Repair of Mybpc3 mRNA by 5′-trans-splicing in a Mouse Model of Hypertrophic Cardiomyopathy

Giulia Mearini1,2, Doreen Stimpel1,2, Elisabeth Krämer1,2, Birgit Geertz1,2, Ingeke Braren1,3, Christina Gedicke-Hornung1,4, Guillaume Précigout4, Oliver J Müller5,6, Hugo A Katus5,6, Thomas Eschenhagen1,2, Thomas Voit1, Luis García1, Stéphanie Lorain4 and Lucie Carrier1,2,4

RNA trans-splicing has been explored as a therapeutic option for a variety of genetic diseases, but not for cardiac genetic disease. Hypertrophic cardiomyopathy (HCM) is an autosomal-dominant disease, characterized by left ventricular hypertrophy (LVH) and diastolic dysfunction. MYBPC3, encoding cardiac myosin-binding protein C (cMyBP-C) is frequently mutated. We evaluated the 5′-trans-splicing strategy in a mouse model of HCM carrying a Mybpc3 mutation. 5′-trans-splicing was induced between two independently transcribed molecules, the mutant endogenous Mybpc3 pre-mRNA and an engineered pre-trans-splicing molecule (PTM) carrying a FLAG-tagged wild-type (WT) Mybpc3 cDNA sequence. PTMs were packaged into adeno-associated virus (AAV) for transduction of cultured cardiac myocytes and the heart in vivo. Full-length repaired Mybpc3 mRNA represented up to 66% of total Mybpc3 transcripts in cardiac myocytes and 0.14% in the heart. Repaired cMyBP-C protein was detected by immunoprecipitation in cells and in vivo and exhibited correct incorporation into the sarcomere in cardiac myocytes. This study provides (i) the first evidence of successful 5′-trans-splicing in vivo and (ii) proof-of-concept of mRNA repair in the most prevalent cardiac genetic disease. Since current therapeutic options for HCM only alleviate symptoms, these findings open new horizons for causal therapy of the severe forms of the disease.

Molecular Therapy—Nucleic Acids (2013) 2, e102; doi:10.1038/mtna.2013.31; published online 2 July 2013

Subject Category: Therapeutic proof-of-concept Gene insertion, deletion & modification

Introduction

In the last decade repair of mRNA by spliceosome-mediated RNA trans-splicing has raised interests as a novel therapeutic intervention (for reviews, see refs. 1, 2). Trans-splicing has many attractive features such as preservation of the endogenous regulation, replacement of selected portions of the target gene and, most importantly, corrections of dominant-negative mutations.3,4 The 5′, 3′- or even internal exons of a target pre-mRNA can be replaced by trans-splicing using engineered pre-trans-splicing molecules (PTMs). PTMs carry the wild-type (WT) sequence, a binding domain complementary to the endogenous target and an appropriate set of splicing elements. After nuclear import, PTMs are transcribed and can specifically hybridize the target mutant pre-mRNA via their binding domain, giving rise to a repaired mRNA molecule (Figure 1a). As a positive side effect of trans-splicing, cis-splicing should be reduced due to competition for access to the RNA splicing machinery. So far, successful trans-splicing between PTMs and endogenous targets has been described for different genetic diseases such as hemophilia A,5 cystic fibrosis,6 spinal muscular atrophy,7,8 hyper-IgM-X-linked immunodeficiency,9 frontotemporal dementia with Parkinsonism linked to chromosome 17,10,11 epidermolysis bullosa with muscular dystrophy,12 and Huntington’s disease,13 most of them being 3′-trans-splicing approaches.

To the best of our knowledge, no study has provided evidence for successful 5′-trans-splicing in vivo yet and this promising strategy has not been evaluated for cardiac genetic diseases. The aim of the present study was therefore to investigate this approach in hypertrophic cardiomyopathy (HCM). HCM is a myocardial disease mainly characterized by left ventricular hypertrophy (LVH) and diastolic dysfunction.14–16 The clinical outcome of HCM is highly variable and ranges from an asymptomatic benign course to heart failure, atrial fibrillation and sudden cardiac death caused by arrhythmias.14,15 HCM is a genetic disease transmitted as an autosomal-dominant trait and caused by mutations in genes encoding sarcomeric proteins.17 Among them, mutations in MYBPC3 encoding cardiac myosin-binding protein C (cMyBP-C) are the most frequent ones.18,19 cMyBP-C is a component of the thick filaments of the sarcomere, and plays important structural and functional roles.18,20,21

In the present study the feasibility of 5′-trans-splicing to repair HCM-mutant mRNA was assessed in isolated cardiac myocytes and in vivo in Mybpc3-targeted knock-in (KI) mice that have been generated previously.22 KI mice carry a G>A transition on the last nucleotide of exon 6, which is...
associated with a severe phenotype and a poor prognosis in humans and occurs in 13% of all HCM patients in Toscany. KI mice exhibit LVH with systolic and diastolic dysfunction.

**Results**

**Design of PTMs**

The G>A transition mutation leads to three different mutant mRNAs in homozygous KI mice (**Supplementary Figure S1**). Mutant-1 contains the mutation (missense), whereas mutant-2 (nonsense) and mutant-3 (deletion/insertion) are due to skipping of exon 6.

We generated different PTMs encoding exons 1–6 of WT *Mybpc3* under the control of a ubiquitous (cytomegalovirus) or cardiac myocyte-specific (*TNNT2*, human cardiac troponin T) promoter (**Figure 1b**). To specifically detect repaired *Mybpc3* mRNA and protein, an N-terminal FLAG-tag was introduced in the coding sequence. In addition, an intron was

---

**Figure 1 RNA trans-splicing strategy.** (a) Pre-trans-splicing molecules (PTMs) are transferred into cells using plasmids or virus particles, and are transcribed in the nucleus. They target the pre-mRNA of the gene of interest and produce repaired mRNA and protein. This process is competing with cis-splicing, which gives rise to endogenous mRNA and proteins. (b) Schematic illustration of PTMs. We generated several PTMs containing the same *Mybpc3* wild-type sequence (exons 1–6) but different binding domains covering the *Mybpc3* intron 6. PTMs also included a chimeric intron, containing sequences from the human β-globin and immunoglobulin (IgG) genes, and a DISE from the rat fibroblast growth factor receptor 2 gene. (c) Schematic representation of 5'-trans-splicing bypassing a *Mybpc3* point mutation (not in scale). The endogenous KI *Mybpc3* pre-mRNA target containing the mutation on the last nucleotide of exon 6 is shown together with the PTM and the resulting transcripts obtained by cis- or trans-splicing. (d) Schematic illustration of repaired FLAG-tagged cMyBP-C protein. The protein is composed of 10 Ig-1 and Fn-3 domains and has 4 phosphorylation sites (PPPP) in the MyBP-C motif. The C0–C1 domains, including the Pro-Ala-rich region derived from the PTM (white), BD, binding domain; CMV, cytomegalovirus; DISE, downstream intronic splicing enhancer; E, exon; P, promoter; TNNT2, human cardiac troponin T; 5'SS, 5'splice donor site.
inserted right after the promoter to increase mRNA stability and enhance expression of the constructs. The splicing domain included a canonical 5′ splicing donor site sequence followed by a downstream intronic sequence enhancer element, which has been shown to markedly increase the transsplicing efficiency. Importantly, the binding domain of the PTM is an essential part because it confers specificity to the target pre-mRNA. To evaluate the feasibility and efficacy of 5′-trans-splicing, we designed several constructs differing only with respect to the length of the binding domain and to the target site in Mybpc3 intron 6 (Supplementary Table S1). The binding domains are complementary to this intron, but leave out its 3′ splicing elements. Moreover, to maintain the PTM in the nucleus and reduce its translation we deleted the SV40 polyadenylation (polyA) signal, which is known to contribute to mRNA stability and nuclear export (Figure 1b). As negative controls we designed PTMs with reversed binding domains (PTM-R), which should not induce 5′-trans-splicing events. PTM-driven 5′-trans-splicing on the endogenous KI Mybpc3 pre-mRNA target should produce a full-length repaired Mybpc3 mRNA, in which the mutation is bypassed, resulting in a FLAG-tagged WT repaired cMyBP-C protein, and simultaneously cis-splicing should be reduced (Figure 1c,d).

Evidence for repair of Mybpc3 mRNA by 5′-trans-splicing in vitro

To allow gene transfer in neonatal mouse cardiac myocytes (NMCs), PTMs were packaged into self-complementary adeno-associated virus serotype 6 (AAV6), a serotype known to efficiently transduce cardiac myocytes in culture. NMCs were isolated from KI mice and transduced with AAV6-PTMs either with or without polyA signal (ΔpA) or AAV6-green fluorescent protein (GFP) as a control. After 4 days of transduction (multiplicity of infection (MOI): 3,000) about 80% of cells expressed GFP (Supplementary Figure S2). Using PCR primers that specifically amplify the repaired Mybpc3 mRNA (Supplementary Figure S1 and Supplementary Table S2), we obtained a specific signal in AAV6-PTM- and AAV6-PTMΔpA-transduced NMCs, but not in untransduced or PTM-R-transduced NMCs (Figure 2a). The absence of 5′-trans-splicing in AAV6-PTM-R-transduced NMC excluded the possibility that recombination occurred between the highly homologous sequences of PTMs and endogenous Mybpc3. The amount of repaired Mybpc3 was higher in the absence than in the presence of the polyA signal in the PTM. To evaluate whether cis-splicing was reduced, we used Mybpc3 primers binding in exons 1 and 9, which amplify total (repaired plus mutant) Mybpc3 mRNA. Although no major difference was detected between samples, reduced signals were observed for certain Mybpc3 mRNA species in AAV6-PTM- and in AAV6-PTMΔpA-transduced NMCs. This suggests a reduction in Mybpc3 cis-splicing when 5′-trans-splicing occurred (Figure 2a). Sequencing of repaired Mybpc3 mRNA amplicons confirmed the presence of the WT guanine (G) at the exon 6–exon 7 junction (Figure 2b). Conversely, sequencing of the upper 896-bp band of total Mybpc3 mRNA in AAV6-PTMΔpA- and AAV6-PTM-R-transduced NMCs showed the presence of the mutant adenine (A) at the same position (Figure 2b). To estimate the amount of repaired Mybpc3 mRNA, we performed two rounds of PCR to amplify either total or only repaired Mybpc3 mRNA (Figure 2c). Comparison of amplicon intensities revealed that up to 33% of total Mybpc3 transcripts were repaired. To evaluate whether the efficiency of 5′-trans-splicing can be improved by increasing the dose of virus, we generated bicistronic recombinant adenovirus (AdV) encoding the PTMΔpA and GFP both under the control of the TNN72 promoter. KI NMCs were transduced with different MOI of AdV-PTMΔpA and analyzed 7 days after. Repaired Mybpc3 mRNA was detected in all transduced samples and its amount increased with increasing MOI (Figure 2d). The pattern of total Mybpc3 mRNA did not reveal major difference from one MOI to another, except at a MOI of 100 at which the intensity of the mutant-3 and mutant-2 mRNAs was lower than in untransduced cardiac myocytes (Figure 2d). Fluorescence analysis of AdV-GFP transduced cardiomyocytes confirmed a complete transduction with a MOI of 100 (Supplementary Figure S2). We further determined the efficiency of 5′-trans-splicing in several samples with AdV-PTMΔpA at a MOI of 100, and estimated by semi-quantitative analysis that 51 ± 7% of total Mybpc3 mRNA was repaired (Figure 2e).

We then investigated whether the repaired Mybpc3 mRNA is translated into protein and whether the repaired cMyBP-C is properly incorporated into the sarcomere. The presence of the FLAG-tag allowed specific detection of repaired cMyBP-C. Whereas repaired cMyBP-C was not detected by standard western blot with the anti-FLAG antibody, it was detected at the correct molecular weight after FLAG-immunoprecipitation (Figure 3a,b), confirming that 5′-trans-splicing occurred in cardiac myocytes. FLAG-immunoprecipitation of AAV6-PTM-R-transduced NMCs did not show any band at 150kDa, while FLAG-Mybpc3 transfected HEK293 cells, used as a positive control, did show it (Figure 3b). On the other hand, we detected a major FLAG-positive band around 35kDa in AAV6-PTM- and AAV6-PTM-R-transduced NMCs, which corresponds to the translated PTM transcripts (Figure 3a). This band was barely detected in AAV6-PTMΔpA-transduced NMCs, supporting the view that the absence of the polyA signal prevented translation and putative accumulation of toxic PTM proteins in cells. Endogenous and/or repaired cMyBP-C, but not translated PTMs were stained with a specific cMyBP-C antibody, which recognizes the MyBP-C motif (Figures 3a and 1d). To investigate whether the repaired cMyBP-C was incorporated into the sarcomere, we performed immunofluorescence analysis of transduced cardiac myocytes. About 9% of cMyBP-C-positive cells (= cardiac myocytes) were co-stained with the anti-FLAG antibody, and the repaired cMyBP-C showed the expected doublets in the A-band of the sarcomeres, indicating correct incorporation of endogenous cMyBP-C (Figure 3c).

Evidence for repair of Mybpc3 mRNA by 5′-trans-splicing in vivo

We next assessed the feasibility of PTM-driven 5′-trans-splicing in KI mice in vivo. The PTMΔpA and Renilla luciferase (RLuc) were inserted in the pdsAAV transfer vector under the control of the TNN72 promoter and were packaged in AAV
serotype 9 (AAV9), which has proven efficient cardiac transduction in mice in vivo. AAV9 (mean dose $5.2 \times 10^{12}$ vg/kg of body weight (BW)) was administered systemically into 7-week-old animals. Echocardiographic analysis performed during one month after injection did not display major differences in cardiac function between mice that received either AAV9 or NaCl (Supplementary Table S3). After 28 days, luciferase expression was evaluated by in vivo bioluminescence imaging and luminescence was recorded only in the heart of the AAV9-RLuc–injected mouse (Supplementary Figure S3). Accordingly, luciferase mRNA level was high in the heart and very low in the liver of the mouse that received

Figure 2 Detection of repaired Mybpc3 mRNA induced by 5′-trans-splicing in vitro. (a) KI NMCMs were transduced with AAV6 (MOI: 3,000–30,000) for 7 days before harvesting. RT-PCR analysis using different primer pairs: FLAG/E9-R primers amplified a specific 921-bp fragment that correspond to the repaired Mybpc3 mRNA in KI NMCMs transduced with AAV6 encoding PTM or PTMΔpA that contain a complementary binding domain to intron 6, but not in NMCMs transduced with a PTM containing a reversed binding domain (PTM-R), in not-transduced NMCMs (NT), nor in the amplifications without RT (−). With E1-F/E9-R primers bands of 896, 824, and 778 bp were amplified, corresponding to mutant-1/repaired, mutant-3 and mutant-2, respectively. Amplification of Myh6 mRNA encoding α-myosin heavy chain was used as a loading control. (b) Sequencing of the FLAG/E9-R amplicons from AAV6-PTM and AAV6-PTMΔpA transduced NMCMs confirmed replacement of the mutant adenine with the wild-type guanine (G, pink box) at the exon 6–exon 7 junction. In addition sequencing of the gel-excised 896-bp band obtained by RT-PCR with primers E1-F/E9-R from AAV6-PTMΔpA and AAV6-PTM-R transduced NMCMs validate the presence of the endogenous mutant adenine (A, red box) on the last nucleotide of exon 6. (c) Determination of the percentage of repaired Mybpc3 mRNA by semi-quantitative RT-PCR. cDNA from AAV6-PTMΔpA-transduced KI NMCMs was used to amplify total (E1-F/E9-R primers) or only repaired (FLAG/E9-R primers) Mybpc3 by PCR (25 cycles). PCR fragments were column-purified and serially diluted (1:3). In a second round of PCR, a common primer pair (E1-F/E2-R) was used to amplify a 242-bp fragment in all Mybpc3 transcripts. The percentage of total Mybpc3 mRNA that was repaired was estimated by comparing bands of similar intensities (black rectangles), that is, lane 9 (dilution 38) of repaired Mybpc3 mRNA and lane 10 (dilution 39) of total Mybpc3 mRNA. (d) KI NMCMs were transduced with AdV-PTMΔpA (MOI: 10–100). RT-PCR analysis was performed using same primer pairs as in a. Repaired Mybpc3 mRNA was amplified in all transduced samples but not in NT or in −RT (amplification without RT) samples. (e) KI NMCMs were transduced with AdV-PTMΔpA (MOI: 100). Bar represents repaired Mybpc3 mRNA expressed as percentage of total Mybpc3 mRNA (mean ± SEM) obtained by semi-quantitative analysis (as described above but with 1:1.5 dilutions). Number of samples is indicated in the bar. MOI, multiplicity of infection; RT-PCR, reverse transcription-PCR.
AAV9-RLuc (Figure 4a), validating efficient and preferential cardiac transduction with AAV9. Importantly, the repaired *Mybpc3* mRNA was detected in the heart of the mouse that received AAV9-PTM*ΔpA*, but not in the others (Figure 4a). No effect on cis-splicing was discernible (Figure 4a). Semi-quantitative analysis showed that 0.05% of total *Mybpc3* mRNA was repaired (Figure 4b).

To augment the dose of virus and thus the 5′-trans-splicing efficiency, we performed experiments in neonates (Figure 5). Longitudinal echo analysis in neonatal mice revealed that KI mice developed first systolic dysfunction, as shown by lower fractional area shortening than WT mice at day 2, followed by LVH, as shown by higher left-ventricular-mass-to-BW than WT mice at day 3 (Figure 5a). We then systemically administered AAV9-PTM*ΔpA* into 1-day-old KI mice (3.4×10^{14} vg/kg BW). This dose of AAV9 resulted in an almost complete transduction of cardiac tissue at postnatal day 7 (Supplementary Figure S4). Although the dose per BW was ~65-fold higher than in the adult mouse, no beneficial effect on left ventricular mass/BW and on fractional area shortening were observed at day 4 and 7 (Figure 5b) as well as 7 weeks after injection (Supplementary Table S3). Despite the absence of rescue, we evaluated the 5′-trans-splicing efficiency in one mouse 7 weeks after injection. The full-length repaired *Mybpc3* mRNA was detected by reverse transcription-PCR only in the heart of the AAV9-PTM*pA*-injected mouse and represented 0.14% of total *Mybpc3* transcripts (Figure 5c,d and Supplementary Figure S5), which thus showed 2.8-fold higher 5′-trans-splicing event in the newborn than in the adult mouse. In addition, the repaired cMyBP-C was detected, although faintly after FLAG-immunoprecipitation (Figure 5e).

**Discussion**

RNA trans-splicing as a potential therapeutic technology has been applied to several diseases both in cell systems and in mouse models (for reviews, see refs. 1,2). The present study
provides the first evidence of successful 5'-trans-splicing both in cardiac myocytes and in the heart in vivo for the most prevalent cardiac genetic disease.

The percentage of total Mybpc3 mRNA that was repaired was estimated to be between 33 and 66% in transduced KI NMCMs. This is much higher than what has been reported in previous studies using endogenous targets.5,33 However, despite the high efficiency of Mybpc3 5'-trans-splicing at the mRNA level, the amount of repaired cMyBP-C protein was rather low. This suggests a low efficiency of translation and underlines that mRNA copy number and protein levels do not need to be correlated.24 On the other hand, the amount of total Mybpc3 mRNAs is 80% and the level of cMyBP-C protein 90% lower in KI than in WT mice.22 Therefore, 33% of total Mybpc3 mRNA is repaired, it represents less than 7% of the Mybpc3 mRNA amount and less than 4% of the cMyBP-C protein amount found in WT mice. This may well be under the limit of detection by western blot.

Our study provides additional evidence for removing the polyA signal in the PTM construct to prevent translation and therefore accumulation of PTM proteins that could exert a dominant-negative effect on the structure and/or function of cardiac myocytes.

Recently, in vivo 3'-trans-splicing has been shown to improve the phenotype of a mouse model of spinal muscular atrophy.8 The present study provides the first evidence for successful mRNA repair by 5'-trans-splicing in vivo. The combination of AAV9 and TNNT2 promoter allowed efficient cardiac transduction in vivo. Although this resulted in detectable levels of repaired cMyBP-C, the amount was still too low to ameliorate the cardiac phenotype. Thus, further optimization of the technique is needed to increase the amount of "therapeutic" protein.

Among strategies that aim at specifically targeting mutant mRNA in dominant genetic disease, such as mutant-specific RNA interference,35 trans-splicing has potential advantages for the therapy of HCM. First, it allows the repair of even complex consequences on RNA splicing that, as exemplified in the present HCM mouse model,22 can result from a single point mutation and will be difficult/impossible to target with siRNA without affecting WT mRNA. Second, and in contrast to RNA interference therapies targeting a specific mutation, two different PTMs would be enough to treat the 40–60% of HCM patients who carry a MYBPC3 mutation18,19,36–38—one targeting the 5’ mutations and the other the 3’ mutations. Therefore, trans-splicing represents a promising, potentially causal therapy of severe forms of HCM.

Materials and methods

Animals. The investigation conforms to the guidelines for the care and use of laboratory animals published by the National Institutes of Health (Publication no. 85-23, revised 1985). The experimental procedures were in accordance with the German Law for the Protection of Animals and accepted by the Ministry of Science and Public Health of the City State of Hamburg, Germany (Nr. 69/10). Mybpc3-targeted KI mice were generated previously,22 and maintained on a Black Swiss background.
Design of PTMs. The sequences of primers used are listed in Supplementary Table S2. The coding sequence of the PTMs was generated by PCR from WT Mybpc3 cDNA with a forward primer (PTM F) containing an XhoI restriction site, the ATG followed by the FLAG sequence and the first 20 nucleotides of Mybpc3 exon 1. The reverse primer (PTM R) contained a BamHI restriction site and the 5’ canonical splice donor site sequence followed by a downstream intrinsic splicing enhancer element/sequence from the rat fibroblast growth factor receptor 2 gene and last 23 nucleotides of Mybpc3 exon 6. The binding domains were obtained by PCR on genomic KI DNA using a forward primer (BD F) containing a BamHI restriction site, and 21 nucleotides of Mybpc3 intron 6. The reverse primer (BD R) contained a NotI restriction site and 28 nucleotides complementary to Mybpc3 intron 6. The reverse binding domain was amplified in the same way (primers BD-R F and BD-R R) but reverse complemented. PCR products were sequentially cloned into pdsAAV6-TNNT2 vector (human TNNT2 promoter) and accuracy of the insertion was verified by DNA sequencing analysis. The SV40 polyA signal was removed in one of the PTM plasmids by digestion with NotI and Mva1269I followed by religation of the plasmid.

Production and titration of AAV particles. AAV6 pseudotyped vectors were generated by cotransfection of HEK293-AAV cells (Biocat, Heidelberg, Germany) with the pdsAAV-TNNT2 vector and the AAV packaging plasmid pDP6s, which provides the AAV2 rep and AAV6 cap genes and adenoviral helper functions. AAV9 pseudotyped vectors were generated by triple-transfection of pdsAAV-TNNT2 transfer plasmid with pAAV2/9 and pHelper encoding adenoviral helper functions (Biocat). Generation of recombinant AAV6 and AAV9 particles was carried out as described previously, with some modifications. HEK293-AAV cells were cultivated in Dulbecco’s modified Eagle’s medium (high glucose) supplemented with 10% (vol/vol) heat-inactivated fetal calf serum, 0.1 mmol/l MEM non-essential amino acids, 2 mmol/l L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. Tissue culture reagents were obtained from Life technologies. Briefly, 1.5 x 10⁷ HEK293-AAV cells were seeded on 15-cm plates and transfected with polyethyleneimine. After 72 hours, cells were harvested, washed three-times with phosphate-buffered saline (PBS) and resuspended in PBS. After freeze–thaw cycles, benzonase (Merck, Darmstadt, Germany) was added and the lysates incubated for 1 hour at 37 °C. Cell debris was pelleted and vector-containing lysates were purified using iodixanol step gradients.

The genomic titers of DNase-resistant recombinant AAV particles were determined by quantitative PCR using the SYBR Green qPCR Master MIX 2 (Fermentas, Darmstadt, Germany) and an ABI PRISM 7900HT cycler (Applied Biosystems, Foster City, CA). Vectors were quantified using primers specific for the TNNT2 promoter sequence. Real-time PCR was performed in a total volume of 10 µl with 0.3 µmol/l for each primer. pdsAAV-GFP plasmid was used as a copy number standard. A standard curve for quantification was generated by serial dilutions of the respective plasmid DNA. The cycling conditions were as follows: 50 °C for 2 minutes, 95 °C for 10 minutes, followed by 35 cycles of 95 °C for 15 seconds and 60 °C for 60 seconds.

Calculations were done using the SDS 2.4 software (Applied Biosystems).

Generation of recombinant AdV. To generate the AdV-PTMΔpA under the control of TNNT2 promoter, we used the In-fusion kit (Clontech, St Germain-en-Laye, France) to fuse together the two cassettes into pShuttle85706. The pShuttle containing the TNNT2-PTMΔpA insert as well as the TNNT2-GFP in a bicistronic manner was electroporated into Escherichia coli BJS5183-D1 (Stratagene, Darmstadt, Germany) to produce adenoviral DNA through recombination. This DNA was used to transfect HEK293 cells and recombinant AdV was amplified using standard techniques.

Isolation, culture, and transduction of neonatal mouse cardiac myocytes. We isolated and cultured NMCs using a well-established protocol. AAV6-mediated transductions of cardiac myocytes were performed for 30 minutes at 37 °C in suspension before plating (4.4 x 10⁷ cells/well) at a MOI of 3,000 (AAV6-PTMΔpA, both RNA and protein analysis, AAV6-PTM and AAV6-PTM-R for protein analysis) or 30,000 (AAV6-PTM and AAV6-PTM-R for RNA analysis). Cardiac myocytes were kept in culture for 7 days at 37 °C and 10% CO₂ before harvesting.

In vivo AAV9 administrations. Seven-week-old KI mice received AAV9-PTMΔpA (1.04 x 10¹¹ vg), AAV9-RLuc (1.36 x 10¹¹ vg) or NaCl via systemic administration into the tail vein with a 29-G needle. Intravenous injections of neonatal KI mice (postnatal day 1) with AAV9-PTMΔpA (4.7 x 10¹¹ vg) or PBS were performed into the temporal vein using a 30-G needle. All mice recovered quickly from the injection.

In vivo bioluminescence imaging. Luciferase activity in the mouse heart was non-invasively assessed by in vivo bioluminescence imaging 4 weeks after AAV9 injection. AAV9-RLuc–injected mouse and the NaCl-injected mouse were anesthetized with 1.8% isoflurane. Thereafter the substrate coelenterazine (Biosynth, Stad, Switzerland) dissolved in methanol and further diluted in sodium phosphate buffer pH 7, was injected intraperitoneally (i.p.) at a dose of 2.5 mg/kg body weight in both mice. The mice were then placed in the chamber of a Xenogen in vivo Imaging System under continuous anesthesia. The oxidation of coelenterazine by Renilla luciferase releases coelentarmide and blue light at 480 nm. This bioluminescence was recorded in a manually-selected region of interest centered over the mouse heart, using 3-minute scans.

Echocardiographic analysis. Transthoracic echocardiography was performed using the Vevo 2100 System (VisualSonics, Toronto, Ontario, Canada). KI mice were anesthetized with isoflurane (1–2%) and fixed to a warming platform in a supine position. B-mode images were obtained using a MS400 transducer for adult mice and a MS550 transducer for neonatal mice. Images were obtained in a parasternal short and long-axis view and dimensions of the left ventricle were measured in a short-axis view in diastole and systole.

Reverse transcription-PCR analysis. Total RNA was isolated from cultured NMCs or ventricular tissue (30 mg) using the SV Total RNA Isolation System Kit (Promega, Madison, WI)
Western blot and immunoprecipitation analyses. Crude protein extract from cultured NMCs or HEK293 cells were extracted in lysis buffer (30 mmol/l Tris base pH 8.8, 5 mmol/l EDTA, 30 mmol/l NaF, 3% SDS, 10% glycerol) and protein concentration was determined by Bradford protein assay (Bio-Rad, Hercules, CA). Total proteins (NMCs 30 µg/lane, HEK293 2.5 µg/lane) were separated on 10% SDS-polyacrylamid (Bio-Rad, Hercules, CA) prior a second touchdown PCR (67–62 °C) with different primer pairs (Supplementary Table S2) were performed using AmpliTaq Gold Polymerase (Applied Biosystems) in a total volume of 20 µl for 35 cycles. PCR products were visualized on 1.5% agarose gels. The full-length repaired Mybpc3 mRNA was amplified by touchdown PCR (67–62 °C) with Phusion Hot StarTII High-Fidelity DNA polymerase (Biozym, Hessisch Oldendorf, Germany) for 31 cycles. For semi-quantitative analysis a touchdown PCR (65–60 °C) for 25 cycles was used to amplify either total (primers E1-F, E9-R) or repaired (primers FLAG, E9-R) Mybpc3 mRNA. PCR products of the first PCR round were purified on a column (QiAquick PCR Purification Kit; QiAGEN, Valencia, CA) prior a second touchdown PCR (65–60 °C, primers E1-F, E2-R) for 35 cycles.

Acknowledgments. We thank Evelyn Bendrat and Michaela Miehe (UKE-HEXT, Hamburg, Germany), and Mareen Welzel and Andreas Jungmann (University Hospital Heidelberg, Heidelberg, Germany) for their excellent technical support in adeno-associated virus production. We thank Christiane Pharrmann and Sonja Schreper (UKE-Transplant and Stem cell immunobiology lab, Hamburg, Germany) for help in cardiac myocytes preparation and confocal imaging. We also thank Jürgen Kleinschmidt (DKFZ Heidelberg, Heidelberg, Germany) and Julie Johnston (Penn Vector Core, Pennsylvania University, Philadelphia, PA, USA) for supplying the pDP6rs and the pAAV2/9 plasmids, respectively, and Christian Witt (University of Heidelberg, Heidelberg, Germany) for the anti-cMyBP-C antibody. This work was supported by the Fritz Thyssen Stiftung (Az. 10.09.1.139), the seventh Framework Program of the European Union (Health-F2-2009–241577; Big-Heart project), the DZHK (German Center for Cardiovascular Research), and the German Federal Ministry of Research and Education (BMBF). The authors declared no conflict of interest.


