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I-1-deficiency negatively impacts survival in a cardiomyopathy mouse model

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ABSTRACT

Aims: Hypertrophic cardiomyopathy (HCM) is characterized by left ventricular hypertrophy, diastolic dysfunction and increased interstitial fibrosis. Current treatment is based on beta-adrenoceptor (AR) and calcium channel blockers. Since mice deficient of protein phosphatase-1 inhibitor-1 (I-1), an amplifier in beta-AR signalling, were protected from pathological adrenergic stimulation in vivo, we hypothesized that I-1 ablation could result in an improved outcome in a HCM mouse model.

Methods and results: We crossed mice deficient of I-1 with homozygous myosin-binding protein C knock-out (*Mybpc3* KO) mice exhibiting cardiac dilatation and reduced survival. Unexpectedly, survival time was shorter in double *I-1*/*Mybpc3* KO than in single *Mybpc3* KO mice. Longitudinal echocardiographic assessment revealed lower fractional area change, and higher diastolic left ventricular inner dimensions and end-diastolic volumes in *Mybpc3* KO than in WT mice. In comparison to *Mybpc3* KO, double *I-1*/*Mybpc3* KO presented higher left ventricular end-diastolic volumes, inner dimensions and ventricular surface areas with increasing differences over time. Phosphorylation levels of PKA-downstream targets and mRNA levels of hypertrophic markers did not differ between *I-1*/*Mybpc3* KO and single *Mybpc3* KO mice, except a trend towards higher beta-myosin heavy chain levels in double *I-1*/*Mybpc3* KO.

Conclusion: The data indicate that interference with beta-AR signalling has no long-term benefit in this severe *MYBPC3*-related cardiomyopathy mouse model.

1. Introduction

Hypertrophic cardiomyopathy (HCM) is mainly characterized by asymmetric left ventricular hypertrophy, diastolic dysfunction and myocardial disarray [1]. To date, HCM is known to be caused by mutations in at least 23 different genes encoding mainly sarcomeric proteins [2]. An early concept of HCM is a hyper-adrenergic state [3–6], which is a rationale for beta-adrenoceptor (AR) blockade as treatment option for HCM in humans. Beta-AR blocker treatment has proven to be beneficial in patients with angina or dyspnea on exertion, especially when associated with left ventricular outflow tract (LVOT) obstruction, and is frequently used to lower the occurrence of non-sustained ventricular arrhythmias in HCM patients [7–9]. On the cellular level, beta-AR blockade leads to lower cyclic AMP production. In pacemaker cells, this lowers spontaneous beating activity with the consequence of a lower heart rate, whereas in ventricular myocytes, protein kinase A (PKA) activity is alleviated, leading to a lower phosphorylation status of L-type Ca^{2+} channels (LTCC), phospholamban (PLB), cardiac troponin I (cTnI), cardiac myosin-binding protein C (cMybpc) and cardiac ryanodine receptors (RyR2).

A downstream element of the cardiac beta-AR signalling pathway is phosphatase-1 inhibitor-1 (I-1). I-1 inhibits protein phosphatase type-1 (PP1), the major isoform of Ser-Thr-protein phosphatases in the heart, and thereby enhances PKA-mediated protein phosphorylation [10–12]. In adult rat and in failing human cardiac myocytes, overexpression of wild-type (WT) I-1 or a constitutively active I-1 (I-1c) mutant protein enhanced beta-AR/cAMP/PKA-dependent contractile responses, accentuating its amplifier role in the beta-AR signalling pathway [5,13]. In addition, inducible cardiac I-1c expression in adult mice protected against ischemia–reperfusion-injury and was connected with smaller

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infarct size and apoptotic injuries [14]. Recently, a study reported that I-1c overexpression with adeno-associated virus (AAV) improved cardiac function in a pig model of ischemic heart failure [15]. In contrast, in a chronic isoprenaline infusion model, I-1 overexpression induced detrimental effects, whereas I-1 deficiency was associated with beneficial effects [16]. These effects were associated with lower steady-state phosphorylation levels of both RyR2 and PLB, without an influence on basal cardiac function, contractile reserve, phosphorylation status of LTCC and key myofibrillar proteins suggesting that PP1 and I-1 strongly control RyR2 and PLB, apparently more than other PKA targets. The data indicate that under conditions of increased adrenergic drive the absence of I-1 is cardioprotective due to the lack of the I-1-mediated intracellular amplification loop. It was therefore suggested that I-1 deficiency acts as an 'internal beta-blocker' without influence on heart rate and phosphorylation of LTCC and myofilament proteins [17].

Here, we tested the hypothesis that this I-1 deficiency profile would have also a beneficial effect in a mouse model of HCM. We crossed I-1 knock-out (KO) mice with a HCM *Mybpc3* KO mouse model and assessed the effect of I-1 deficiency on prognosis and cardiac function. For comparison, we treated *Mybpc3* knock-in mice (KI), another HCM model with more similarity to human HCM [18], chronically with metoprolol. In contrast to our hypothesis, we observed higher mortality combined with worse functional parameters in double *I-1*/*Mybpc3* KO (DKO) than in single *Mybpc3* KO (SKO) mice and no apparent beneficial effect of metoprolol on KI mice.

2. Materials and methods

2.1. Experimental animals and survival curve

The study complies with the Guide for the Care and Use of Laboratory Animals published by the NIH (Publication No. 85-23, revised 1985). Mice were handled and maintained according to approved protocols of the animal welfare committee of the University of Hamburg. For establishing the DKO mouse line, homozygous SKO mice [19–21] were crossed with *I-1* KO mice [22]. Mice were maintained on the C57/BL6J genetic background. For the survival curve, 61 DKO, 58 SKO and 22 WT mice were included. *Mybpc3*-targeted knock-in (KI) mice were developed previously and maintained on the Black Swiss genetic background [18].

2.2. Transthoracic echocardiography

For the longitudinal study with DKO and SKO mice, cardiac functional parameters of 6 mice per group (WT, DKO, SKO) were analyzed by transthoracic echocardiography at 7, 13, 20, 25 and 32 weeks of age using the Vevo 770™ high resolution imaging system (Visual Sonics Inc., Toronto, Canada) as previously described [23].

2.3. Long-term metoprolol treatment and echocardiography

Groups of 10 *Mybpc3* WT or KI mice received either drinking water without (control group) or with metoprolol (treatment group) starting at the age of 6–8 weeks for a period of 6 months. Based on water consumption, mice were dosed with 100 mg/kg/day of metoprolol.

Echocardiography was performed every 8–9 weeks using the Vevo 2100 System (VisualSonics, Toronto, Canada). The last echo was performed after 6 months of treatment. Then animals were killed by cervical dislocation and body parameters were obtained.

2.4. Expression analysis

For molecular biology analysis, 34–35-week old WT, SKO and DKO mice were sacrificed by cervical dislocation; hearts were extracted and frozen in liquid-nitrogen cooled isopentane for subsequent molecular-biological analysis. RNA was isolated from powdered mouse ventricular samples using the SV Total RNA Isolation kit (Promega) and 200 ng transcribed into cDNA using the SuperScript® III Reverse Transcriptase kit (Life Technologies) [24,25]. Quantitative determination of atrial natriuretic peptide (*Nppa*), brain natriuretic peptide (*Nppb*), α-skeletal actin (*Acta1*) and beta-myosin heavy chain (*Myh7*) mRNA levels was performed by real-time PCR using the Maxima SYBR Green/Rox qPCR Master Mix (Thermo Scientific) and primers specific for every sequence (Supplemental Table 1). Ct values were normalized to G alpha s (*Gαs*). ΔΔCt values were related to WT.

Western blotting was performed as described previously [13,26]. Details on primary antibodies are provided in Supplemental Table 2.

2.5. Statistical analysis

Data are reported as mean ± S.E.M. Statistical differences between the groups of mice were either calculated by one-way or two-way ANOVA as indicated in the figure legends, using the GraphPad software (GraphPad Software Inc.), version 5.02. Survival analysis of DKO vs. SKO was calculated by the Kaplan–Meier method. A value of $p < 0.05$ was considered significant.

3. Results

3.1. I-1-deficiency in a Mybpc3 KO mouse model impacts negatively on survival

For long term evaluation of I-1-deficiency in the *Mybpc3* KO mouse model we performed a survival study with homozygous SKO, DKO and WT mice. Both DKO and SKO had shorter survival rates than WT mice (Fig. 1). Unexpectedly, DKO presented a significantly shorter median survival than SKO mice (39 vs. 48 weeks, $p < 0.05$), despite unchanged survival rates of single I-1 deficient mice compared to WT mice (data not shown). There was no gender difference (data not shown). None of the WT mice died during the study period.

This outcome suggests that I-1-deficiency is not beneficial in this *Mybpc3* KO mouse model of severe HCM.

3.2. DKO mice show larger ventricles and higher diastolic volume than SKO mice

To investigate why I-1 deficiency impacts negatively on survival in our model we performed a longitudinal echocardiography study on animals of each genotype (7 until 32 weeks of age). Echo analysis over the

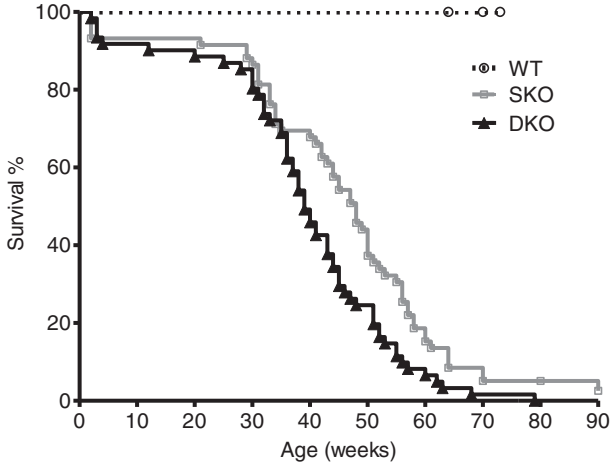


Fig. 1. Analysis of survival of WT, single *Mybpc3* KO (SKO) and double I-1/*Mybpc3* KO (DKO) mice. Kaplan–Meier cumulative survival curves of wild type (WT), single *Mybpc3* KO (SKO) and double I-1/*Mybpc3* KO (DKO) mice from birth on. Median survival rates were: SKO = 48 weeks, DKO = 39 weeks, log-rank (Mantel–Cox) test, $p < 0.001$ vs. WT for SKO/DKO, $p < 0.05$ vs. SKO. None of the WT mice died during the study period.

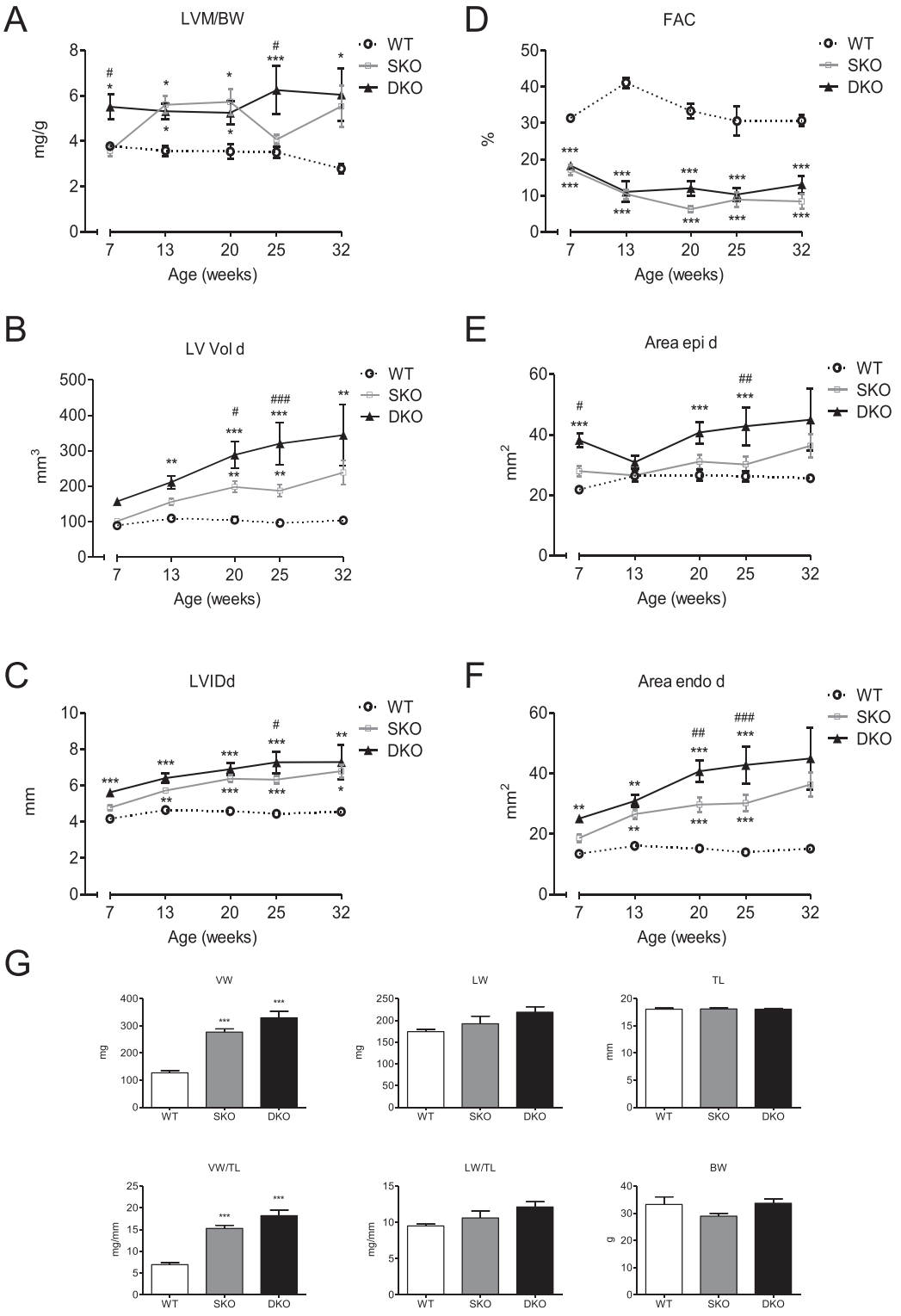


Fig. 2. Longitudinal echocardiography study and body parameters of WT, SKO and DKO mice. A, Left ventricular mass/body weight ratio [LVM/BW], B, fractional area change [FAC], C, left ventricular enddiastolic volume [LV Vol d], D, left ventricular internal enddiastolic diameter [LVIDd], E and F, external (Area epi d) and internal (Area endo d) left ventricular enddiastolic areas were measured in WT, SKO and DKO, n = 6. G, ventricular weight (VW), lung weight (LW), tibia length (TL), ventricular weight/tibia length ratio (VW/TL), lung weight/tibia length ratio (LW/TL) and body weight (BW) were assessed in 34–35-week old WT, SKO and DKO mice, n = 6–9. Data are expressed as mean ± SEM, $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. WT, #p < 0.05, ##p < 0.01, and ###p < 0.001 vs. SKO, two-way ANOVA followed by Bonferroni comparison post-test or one-way ANOVA followed by Dunnett's comparison post-test.

course of time revealed no difference in fractional area change (FAC) at the different ages between SKO and DKO (both were markedly reduced compared to WT), but a higher left ventricular mass to body weight ratio (LVM/BW) for DKO than SKO mice at the age of 7 and 25 weeks, but no difference at 32 weeks of age (Fig. 2A, D).Furthermore, left ventricular end-diastolic volume (LV Vold) and left ventricular inner dimensions in diastole (LVIDd) were higher in DKO than in SKO mice. This difference increased over the course of time, illustrating a dilated

phenotype with increased volume retention (Fig. 2B, C). External and internal left ventricular areas in diastole (Area epi/endo d) were also higher in DKO mice, supporting the observations of higher chamber dimensions and a more pronounced dilation phenotype than in SKO (Fig. 2E, F). Since the median survival of DKO mice was 39 weeks (Fig. 1), we assessed ventricular, lung and body weight parameters in 34–35-week old mice of all genotypes. Body and lung weights did not differ between the different genotypes (Fig. 2G). SKO and DKO mice showed a cardiomyopathic phenotype with higher ventricular weight (VW) and ventricular weight to tibia length ratios (LV/TL) than the WT. Ventricular and lung parameters (VW, VW/TL, LW, LW/TL) showed a tendency to slightly higher values in DKO than in SKO mice.

3.3. No difference in levels of hypertrophic markers and calcium-handling proteins between DKO and SKO

Transcript levels of the hypertrophic markers *Nppa*, *Nppb*, *Acta1*, and *Myh7* were markedly higher in SKO and DKO than in WT, confirming the disease phenotype, but did not differ between SKO and DKO which is in line with unchanged *Nppa* and *Nppb* levels between I-1 deficient and WT mice [16]. An exception could be detected for *Myh7* levels that were higher by trend in DKO (Fig. 3A–D).

Both SKO and DKO showed higher beta-myosin heavy chain steady-state protein levels than WT (Fig. 4A). Quantification of sarcoplasmic reticulum Ca^{2+}-ATPase pump (SERCA) and total PLB protein levels revealed no differences between the three genotypes (Fig. 4B, C). PLB phosphorylation levels at Ser16 and Thr17 showed a stronger signal in SKO and DKO than in WT. Analysis of the total and phosphorylated states of the sarcomeric PKA target cardiac cTnI showed no difference in total levels, but a tendency to higher phosphorylation in SKO and DKO than in WT (Fig. 4D). This hyperphosphorylation could indicate an increased adrenergic signalling. *I-1* mRNA levels were undetectable in DKO mice, but ~40% lower in SKO than in WT mice which is in agreement with lower I-1 mRNA and protein levels in failing human hearts and in response to chronic isoprenaline treatment [27,28]. There was no difference in *PP1* mRNA levels indicating no compensatory changes on gene expression level (Supplemental Fig. 1).

3.4. Long-term metoprolol treatment has no effect on cardiac function in Mybpc3 KI mice

In order to evaluate whether a classical beta-AR blockade has a similarly detrimental effect on cardiac function as I-1 deficiency in the *Mybpc3* KO mouse model, we supplemented the drinking water of *Mybpc3* KI mice, another HCM mouse model, with the beta-AR antagonist metoprolol, starting at 6–8 weeks of age. Homozygous KI mice show only 10–20% of *Mybpc3* protein and mRNA levels, and their disease phenotype mimics *Mybpc3* KO and severe HCM cases [3,29,30]. Cardiac function was measured by echocardiography before, during and at the end of the study. During the study none of the KI mice died. After 6 months of metoprolol treatment, no differences in LVM/BW, LV Vol d, LVIDd, FAC, Area epi and endo d were observed between treated and untreated KI mice (Fig. 5A–F). We also assessed ventricular, lung and body weight parameters at the end of the study. Similarly, no differences between treated and untreated KI mice could be seen (Fig. 5G).

4. Discussion

Current treatment of human HCM is based on the use of beta-AR and calcium channel blockers. Both have negative inotropic effects and can reduce outflow tract obstruction. Moreover, they could be advantageous in light of the early concept of hyper-adrenergic state in HCM [7,8,31]. Beta-AR blockers in particular have shown to be beneficial in patients with angina or dyspnea, particularly when associated with LVOT obstruction [9]. These effects are induced by lowering heart rate, contractility and stiffness of the ventricle, which results in improved ventricular relaxation, increased time for diastolic filling, and reduced tendency to arrhythmias [32,33]. Despite these beneficial effects, the ultimate impact of beta-AR blockers on outcome in HCM patients remains undefined [34].

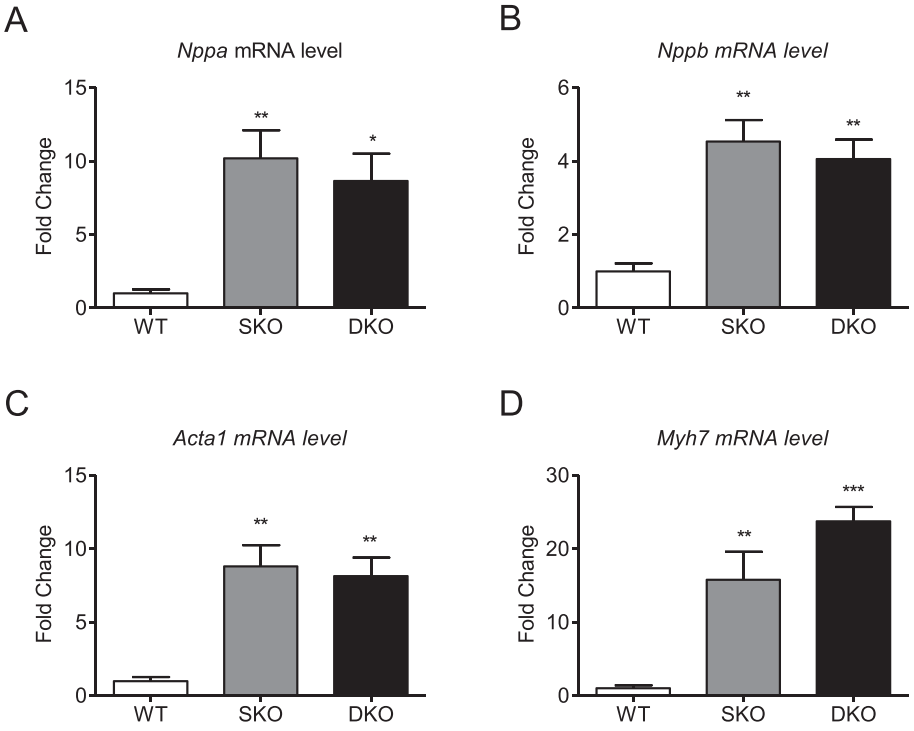


Fig. 3. Expression of markers of the hypertrophic gene program. mRNA levels of A, *Nppa*, B, *Nppb*, C, *Acta1*, D, *Myh7* in 34–35-week old WT, SKO and DKO mice. Values are related to WT mice. Data are expressed as mean ± SEM. *p < 0.05, **p < 0.01 and ***p < 0.001 vs. WT, one-way ANOVA followed by Dunnett's comparison post-test, n = 5–8 per group.

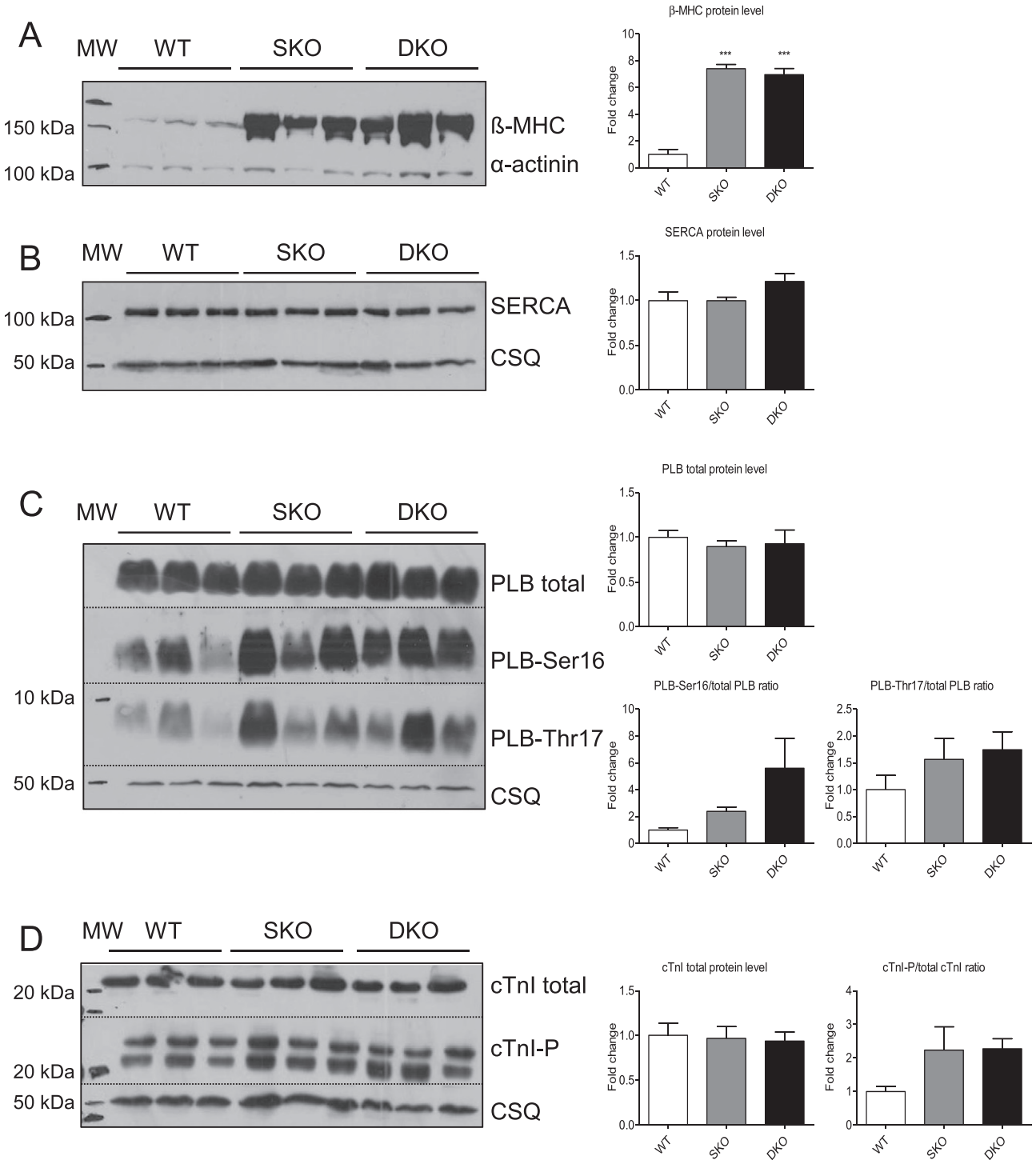
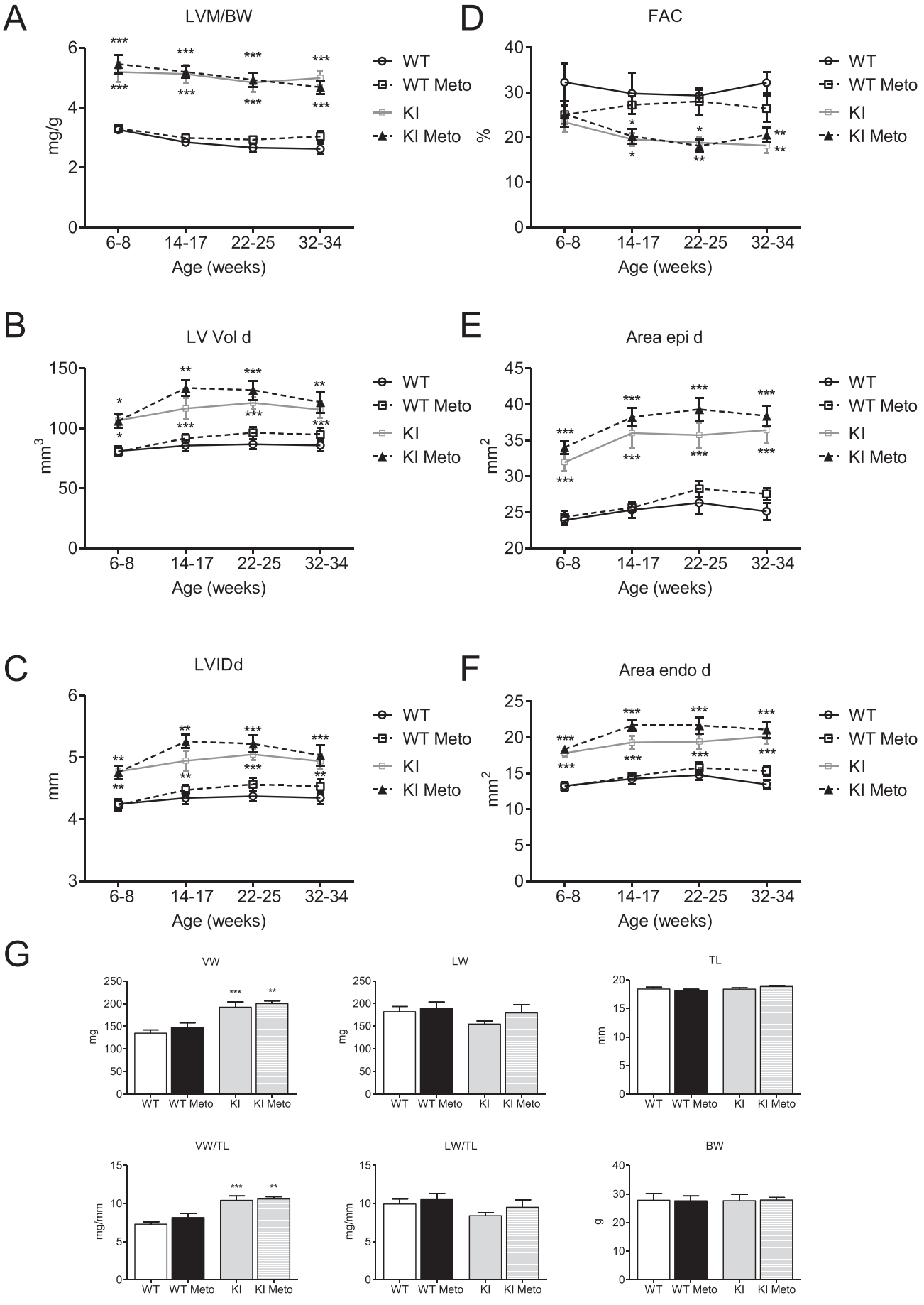


Fig. 4. Analysis of calcium-handling proteins in WT, SKO and DKO mice. Representative Western blots stained with antibodies directed against A, β-myosin heavy chain (β-MHC), B, sarcoplasmic reticulum Ca^{2+}-ATPase pump (SERCA), C, total and phosphorylated phospholamban (PLB), and D, total and phosphorylated cardiac troponin I (cTnI) or alpha-actinin/calsequestrin (protein loading control). Molecular weight markers (MW) as indicated. Protein levels were normalized to alpha-actinin/calsequestrin and related to WT protein levels. Data are expressed as mean ± SEM, ***$p < 0.001$ vs. WT, one-way ANOVA followed by Dunnett's comparison post-test, $n = 3–6$.

Experimental approaches in HCM mouse models have produced positive and negative results. Whereas treatment with drugs as diltiazem, losartan, spironolactone and HMG-CoA-reductase inhibitors has shown beneficial effects, application of calcineurin inhibitors worsened the phenotype [35–39]. Lately, it was reported that genetic normalization of myofilament Ca^{2+} sensitivity by pseudophosphorylated TnI inhibits disease development in HCM tropomyosin mice [40]. Gene therapy approaches directed towards normalization of pathologically low mRNA and protein levels in *Mybpc3* KI mice, either by repair of mutated RNA [41–43] or by introduction of the correct full-length *Mybpc3* cDNA have provided significant disease prevention [3].

This study shows the importance of a more comprehensive evaluation of a potential "therapeutic target/strategy" in different types of heart diseases to be really able to assess the therapeutic potential of such strategy. We tested the hypothesis that intracellular


A
LVM/BW
mg/g
Age (weeks)
6-8
14-17
22-25
32-34
WT
WT Meto
KI
KI Meto
D
FAC
%
B
LV Vol d
mm3
E
Area epi d
mm2
C
LVIDd
mm
F
Area endo d
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VW
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VW/TL
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modulation of the beta-AR cascade by I-1 ablation could be advantageous in a *Mybpc3* KO mouse model by reducing the sensitivity of beta-AR signalling without lowering heart rate or contractility (e.g. by lower LTCC and myofilament phosphorylation), but rather by targeting intracellular calcium handling. Similar to results we obtained in human heart failure samples and in rats after chronic ISO stimulation [27,28], SKO mice demonstrated lower *I-1* mRNA levels than WT mice, which could indicate a protective mechanism against excessive adrenergic drive in heart failure. We previously demonstrated that I-1 deficiency slightly reduced the inotropic response to beta-AR stimulation in isolated heart muscle preparations, but did not affect basal contractility [16]. Accordingly, in the current study we did not observe a difference in FAC between SKO and DKO mice. However, DKO exhibited even more pronounced LV dilatation and earlier death, clearly indicating that, in this *Mybpc3* KO mouse model, I-1 deficiency has adverse effects albeit unchanged LV dimensions in single I-1 deficient mice [16]. There are two possible explanations. In heart failure, beta-AR blockers should not be initiated in acutely decompensated patients because of further worsening of pump function [44]. Both *Mybpc3* mouse models which were used in the study show severe systolic and diastolic dysfunction and ventricular dilatation at the homozygous state. A high-dose beta-AR blocker treatment could therefore promote heart failure decompensation as seen in the case of the DKO. At first sight, the observation that beta-AR blockade by metoprolol did not worsen cardiac function in *Mybpc3* KI does not support this theory. However, KI in this genetic background (Black Swiss) had a much better contractile function than the SKO, suggesting that this may well be due to a difference in severity of the cardiomyopathy phenotype in the two mouse models. And it was apparent that metoprolol treatment did not improve cardiac function or reduce the degree of hypertrophy in *Mybpc3* KI mice. Thus, most likely, *Mybpc3* KO mice with a severe form of cardiomyopathy need a certain level of adrenergic signalling downstream of the beta-AR receptor for compensation and this reserve was depressed by I-1 ablation.

An alternative or additional explanation for the adverse consequences of I-1 ablation in the DKO relates to diastolic function. Formerly we reported that SKO mice showed a relaxation deficit and higher PLB phosphorylation levels. In this study, SKO and DKO showed higher PLB and cTnI phosphorylation levels than the WT as well. This was unexpected for DKO mice since in a former study single *I-1* KO mice presented PLB hypophosphorylation [16]. This higher phosphorylation status could be a compensational mechanism to accelerate Ca^{2+} uptake into the SR and therefore hasten relaxation [21]. In this respect, stimulation of beta-AR in *Mybpc3*-related HCM could be a beneficial compensatory mechanism that alleviates diastolic dysfunction caused by the relative or complete lack of *Mybpc3*. I-1 deficiency in the *Mybpc3* KO model would then negatively interfere by misbalancing beta-adrenergic signalling and causing exaggerated cardiac hypertrophy and lower survival rates in DKO compared to SKO.

4.1. Study limitations

The study has several limitations. 1) While we used different approaches to understand the nature of the augmented phenotype in (*I-1*/*Mybpc3* KO) mice, including gene and protein expression levels of genes usually deregulated in cardiomyopathy, we were not able to identify critical molecular difference between SKO and DKO mice with respect to the observed phenotype. An unbiased approach to identify potential genetic modifiers, e.g. by whole-genome transcriptomics or proteomics, was however beyond of scope of the current study. 2) Most importantly, *Mybpc3* KO and KI mice do not exhibit a typical HCM phenotype. Whereas humans generally develop a HCM phenotype with only one allele mutated, heterozygous *Mybpc3* KO and KI exhibit only mild signs of diastolic dysfunction, but no cardiac hypertrophy. The present experiments were therefore performed with homozygous KO/KI mice which are severely sick, but rather with a dilated cardiomyopathy phenotype and severe systolic and diastolic dysfunction. This mimics the rare cases of neonates with two diseased alleles [45,46]. Both models are obviously not perfect for the more common form of heterozygous HCM with normal or even supra-normal ejection fraction. The results can therefore not be directly transferred to the human situation and further studies using heterozygous *Mybpc3* KO and KI mouse models on an I-1 deficient background might help to elucidate the full picture of beta-AR signalling blockade in clinical settings of HCM. Yet, the clearly harmful effects of I-1 ablation in the *Mybpc3* KO model indicate that beneficial effects seen previously under strong beta-AR stimulation [16] cannot be simply transferred to all cardiac pathologies. And, together with the lack of beneficial effect of beta-AR blocker treatment in the KI model, the data argue against a beneficial effect of beta-AR treatment on severe *Mybpc3*-related cardiomyopathies. 3) Finally we cannot rule out any adverse effects of I-1 ablation on extra cardiac tissue which might contribute to the observed phenotype in the DKO. Construction of a tissue-restricted I-1 KO mouse model may help in clarifying this issue in the future.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ijcha.2015.05.010.

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Disclosures

None.

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Fig. 5. Longitudinal echocardiography study and body parameters of *Mybpc3* WT and KI mice ± metoprolol. Heart function was measured in *Mybpc3* WT and KI mice by echocardiography before (6–8 weeks of age) and during metoprolol treatment (14–17, 22–25, 32–34 weeks of age). A, Left ventricular mass/body weight ratio [LVM/BW], B, fractional area change [FAC], C, left ventricular enddiastolic volume [LV Vol d], D, left ventricular internal enddiastolic diameter [LVIDd], E and F, external (Area epi d) and internal (Area endo d) left ventricular enddiastolic areas were measured in *Mybpc3* WT and KI mice ± metoprolol (Meto), n = 8–10. G, ventricular weight (VW), lung weight (LW), tibia length (TL), ventricular weight/tibia length ratio (VW/TL), lung weight/tibia length ratio (LW/TL) and body weight (BW) were assessed after 6 months of treatment, n = 8–10. Data are expressed as mean ± SEM, $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. WT, two-way ANOVA followed by Bonferroni comparison post-test or one-way ANOVA followed by Dunnett's comparison post-test.

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