

SUPPLEMENTAL MATERIAL

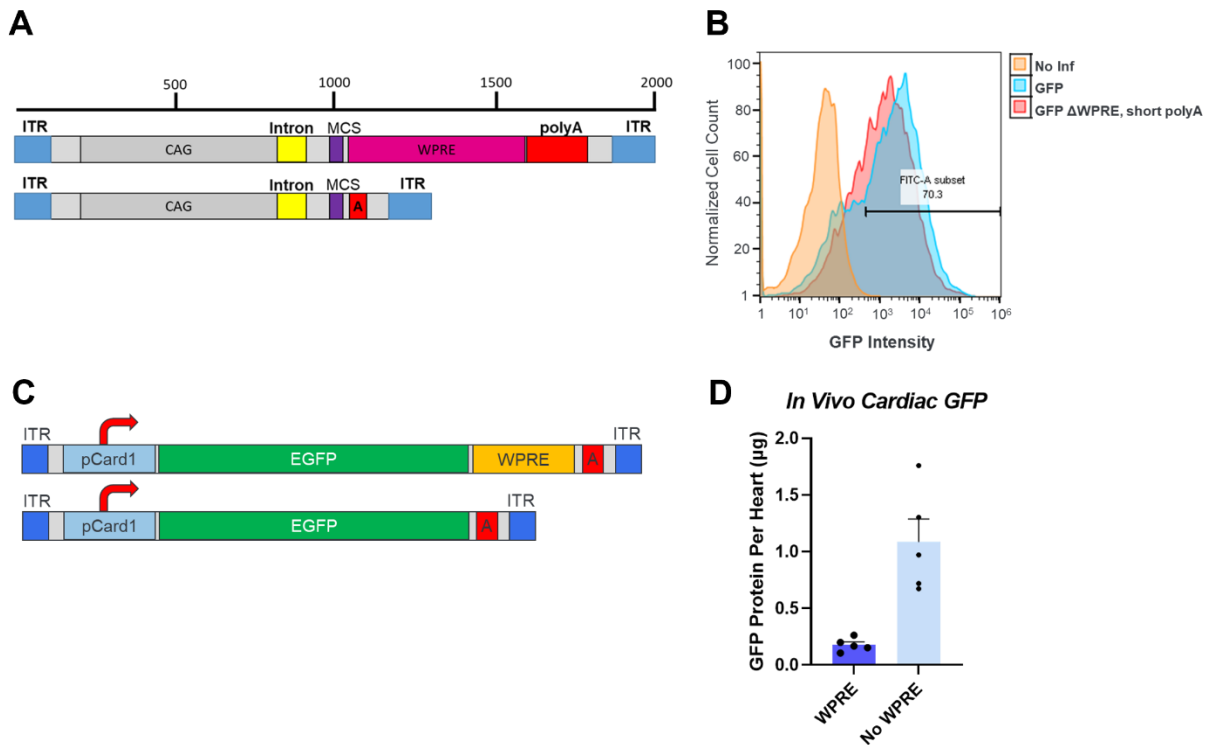


Figure S1. Minimized *cis*-regulatory AAV transgene cassettes. **a** Schematic of *cis*-regulatory cassette modifications. Starting with a standard AAV vector having a CAG promoter, intron, WPRE, and standard polyA sequence totaling ~2 kb of regulatory sequence, we assessed the impact of woodchuck hepatitis virus posttranscriptional regulatory element (WPRE, 589 bp) removal, as well as polyadenylation signal abbreviation (“A”) to save an additional 170 bp. Multiple Cloning Site, MCS. **b** Expression of a GFP reporter cloned into the multiple cloning site (MCS), as assessed by flow cytometry analysis 2 days post-infection, indicated slightly decreased but robust expression in human cardiac fibroblasts (n = 2), MOI 160, 000. **c** Schematic of *cis*-regulatory cassette modifications in the context of pCard1. **d** To determine the cardiac impact of WPRE removal in the context of pCard1, wild-type mice were dosed retro-orbitally IV with AAV9 containing the cassettes illustrated in C at 1E13 vg/kg (n = 5/group) and harvested 3.5 weeks later. Cardiac GFP protein abundance was measured using Abcam GFP ELISA kit

(ab171581). This analysis clearly indicated there were no detrimental effects on protein expression for the “No WPRE” cassette.

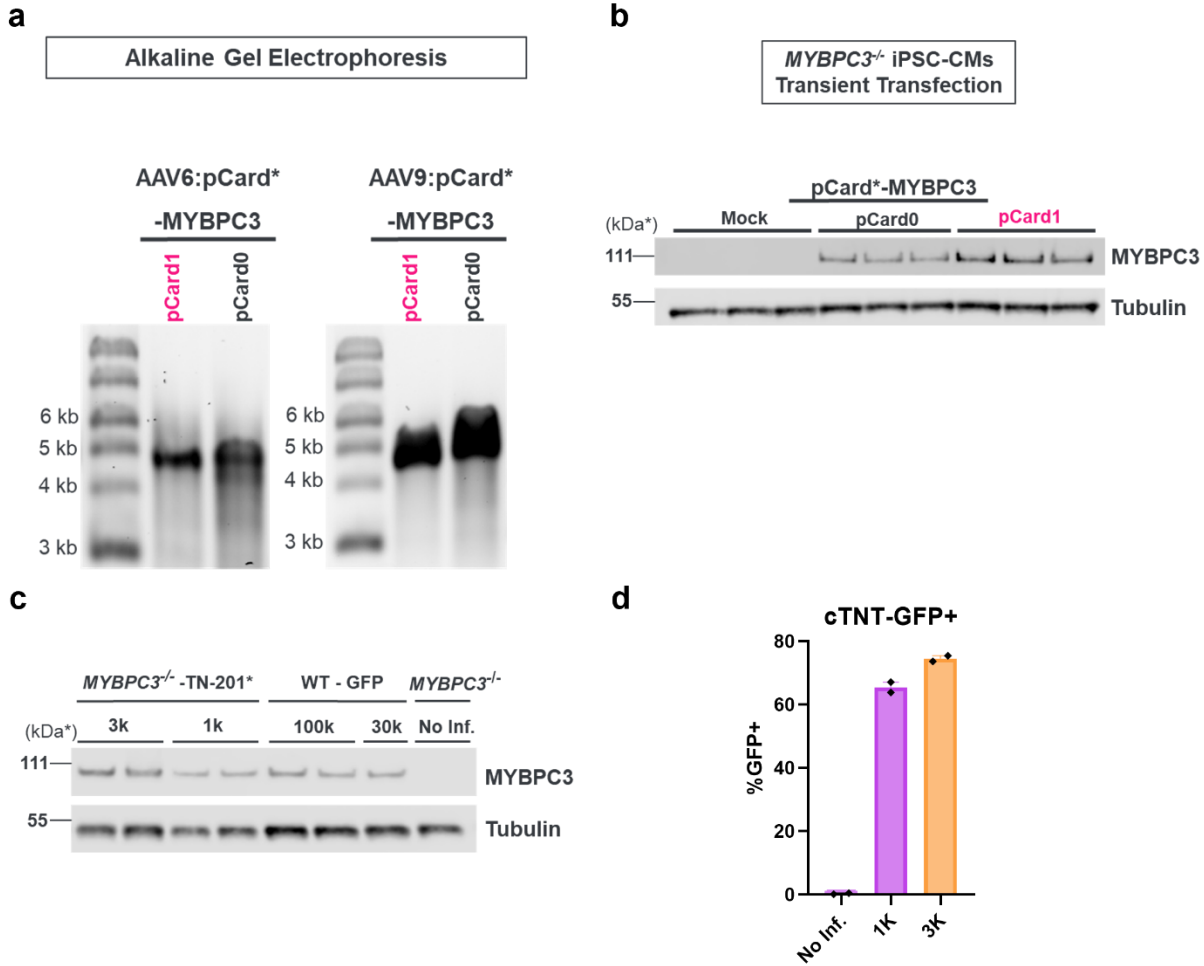


Figure S2. Promoter Assessment in Human iPSC-CMs. **a** Denaturing alkaline gel electrophoresis of purified AAV vector genomes of matched AAV6: pCard*-MYBPC3 (left) and AAV9: pCard*-MYBPC3 (right) viral preps. The ladder was similarly denatured such that the size of ssDNA viral genomes is represented. While pCard1 viral genomes ran at the expected 4.7 kb size, pCard0 preps demonstrated more heterogeneity on the lower end than the expected 4.9 kb size. Equal amounts of DNA were loaded for the matched serotype preps. **b** The expression driven off of the unpackaged plasmid DNA of pCard0 and pCard1 constructs was assessed by transient transfection of *MYBPC3*^{-/-} iPSC-CMs seven days after transfection (n = 3/condition assayed). The increased expression of MYBPC3 driven off of the pCard1 plasmid indicated the

enhanced potency of the DNA sequence relative to pCard0. **c** Representative immunoblot of Fig 1f-h matched transduction, RNA and protein analysis in *MYBPC3*^{-/-} iPSC-CMs, showing near-WT levels of protein expression at 1 vg/dg compared to parental wild-type control iPSC-CMs one week post-infection. *Please note that MYBPC3 exhibits accelerated migration on Tris-acetate gels. **d** Flow cytometry analysis of iPSC-CMs transduced with CR9-01:pCard1-GFP one week post-infection (n = 2/condition assayed). Consistent with vector genome analysis, flow cytometry one week post-infection with CR9-01:pCard1-GFP, a green fluorescent protein reporter, demonstrated that at the lowest dose used (1K) ~60% of iPSC-CMs were transduced indicating an infectious multiplicity of infection equivalent to one. Data are shown as means ± SEM.

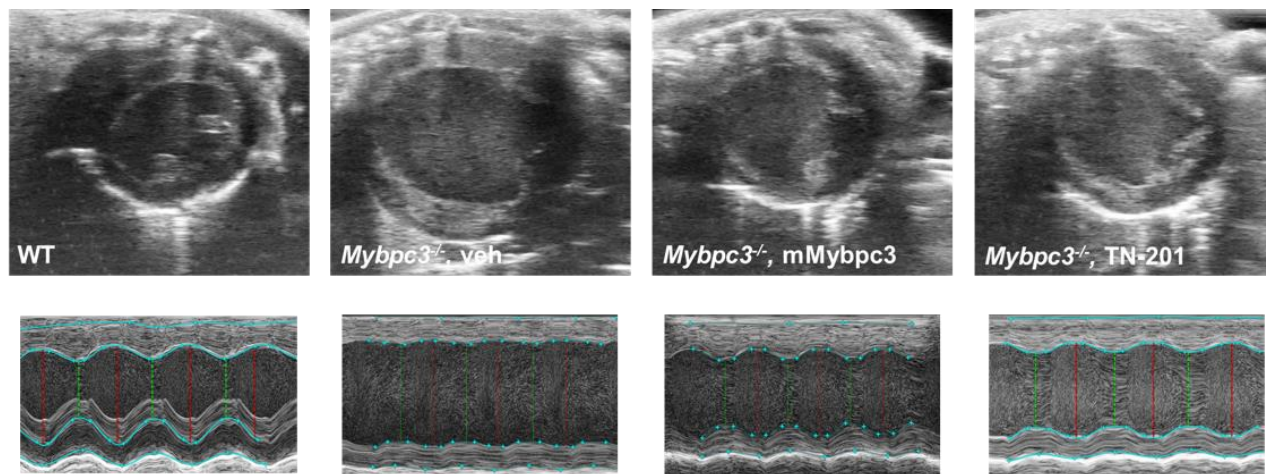


Figure S3. Representative B-mode (top) and M-mode (bottom) ultrasound images. WT (far left); *Mybpc3*^{-/-} vehicle (center left); *Mybpc3*^{-/-}, m*Mybpc3* (center right); and *Mybpc3*^{-/-}, TN-201 (far right) shown.

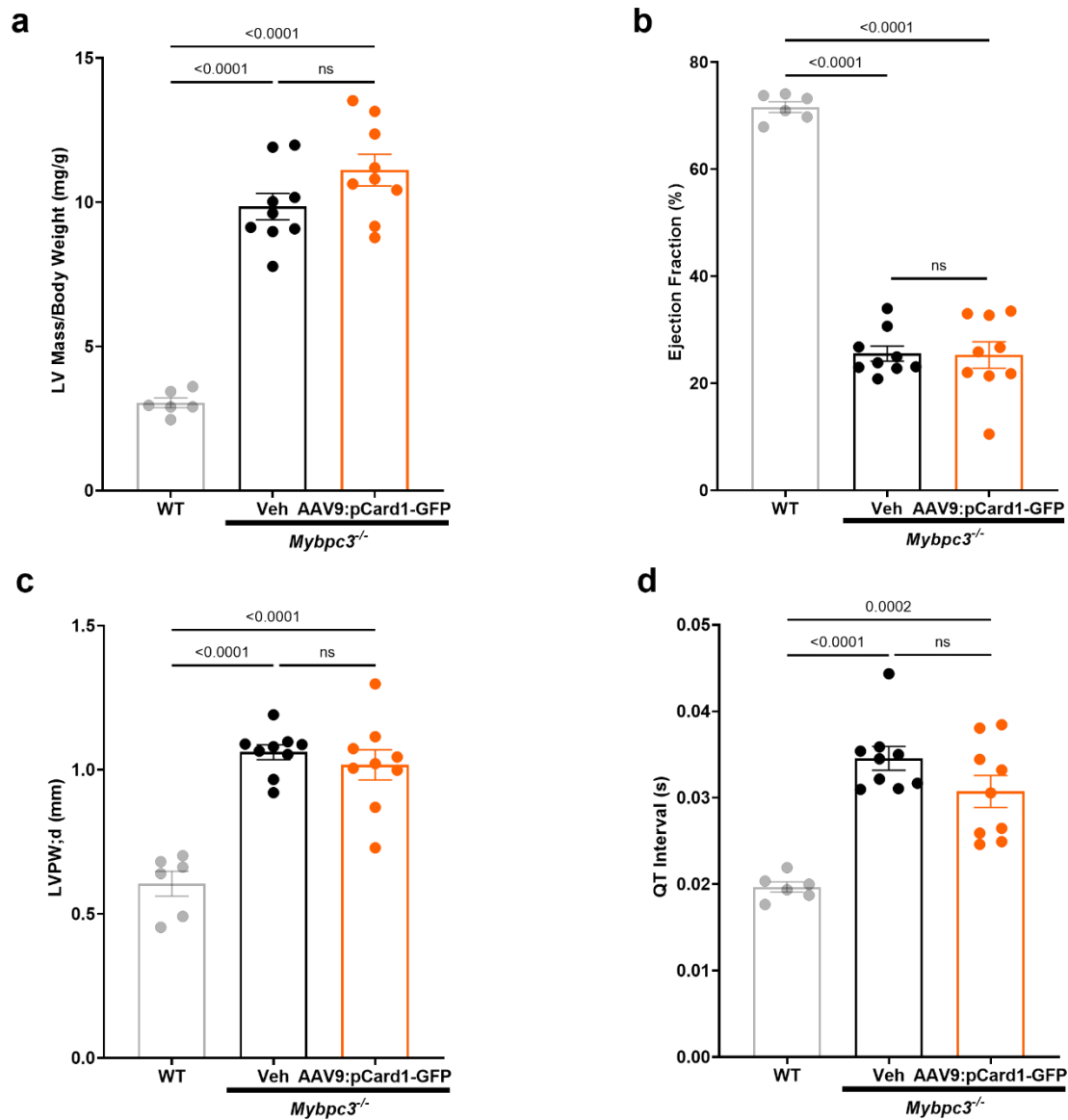


Figure S4. Dosing with AAV9: pCard1-GFP Results in No Significant Effects on Cardiac Function. To control for the effect of AAV capsid proteins and other components of the vector expression cassette on the heart, homozygous mice were dosed retro-orbitally IV with vehicle or AAV9 with the same cassette but encoding GFP (AAV9:pCard1-GFP) at the efficacious dose of 3E13vg/kg. Age-matched WT littermates were used as controls. In contrast to AAV9:mMybpc3, echocardiographic and electrocardiographic assessments six weeks post-injection demonstrated

no significant benefit of animals dosed AAV9:pCard1-GFP compared to Vehicle-treated animals in **(a)** LV mass normalized to body weight, **(b)** ejection fraction (EF), **(c)** Left ventricular posterior wall thickness in diastole (LVPW;d), and **(d)** QT interval. WT (n=6), *Mybpc3*^{-/-}, Veh (n=9), *Mybpc3*^{-/-}, AAV9:pCard1-GFP (n=9). P-value per one-way ANOVA with Tukey's multiple comparisons test.

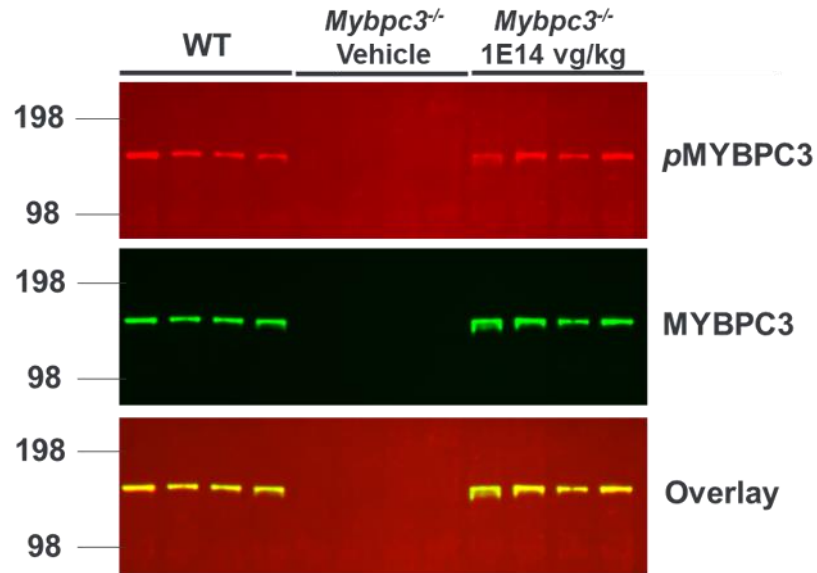


Figure S5. PKA-Mediated Phosphorylation of Restored Cardiac MYBPC3. Homozygous mice were dosed retro-orbitally IV at two weeks of age with vehicle or 1E14vg/kg of AAV9:mMybpc3 and cardiac protein analyzed six weeks post-injection. Age-matched WT littermates were used as controls. Following BCA quantification, equivalent levels of protein were loaded and analyzed by immunoblotting for Phospho-PKA Substrate (RRXS*/T*)⁶⁵ (top) detected in the 680 channel and total MYBPC3 protein (middle) detected in the 800 channel. Overlay bottom, n = 4/condition.

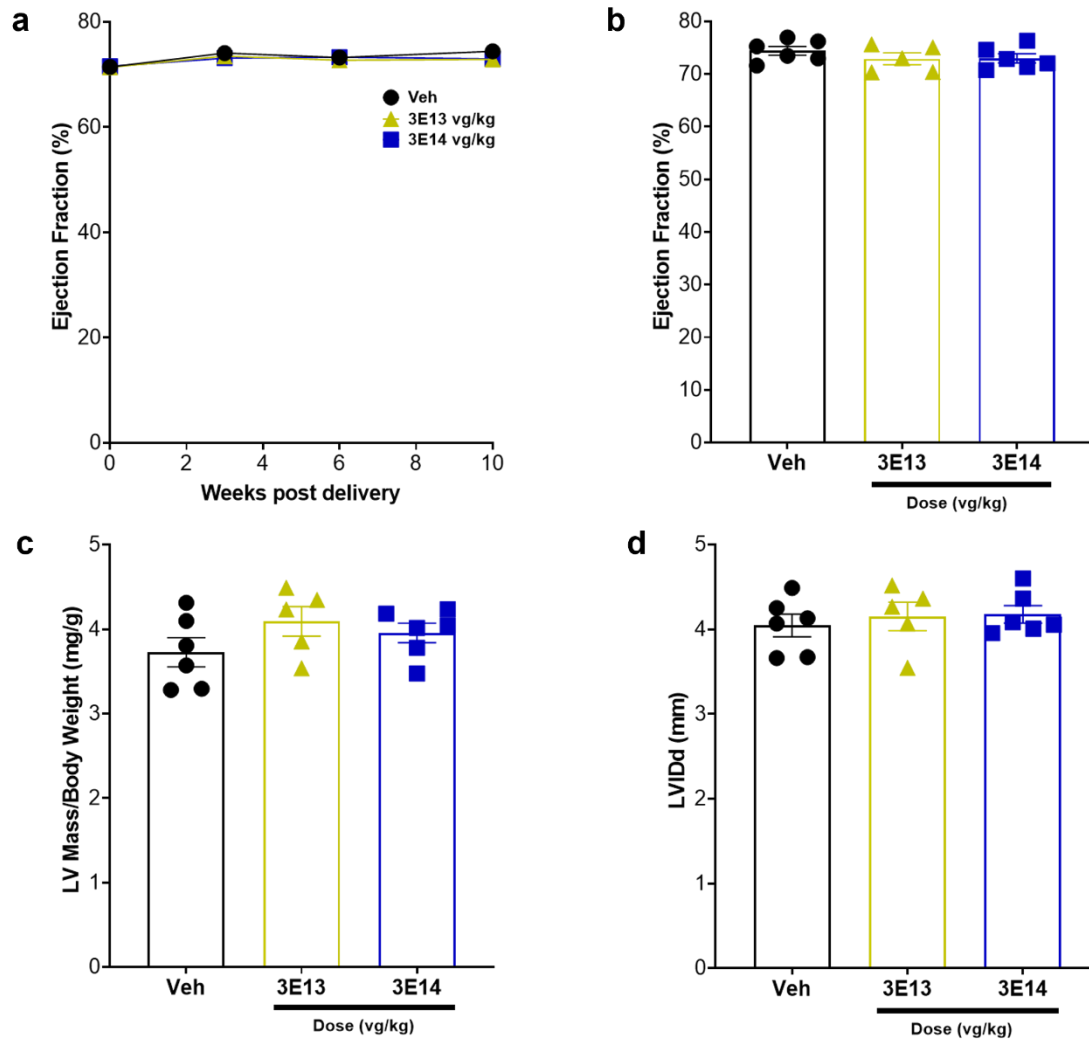


Figure S6. AAV9:mMybpc3 Did Not Affect Cardiac Function of Naïve Male CD-1 Mice at 30X an Efficacious Dose. **a** EF progression and **(b)** EF at 10 weeks post-delivery. Gene delivery did not result in any changes in EF. **c** Left ventricular (LV) mass was measured to represent hypertrophy and was unchanged 10 weeks post-delivery. **d** Left ventricular internal diameter during diastolic (LVID;d) was measured to represent dilation and was unchanged 10 weeks post-delivery. Vehicle, Veh (n = 6), 3E13 vg/kg (n = 5) and 3E14 vg/kg (n = 6). P-value per one-way ANOVA with Tukey's multiple comparisons test.

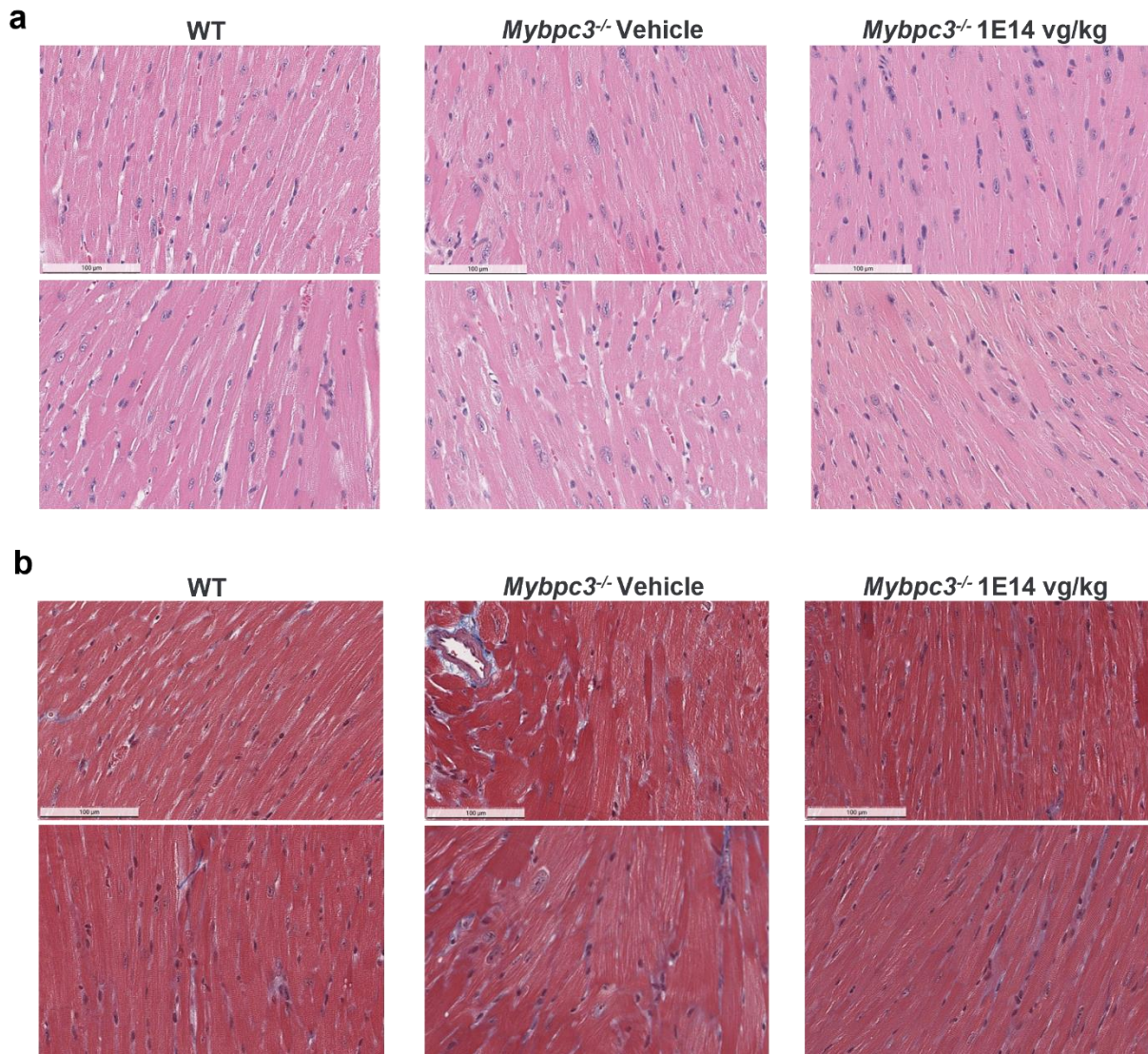


Figure S7. Representative H&E and Masson's Trichrome Histology. Homozygous mice were dosed retro-orbitally IV at two weeks of age with vehicle or 1E14 vg/kg of AAV9:mMybpc3. Age-matched WT littermates were used as controls. Animals were euthanized at four months of age and their hearts fixed and paraffin embedded for (a) H&E staining and (b) Masson's trichrome analysis (scale bars, 100 μm).

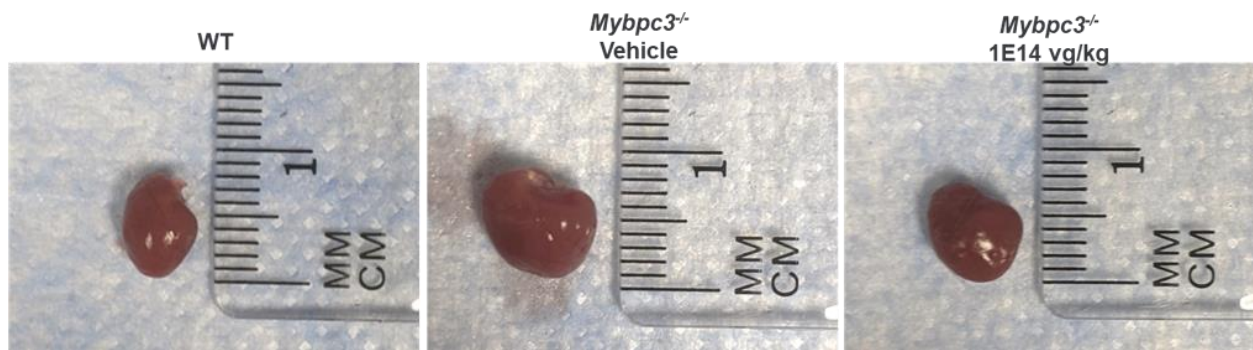


Figure S8. Representative Heart Images at Eight Weeks of Age. Homozygous mice were dosed retro-orbitally IV at two weeks of age with vehicle or 1E14 vg/kg of AAV9:mMybpc3. Age-matched WT littermates were used as controls. Animals were euthanized at eight weeks of age, or six weeks following viral injection.

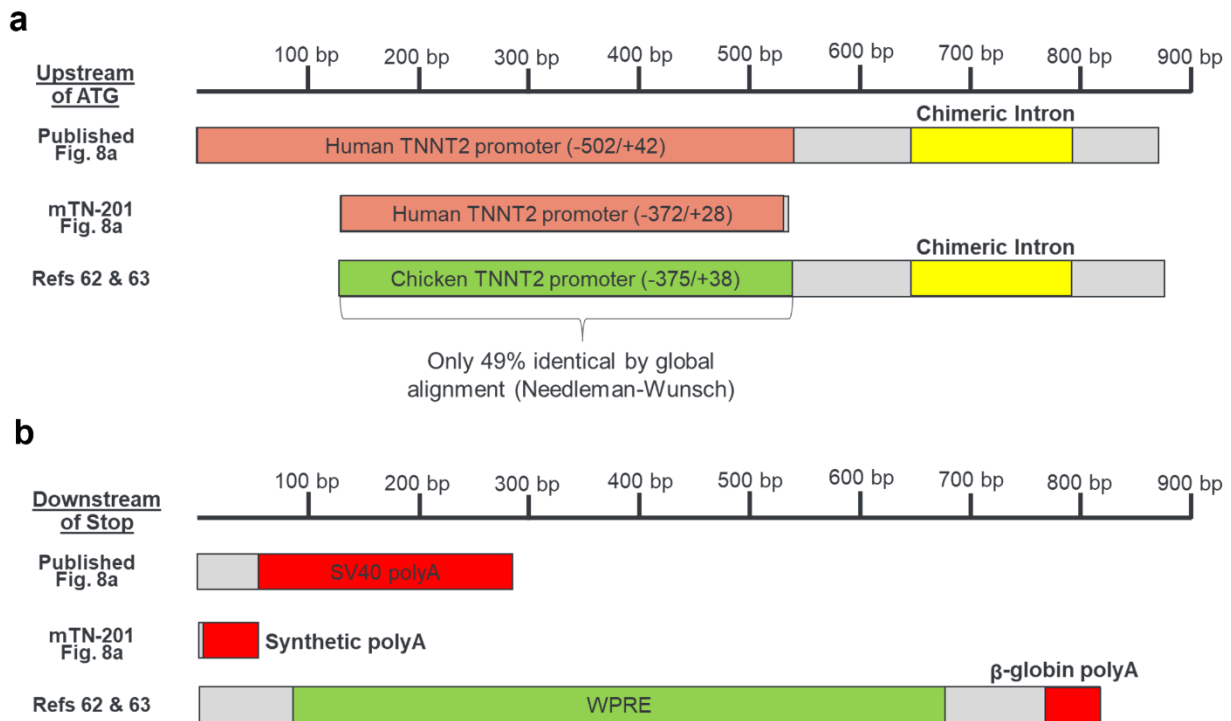


Figure S9. Comparison of Published Upstream and Downstream Regions for MYBPC3 Preclinical Gene Therapy. **a** Upstream regions used in Figure 8a for the published 5.4 kb cassette (denoted “Published”) or the TN-201 mouse surrogate (denoted mTN-201), as well as another standard used in References 62 and 63. This figure visually delineates the genetic elements upstream of the ATG and provides the precise promoter sequence location with respect to the native transcription start site (-/+). The chicken TNNT2 promoter sequence is only 49% identical (global alignment Needleman-Wunsch) to the human TNNT2 promoter sequence of pCard1 (of mTN-201). pCard0 is equivalent to human TNNT2 promoter (-572/+28). **b** As above, showing the differences in expression cassette regions downstream of the stop codon.

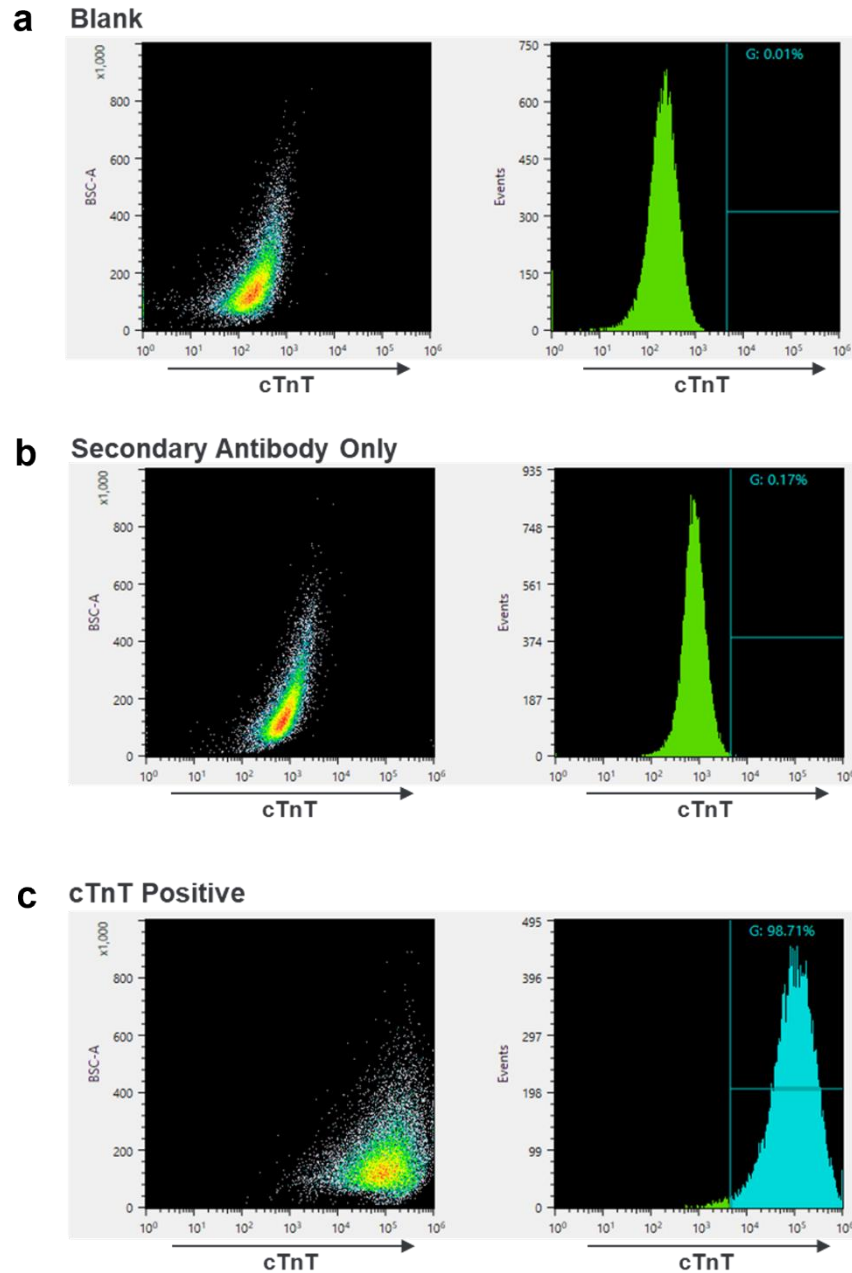


Figure S10. Representative Flow Cytometry of Human iPSC-CM Differentiations.

Differentiated iPSC-CMs were thawed and a million live cells were fixed, permeabilized and assessed by flow cytometry as follows: **(a)** no antibody control, **(b)** secondary antibody only control, and **(c)** cTnT primary (ThermoFisher MA5-12960) and secondary antibodies, demonstrating a homogenous >98% cTnT+ population of iPSC-CMs.

Table S1. Echocardiography Parameters for Figure 2

	WT	<i>Mybpc3</i> ^{+/-}	<i>Mybpc3</i> ^{-/-}
HR (bpm)	509.0±19.8	496.3±18.4	493.0±9.0
LVID;s (mm)	1.57±0.04	1.63±0.03	3.22±0.08****
LVID;d (mm)	2.64±0.05	2.80±0.15	3.85±0.08****
SV (μL)	18.9±0.9	22.1±1.0	22.5±1.0*
EF (%)	73.4±0.9	74.4±0.4	35.2±1.0****
FS (%)	40.7±0.7	41.7±0.3	16.5±0.5****
LV Mass (mg)	29.0±2.0	35.6±1.4	110.2±6.9****
LV Mass/BW (mg/g)	3.4±0.3	4.0±0.2	12.8±0.8****
LVPW;d (mm)	0.54±0.03	0.64±0.03	0.85±0.04**

Heart rate (HR), left ventricular inner diameter during systole (LVID;s), left ventricular inner diameter during diastole (LVID;d); stroke volume (SV), ejection fraction (EF), fractional shortening (FS); body weight (BW), left ventricular poster wall thickness during diastole (LVPW;d). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 relative to WT. Data shown as Mean ± SEM.

Table S2. Echocardiography Parameters for Figure 3

	WT	<i>Mybpc3</i> ^{-/-} , Veh	<i>Mybpc3</i> ^{-/-} , m <i>Mybpc3</i>	<i>Mybpc3</i> ^{-/-} , TN-201
HR (bpm)	520.3±12.9**	578.2±13.5	522.4±9.9*	482.5±10.9****
LVID;s (mm)	2.18±0.08****	5.16±0.13	3.38±0.10****	4.05±0.21****
LVID;d (mm)	3.75±0.10****	5.69±0.12	4.54±0.11****	4.95±0.22**
SV (μL)	44.6±2.5**	32.0±1.9	47.7±2.2***	43.7±3.5*
EF (%)	73.6±1.0****	20.2±1.2	50.5±1.1***	38.0±1.5****
FS (%)	42.0±0.8****	9.3±0.6	25.6±0.7****	18.4±0.8****
LV Mass (mg)	121.8±5.7****	383.1±20.8	220.5±10.3****	239.5±14.4***
LV Mass/BW (mg/g)	4.5±0.1****	13.2±0.6	8.0±0.3****	9.0±0.4****
LVPW;d (mm)	0.74±0.03****	1.13±0.05	0.94±0.03**	0.90±0.05**

Heart rate (HR), left ventricular inner diameter during systole (LVID;s), left ventricular inner diameter during diastole (LVID;d); stroke volume (SV), ejection fraction (EF), fractional shortening (FS); body weight (BW), left ventricular poster wall thickness during diastole (LVPW;d). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 relative to *Mybpc3*^{-/-}, veh. Mean ± SEM shown.

Table S3. Echocardiography Parameters for Figure 6

	WT	<i>Mybpc3</i> ^{-/-} , Veh	<i>Mybpc3</i> ^{-/-} , m <i>Mybpc3</i>
e' (mm/s)	-23.8±1.0****	-8.2±0.9	-15.4±1.1****
MV E (mm/s)	725.6±39.3***	452.7±36.8	675.2±44.0**
MV A (mm/s)	385.2±38.8	413.3±43.2	347.2±51.5
IVRT (ms)	14.5±0.4****	31.2±1.2	24.1±0.8****
MV E/A	2.02±0.20	1.14±0.06	2.55±0.60*
MV E/E'	-31.1±2.6***	-57.9±5.6	-45.2±3.7

Mitral Annular e' Velocity (e'), Maximal Transmitral E-wave velocity (MV E), Maximal A-wave velocity (MV A), Isovolumic Relaxation Time (IVRT) *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 relative to *Mybpc3*^{-/-} veh. Mean ± SEM shown.

Table S4. Taqman Probes for RNA Detection

Target Gene	Species	Taqman Probe ID	Sequence
<i>Mybpc3</i>	Mouse	Mm00435104_m1	
<i>Gapdh</i>	Mouse	Mm99999915_g1	
<i>Nppa</i>	Mouse	Mm01255747_g1	
<i>Nppb</i>	Mouse	Mm01255770_g1	
<i>Myh7</i>	Mouse	Mm01319006_g1	
<i>Col1a1</i>	Mouse	Mm00801666_g1	
<i>Col3a1</i>	Mouse	Mm01254476_m1	
<i>Col4a1</i>	Mouse	Mm01210125_m1	
<i>Postn</i>	Mouse	Mm00450111_m1	
<i>MYBPC3</i>	Human	Custom	Forward Primer: GGTGGAGTTTGAGTGTGAAGTA Probe: CGCAAGTCAAATGGCTGAAGGACG Reverse Primer: TGAACCGGTATTTGAAGGTCTC

Table S5. Primers Used With SYBR Detection

Primer Name	Primer Sequence	Application
oLL285	cgcacattcctccctccg	mTN-201 vector genome detection
oLL370	aggcgtctgctcagtctcag	
oLL341	taggaagatcggaattcgccc	TN-201 vector genome detection
oLL382	gcagggaacagtcacgaac	
oLL290	gccagagctcatcattggc	Human <i>MYBPC3</i> RNA detection
oLL353	ggctcataggtgatgcctgg	

Table S6. Viral Lots

Virus	AKA	Lot	Figure
AAV6: pCard0-MYBPC3		CR #107	1C, S2
AAV6: pCard1-MYBPC3		014VC19AUG28-1	1C, S2
AAV6: pCard2-MYBPC3		014VC19AUG28-2	1C
CR9-01: pCard0-MYBPC3		CR012820-142	1D
CR9-01: pCard1-MYBPC3		CR012820-143	1D
AAV9: pCard0-MYBPC3		012VC19Aug07-1	1E
AAV9: pCard0-MYBPC3		023VC19Oct16-3	S2
AAV9: pCard1-MYBPC3		021VC19Oct02	1E, S2
CR9-01: pCard1-MYBPC3	TN-201*	AP042822	1F-1I
CR9-01: pCard1-GFP		HZ011121	1F-1I
CR9-01: pCard1-MYBPC3	TN-201*	EE061022	1A, 9
TN-201		002VC08Jan2020-3	3
AAV9:mMybpc3	mTN-201	002VC08Jan2020-5 & 002VC08Jan2020-7	3, 5D, 8D-8F
AAV9:mMybpc3	mTN-201	006VC19Feb2020-4 & 007VC26Feb2020-1	4, 5A-5C, 5E-5G, S4, S5, S7
AAV9:mMybpc3	mTN-201	ZC #821	6
AAV9:mMybpc3	mTN-201	009VC24Apr2020-1	7
AAV9:mMybpc3	mTN-201	020VC02Sep2020-9	8B, 8C
AAV9:mMybpc3 5.4kb	Published	020VC02Sep2020-6 & 021VC09Sep2020-7	8B, 8C
AAV9:mMybpc3	mTN-201	PD210608	S6