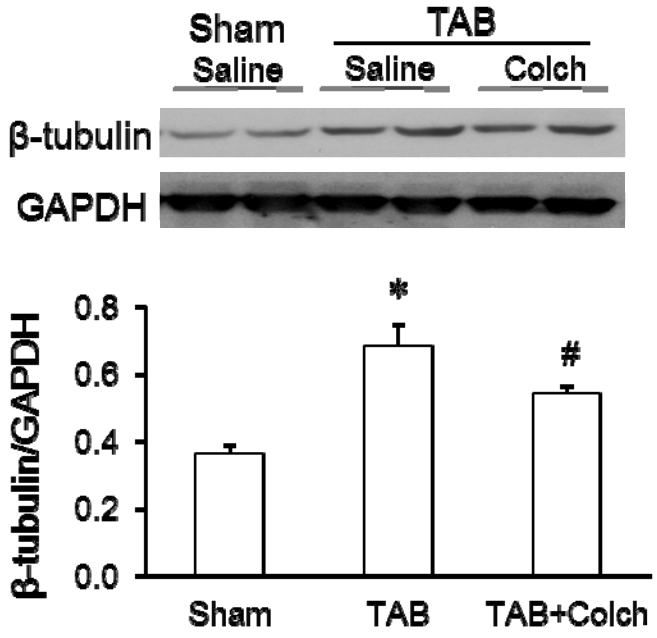

Figure S1. Pressure overload increases the protein expression of β-tubulin. Representative Western blot (upper) and summary data (lower) of β-tubulin expression in LV of sham mice and TAB mice injected with saline or Colchicine (Colch). n = 4 hearts per group. * $p< 0.05$, compared to sham. $^{\#}p< 0.05$, compared to saline TAB.

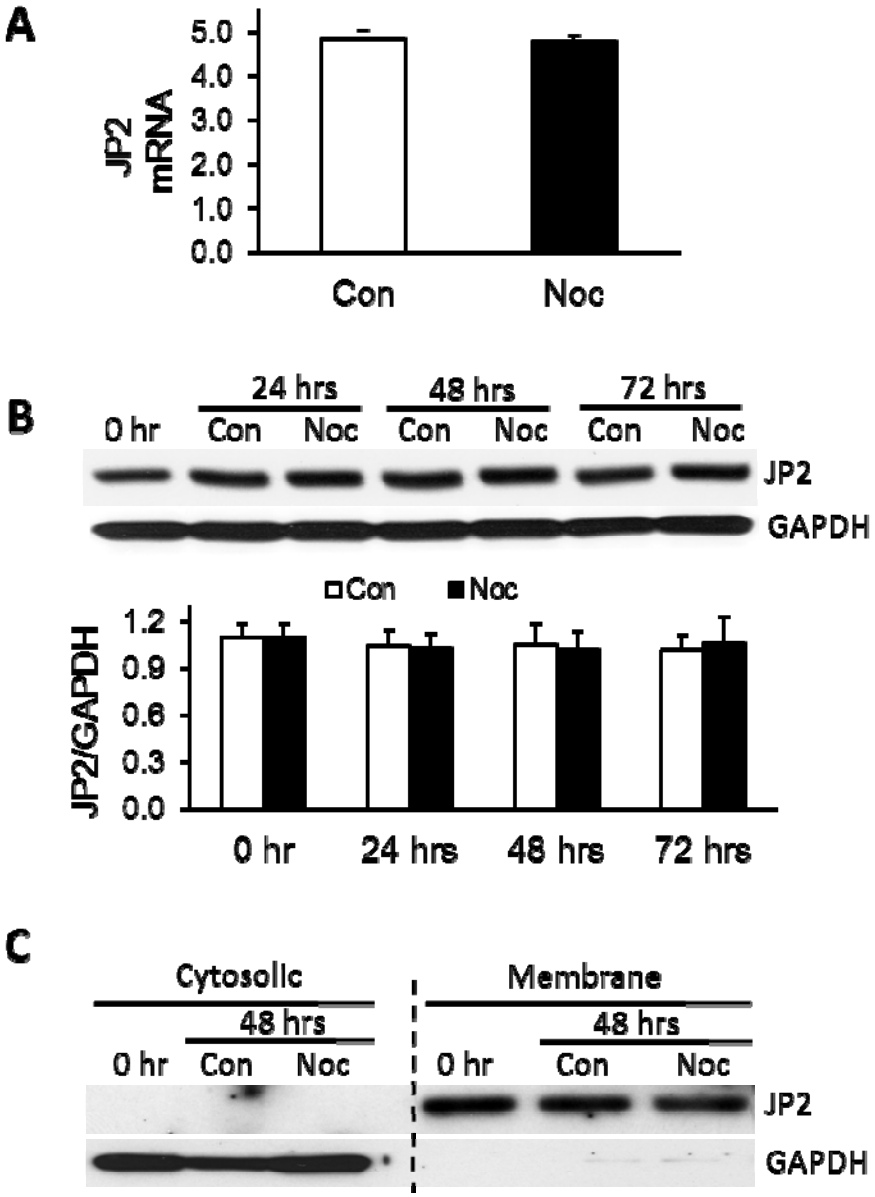

Figure S2. Microtubule depolymerizing agent does not affect JP2 mRNA and protein levels.A. JP2 mRNA levels in cultured cardiomyocytes with or without 10 μM nocodazole for 48 hrs (n = 4 hearts per group). **B**. Representative Western blot (upper) and summary data (lower) of JP2 protein levels after treatment with 10 μM nocodazole. Data were normalized to GAPDH and quantitated relative to control levels at 0 hr. **C**. JP2 protein expression in cytosolic and membrane fractions of cardiomyocytes cultured for 48 hrs in the absence or presence of 10 μM nocodazole. Con: DMSO control; Noc: nocodazole. n = 3 hearts per group.

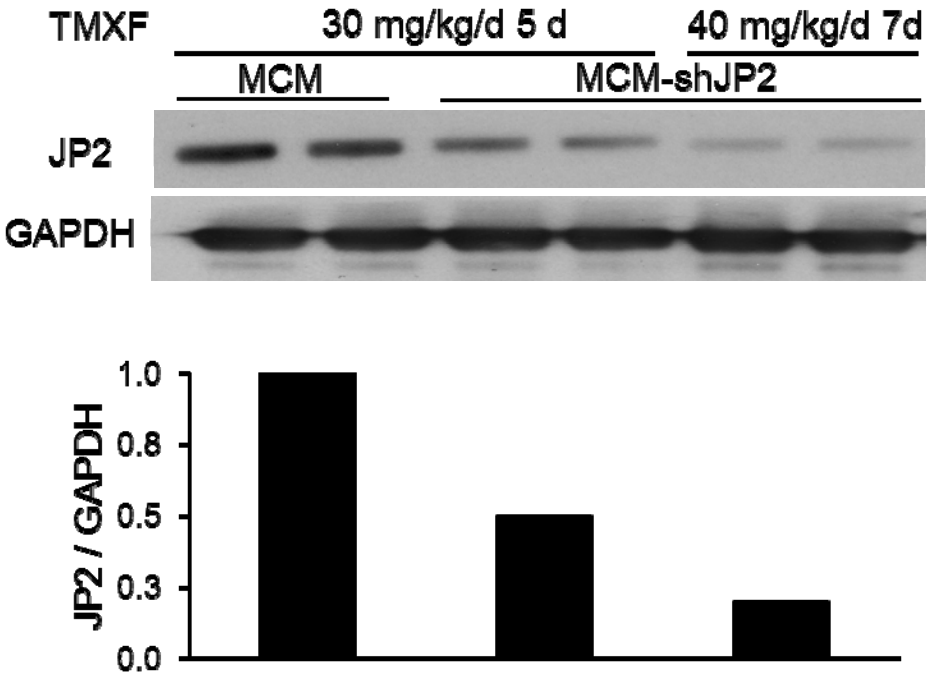

Figure S3. JP2 protein expression in the myocardium of MCM mice and MCM-shJP2 mice after tamoxifen (TMXF) injection. Upper: representative Western blot; Lower: summary data. TMXF were administrated by intraperitoneal injection ~30 mg/kg for 5 consecutive days or ~40 mg/kg for 7 consecutive days. n = 4 hearts per group.