

Title page

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

Buyandelger et al., ZBTB17 mutations cause cardiomyopathy

Authors

Byambajav Buyandelger, PhD^{1,2}; Roddy Walsh, PhD¹; Sawa Kostin, PhD³; Catherine Mansfield, PhD¹; Onjee Choi, PhD¹; Angharad M. Roberts, PhD¹; James S. Ware, PhD¹; Francesco Mazzarotto, PhD¹; Francesco Pesce, PhD¹; Rachel Buchan, PhD¹; Rivka L. Isaacson, PhD⁴; Josee Vouffo, PhD²; Sylvia Gunkel, PhD²; Gudrun Knöll^{1,2}; Sara J. McSweeney, PhD¹; Heming Wei, PhD^{20,21}; Andreas Perrot, MD^{5,6}; Conny Pfeiffer²; Mohammad Reza Toliat, MD⁷; Kristina Ilieva⁴; Ewelina Krysztofinska⁴; Marina M. López-Olañeta⁹; Jesús M. Gómez-Salinerio⁹; Albrecht Schmidt²; Keat-Eng Ng, PhD¹; Niels Teucher, MD²; Ju Chen, PhD¹⁰; Martin Teichmann, PhD¹¹; Martin Eilers, PhD¹²; Wilhelm Haverkamp, MD⁶; Vera Regitz-Zagrosek, PhD¹³; Thomas Braun, PhD³; Dudley J. Pennell, MD¹⁴; Ian Gould, PhD¹⁵; Paul J.R. Barton, PhD¹⁴; Enrique Lara-Pezzi, PhD^{1,9}; Sebastian Schafer^{22,23}; Norbert Hubner^{22,23}; Leanne A. Felkin¹⁸; Declan P. O'Regan¹⁶; Enrico Petretto, PhD¹⁶; Thomas Brand, PhD¹⁸; Hendrik Milting, PhD¹⁷; Peter Nürnberg, PhD^{7,8,19}; Michael D. Schneider, PhD¹; Sanjay Prasad, MD¹⁴; Stuart Cook*, PhD^{14,20,21}; and Ralph Knöll*, MD. PhD^{1,2}

Conflict of interest

The authors have declared that no conflict of interest exists.

Affiliations

¹National Heart & Lung Institute, Faculty of Medicine, Imperial College London, Hammersmith Campus, Imperial Centre for Translational and Experimental Medicine, London W12 0NN, UK.

²Heart Centre, Georg-August University of Göttingen, Robert-Koch-Str. 40, 37075 Göttingen, Germany.

³Max-Planck-Institute for Heart and Lung Research, 61231 Bad Nauheim, Germany.

⁴Division of Molecular Biosciences, Department of Life Sciences, Faculty of Natural Sciences, Imperial College London, London, UK.

⁵Charité-Universitätsmedizin Berlin, Experimental and Clinical Research Center (ECRC), a joint cooperation between the Charité Medical Faculty and the Max-Delbrück Center for Molecular Medicine, 13125 Berlin, Germany.

⁶Charité-Universitätsmedizin Berlin, Dept. of Cardiology at Campus Virchow-Klinikum, 13353 Berlin, Germany.

⁷Cologne Center for Genomics, University of Cologne, Cologne, Germany.

⁸Cologne Excellence Cluster on Cellular Stress Responses in Aging-Associated Diseases (CECAD), University of Cologne, Cologne, Germany.

⁹Cardiovascular Development and Repair Department, Centro Nacional de Investigaciones Cardiovasculares (CNIC), Melchor Fernández Almagro 3, 28029 Madrid, Spain.

¹⁰Department of Medicine, University of California at San Diego (UCSD), 9500 Gilman Drive, San Diego, CA, USA.

¹¹Institut Européen de Chimie et Biologie, INSERM U869, Bordeaux, France.

¹²Theodor Boveri Institute, Biocenter, University of Würzburg, Am Hubland, 97074 Würzburg, Germany.

¹³Berlin Institute of Gender in Medicine, Charité-Universitätsmedizin Berlin, Germany.

¹⁴NIHR Cardiovascular Biomedical Research Unit at Royal Brompton & Harefield NHS Foundation Trust and Imperial College London, London SW3 6NP, UK

¹⁵Department of Chemistry and Institute of Chemical Biology, Imperial College London, South Kensington, SW7 2AZ, UK.

¹⁶Medical Research Council (MRC) Clinical Sciences Centre, Faculty of Medicine, Imperial College London, Hammersmith Hospital, Du Cane Road, London W12 0NN, UK.

¹⁷Ruhr-Universität Bochum, Herz & Diabeteszentrum NRW, Erich & Hanna Klessmann-Institut, Bad Oeynhausen, Germany.

¹⁸Heart Science Centre, National Heart and Lung Institute, Imperial College London, Harefield Middlesex UB9 6JH, UK.

¹⁹Center for Molecular Medicine Cologne (CMMC), University of Cologne, Cologne, Germany.

²⁰Duke-NUS Graduate Medical School, 8 College Road, 169857 Singapore.

²¹National Heart Centre, Singapore, 17 Third Hospital Avenue, 168752 Singapore.

²²Max-Delbrück-Center for Molecular Medicine (MDC), 13125 Berlin, Germany.

²³DZHK (German Centre for Cardiovascular Research), Partner Site Berlin, Germany.

***Correspondence to:**

Professor Ralph Knöll, MD, PhD

Chief Scientist

AstraZeneca R&D Mölndal

R&D | Innovative Medicines & Early Development | Cardiovascular & Metabolic Diseases iMed

Pepparedsleden 1

SE-431 83 Mölndal, Sweden

Phone: (+46) (0) 706 165801

Email: ralph.knoell@astrazeneca.com

ICMC (Integrated Cardio Metabolic Centre)

Karolinska Institutet

Karolinska University Hospital in Huddinge (M54)

SE 141 86 Stockholm, Sweden

Email: ralph.knoell@ki.se

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Abstract

Background- Mutations in sarcomeric and cytoskeletal proteins are a major cause of hereditary cardiomyopathies, but our knowledge remains incomplete as to how the genetic defects execute their effects.

Methods and Results- We used cysteine and glycine-rich protein 3 (CSRP3), a known cardiomyopathy gene, in a yeast two-hybrid screen and identified zinc finger and BTB domain containing protein 17 (*ZBTB17*) as a novel interacting partner. *ZBTB17* is a transcription factor that contains the peak association signal (rs10927875) at the replicated 1p36 cardiomyopathy locus. *ZBTB17* expression protected cardiac myocytes from apoptosis *in vitro* and in a mouse model with cardiac myocyte-specific deletion of *Zbtb17*, which develops cardiomyopathy and fibrosis after biomechanical stress. *ZBTB17* also regulated cardiac myocyte hypertrophy *in vitro* and *in vivo* in a calcineurin-dependent manner.

Conclusions- We revealed new functions for *ZBTB17* in the heart, a transcription factor which may play a role as a novel cardiomyopathy gene.

Key words- Heart Failure; Cardiomyopathy; Genetics

Introduction

Mutations in over 50 genes, mostly encoding sarcomeric and Z disc proteins, cause hypertrophic cardiomyopathy (HCM) and dilated cardiomyopathy (DCM) that lead to heart failure.¹ Genetic variation in a Z disc gene can cause either HCM or DCM perhaps due to pleiotropic effects on survival and/or hypertrophic pathways.² The muscle LIM protein (MLP, CSRP3) is a Z disc protein involved in cardiac mechanosensation that is important for myocyte-specific survival pathways through interactions with other Z disc proteins.³⁻⁵ *CSRP3* mutations have been found in both HCM and DCM patients^{3,4,6} and we used protein-protein interaction studies to identify new CSRP3-interacting partners that may be important for cardiac myocyte survival and/or cardiomyopathy.

Methods

All primers and antibodies used are listed in Supplemental Tables. Tissue culture experiments were performed using primary neonatal and adult rat cardiac myocytes and animal experiments were performed using conditional knockout (cKO)-Zbtb17, overexpressing transgenic (TG)-ZBTB17 and TG-ZBTB17/knockout (KO)- protein phosphatase 3, catalytic subunit, beta isozyme (calcineurin A β , Ppp3cb) animals. Yeast two-hybrid, Western blot, quantitative Real-time PCR (qRT-PCR) and other assays were performed as described elsewhere.^{3-5, 7} Statistical evaluations were performed by Fisher exact, ANOVA or Student's t-test. (For more details please see the online supplemental information.)

Results

MLP interacts with ZBTB17

Yeast two-hybrid screens using CSRP3 as bait³ classified α -actinin and telethonin as CSRP3-interacting proteins that are important determinants of cardiac function^{5, 8} and also identified ZBTB17 (Figure 1A). ZBTB17 is a member of the poxvirus and zinc-finger (POZ or BTB) domain/zinc-finger transcription factor family with no previously described role in the heart.⁹ We confirmed the CSRP3-ZBTB17 interaction by co-immunoprecipitation *in vivo* (Figure 1B), cross-linking of recombinant proteins (Figure 1C) and immunohistochemistry studies (Figure 1D and Supplemental Figure 1). These data identify ZBTB17 as a novel CSRP3 interacting protein.

Analysis of a human ZBTB17 variant (T106M)

ZBTB17 is encoded on human chromosome 1 at the replicated 1p36 cardiomyopathy locus.¹⁰⁻¹² The peak DCM association at this locus (rs10927875, $P = 1.3 \times 10^{-7}$)¹² is located in intron 2 of *ZBTB17*, with further evidence of association of this SNP with DCM in Chinese population.^{12, 13} Considering rare polymorphisms (allele frequency <0.1%; population prevalence of cardiomyopathies ~1:500¹), we identified a p.T106M variant in the Exon variant server (ESP). The p.T106M variant could not be stably expressed in cardiac myocytes (Figure 2A), bound poorly to CSRP3 (Figure 2B) and caused structural abnormalities to the POZ domain (Figure 2C, D), and may represent a rare haplo-insufficient polymorphism. Together, these data point to *ZBTB17* as a possible disease gene at the 1p36 cardiomyopathy locus and suggest a role for a

transcription factor in heart failure, as recently shown for left ventricular non compaction cardiomyopathy.¹⁴

Analysis of ZBTB17 function *in vitro*

To study function *in vitro* we overexpressed *ZBTB17* in cardiac myocytes and found that it induced hypertrophy (Figure 3A, B and Supplemental Figure 2A). CSRP3 and other CSRP3-interacting proteins have important roles in cardiac myocyte survival, and this was also observed for *ZBTB17* (Figure 3C and Supplemental Figure 2B). Calcineurin activity is of central importance for pro-hypertrophic signaling and myocyte survival^{15, 16} and in both the hypertrophy and the cell death experiments inhibition of calcineurin attenuated the effects of *ZBTB17*, suggesting an interaction (Figure 3A, C). Hence, *ZBTB17* regulates cardiac hypertrophy and cell survival, a recognized dual-property of genes that are of central importance for cardiac myocyte biology.¹⁵⁻¹⁸

Analysis of ZBTB17 function *in vivo*

Mice globally deleted for *Zbtb17* die due to a gastrulation defect.¹⁹ We therefore crossed floxed *Zbtb17* mice²⁰ with the MLC2v Cre deleter line²¹ to test *Zbtb17* gene function in cardiac myocyte-specific conditional knockout mice (cKO) (Supplemental Figure 3A-D). cKO mice were viable and, in the absence of a spontaneous functional cardiac phenotype, we employed the established model of transverse aortic constriction (TAC)⁵ to unveil potential gene effects. After

TAC the left ventricles of cKO mice became dilated and exhibited impaired contractile performance, which are the cardinal features of DCM (Table 1). As compared to controls, failing cKO hearts had higher apoptotic events (8.5-fold, $P < 0.001$), increased activated caspase 3 (6.3-fold, $P < 0.001$) and marked replacement fibrosis (Figure 4A, Supplemental Figure 3E and Table 3). These data show that *Zbtb17* modulates biomechanical stress-induced apoptosis and interstitial fibrosis *in vivo*, which are important in the pathobiology of both HCM and DCM.

To begin to understand the molecular mechanisms by which ZBTB17 regulates cardiac myocyte survival, based on the functions of ZBTB17 in other tissues¹⁹, we applied apoptosis gene-focused PCR array analysis combined with genome wide chromatin immunoprecipitation-chip (ChIP-chip). In cKO hearts subjected to TAC, pro-apoptotic gene expression was increased with evidence of direct regulation of a subset of these genes in ChIP-chip studies (Table 2). Hence ZBTB17 regulates a transcriptional program that protects cells against apoptosis.

In addition, ZBTB17 overexpression drives hypertrophy *in vitro* (Figure 3). To examine further the effects of *ZBTB17* on hypertrophy we overexpressed *ZBTB17* in mice using a cardiac myocyte specific promoter²² (Supplemental Figure 4). Transgenic mice exhibited spontaneous cardiac hypertrophy with enlarged cardiac myocytes and increased *Nppa* expression (Table 3; Figure 4B, C). *In vitro* experiments suggested a link between ZBTB17 and calcineurin and ChIP-chip data showed binding of *Zbtb17* to the calcineurin A β (*Ppp3cb*) promoter that we confirmed by ChIP-qRT-PCR and reporter assays (Figure 4D, E). There was increased expression of *Ppp3cb* and *Nfatc2* in TG-ZBTB17 hearts and decreased expression in cKO (Figure 4F and Supplemental Figure 5A). We crossed *ZBTB17* transgenic mice into a *Ppp3cb* deficient

background that prevented cardiac myocyte hypertrophy confirming the interaction of ZBTB17 with the calcineurin pathway (Figure 4B and Supplemental Table 1).

Discussion

CSRP3 mutations cause heart failure in various animal models^{4, 23} and have been identified in various patients affected by DCM^{3, 24} or HCM²⁵⁻²⁷. Although many cardiomyopathy and heart failure models have been developed, the molecular mechanisms which link sarcomeric and Z disc proteins to these phenotypes remain not well understood. Here we show that *CSRP3* interacts with *ZBTB17* and provide the first detailed analysis of this transcription factor in the cardiovascular system. In particular we demonstrate that *ZBTB17* causes cardiac myocyte hypertrophy and is essential for cell survival. The extent of hypertrophy in cardiac myocytes is comparable to the effects seen with trophic signals including mitogenic serum in these post-mitotic cells.^{28, 29} So far, *ZBTB17* is the only known transcription factor to interact with *CSRP3* and to be expressed in cardiac myocytes (for a brief review:³⁰). The effects of *ZBTB17* on hypertrophy and survival are not restricted to the *in vitro* situation, but can also be observed in genetically altered animals under *in vivo* conditions. Interestingly, *ZBTB17* overexpressing transgenic animals did not develop heart failure due to massive increase in apoptosis as frequently seen in other genetically altered mouse models (for a review:³¹).³²⁻³⁴

The combination of ChIP-chip, whole gene expression- and apoptosis array data clearly point to an important role of *ZBTB17* in orchestrating a cardio-protective gene expression program. This is indicated by the fact that pro-apoptotic genes were consistently upregulated, while anti-apoptotic genes were down regulated in cKO hearts following biomechanical stress (Table 2).

We also show that *ZBTB17* targets various calcineurin/NFAT genes and activates this pathway. This notion is supported by our whole gene expression analysis, ChIP-chip assays, analysis of the calcineurin/NFAT pathway activation, promoter assays and by the significant loss of hypertrophy when TG-*ZBTB17* animals are crossed into the calcineurin *A β* deficient background

(Figure 3, 4D and E). These data are also supported by our finding of enhanced calcineurin *Aβ* and *Nfatc2* mRNAs in hearts of *ZBTB17* transgenic animals and decreased calcineurin *Aβ* and *Nfatc2* mRNAs in hearts of *Zbtb17* cKO animals after TAC (Figure 4F and Supplemental Figure 5A).

CSRP3 translocates into the nucleus upon TAC³⁵ and is essential for the adaptation of cardiac myocytes to biomechanical stress.³⁶ CSRP3, like telethonin, is primarily expressed in muscle tissues. By interacting with ZBTB17, CSRP3 plays a role in the initiation of myocyte specific survival pathways or mechanoptosis (mechanosensitive types of cell death⁵). This notion is supported by the fact that *Csrp3* deficient hearts exhibit increased rates of apoptosis³⁷ hence the interaction of CSRP3 with ZBTB17 can also be described as a form of Z disc transcriptional coupling.³⁸

Calcineurin/NFAT signaling is an important mediator of cardiac hypertrophy^{39, 40} and this pathway is also known to exert cardio-protective effects by avoiding apoptosis during ischemia reperfusion¹⁵, which emphasizes the effects of ZBTB17 on cell survival.

CSRP3 directly interacts with calcineurin and is required for its activation⁴¹, thus, by interacting with ZBTB17, CSRP3 introduces an additional level of control which ensures a medium level of calcineurin/NFAT activation (Figure 4G). For a long time c-myc is known to be induced during TAC⁴², but its downstream effects in cardiac myocytes remained elusive. However, c-myc is known to inhibit ZBTB17⁴³ and therefore may have adverse effects on survival and hypertrophy in cardiac myocytes. Interestingly, CSRP3 interacts with the aminoterminal POZ domain which is predicted to interfere with ZBTB17 tetramerization.

An additional layer of complexity is added by the fact that ZBTB17 can be phosphorylated at position S428 by the Akt kinase, which is another important mediator of hypertrophy.⁴⁴ This

post-translational modification enables the interaction of ZBTB17 with 14-3-3 η and leads to its inactivation, thus avoiding prolonged activation of ZBTB17, which can also have adverse effects.⁴⁵

In the Exome Variant Server (EVS) database there are 10 T106M variants annotated out of at least 13006 alleles of European-American and Afro-American origin combined. The EVS does not provide data on individual phenotypes, but the frequency for this variant is certainly very low and a disease causing or at least modifying role cannot be excluded. Nevertheless, a two-hit hypothesis whereby a structural perturbation combines with a defect in a ZBTB17 mediated cell survival pathway could also lead to heart failure.

As a result we expect this variant to impair calcineurin dependent hypertrophy and being unable to efficiently protect cardiac myocytes from apoptosis. However, this variant may also have specific effects on remodeling and hypertrophy⁴⁶ by specifically affecting protein degradation pathways, for example via the ubiquitin proteasome system (UPS)⁴⁷, and aside from affecting its interaction with CSRP3 (Figure 2B), may specifically interfere with other protein / protein interactions.

ZBTB17 is a transcription factor that contains the peak association signal (rs10927875) at the replicated 1p36 cardiomyopathy locus. Although other genes in the genomic region of *ZBTB17*, such as *HSPB7* and *CLCNKA*, have been suggested as possible candidate genes by GWAS^{10, 48}, *ZBTB17* might well be another novel DCM-associated gene. However, except for this report, the effects of *ZBTB17* on the cardiovascular system have never been analyzed before. It is interesting to note that *HSPB7* which encodes heat shock protein 27, a factor involved in the immediate stress response, *CLCNKA*, which encodes a K_a renal chloride channel involved in the

regulation of hypo-osmotic cell stretch, as well as *ZBTB17* are all involved in cellular primary stress response and survival signaling.

At least 50 single genes have been identified as linked to familial DCM, some of which encode proteins of the sarcomere⁴⁹, costamere, Z disc^{3, 4} and nuclear membrane⁵⁰ while others function as phosphatases and transcriptional activators (EYA4).^{1, 51} Unfortunately not much is known about how these effects are linked to changes in gene transcription. An elegant recent study found pro-fibrotic gene expression profiles in hypertrophic cardiomyopathy mouse models and identified Transforming growth factor beta 1 (*TGFβ1*) as the initiating event, but the underlying transcriptional events remained elusive.⁵² However, *ZBTB17* may well play a role in these genetic circuits. Defective Z disc mediated survival signaling may also contribute to the DCM phenotype observed in patients carrying truncating Titin (*TTN*) mutations.⁴⁹

In summary, we identify *ZBTB17* as a candidate for a new cardiomyopathy gene, which may also be important for heart failure syndromes in general, and suggest that its primary function is to protect cardiac myocytes from apoptosis⁵ through modulation of both hypertrophic and cell death pathways (Figure 4G).

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Disclosures

None

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Figure legends

Figure 1. A, Analysis of the interaction between CSRP3 and ZBTB17 by yeast two-hybrid assays. No interaction between proteins when the ZBTB17-POZ domain is deleted (lower right quadrant). A total of 10^6 clones were screened and 650 positive clones were identified³, one of which encoded the 5' sequence of *Zbtb17*. **B,** Immunoprecipitation of ZBTB17 from heart lysates results in the co-precipitation of CSRP3. Input indicates mouse heart extract, a Csrp3 antibody (Santa Cruz Biotechnology; sc-30274) has been used for immunoprecipitation and a well characterized Zbtb17 (Santa Cruz H-190; sc 22837) antibody has been used to detect the protein. No antibody or an unrelated anti-Kir2.1 antibody served as negative controls. **C,** Cross-linking experiments of recombinant proteins confirm binding between ZBTB17 and CSRP3. Top panel: With increasing concentration of cross-linking reagent the individual components decrease while a large complex emerges. The complex contains multiple copies of each protein reflecting the oligomeric nature of the individual components. Bottom left: Positive control showing cross-linking between CSRP3 and Telethonin (TCAP), a known interaction. Bottom right: Negative controls - we attempted to cross-link CSRP3 separately with two different proteins that have no documented association but are known to cross-link well to their binding proteins. These were small glutamine-rich tetratricopeptide repeat-containing protein alpha (SGTA) and HLA-B associated transcript 3 (BAT3). As expected, increasing cross-linking reagents showed no binding between CSRP3 and the negative control proteins but only the usual CSRP3 oligomers indicated with stars. **D,** Co-localization of ZBTB17 and CSRP3 in the nuclei of human cardiac myocytes. Arrows indicate typical cardiac myocyte nuclei that are positive for both ZBTB17 and CSRP3. Lower left panel is the three-dimensional image of the boxed region (left middle panel). Lower right panel is a three-dimensional co-localized color intensity image

of ZBTB17 with CSRP3: mean percentage of nuclear ZBTB17 co-localized with nuclear CSRP3 = $79.4\% \pm 5.5$; mean Pearson's coefficient of co-localized volumes = 0.88 (n = 4, ~125 nuclei per heart).

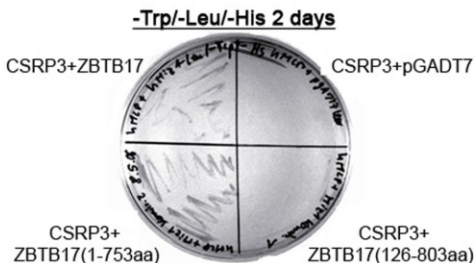
Figure 2. A, Analysis of WT- and T106M-ZBTB17 proteins via adenoviral overexpression in NRCMs. While WT-ZBTB17 protein is readily detectable, ZBTB17:p.T106M was not and is most likely unstable in eukaryotic cells. **B,** Interaction of T106M-ZBTB17 with CSRP3 as studied via cross-link interaction. CSRP3 interacts with WT-ZBTB17 (left), but interacts less well with T106M-ZBTB17 (right). **C,** NMR ^1H - ^{15}N HSQC spectrum of WT (red) and T106M (black) ZBTB17-POZ domain with pertinent residues assigned based on predicted chemical shift. The mutation causes significant structural disruption to the POZ domain. **D,** The available ZBTB17-POZ structure was used to model the T106M mutation. 50 nanoseconds of simulation of the WT and the T106M structures which have been run as the full quadrameric structures as in the PDB 2Q81. Red indicates the native and yellow the mutant ZBTB17, spheres represent the T and M residues. It can clearly be seen that the structures of the alpha-6, where the mutation is located, have changed. Also the simulations reveal that there is a much higher level of structural mobility for the mutant case. The mutation causes significant structural disruption to the POZ domain, a finding consistent with our NMR data, arrowheads indicate position 106, arrows indicate a position far away).

Figure 3. **A**, Analysis of ZBTB17 effects on hypertrophy as determined by impedance measurements. Bar graphs represent cell index values after 24 hours (n = 10, experiments done in duplicate). Dotted lines indicate cell indices in calcineurin inhibition experiments (CsA: 0.2 μ M; n = 4; * P <0.05, ** P <0.01). **B**, Representative images of rat cardiac myocytes with and without ZBTB17 adenoviral gene transfer. **C**, Effects of *ZBTB17* overexpression on doxorubicin (DOX, 1 μ M)-induced cell death analysis (n = 7). Dotted lines indicate data from inhibition experiments (CsA: 0.2 μ M; n = 4; * P <0.05)

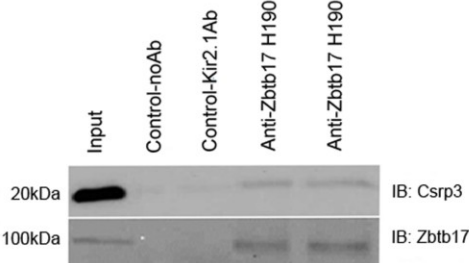
Figure 4. **A**, Representative micrographs of fibrosis staining of mouse hearts following sham or TAC procedures and quantification of fibrosis (4 fields; 2 independent animals per group). **B**, Analysis of cardiac myocyte cross sectional area under spontaneous conditions in WT, cKO-Zbtb17, calcineurin $A\beta^{-/-}$ (KO-Ppp3cb), TG-ZBTB17, TG-ZBTB17/KO-Ppp3cb (~150 cardiac myocytes per animal; n = 5 per group). **C**, Upregulation of the pro-hypertrophic biomarker Nppa in TG-ZBTB17 hearts (n = 3 per group). **D**, ChIP qRT-PCR analysis of Zbtb17 binding to the Ppp3cb promoter *in vivo*. **E**, Luciferase reporter assay of ZBTB17 activation of the Ppp3cb promoter via transient transfection. **F**, Increased *Ppp3cb* and *Nfatc2* mRNA expression levels in TG-ZBTB17 (n = 3 per group; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; error bars represent mean $\pm$ SD).

Figure 1

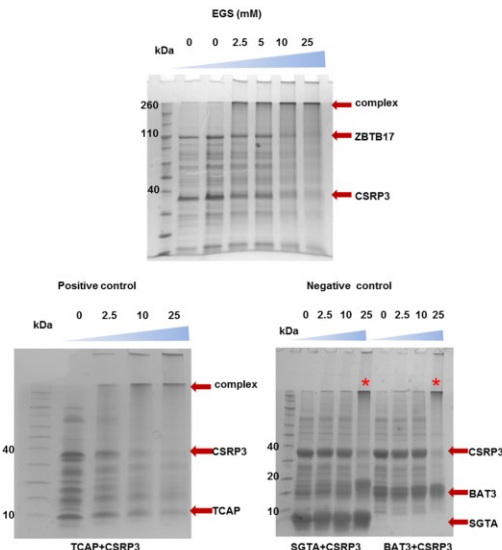
A



B



C



D

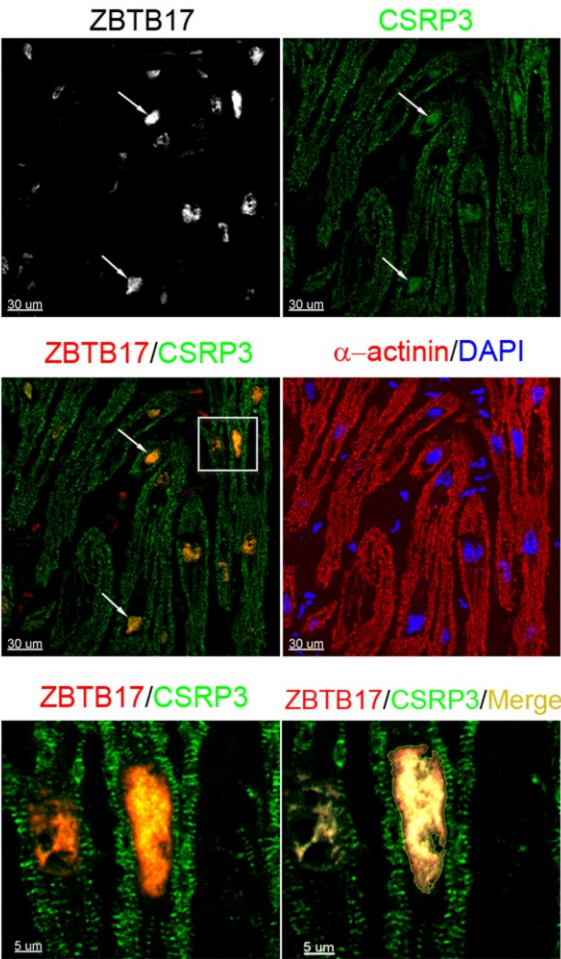


Figure 2

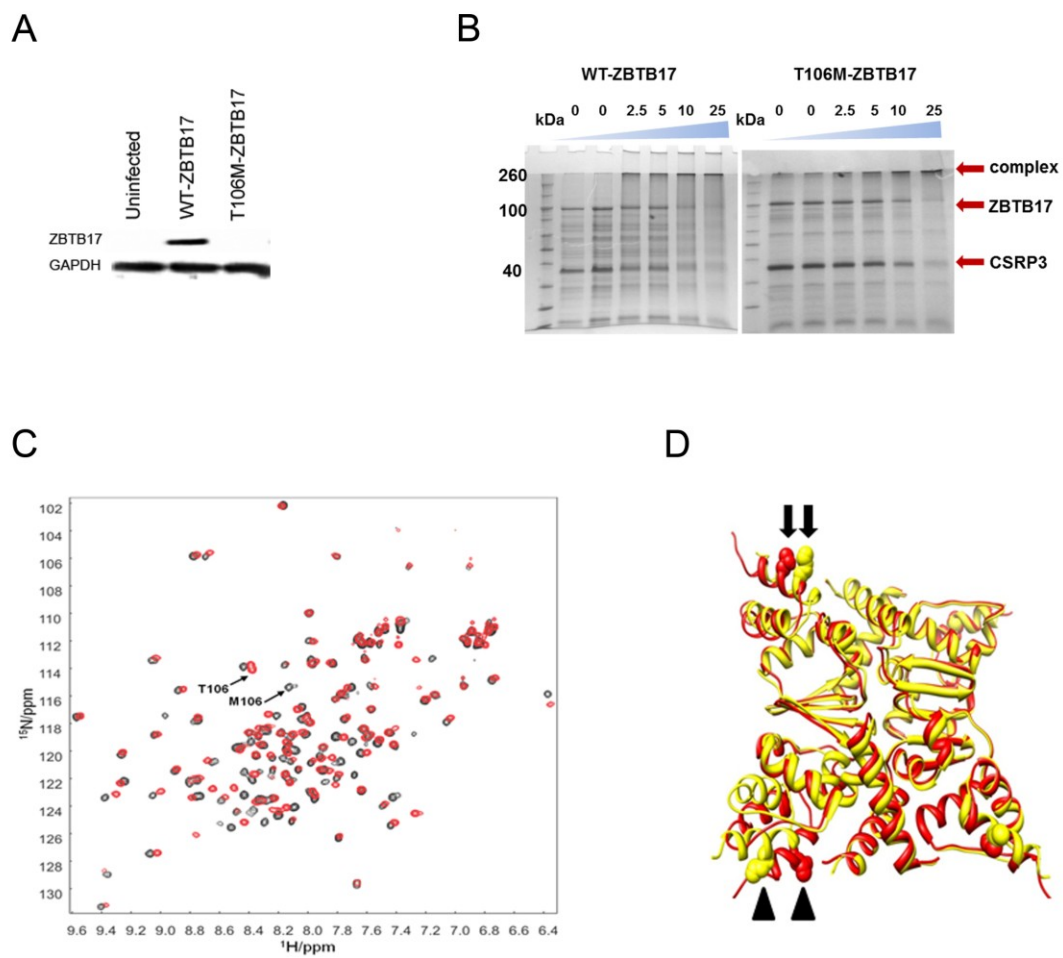
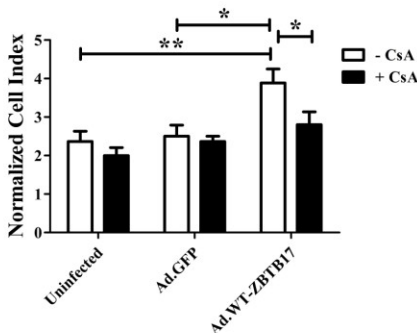
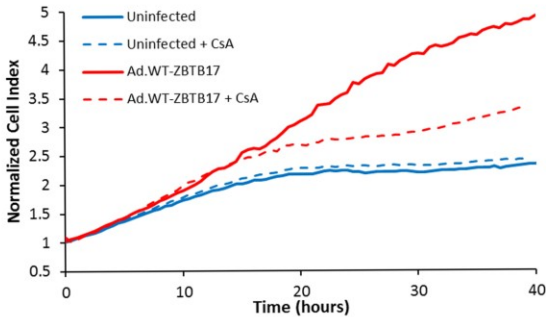


Figure 3

A



B



C

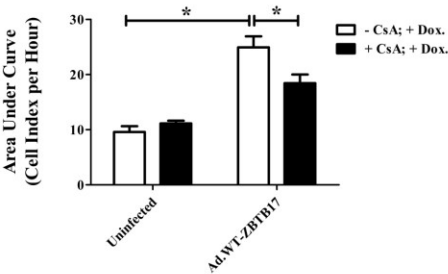
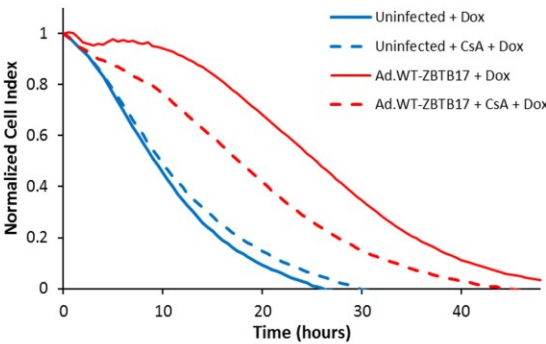


Figure 4

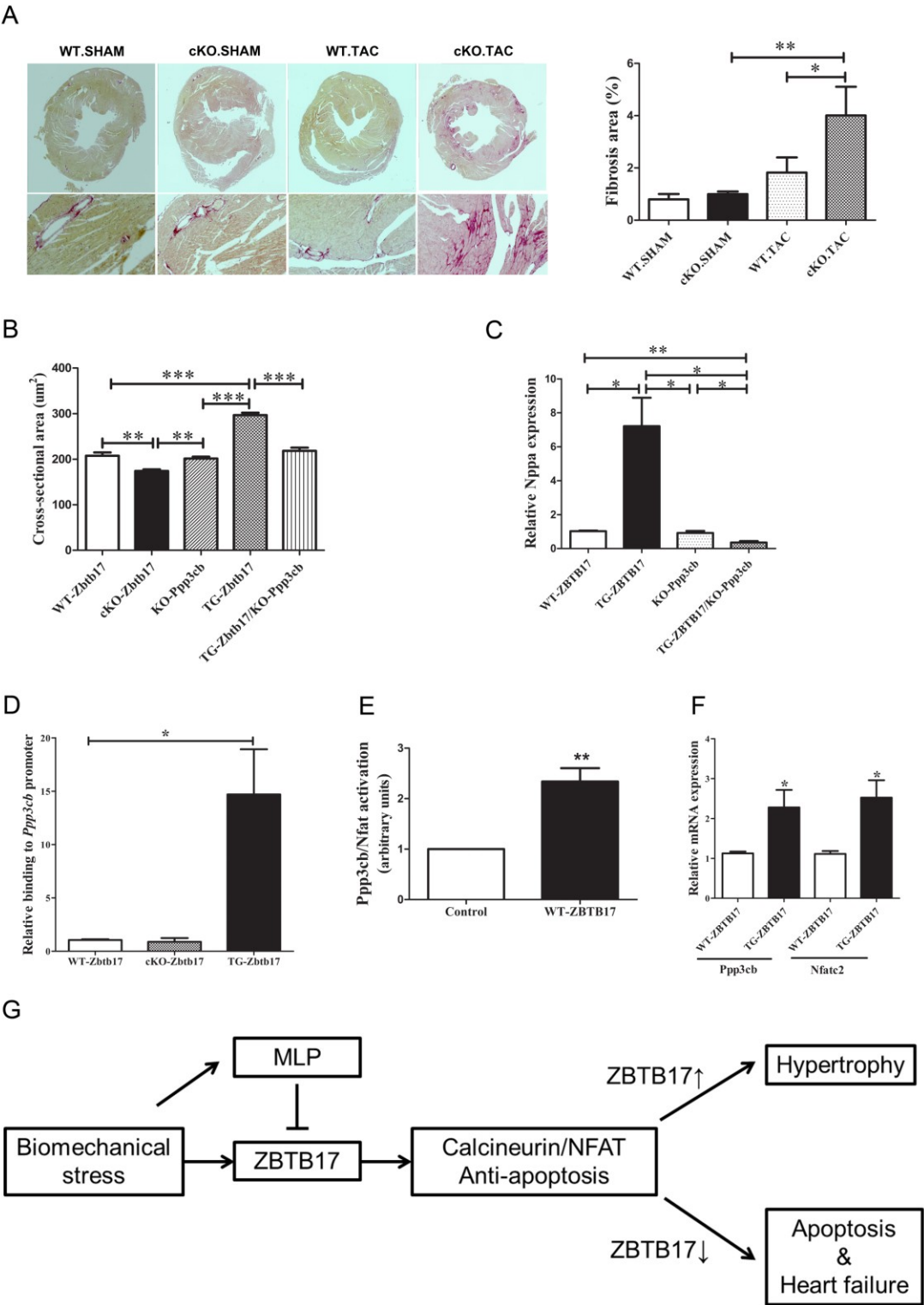


Table 1. Echocardiography of cKO-*Zbtb17* animals

Genotype/ Intervention	BW g	LVID;d mm	LVID;s mm	FS %	h/r	HW/B W mg/g	LV Vol;s	LV Vol;d	EF %
Control.SHAM	31.90	3.77	2.06	45.61	0.54	4.26	14.40	61.39	45.62
Control.TAC	31.01	3.44*	1.65*	52.01*	0.63*	5.03*	7.86*	49.04*	52.04*
cKO.SHAM	32.04	3.71	2.05	45.71	0.56	4.07	15.30	59.79	45.73
cKO.TAC	32.55	3.74 ^{††}	2.00 [†]	46.46 [†]	0.57	4.82	13.22 [†]	59.69 [†]	46.46 [†]

Functional data obtained via echocardiography 4 weeks after sham operations (SHAM) or 4 weeks after transverse aortic constriction (TAC) in *Zbtb17* cKO and control animals. After TAC cKO hearts enlarge (LVID;d and LVID;s) and decrease in function (%FS) (Control = flox/flox, Cre⁻; cKO = flox/flox, Cre⁺; * $P < 0.05$, ** $P < 0.01$ for Control.SHAM vs Control.TAC; [†] $P < 0.05$, ^{††} $P < 0.01$ for Control.TAC vs cKO.TAC; data = mean).

Table 2. Integrated analysis of apoptotic gene expression and ChIP-chip

Gene	Fold Change	p-value	Function	ZBTB17 Binding site (FDR < 0.05)
<i>Fadd</i>	0.4	0.0017	pro-apoptotic	Y
<i>Casp2</i>	2.2	0.0023	pro-apoptotic	Y
<i>Traf3</i>	1.6	0.0076	pro-apoptotic	Y
<i>Rnf7</i>	1.8	0.0106	anti-apoptotic	Y
<i>Casp14</i>	17.2	0.0255	pro-apoptotic	Y
<i>Casp1</i>	1.8	0.0280	pro-apoptotic	Y
<i>Bnip3</i>	1.6	0.0285	pro-apoptotic	Y
<i>Cradd</i>	1.8	0.0364	pro-apoptotic	Y
<i>Cd70</i>	4.3	0.0003	pro-apoptotic	N

Summary of significantly differentially expressed apoptotic genes and Zbtb17 binding sites of genes involved in cardiac myocyte programmed cell death in cKO animals as compared to control using qRT-PCR arrays. Apoptotic qRT-PCR array and ChIP-chip methods are described in the Supplementary Information. Abbreviations: FDR = false discovery rate; Y = ZBTB17 binding site; N = no ZBTB17 binding site at FDR < 0.05.

Table 3. In vivo analysis of TUNEL positive cells, activated caspase 3 and collagen I

Animals	TUNEL (n/1000 cardiomyocytes)		Activated caspase 3 (n/1000 cardiomyocytes)		Collagen I (% tissue area)	
	Control.TAC	cKO.TAC	Control.TAC	cKO.TAC	Control.TAC	cKO.TAC
1	1.04	11.35	3.53	17.73	5.89	10.71
2	1.73	12.55	3.86	21.81	6.63	12.53
3	1.49	9.41	4.01	19.55	7.22	16.24
4	2.38	15.44	3.37	19.92	5.14	11.25
5	0.95	13.87	3.88	25.77	7.73	15.02
6	1.72	8.08	1.85	23.19	6.85	13.09
mean ± SD	1.55 ± 0.5	11.78 ± 2.5***	3.42 ± 0.73	21.32 ± 2.63***	6.57 ± 0.85	13.29 ± 1.98***

Analysis of TUNEL positive cells, activated caspase 3 (n/1000 cardiomyocytes) and collagen I (percent tissue area); data = mean ± SD; Control.TAC: flox/flox, Cre⁻; cKO.TAC: flox/flox, Cre⁺; n=6 per group, *** $P < 0.001$).