**ZBTB17 (MIZ1) Is Important for the Cardiac Stress Response and a Novel Candidate Gene for Cardiomyopathy and Heart Failure**

Running title: Buyandelger et al., ZBTB17 plays a role in cardiomyopathy

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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, animal models
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. Genetic variation in a Z disc gene can cause either HCM or DCM perhaps due to pleiotropic effects on survival and/or hypertrophic pathways. 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. CSRP3 mutations have been found in both HCM and DCM patients 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 double transgenic (TG-ZBTB17/KO-Ppp3cb) protein phosphatase 3, catalytic subunit, beta isozyme (calcineurin Aβ, Ppp3cb) animals.

The procedures in animal studies have been followed in accordance with institutional guidelines. Human studies have been approved by an institutional review committee and that the subjects gave informed consent.

Yeast two-hybrid, Western blot, quantitative Real-time PCR (qRT-PCR) and other assays were performed as described elsewhere. Statistical evaluations were performed by non-parametric Mann-Whitney tests and unpaired Student t-tests. (For more details please see the online Supplemental information.)
Results

CSRP3 interacts with ZBTB17

Yeast two-hybrid screens using CSRP3 as bait classified α-actinin and telethonin as CSRP3-interacting proteins that are important determinants of cardiac function 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.

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. The peak DCM association at this locus (rs10927875, \(P = 1.3 \times 10^{-7}\)) is located in intron 2 of ZBTB17, with further evidence of association of this SNP with DCM in Chinese population.

Considering rare polymorphisms (allele frequency <0.1%; population prevalence of cardiomyopathies ~1:500), we identified a p.T106M variant in the Exome Variant Server (EVS). The p.T106M variant could not be stably expressed in cardiac myocytes (Figure 2A), bound poorly to CSRP3 (Figure 2B), 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.

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\textsuperscript{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.\textsuperscript{15-18}

**Analysis of ZBTB17 function in vivo**

Mice with global deletion of *Zbtb17* die due to a gastrulation defect.\textsuperscript{19} We therefore crossed floxed *Zbtb17* mice\textsuperscript{20} with the MLC2v Cre deleter line\textsuperscript{21} 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)\textsuperscript{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 2). 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 tissues\textsuperscript{19}, we combined our microarray data with apoptosis specific gene expression array analysis (qRT-PCR based,
n=84) in cKO.TAC and control.TAC mouse hearts. We used Gene Set Enrichment Analysis (GSEA)\textsuperscript{22} to test whether differentially expressed genes by genome-wide microarray analysis were significantly enriched in the set of differentially expressed genes detected by apoptosis specific qRT-PCR array analysis and found significant concordance for a set of eight genes (p<0.05, false discovery rate (FDR)<1%; Table 3). Amongst these, the pro-apoptotic genes were consistently upregulated in cKO, while the anti-apoptotic genes, Nol3 and Tnfrsf1a, were downregulated in cKO hearts following biomechanical stress, supporting a role for Zbtb17 in the regulation of apoptosis. Six out of eight differentially expressed genes were also detected by ChIP-chip analysis to have binding sites (Table 3). Hence ZBTB17 regulates a transcriptional program that protects cells against apoptosis.

In addition, ZBTB17 overexpression drives hypertrophy \textit{in vitro} (Figure 3). To examine further the effects of ZBTB17 on hypertrophy we overexpressed ZBTB17 in mice using a cardiac myocyte specific promoter\textsuperscript{23} (Supplemental Figure 4). Transgenic mice (TG-ZBTB17) exhibited spontaneous cardiac hypertrophy with enlarged cardiac myocytes and increased Nppa expression (Supplemental Table 1; Figure 4B, C). \textit{In vitro} experiments suggested a link between ZBTB17 and calcineurin, and ChIP-chip data showed binding of ZBTB17 to the calcineurin Aβ (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 and decreased Nppa expression, 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\(^4,\)\(^24\) and have been identified in various patients affected by DCM\(^3,\)\(^25\) or HCM\(^26-28\). 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 postmitotic cells\(^29,\)\(^30\). So far, ZBTB17 is the only known transcription factor to interact with CSRP3 and to be expressed in cardiac myocytes (for a brief review:\(^31\)). The effects of ZBTB17 on hypertrophy and survival are not restricted to the \textit{in vitro} situation, but can also be observed in genetically altered animals under \textit{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:\(^32\),\(^33-35\)).

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

CSRP3 translocates into the nucleus upon TAC$^{36}$ and is essential for the adaptation of cardiac myocytes to biomechanical stress.$^{37}$ 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 death$^5$). This notion is supported by the fact that Csrp3 deficient hearts exhibit increased rates of apoptosis$^{38}$ hence the interaction of CSRP3 with ZBTB17 can also be described as a form of Z disc transcriptional coupling.$^{39}$

Calcineurin/NFAT signaling is an important mediator of cardiac hypertrophy$^{40,41}$ and this pathway is also known to exert cardio-protective effects by avoiding apoptosis during ischemia reperfusion$^{15}$, which emphasizes the effects of ZBTB17 on cell survival.

CSRP3 directly interacts with calcineurin and is required for its activation$^{42}$, thus, by interacting with ZBTB17, CSRP3 introduces an additional level of control which ensures an intermediate level of calcineurin/NFAT activation (Figure 4G). For a long time c-myc is known to be induced during TAC$^{43}$, but its downstream effects in cardiac myocytes remained elusive. However, c-myc is known to inhibit ZBTB17$^{44}$ 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.\textsuperscript{45} This post-translational modification enables the interaction of ZBTB17 with 14-3-3\(\eta\) and leads to its inactivation, thus avoiding prolonged activation of ZBTB17, which can also have adverse effects.\textsuperscript{46}

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 hypertrophy\textsuperscript{47} by specifically affecting protein degradation pathways, for example via the ubiquitin proteasome system (UPS)\textsuperscript{48}, and aside from affecting its interaction with CSRP3 (Figure 2B), may specifically interfere with other protein/protein interactions.

\textit{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 \textit{ZBTB17}, such as \textit{HSPB7} and \textit{CLCNKA}, have been suggested as possible candidate genes by GWAS\textsuperscript{10,49}, \textit{ZBTB17} might well be another novel DCM-associated gene. However, except for this report, the effects of \textit{ZBTB17} on the cardiovascular system have never been analyzed before. It is interesting to note that \textit{HSPB7} which encodes heat shock protein 27, a factor involved in the immediate stress response, \textit{CLCNKA}, which encodes a \(K_a\) renal chloride channel...
involved in the regulation of hypo-osmotic cell stretch, as well as \textit{ZBTB17} are all involved in the 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 sarcomere\textsuperscript{50}, costamere, Z disc\textsuperscript{3,4} and nuclear membrane\textsuperscript{51} while others function as phosphatases and transcriptional activators (EYA4).\textsuperscript{1,52} Unfortunately not much is known about how these effects are linked to changes in gene transcription. A 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.\textsuperscript{53} 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.\textsuperscript{50}

In summary, we identify \textit{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 apoptosis\textsuperscript{5} through modulation of both hypertrophic and cell death pathways (Figure 4G).

\textbf{Acknowledgments:} Dr J. Molkentin is kindly acknowledged for providing the calcineurin Aβ Promotor-Luciferase plasmid and calcineurin Aβ (Ppp3cb) deficient animals. Dr S.C. Wright is thanked for providing the ZBTB17-POZ plasmid.

\textbf{Funding Sources:} R. Knöll is supported by Leducq, DFG Kn 448/9-1, /10-1, /10-2, Fritz Thyssen Stiftung, FP7-PEOPLE-2011-IRSES Proposal No 291834 – Acronym: SarcoSi, and British Heart Foundation (BHF) grants PG/11/34/28793, RG 11/20/29266 and SI/11/2/28875. R. Isaacson is supported by an MRC New Investigator Research Grant. A. Perrot is funded by ECRC. M. Schneider is the British Heart Foundation Simon Marks Chair in Regenerative Cardiology and supported by BHF grants RE/08/002 and SI/11/2/28875. P Barton, J Ware and A Roberts are supported by grants from the MRC UK, the NMRC Singapore, the British Heart Foundation, the Wellcome Trust and the Leducq Foundation, Heart Research UK, The Academy of Medical Sciences and Arthritis Research UK. This project was also supported by the NIHR Cardiovascular Biomedical Research Unit of Royal Brompton and Harefield NHS Foundation.
Trust and Imperial College London. P. Barton, and E Lara-Pezzi are supported by EU FP7 (CardioNeT-ITN-289600). M. Schneider is the British Heart Foundation Simon Marks Chair in Regenerative Cardiology and supported by BHF grants RE/08/002 and SI/11/2/28875.

Conflict of Interest Disclosures: None.

References:


**Table 1:** Echocardiography of cKO-Zbtb17 animals

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<tr>
<th>Genotype/Intervention</th>
<th>BW g</th>
<th>LVID;d mm</th>
<th>LVID;s mm</th>
<th>FS %</th>
<th>h/r</th>
<th>HW/BW mg/g</th>
<th>LV Vol;s</th>
<th>LV Vol;d</th>
<th>EF %</th>
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<td>2.06</td>
<td>45.61</td>
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<td>4.26</td>
<td>14.40</td>
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<td>Control.TAC</td>
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<td>3.44*</td>
<td>1.65*</td>
<td>52.01*</td>
<td>0.63*</td>
<td>5.03*</td>
<td>7.86*</td>
<td>49.04*</td>
<td>52.04*</td>
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<td>15.30</td>
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<td>45.73</td>
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<td>2.00 †</td>
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<td>0.57</td>
<td>4.82</td>
<td>13.22†</td>
<td>59.69††</td>
<td>46.46†</td>
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</table>

Functional data obtained via echocardiography 4 weeks after sham (SHAM) and transverse aortic constriction (TAC) operations 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, for Control.SHAM vs Control.TAC; †P < 0.05, ‡P < 0.01 for Control.TAC vs cKO.TAC; n = 6 per group; Student t-test was used for the comparison of groups).
Table 2: In vivo analysis of TUNEL positive cells, activated caspase 3 and collagen I

<table>
<thead>
<tr>
<th>Animals</th>
<th>TUNEL (n/1000 cardiomyocytes)</th>
<th>Activated caspase 3 (n/1000 cardiomyocytes)</th>
<th>Collagen I (% tissue area)</th>
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<td>5</td>
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<td>6</td>
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<td>mean ± SD</td>
<td>1.55 ± 0.5</td>
<td>11.78 ± 2.5***</td>
<td>3.42 ± 0.73</td>
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Analysis of TUNEL positive cells, activated caspase 3 (n/1000 cardiomyocytes) and collagen I (percent tissue area). Student t-tests were used for the comparison of groups, data=mean ± SD; Control.TAC: flox/flox, Cre−; cKO.TAC: flox/flox, Cre+. n = 6 per group, ***P < 0.001.
Table 3: Summarized expression profiles and Zbtb17 binding sites of target genes involved in cardiac myocyte specific programmed cell death in cKO animals (n = 6 per group). Directional change of genes is consistent with the anti-apoptotic role of endogenous Zbtb17.

<table>
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<tr>
<th>Gene</th>
<th>Apoptosis RT² Profiler PCR Array data (SA Biosciences)</th>
<th>Whole-transcript array data (Affymetrix microarray)</th>
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<th>ChIP - chip</th>
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Abbreviations: NBS = no ZBTB17 binding site
Figure Legends:

Figure 1: A. Analysis of the interaction between CSRP3 and ZBTB17 by yeast two-hybrid assays. No interaction occurs between proteins when the ZBTB17-POZ domain is deleted (lower right quadrant). A total of 10⁶ 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% ± 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 neonatal rat cardiac myocytes (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 $^1$H-$^{15}$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 quaternary 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 overexpression in neonatal rat cardiac myocytes (NRCMs) as determined by impedance measurements. Scatter dot plot represents cell index values after 24 hours (n = 10, experiments done in duplicate). Dotted lines indicate cell indices in calcineurin inhibition experiments (Cyclosporin A (CsA): 0.2μM; n = 4; Mann-Whitney test was used for
the comparison of groups, **P<0.01). B. Representative images of NRCMs with and without ZBTB17 adenoviral gene transfer. C. Effects of ZBTB17 overexpression on doxorubicin (DOX, 1μM)-induced cell death (n = 4). Dotted lines indicate data from calcineurin inhibition experiments (CsA: 0.2μM; n = 4; Mann-Whitney test was used for the comparison of groups, *P<0.05).

Figure 4: A. Representative micrographs of fibrosis staining of mouse hearts following SHAM or TAC procedures and quantification of fibrosis (4 data points per animal, one representative experiment of two shown, Mann-Whitney test was used for the comparison of groups, *P<0.05).

B. Analysis of cardiac myocyte cross sectional area under spontaneous conditions in WT-ZBTB17, cKO-Zbtb17, TG-ZBTB17 (~150 cardiac myocytes per animal; n = 5 per group, Mann-Whitney test was used for the comparison of groups, *P<0.05, **P<0.01), and KO-Ppp3cb, TG-ZBTB17/KO-Ppp3cb (~150 cardiac myocytes per animal; n = 2 per group). C. Upregulation of the pro-hypertrophic biomarker Nppa in TG-ZBTB17 hearts (n = 2 per group, experiments done in duplicate). D. ChIP qRT-PCR analysis of Zbtb17 binding to the Ppp3cb promoter in vivo (one representative experiment of two shown, Mann-Whitney test was used for the comparison of groups, *P<0.05). E. Luciferase reporter assay of ZBTB17 activation of the Ppp3cb promoter via transient transfection (n = 3 - 4 per group, Mann-Whitney test was used for the comparison of groups, *P<0.05). F. Increased Ppp3cb and Nfatc2 mRNA expression levels in TG-ZBTB17 (one representative experiment of two shown, Mann-Whitney test was used for the comparison of groups, *P<0.05). G. ZBTB17 is important for the adaptation of cardiac myocytes to biomechanical stress. Loss of ZBTB17 is associated with an increase in cardiac myocyte apoptosis and heart failure, whereas overexpression leads to activation of the calcineurin / NFAT pathway and cardiac myocyte hypertrophy.
**ZBTB17 (MIZ1) Is Important for the Cardiac Stress Response and a Novel Candidate Gene for Cardiomyopathy and Heart Failure**


_Circ Cardiovasc Genet._ published online July 14, 2015; _Circulation: Cardiovascular Genetics_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

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Print ISSN: 1942-325X. Online ISSN: 1942-3268

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SUPPLEMENTAL MATERIAL

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)\textsuperscript{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.\textsuperscript{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) was analyzed via cross-linking experiments with ethylene glycol bis(sulfosuccinimidylsuccinate) (sulfo-EGS) (Thermo Scientific) solution in DMSO. The experiment was performed in 40 µl 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 1µl 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 and as these are non-proliferating cells any increase in cell index represents an
increase in cell size. 48 hrs after adenoviral infection 1µM of Doxorubicin (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<sup>−/−</sup> (kindly provided by Dr J. Molkentin<sup>6</sup>).

**Echocardiography and surgery**

Surgery was carried out using a modified minimally invasive approach as previously described<sup>3</sup>. Mice were anesthetized with Isoflurane and echocardiography was performed as described previously<sup>3</sup>. 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 and microarray**

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 1µg 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).

For genome-wide microarray: Total RNA was quantified by a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE), and quality was assessed by a 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). All samples displayed an RNA integrity number >8.0. Double-stranded cDNA synthesis and in vitro transcription (IVT) were carried out according to Affymetrix protocols. In brief, 1 μg of total RNA was reverse transcribed with SuperScript II and T7-Oligo(dT)24 Promoter Primer for 1 h at 42°C, followed by 2 h at 16°C RNase H-mediated second-strand cDNA synthesis. After sample clean-up the cDNA served as a template for subsequent IVT. The IVT reaction was carried out in the presence of T7 RNA polymerase and a biotinylated nucleotide analog/ribonucleotide mix for complementary RNA (cRNA) amplification and biotin labeling for 16 h at 37°C. The quality of cRNA was assessed by a 2100 Bioanalyzer. Fragmentation of antisense cRNA and hybridization to GeneChip Mouse Exon 1.0 ST Array were performed at the Genomics Laboratory, MRC according to their protocol. Affymetrix .CEL files were imported into GeneSpring 12.5-GX-PA (Agilent Technologies) and processed with RMA followed by apoptosis related genes.

**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 Laboratories.
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 was 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 $\log_2 (\text{ChIP/input})$ 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-tests were used for the comparison of groups with $n > 5$ and, unless otherwise indicated, error bars represent mean ± SEM. Mann-Whitney tests were used for the comparison of groups with $n \leq 5$.

Data were formatted in MS Excel and statistical analyses were carried out using GraphPad Prism.

Gene Set Enrichment Analysis (GSEA) was used to test whether differentially expressed genes by genome-wide microarray analysis were significantly enriched in the set of differentially expressed genes detected by apoptosis specific qRT-PCR array analysis and used to find significant concordance for a certain set of genes.
References


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 (1µM of SP for 24 hrs, AdLacZ 1.0 ± 0.02, n = 653 cells; AdZBTB17 1.4 ± 0.03, n = 589 cells). Student t-tests were used for the comparison of groups, †p<0.05; †††p<0.001, error bars represent mean ± SEM.
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 (f/f, Cre−; n = 4) and homozygous knockout animals (f/f, Cre+; n = 9). There is a significant loss of this genomic region in the homozygous knockout animals. Mann-Whitney test was used for the comparison of groups, *P<0.05, **P<0.01. 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. Mann-Whitney test was used for the comparison of groups. 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; Mann-Whitney test was used for the comparison of groups) (lower panel). B, Fibrosis as determined by PSR staining in TG-ZBTB17 animals (4 data points per animal, one representative experiment of two shown, Mann-Whitney test was used for the comparison of groups, *P<0.05). 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

Decreased *Ppp3cb* and *Nfatc2* mRNA expression levels in cKO (one representative experiment of two shown, Mann-Whitney test was used for the comparison of groups).
Supplemental Figure 1

A

B

C
Supplemental Figure 4

A

WT-ZBTB17  TG-ZBTB17

overexpressed ZBTB17  endogenous ZBTB17

100kDa  37kDa

GAPDH

ZBTB17/GAPDH

WT-ZBTB17  TG-ZBTB17

0.20

0.15

0.10

0.05

0.00

C

D

FLAG  F-actin/DAPI

FLAG/F-actin/DAPI

FLAG/F-actin/DAPI

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

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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

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Supplemental Table 3. Primary antibodies used in this study

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