

SUPPLEMENTAL MATERIAL

***ZBTB17 (MIZ1)* is important for the cardiac stress response and a novel candidate gene for cardiomyopathy and heart failure**

Supplemental Methods

Yeast two-hybrid, Immunoprecipitation and Western blots

A Matchmaker Gal4 two-hybrid system 3 (Clontech) was used for interaction analyses and an adult mouse heart library was screened. A total of 10^6 clones were screened, all 650 positive clones were isolated, transformed and sequenced following the manufacturer's instructions. Among the positive clones were cDNAs encoding alpha actinin, a previously known CSRP3 interacting protein or telethonin (TCAP)¹⁻³. We also identified a clone encoding the 5' sequence of Zbtb17.

Mouse heart tissue (WT) was homogenized in ice-cold RIPA buffer containing 10 mM Tris, pH7.4, 150 mM NaCl, 0.2% Triton X-100, 2mM EDTA, 1 mM PMSF, plus 1× protease inhibitor mixture. The heart lysate was centrifuged at 3000 rpm for 10 min at 4°C, and the supernatant collected. 1 mg of total protein was pre-cleared by addition of 100 µl of 1:1 slurry of protein A sepharose beads for 1 hr at 4°C on rotivator and centrifuged at 3000 rpm for 1 min at 4°C. The resulting supernatants were transferred to fresh tubes, and 10 µl of anti-ZBTB17 antibody (Santa Cruz H-190; sc 22837) was added and incubated overnight at 4°C on a rotivator. After incubation, 100 µl of 1:1 slurry of protein A sepharose beads was added to each sample to collect the immunocomplex, and the mixture of lysate and beads was incubated for 2 hrs at 4°C.

Immune precipitates were then washed 3 times with cold lysis buffer, and analyzed by Western blot.¹

Cross-linking experiments

Formation of a complex between ZBTB17 (produced with an N-terminal His-tag in pET46 vector and purified by standard nickel-affinity chromatography) and CSRP3 (MLP)⁴ was analyzed via cross-linking experiments with ethylene glycol bis(sulfosuccinimidylsuccinate)⁴ (sulfo-EGS) (Thermo Scientific) solution in DMSO. The experiment was performed in 40 μ l reactions with increasing concentration of the crosslinker (0, 0.5, 1, 2, and 5 mM sulfo-EGS) and incubated for 15 to 30 min at room temperature. The reaction was quenched by addition of 1 μ l of 1M tris(hydroxymethyl)aminomethane (Tris) buffer at pH8 and analyzed by SDS-PAGE.

Adenoviral ZBTB17 constructs and analysis *in vitro*

WT-ZBTB17 cDNA was cloned into recombinant human adenovirus 5 (Vector Biolabs). The effects of WT-ZBTB17 overexpression on hypertrophy and cell death were analyzed in neonatal rat cardiac myocytes (NRCM) using the xCELLigence Real-Time Cell Analyzer (RTCA; Roche Diagnostics Ltd.) or via cell surface area measurements. The xCELLigence system consists of a 16-well E-plate with the bottom of each well covered by a gold microelectrode array. Electrical impedance (displayed as cell index) is directly proportional to the total area of the well that is covered by cells. Freshly isolated NRCMs in M-199 (Sigma) culture medium were plated at a density of 50,000 per well in a laminin-coated xCELLigence E-plate 16. A background reading is taken on the xCELLigence system prior to plating in the absence of cells. The xCELLigence

instrument is incubated at 37°C and 5% CO₂ and the software programmed to take impedance measurements every 30 mins. 16-18 hrs after plating cells were infected with an adenovirus for either a GFP control or WT-ZBTB17. The effects of ZBTB17 overexpression were recorded for the following 48 hrs, an increase in cell index represents an increase in the area of the well covered by cells as these are non-proliferating cells any increase in cell index represents an increase in cell size. 48 hrs after adenoviral infection 1μM of DOX was added and cell index recorded for a further 48 hrs. Cell death was analyzed by normalizing the data to the point of addition of DOX and calculating the area under the curve (AUC), thus a larger AUC represents a slower rate of cell death. Staurosporin (SP) induced apoptosis was analyzed using fluorescence microscopy and DAPI staining (GenScript). NRCMs were infected with adenoviral constructs using 100 multiplicity of infection (MOI), for Western blot analysis cells were harvested after 48 hrs.

Generation of *Zbtb17* conditional knock out animals, transgenic animals and TG-ZBTB17/KO-Ppp3cb double transgenic animals

Targeted deletion of *Zbtb17* in cardiac tissue was accomplished by crossing mice expressing Cre recombinase under the control of the MLVC2 promoter as described previously⁵ into *ZBTB17* homozygous floxed (flox/flox) mice and backcrossing the resulting heterozygous mice governing the Cre recombinase back to homozygous *Zbtb17* flox/flox, *Cre*⁺ mice. Cre mediated recombination resulted in deletion of the POZ domain.

ZBTB17 transgenic (TG-ZBTB17) mice have been generated by overexpressing the human *ZBTB17* cDNA under the control of the alpha myosin heavy chain promoter (*αMHC*). The transgene construct consists of the murine *αMHC* promoter, FLAG epitope tag, human *ZBTB17*

cDNA, and human growth hormone poly A signal. We established several lines overexpressing *ZBTB17* at different expression levels. All experiments described here were performed with TG-ZBTB17 line 10.

TG-ZBTB17/KO-Ppp3cb double transgenic animals were generated by crossing TG-ZBTB17 into Ppp3cb^{-/-} (kindly provided by Dr J. Molkentin⁶).

Echocardiography and surgery

Surgery was carried out using a modified minimally invasive approach as previously described³. Mice were anesthetized with Isoflurane and echocardiography was performed as described previously³. Briefly, transthoracic echocardiography was performed by an examiner blinded to the genotype of the animals using a Vevo2100 (VisualSonics) system with a 30 MHz centre frequency transducer. Two-dimensional cine loops with frame rates of >200 frames/s of a long axis view and a short axis view at mid-level of the papillary muscles as well as M-mode loops of the short axis view were recorded.

Gene expression analyses in mouse hearts by qRT-PCR

RNA from mouse left ventricles was extracted using the RNeasy Fibrous Tissue Mini Kit (Qiagen GmbH). For single qRT-PCR, reverse-transcription was performed from 500 ng total RNA using the iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's instructions. PCR was performed in duplicate using the Bio-Rad iCycler (Bio-Rad) and SYBR Green as fluorescence.

For the SA Biosciences apoptosis gene expression array: RNA quantity and quality was assayed by the NanoDrop spectrometer and 1 µg of the RNA was used for the cDNA synthesis with the reagents and primers provided (according to the manufacturer's protocol, SA Biosciences). Gene expression of 84 key apoptosis genes was profiled by qRT-PCR based RT2 Profiler Apoptosis PCR Arrays (mouse: PAMM-012) using the RT2 SYBR Green/Rox Master mix. qRT-PCRs were performed in 96-well plate format using the ABI 7900 Fast Real-Time PCR System (Applied Biosystems). All data were analysed by RT2 Profiler Web-Based PCR Array Data Analysis (SA Biosciences).

Immunofluorescence, Apoptosis, Immunolabelling and Cell Size Measurements

Cryosections 5 µm thick were air dried and fixed with 4% paraformaldehyde (PFA). ZBTB17 rabbit polyclonal primary antibody (Miz1, H-190; sc-22837, Santa Cruz, please see also Supplementary Table 7 for all antibodies used) was used. Phalloidin was purchased from Sigma and used to stain for F-actin; DAPI (nuclear staining) was purchased from Vector Laboratories². Apoptosis was analyzed using the "Roche - In Situ Cell Death Detection Kit, (Roche, #116847959110) according to the manufacturer's instructions. We used 6 WT and 6 Zbtb17 conditional knockout hearts (cKO) after transverse aorta constriction (TAC) and results were averaged per mouse and expressed as apoptotic cells per mm².

The quantification process was performed blinded to the type of section, having on the screen only one channel showing F-actin labeling. For each quantification, a specific setting was established and kept constant in all measurements. Quantification was performed by measurements of fluorescence intensity by using a range of 0 to 255 gray values. Arbitrary units of the fluorescence intensity were calculated per unit myocardial area (AU/mm²). The area of

collagen I were calculated as percent of positive labeling per tissue area. Primary antibody: biotinconjugated rabbit anti-collagen type I (Rockland), secondary detection system consisted of anti-mouse IgG and streptavidin linked Cy 3 (Amersham).

Quantification of collagen I was performed as described previously². In brief, cryosections from at least two different levels in each mouse were used. All samples were immunolabeled simultaneously with identical conditions of fixation and dilutions of primary and secondary antibodies. Sections exposed to PBS instead of primary antibodies served as negative controls. For each heart at least 10 random fields of vision were analyzed with a fluorescent microscope Leica (Leitz DMRB) using x40 Planapo objective (Leica). Immunolabeled cryosections were studied using image analysis (Leica) and Image J software (NIH).

The quantification of Picrosirius Red stained (PSR) mouse heart cross sections was performed using Zen software (Zeiss) for x10 tile images and analysed the percentage of fibrosis area for 4 random fields of each section by Fiji software.

NRCM were infected *in vitro* with adenoviral WT-ZBTB17 for 48 hrs and cell surface areas were measured using Image J software.

Cardiomyocyte cross-sectional area *in vivo* was determined at the nuclear level in 100 - 150 cardiomyocytes with clearly defined cellular borders as determined by dystrophin immunolabeling (H-300, Santa Cruz Biotechnology).

Three-dimensional co-localization analysis was performed using the co-localization module of Imaris®, version 7.3 (Bitplane, Zürich, Switzerland).

Calcineurin promoter and calcineurin/NFAT pathway analysis

NFAT activity in cardiomyocytes was measured using a Gal4-NFAT chimera.^{7, 8} NRCMs were isolated as previously described.⁹ Cells were transfected for 6 hrs in serum-free DMEM using Lipofectamine 2000 (Invitrogen) together with a vector expressing a chimeric construct of the DNA binding domain of the yeast transcription factor Gal4 linked to the transcription activation domain of NFAT; a reporter plasmid in which luciferase expression is controlled by four Gal4 responsive sites (UAS) and a WT pcDNA3.1-ZBTB17 expression vector. Empty pcDNA3.1 was used as a negative control for ZBTB17. Medium was replaced after transfection and cells were lysed 48 hrs later. Luciferase activity was measured using a commercial kit (Promega).

ChIP - chip

ChIP-chip analysis was performed using ChIP-chip 3x720K RefSeq Promoter Assay (Roche, genome build mm9). ChIP samples were prepared using the Zbtb17 specific antibody (Miz1, H190, sc22837X, Santa Cruz) and 25 mg of mouse heart combined with Dynabeads sheep anti-rabbit IgG (Life Technology; Nimblegen ChIP-chip service protocol). The hybridization and scanning step were performed by Roche Nimblegen Array Service. Peaks of ChIP-enriched sequences were calculated by $\log_2$ (ChIP/input) method and the FDR value were calculated by NimbleScan software (Nimblegen system). The peak data was analyzed by SignalMap version 1.9 (Nimblegen system) software.

Statistical analysis

Student t-test and 2-tailed P-values were used for comparing groups unless otherwise indicated. Data were formatted in MS Excel and statistical analyses were carried out using SPSS v20 (IBM SPSS statistics). Unless otherwise indicated data are reported as means $\pm$ S.D.

References

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Supplemental Figure 1. Co-localization of Zbtb17 and Csrp3 in the nuclear compartment of the mouse cardiac myocytes

(A) Representative confocal images of Zbtb17 and Csrp3 in WT mouse heart samples. Arrows indicate typical cardiomyocyte nuclei that are positive for both, Zbtb17 and Csrp3. Lower left panel is the three-dimensional image of the boxed region (left middle panel). Shown in the lower right panel is a three-dimensional co-localized color (in intensity units, from blue color as the lowest value and white color, as the highest value) of Zbtb17 with Csrp3. The mean percentage of co-localization of ROI (nucleus) of Zbtb17 with Csrp3 was 83.8 ± 3.4 ($n = 5$ WT hearts, 148 ± 5.1 nuclei per each heart tissue sample). The mean Pearson's coefficient in co-localized volume was 0.9019 ± 0.0024 . (B) Csrp3 knockout hearts are used as negative controls (arrows indicate Zbtb17 in cardiac myocytes). (C) cKO hearts are used as negative controls (arrowheads indicate Zbtb17 in non-cardiac myocytes and arrows indicate Csrp3 in cardiac myocytes).

Supplemental Figure 2. ZBTB17 overexpression in NRCM results in marked hypertrophy

(A) Adenoviral overexpression of WT-ZBTB17 for 48 hrs results in marked hypertrophy in NRCM as measured by cell surface area (AdLacZ 1.0 ± 0.020 n = 653; AdWT-ZBTB17 1.35 ± 0.025 n = 589; 3 independent experiments). **(B)** ZBTB17 overexpression protects NRCM from SP induced apoptosis. Three different experiments were performed (1 μ M of SP for 24 hrs, AdLacZ 1.0 ± 0.02 , n = 653 cells; AdZBTB17 1.4 ± 0.03 , n = 589 cells; *p<0.05; ***p<0.001).

Supplemental Figure 3. Characterization of cKO-Zbtb17 animals

(A) MLC2V–Cre mediated recombination efficiency as determined by Southern blot analysis using genomic DNA derived from myocardial tissue. The three lanes on the right side of the gel marked: “f/f, Cre⁺” (cKO) where floxed recombined and floxed alleles can be compared to each other. It can be estimated that recombination occurred in at least 20% of the cells, which indicates relatively high recombination efficiency in the cardiac myocyte population (M: marker) (upper panel). MLC2V–Cre mediated recombination efficiency as determined by Southern blot analysis using genomic DNA derived from isolated cardiac myocytes and non-myocytes. We observed much stronger signals for the floxed, recombined allele in case of cardiac myocytes isolated from homozygous knockout animals (f/f, Cre⁺) when compared to the low-level background recombination occurring in non-myocytes (the fibroblast fraction might not be absolutely pure, a few cardiac myocytes might have been present) (lower panel). (B) Efficiency of the MLC2V–Cre mediated recombination as determined by qRT-PCR. We amplified the Cre deleted part of the ZBTB17 gene in control (+/+, Cre⁻; n = 4), heterozygous (f/+, Cre⁺; n = 1) and homozygous knockout animals (f/f, Cre⁺; n = 9). There is a significant loss of this genomic region in the homozygous knockout animals. (C) MLC2V–Cre mediated recombination efficiency as determined by immunohistochemistry staining. There is a significant loss of Zbtb17 expression in cardiac myocyte nuclei in cKO animals (red: F-actin; green: Zbtb17; blue: DAPI). (D) Western blot analysis of Zbtb17 protein expression in cKO-Zbtb17 animals. (E) Analysis of apoptosis via TUNEL assay and caspase 3 positivity (pink: TUNEL positive nuclei; DAPI: nuclear staining; red: caspase 3; green: F-actin).

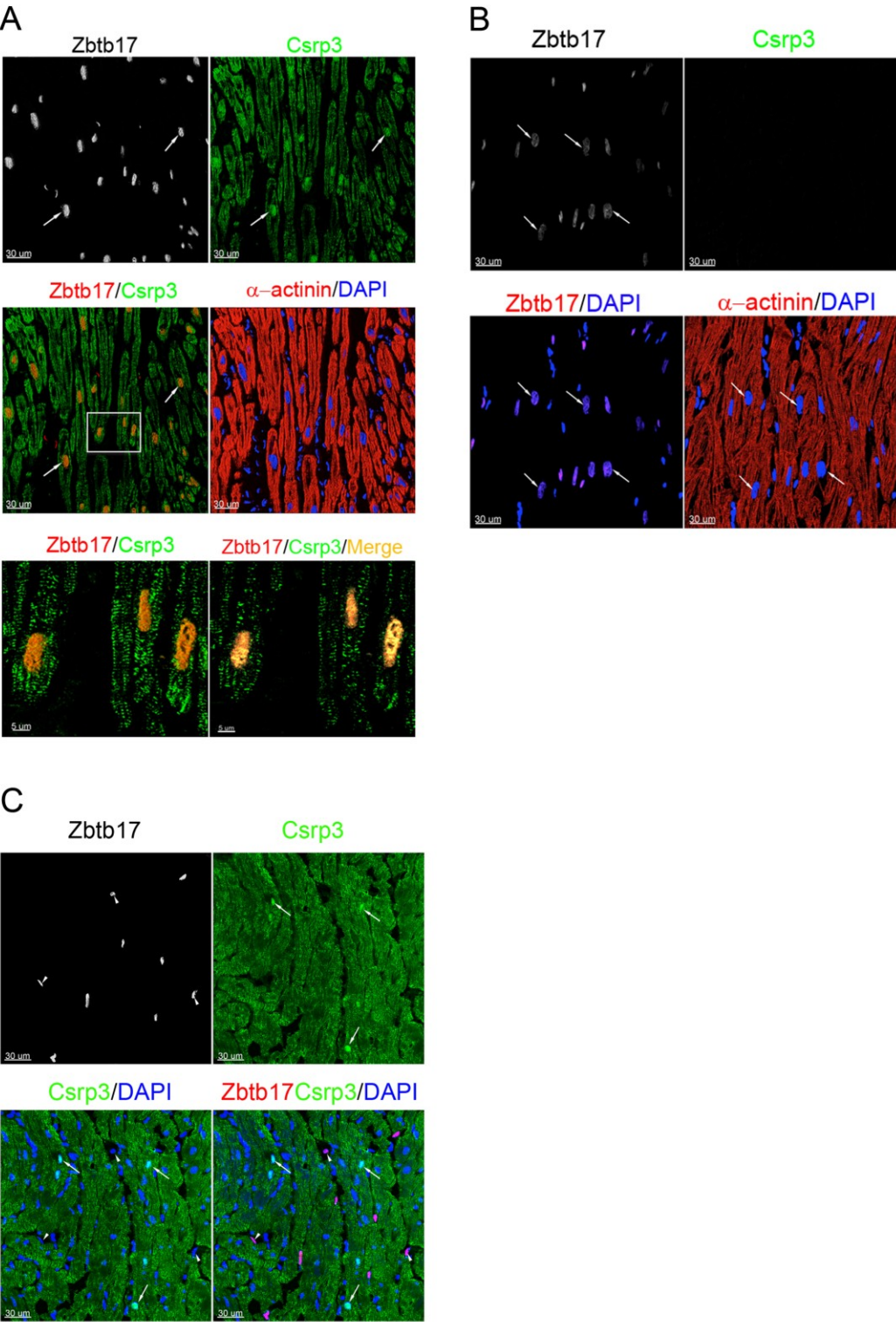
Supplemental Figure 4. Characterization of TG-ZBTB17 animals

(A) Western blot analysis of ZBTB17 in ZBTB17 transgenic animals. The FLAG tagged transgenic protein has a slightly higher molecular mass than the endogenous ZBTB17 protein (upper panel). Quantification of Western blot analysis (WT: wildtype, TG: transgenic, n = 3 per group, data normalized to GAPDH expression; *p<0.05) (lower panel). **(B)** Fibrosis as determined by PSR staining in TG-ZBTB17 animals. **(C)** ZBTB17 protein localization in FLAG tagged TG-ZBTB17 animals. FLAG-tagged ZBTB17, like endogenous Zbtb17, localizes to cardiac myocyte nuclei. **(D)** Negative control (red: anti-FLAG antibody; green: F-actin, blue: DAPI (nuclear staining). All experiments were performed using TG-ZBTB17 line 10.

Supplemental Figure 5. *Ppp3cb* and *Nfatc2* mRNA expression in cKO

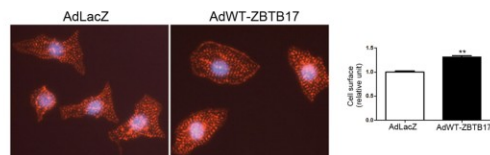
(A) Decreased *Ppp3cb* and *Nfatc2* mRNA expression levels in cKO (experiments were performed twice, n = 3 per group)

Supplemental Figure 1

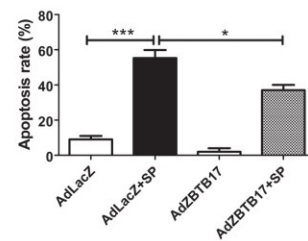


Supplemental Figure 2

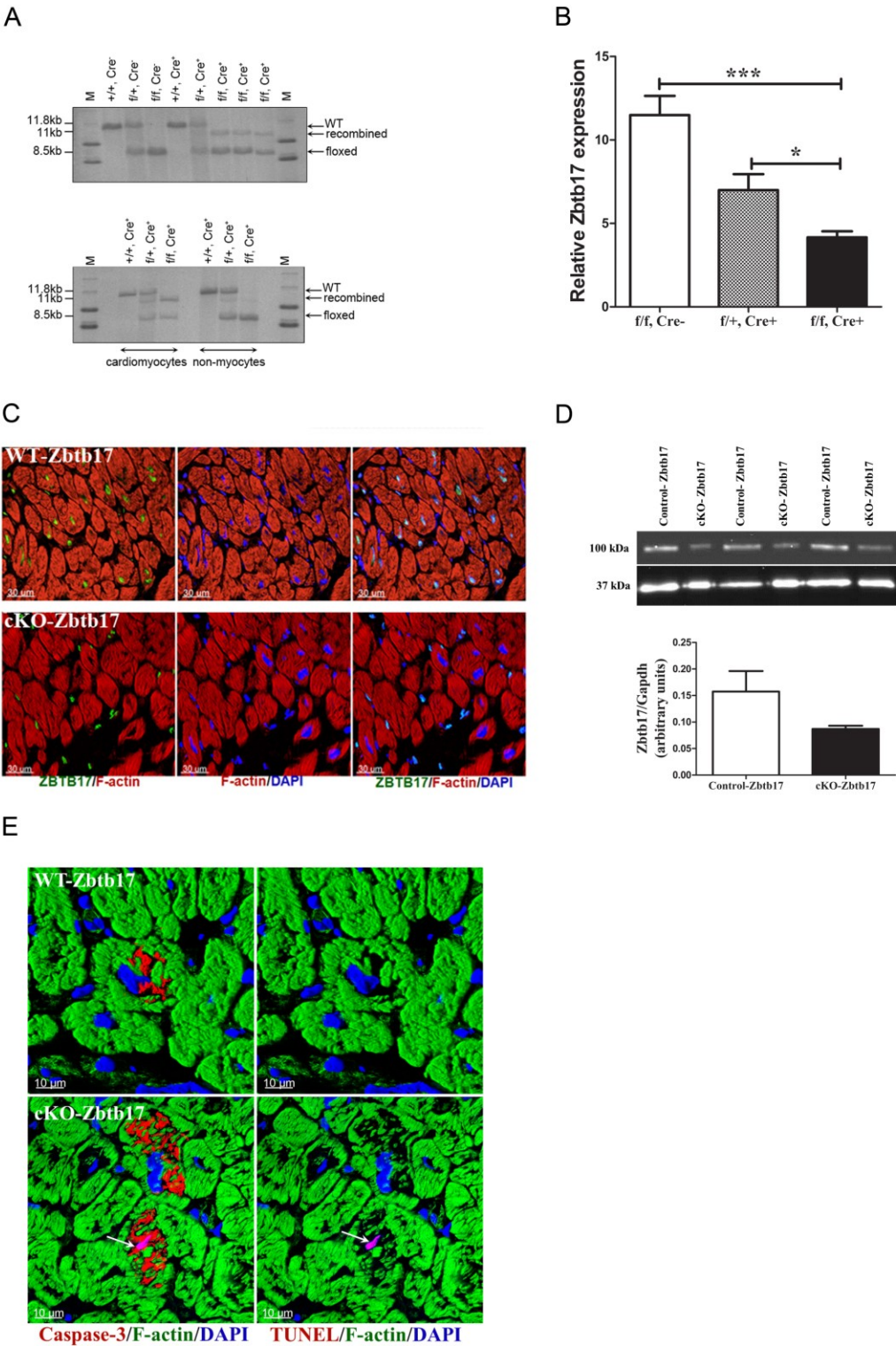
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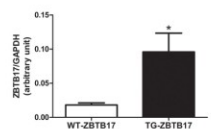
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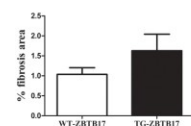
Supplemental Figure 3



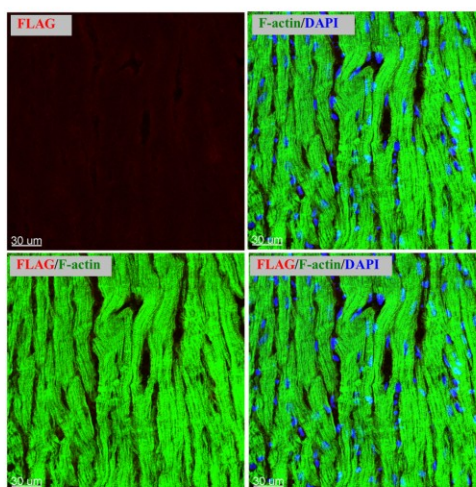
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WT-ZBTB17 TG-ZBTB17

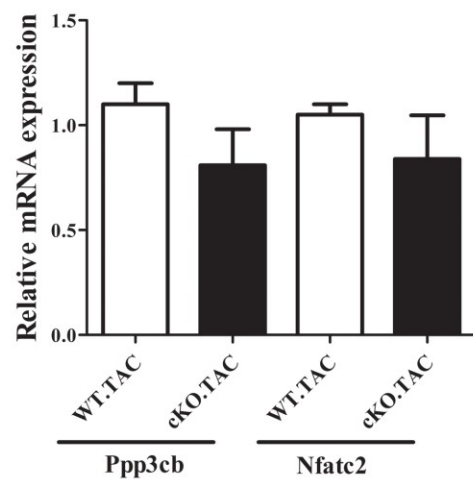


D



Supplemental Figure 5

A



Supplemental Table 1. Echocardiography of TG-ZBTB17/KO-Ppp3cb animals

Echocardiography	LVID;s (mm)	LVID;d (mm)	EF (%)	LV Wall mass/BW (mg/g)
WT-ZBTB17 (n=5)	2.56	3.61	57.02	3.36
TG-ZBTB17 (n=11)	1.99*	3.44	73.76**	4.87*
TG-ZBTB17/KO-Ppp3cb (n=5)	2.01	3.53	74.98 [†]	3.18 [#]

Analysis of the spontaneous phenotype of TG-ZBTB17 and TG-ZBTB17/KO-Ppp3cb double transgenic animals by echocardiography (* $P < 0.05$, ** $P < 0.01$ vs WT-ZBTB17; [†] $P < 0.05$ vs WT-ZBTB17, [#] $P < 0.05$ vs TG-ZBTB17).

Supplemental Table 2. List of primers used in this study

Gene name	Primer name	Primer sequence	Description
Ppp3cb	mCnA β -forward	tgg gat acc cag ggt tga tg	qRT-PCR
	mCnA β -reverse	aat tgg cca tgg atg tca cc	qRT-PCR
Ppp3cb promoter	m_ppp3cb_pro_F	gagtgactggctgcctcc	ChIP-qPCR
	m_ppp3cb_pro_R	cacaggcaaagcaggaaaga	ChIP-qPCR
Nfatc2	mNFATc2-forward	ggg ttc ggt gag tga cag tt	qRT-PCR
	mNFATc2-reverse	cag gaa ggc act tgg gta aa	qRT-PCR
Zbtb17	miz1-P3-forward	cgt tga ctt caa ggc tca ca	qRT-PCR
	miz1-P4-reverse	cgt cca cgt tct cag ggc taa	qRT-PCR
Nppa	RT-m-anf-F	acctgctagaccacctggaggag	qRT-PCR
	RT-m-anf-R	ccttggtgttatcttcggtaccg	qRT-PCR
Gapdh	mGAPDH-F	cgtcccgtagacaaaatggt	qRT-PCR
	mGAPDH-R	ttgatggcaacaatctccac	qRT-PCR

Supplemental Table 3. Primary antibodies used in this study

Primary Antibodies	Companies (Catalogue #)
Caspase 3 (polyclonal)	Cell Signalling (#9661)
Collagen I (polyclonal)	Rockland (600-401-103-0.5)
Csrp 3/Mlp (polyclonal)	Santa Cruz Biotechnology (sc-30274)
DAPI (nuclear staining)	Vector Laboratories (H-1200)
Dystrophin (H-300)	Santa Cruz Biotechnology (sc-15376)
F-actin (Phalloidin)	Sigma (P1951)
anti-FLAG (monoclonal)	Sigma (F3165)
Miz1 (H-190)	Santa Cruz Biotechnology (sc-22837)
GAPDH	Sigma (9545)
Kir2.1	Santa Cruz Biotechnology (sc-28633)
alpha-actinin (monoclonal, clone EA.53)	Sigma (A7811)
Telethonin (G-11)	Santa Cruz Biotechnology (sc-25327)