Supporting Information

Zelarayán *et al.* 10.1073/pnas.0808393105

Materials and Methods

RU486 Administration. Activation of Cre expression in αMHC-CrePR1 mice was induced by i.p. administration of RU486 dissolved in sesame oil (30 μg/g body weight, Sigma-Aldrich). Daily injections were administrated for 5 days in 10–12-week-old mice. Seven days after the last RU486 injection, mice were subjected to experimental myocardial infarction. Littermates negative for either Cre or loxP sites were used as controls.

BrdU Administration. For *in vivo* BrdU labeling, mice received daily injections of BrdU (50 mg/kg BW) 5 days before they were killed.

Primers. List of primers used in real-time PCR: ANP: 5′-CATCACCCTGGGCTTCTTCCT and 3′-TGGGCTCCAATC-CTGTCAATC; BNP: 5′-GCGGCATGGATCTCCTGAAGG and 3′-CCCAGGCAGAGTCAGAAACTG; β-MHC: 5′-ATGT-GCCGGACCTTGGAA and 3′-CCTCGGGTTAGCT-GAGAGATCA; α-skeletal actin: 5′-AGACACCATGTGCGAC-GAAG and 3′-CCGTCCCCAGAATCCAACACA; GATA4: 5′-CTGGAGGCGAGATGG and 3′-GGTGGTGGTAGTCTGG; Tbx5: 5′-ACAGAGCGAGAATAGAAC and 3′-GGATGATG-GAGACTTGC; Tbx20: 5′-AGAAGGAGGCAGCAGAGAA-CAC and 3′-GCACAGAGAGGATGAGGATGGG; Tbx2: 5′-TGGATGGTGGCAGGCAAAGC and 3′-GGTAGGCAGTGA-CAGCGATGAAG; β-catenin: 5′- GCCGAGCCGTCAG and 3′-GTCCAACTCCAT CAGG; TCF-4: 5′-AACGGAACAGACAG-TATAATGG and 3′-GGTCCTCTTCATAGC; LEF-1: 5′-GTCCCTTTCTCCACCCATC and 3′-AAGTGCTCGTCGCTG-TAG; GAPDH: 5′-ATGTTCCAGTATGACTCCACTCACG and 3′-GAAGACACCAGTAGACTCCACGACA. CRE: 5′-TCGCAAGAACCTGATGGAC and 3′-GCCGCATAAC-CAGTGAAAC; Sca-1: 5′-ACTGTGCCTGCAACCTTGTCT-GAGA and 3′-GTCCAGGTGCTGCCTCCATT; c-kit: 5′-CTGCTCTGCGTCCTGTTGGTC and 3′- TTGTGCTGGATG-GATGGATGGC; Oct3/4: 5′-GCTTCAGACTTCGCCTCCT-CACC and 3′-GCCATCCCTCCGCAGAACTCG.

List of Primers Used for Recombination PCR: $\beta\text{-}cat^{\Delta ex3-6}$ mice: β-catenin: 5′-ACTGCCTTTGTTCTCTTCCCTTCTG and 3′-CAGACAGACAGCACCTTCAGCACTC; $\beta\text{-}cat^{\Delta ex3}$ mice: β-catenin: 5′-GCTGCTGTGACACCGCTGCGTGGAC and 3′-CACGTGTGGCAAGTTCCGCGTCATCC.

Myocardial Infarction. Chronic occlusion of the left anterior descending artery (LAD) was performed in RU486-injected male mice at 3 months of age as described previously (1). Permanent ligation of the LAD was induced upon anesthesia (2.4% isofluorane) with a 7–0 prolene suture (Ethicon). After surgery, pain relief was administrated (buprenorfine 2 mg/kg). Myocardial infarction was confirmed by echocardiographic analysis and by visualization of the ischemic area under a binocular (Stemi 2000- C, Zeiss). Two or 4 weeks after surgery, mice were killed and the hearts were removed for further examination.

Echocardiography. Echocardiographic analysis was performed on an Accuson Sequoia (Siemens) instrument equipped with a 13-MHz microprobe scanning head. Mice were anesthesized by ketamine/xylazine (30 mg/10 mg per 1KG BW i.p.), the anterior chest area was shaved and two-dimensional (2D) images and M-mode tracings were recorded as described before (2). Ventricular measurements were taken before and after experimental myocardial with ≥3 readings per mouse. The observer was unaware of the genotypes and treatments.

Histological Techniques. Mouse hearts were fixed in 10% formalin/PBS, embedded in paraffin, and sectioned at 4 μm. For immunohistochemistry, sections were deparaffinized, blocked (5% BSA), microwaved with citrate buffer for antigen retrieval, and incubated overnight with the different primary antibodies: β-catenin (Zymed, 1:200); β-gal (MP Biomedicals, 1:300); cTnT and Tbx5 (Abcam, 1:200); GATA4 (Santa Cruz, 1:100); and Sca-1 (R&D System, 1:50). Masson Trichrome staining was performed on deparaffinized sections. For wheat germ agglutinin (WGA)-FITC staining, deparaffinized sections were O/N with lectin WGA-FITC (20 mg/ml) and mounted with PRO-LONG GOLD (Invitrogen). The HOPE-fixation technique (DCS Innovative Diagnostik-Systeme) was used for detection of Sca-1. Different Alexa antibodies (Invitrogen, 1:200) were used as secondary antibodies for immunofluorescence. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). For peroxidase staining, an anti-rabbit IgG-peroxidase (Sigma) was used and developed with a biotin/avidin amplification system (Vector). Enzymatic lacZ detection was performed in cryosections using X-gal (Roth). Apoptosis was detected by indirect TUNEL with the Apoptosis Detection Kit (Chemicon) and proliferation with the BrdU Assay Kit (Roche). Microscopic images were captured with a digital (IX70, Olympus) or confocal (Fluo View TMFV 1000, Olympus) microscope.

Histological Measurement. The semiautomated immunofluorescence quantification AxioVision software (Zeiss) was used for measurement of the cell area. To calculate the cell area average per animal in WGA-FITC-stained section, 150 cells per animal were selected under the microscope. For area calculation of $cTnT^{pos}/GATA4^{pos}$ cells in the scar, cells with an area <16 μm were counted and matched to total DAPI postive cells in the scar. Infarct size was calculated using longitudinal sections of the heart stained with Masson Trichrom. Planimetry of the photographed sections was measured as described previously (3) using the AxioVision software (Zeiss).

RNA Quantification and Western Blot. Quantitative real-time PCR was performed on total heart RNA (Qiagen) protocol converted to cDNA using SYBR Green primers. All samples were run in triplicates and normalized to GAPDH. mRNA copy number was calculated using a standard curve with the BioRad software. Protein isolation was performed with a cell fractioning kit (Chemicon). Samples were incubated with β-catenin (BD Bioscience) and GAPDH (Advanced ImmunoChemicals) antibodies. HRP-conjugated secondary antibodies were from DAKO.

Flow Cytometry. For cardiac cells, a total cardiac population from 10 to12-week-old mice was isolated, minced, homogenized, and filtered through 40-μm mesh (BD Falcon). After centrifugation, the cell pellet was resuspended in FACS-buffer (PBS + 5% FCS) and filtered through 30-μm mesh (Miltenyi Biotec) to separate cell aggregates and debris. Isolated cells were labeled with the already-cited antibodies. For intracellular labeling, cells were fixed with 0.2% formaldehyde and permeabilized with FACS-buffer containing 0.5% Saponin (Sigma-Aldrich). A donkey anti-rabbit IgG-APC (Jackson Immuno Research) was used as secondary antibody for β-galactosidase and GATA4. A FITC conjugated rat IgG2a (eBioscience) was used as matched isotype

control for Sca-1-FITC antibody and a rabbit isotype control (Zymed) for β-galactosidase or GATA4. Nonviable cells were detected with propidium iodide. Gates were established by nonspecific Ig binding in each experiment. Crosshatch lines were determined by comparing with isotype control antibodies. Spleen was used as positive control for Sca-1 expression and negative control for GATA4 detection. Spleen samples were depleted of red blood cells by incubating with a buffered ammonium chloride solution for 2 minutes at room temperature and the procedure as already described for heart was followed.

Differentiation Assay. For isolation of Sca-1^{pos} cells, adult murine heart (10–12 weeks old) were enzymatical dissociated as described previously. After heart dissociation cell suspension was filtered through 12-μm mesh and incubated with FITC-conjugated anti-Sca-1 antibody (20 min at 4°C). The cell suspension was washed (PBS, 0.5% BSA, 2 mM EDTA) and incubated with anti-FITC microbeads (30 min at 4°C). Cell preparations were systematically subjected to two cycles of MACS selection. Sorted populations were reanalyzed by flow cytometry and >95% purity of Sca-1^{pos} cells was confirmed before use. Sca-1^{pos} cells were labeled with the CM-Dil cell tracer (Molecular Probes). Primary cultures of neonatal cardiomyocytes were prepared by enzymatic digestion of ventricles from 1- to 3-day-old FVB mice as previously described. Purified labeled ScaI^{pos} cells were cocultured in fibronectin-coated chamber slides at a total density of 2×10^4 (1:4 ratio; Sca-1^{pos}/neonatal cardiomyocytes) in DMEM/10% FBS and antibiotics at 37°C in humid air with 5% CO_2. In parallel control experiments transwell permeable support was used for co-incubation, which allows the cells to share the same medium but without physical interaction; no staining of neonatal cardiomyocytes with CM-Dil was observed excluding any leakage of the dye over this time course *in vitro*. After 10 days, cocultured cells were fixed with 4% PFA and analyzed by α-sr ac/CM-Dil immunofluorescence. α-sr ac/CM-Dil cells vs. total CM-Dil cells were quantified under microscope. Further quantifications were performed using GATA4/cTnT costaining. Specific control experiments were performed to exclude any nonspecific CM-Dil labeling of neonatal cardiomyocytes due to leakage of the dye.

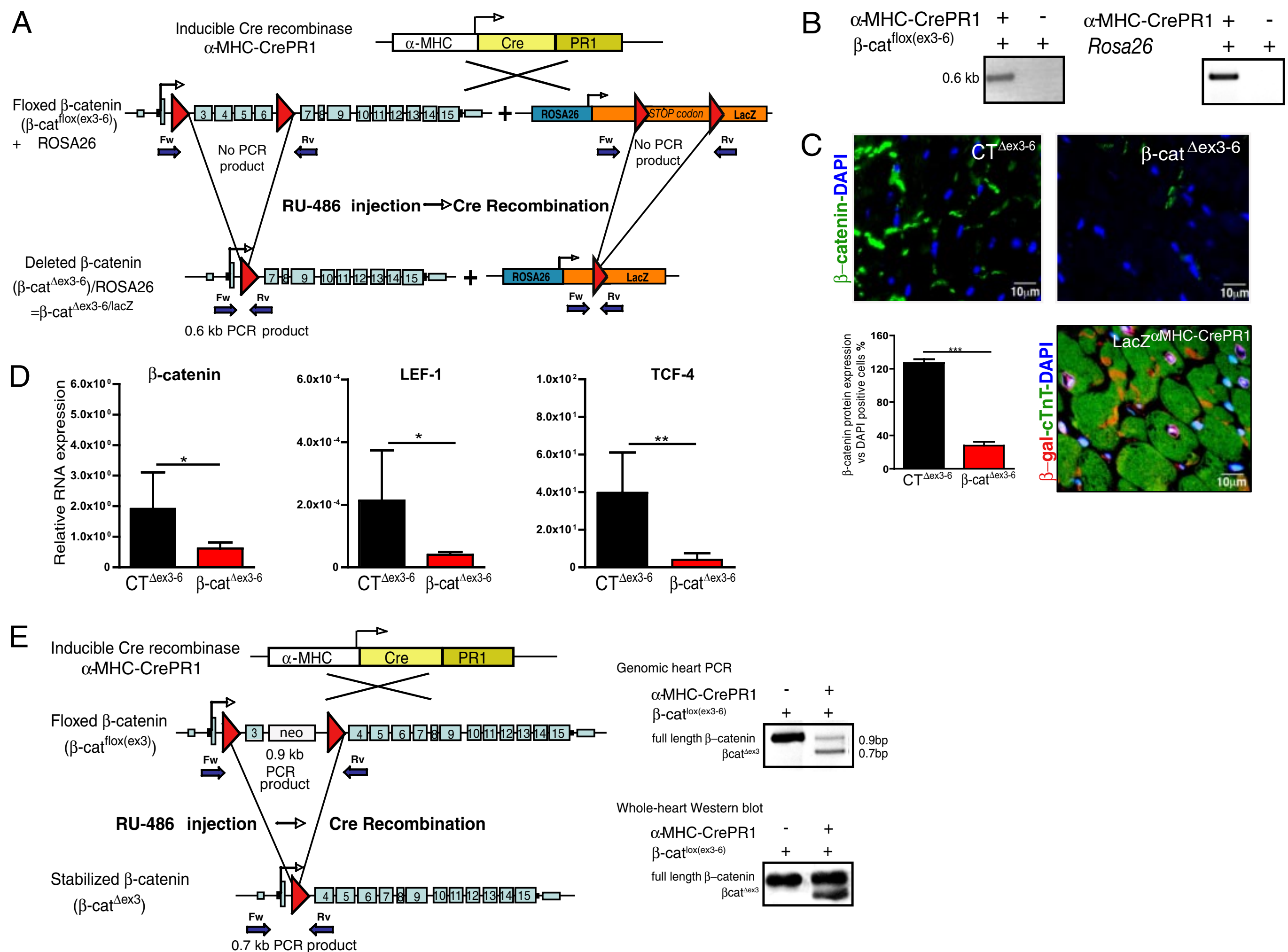


Fig. S1. Generation of mice with inducible, α*MHC*-restricted depletion or stabilization of β-catenin and expression of the reporter gene *lacZ*. (*A*) Mating scheme of the inducible α*MHC-CrePR1* mice with β-*catenin*$^{flox/floxex3-6}$ described before and ROSA26 mice. *Cre* recombinase expression results in β-catenin depletion (β-*cat*$^{\Delta ex3-6}$) and *lacZ* expression (*lacZ*$^{\alpha\text{-MHC-Cre}}$) by removing the *loxP* sites (red arrows) flanking exons 3–6 of β-catenin and a STOP codon controlling *lacZ* gene expression. (*B*) Employing primers as indicated (blue arrows) successful genomic recombination was confirmed in heart tissue. (*C*) β-Catenin protein expression is significantly reduced in β-*cat*$^{\Delta ex3-6}$ mice. β-Gal expression was confirmed by immunofluorescence (*D*) Downregulation of full-length β-catenin and target genes LEF-1 and TCF4 was observed by quantitative real-time PCR 3 weeks after Cre induction. (*E*) Mice with α*MHC*-restricted β-catenin stabilization (β-*cat*$^{\Delta ex3}$) have been described before (9). Recombination was confirmed by genomic PCR. Expression of the truncated β-catenin protein was detected by Western blot of whole heart samples 4 weeks after Cre induction. β-Gal, β-galactosidase; cTnT, cardiac Troponin T; *, $P < 0.05$; **, $P < 0.01$.

Fig. S2. Validation of the transgenic mice by analysis of β-catenin protein expression. (*A*) Decreased expression of full-length β-catenin protein demonstrated by Western blot of whole cell lysate and in the cytosol and nucleus fraction of heart samples from β-$cat^{\Delta ex3-6}$ mice. No truncated product was detected. (*B*) Expression of the truncated stabilized β-catenin protein was found in cytosol and nucleus of β-$cat^{\Delta ex3}$ mice. Wild-type β-catenin is detected due to mixtures of recombined, nonrecombined, and noncardiomyocyte cells.

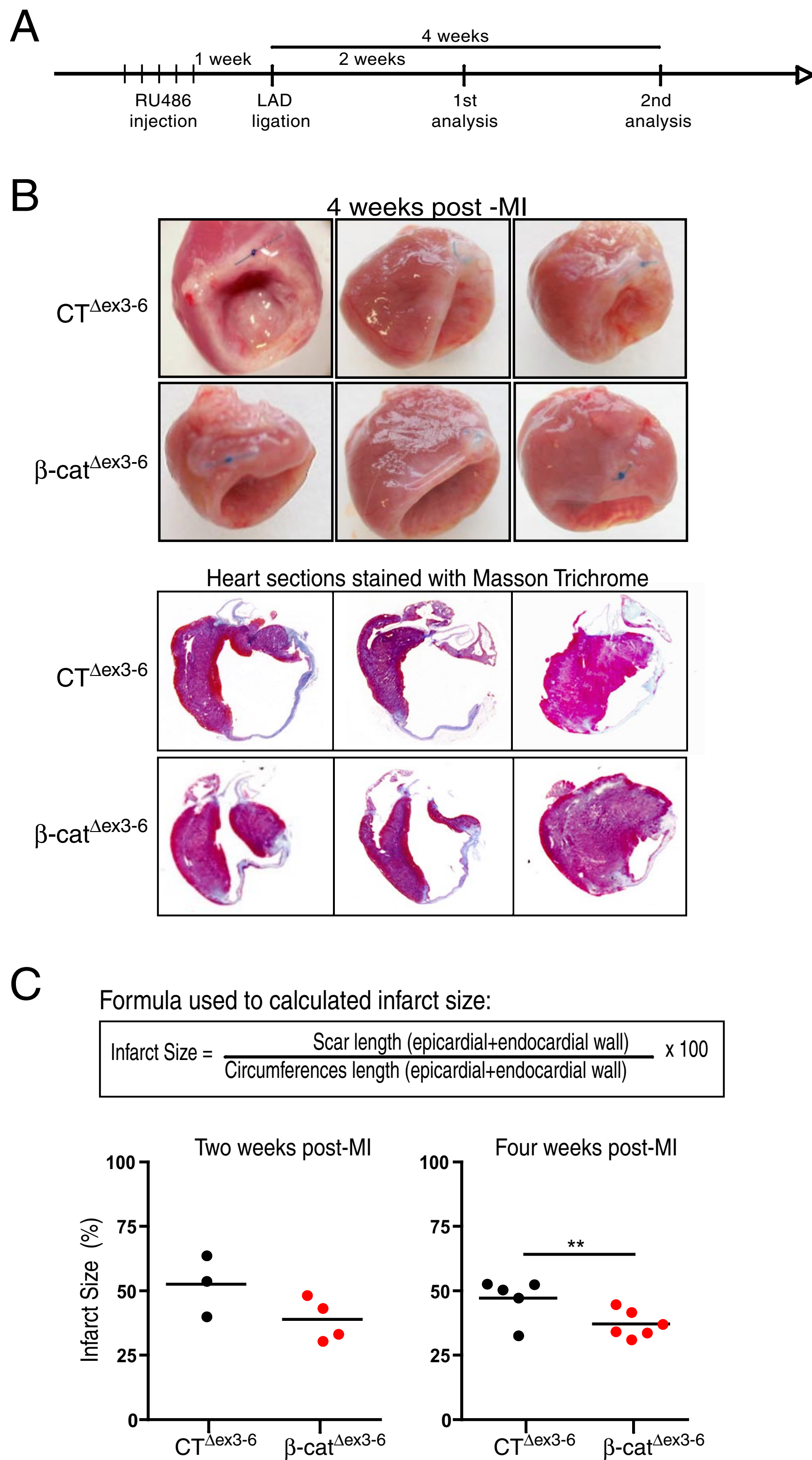


Fig. S3. Infarct size measurement. (*A*) Schematic course of the experimental set. (*B*) Representative pictures of whole heart from *β-cat*$^{\Delta ex3-6}$ mice and controls 4 weeks after experimental infarct showing comparable scar area and therefore selected for analysis. The lower panel shows Masson-Thrichrom stained sections used for scar size calculation. (*C*) Formula used for scar size calculation and posterior statistical analysis of infarcted hearts 2 and 4 weeks after LAD ligation. Only 4 weeks after ischemia the difference is significant between the compared groups.

Fig. S4. Time course of left ventricular remodeling following experimental infarct. Echocardiography analysis after 2 and 4 weeks LAD ligation of β-*cat*$^{\Delta ex3-6}$ and control mice. IVSD and LVD increased as expected already at 2 weeks postischemia in comparison to sham mice but no differences were observed when β-catenin-depleted mice are compared to their respective controls after infarct. Only 4 weeks after experimental infarct, fractional shortening showed a significant improvement in mice with β-catenin depletion supporting an effect on secondary infarct expansion. ***, $P < 0.001$.

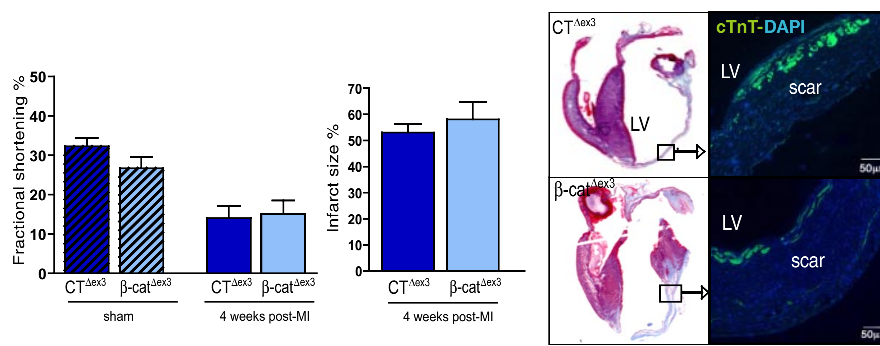


Fig. S5. αMHC-dependent β-catenin stabilization did not affect postinfarct LV remodeling. No difference concerning infarct size and fractional shortening 4 weeks after infarct in β-catenin stabilized mice compared to controls. MI, myocardial infarct; LV, left ventricle; cTnT, cardiac Troponin T.

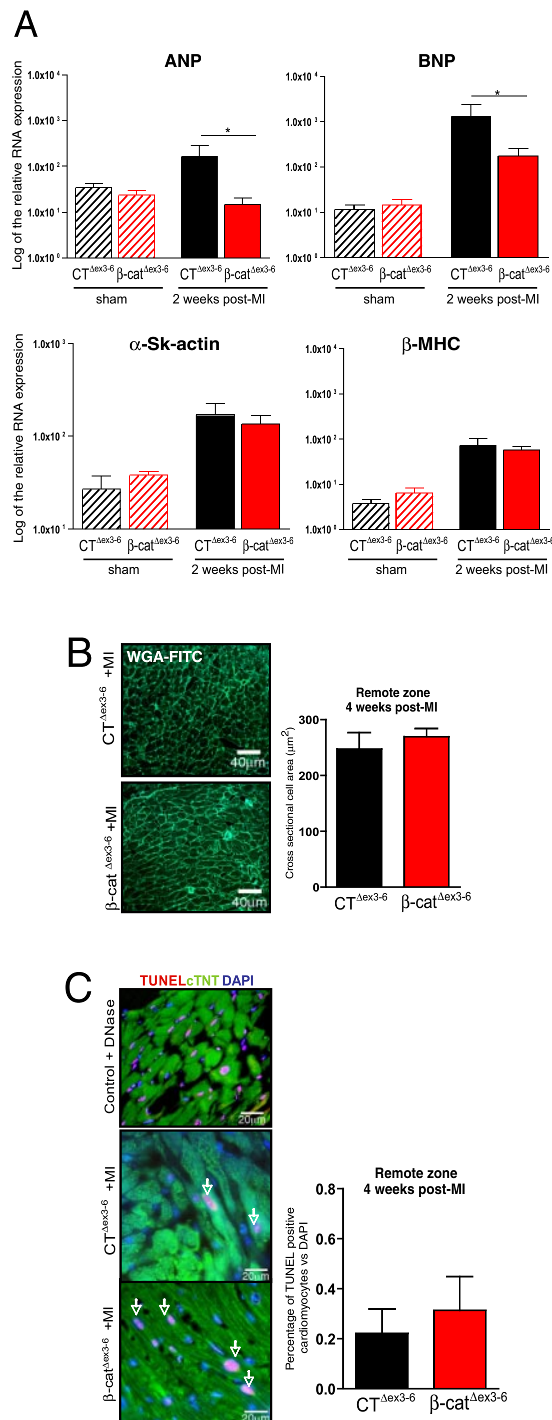


Fig. S6. Inhibition of adult cardiomyocyte hypertrophy or apoptosis does not explain the observed phenotype. (*A*) Quantification of ANP, BNP, α-skeletal-actin, and β-MHC by quantitative real-time PCR vs. GAPDH 2 weeks after infarct. Hypertrophy marker expression was upregulated in mice with LAD ligation but not in sham mice as expected. No significant changes were observed concerning α-skeletal-actin or β-MHC gene expression when comparing *β-cat*$^{\Delta ex3-6}$ and *CT*$^{\Delta ex3-6}$ mice, while heart failure markers ANP and BNP were reduced in *β-cat*$^{\Delta ex3-6}$ mice 2 weeks after infarct. (*B*) Quantification of cardiomyocyte hypertrophy in the remote zone as measured by cross-sectional area revealed no differences between β-catenin-depleted mice and controls. Representative wheat germ agglutinin FITC (WGA-FITC)-staining labeling the cell membrane is shown. (*C*) Apoptosis assessed by TUNEL assay showed no significant difference between β-catenin-depleted mice and controls. Representative immunofluorescence pictures of the TUNEL assay are shown and the positive control with DNase digestion. MI, myocardial infarct; cTnT, cardiac Troponin T; *, $P < 0.05$.

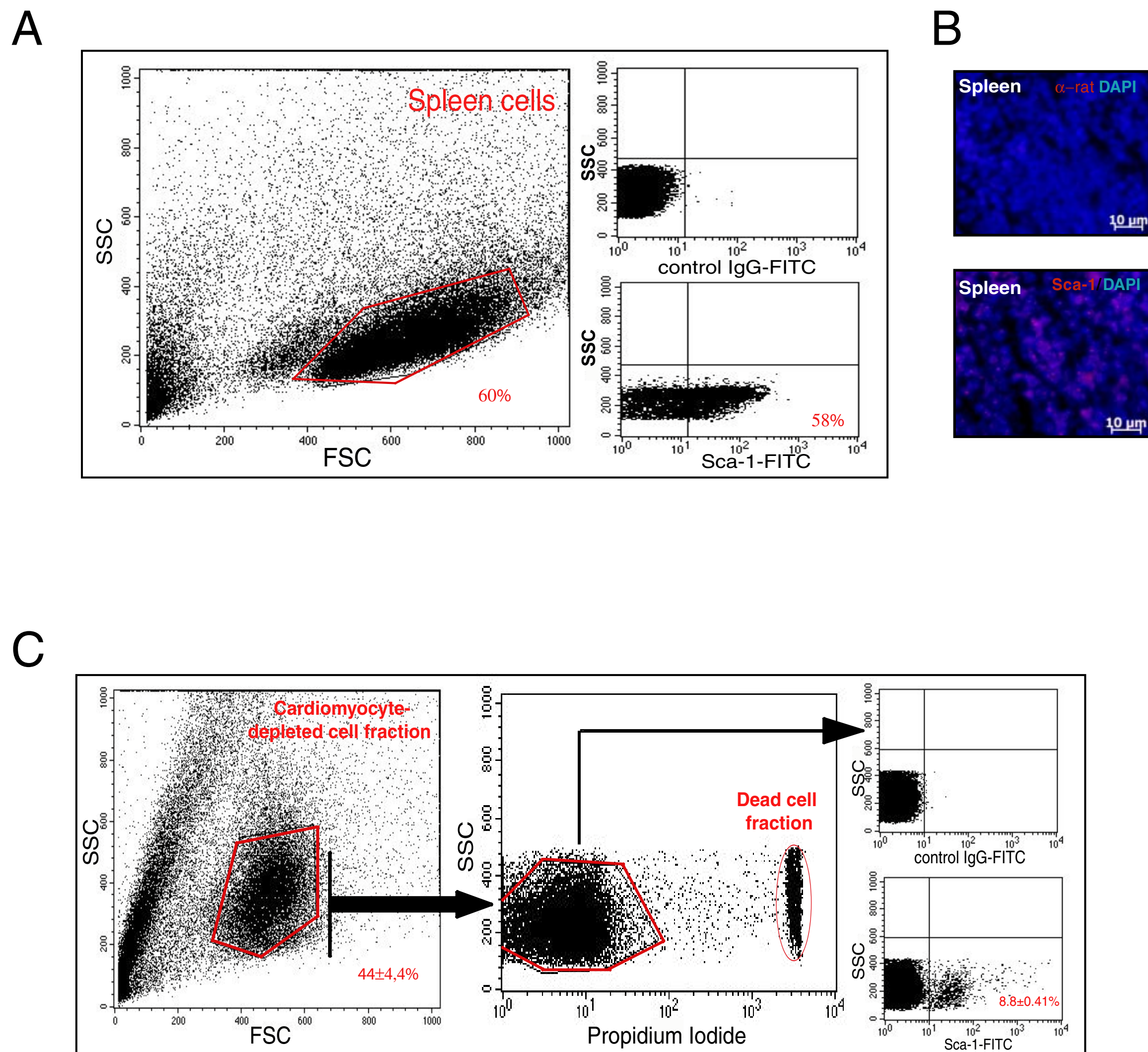
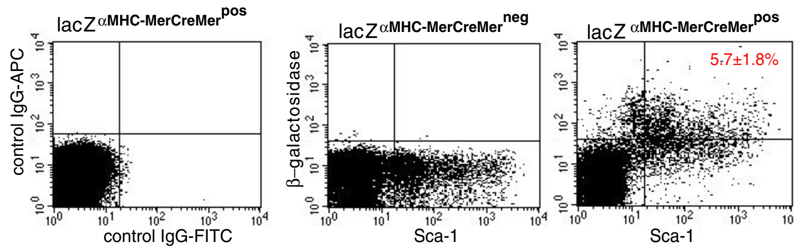
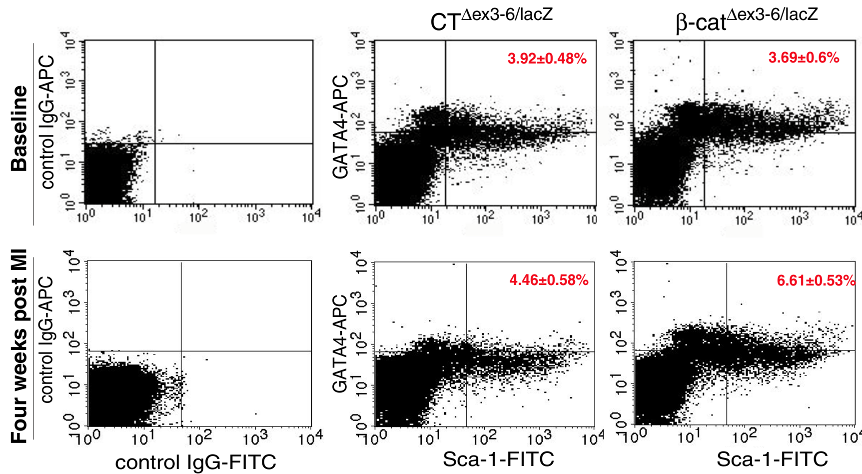
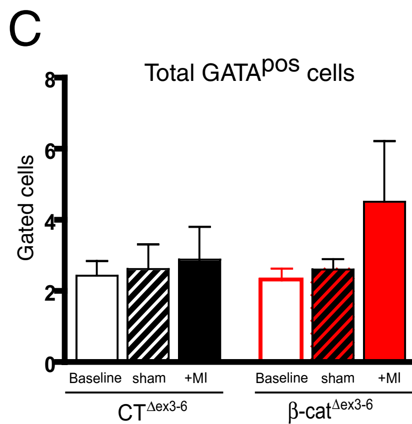


Fig. S7. Validation of Sca-1-specific staining for flow cytometry and immunohistochemistry. Flow cytometry analysis of spleen and cardiomyocyte-depleted fraction. (*A*) Isolated cells from spleen were analyzed as positive control staining for Sca-1 detection by flow cytometry. Cells were gated according to their physical parameters (side and forward light scatter, SSC and FSC, respectively). As expected, approximately 60% of the isolated cells expressed Sca-1. (*B*) Expression of Sca-1 in spleen tissue was also confirmed by immunofluorescence staining. (*C*) Control experiments for analysis of isolated heart cells by flow cytometry: the cell fraction was depleted of adult cardiomyocytes by mesh filtration and gated by SSC and FSC properties (surrounded in red). Propidium iodide was used to detect nonviable cells. Dot plots at the right panels show Sca-1 expression in the cardiomyocyte-depleted cellfraction after exclusion of dead cells. To exclude unspecific cross-reaction, an IgG-FITC antibody was used as control and no signal was detected.







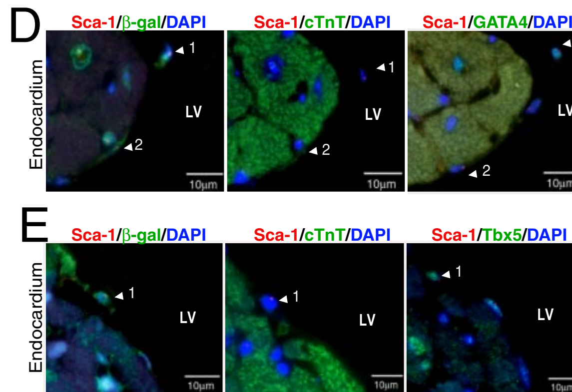


Fig. S8. α-*MHC*-dependent lacZ reporter gene expression by flow cytometry analysis and immunofluorescence. (*A*) Validation of the conditional, α*MHC*-driven Cre expression in cardiac precursor cells employing an alternative conditional Cre line. Analysis of α*MHC*-dependent reporter gene expression in Sca-1^{pos} cells by flow cytometry at baseline in α*MHC-MerCreMer* mice mated to the ROSA26 reporter, (*lacZ*$^{aMHC\text{-}Mer\text{-}Cre\text{-}Mer}$) showed similar results to the data obtained with the α*MHC-CrePR1* mice. (*B*) Flow cytometry analysis of the cardiac cell fraction showing ScaI^{pos} cell fraction coexpressing GATA4 to significantly increase in β-*cat*$^{\Delta ex3}$ mice after cardiac infarct in comparison to the control animals. No significant quantitative difference is observed at baseline. (*C*) Variation of the absolute GATA4 total cell number between β-*cat*$^{\Delta ex3}$ and control mice at baseline and after ischemia showing a nonsignificant increase of GATA4 total cell number after infarct in β-*cat*$^{\Delta ex3}$ mice. (*D* and *E*) Detection of α-*MHC*-dependent lacZ reporter gene expression in cardiomyocyte progenitors as defined by Sca-1^{pos}/gal^{pos}/GATA4^{pos}/Tbx5^{pos}/cTnT^{neg} staining (white arrows). Double staining of consecutive slides is displayed.

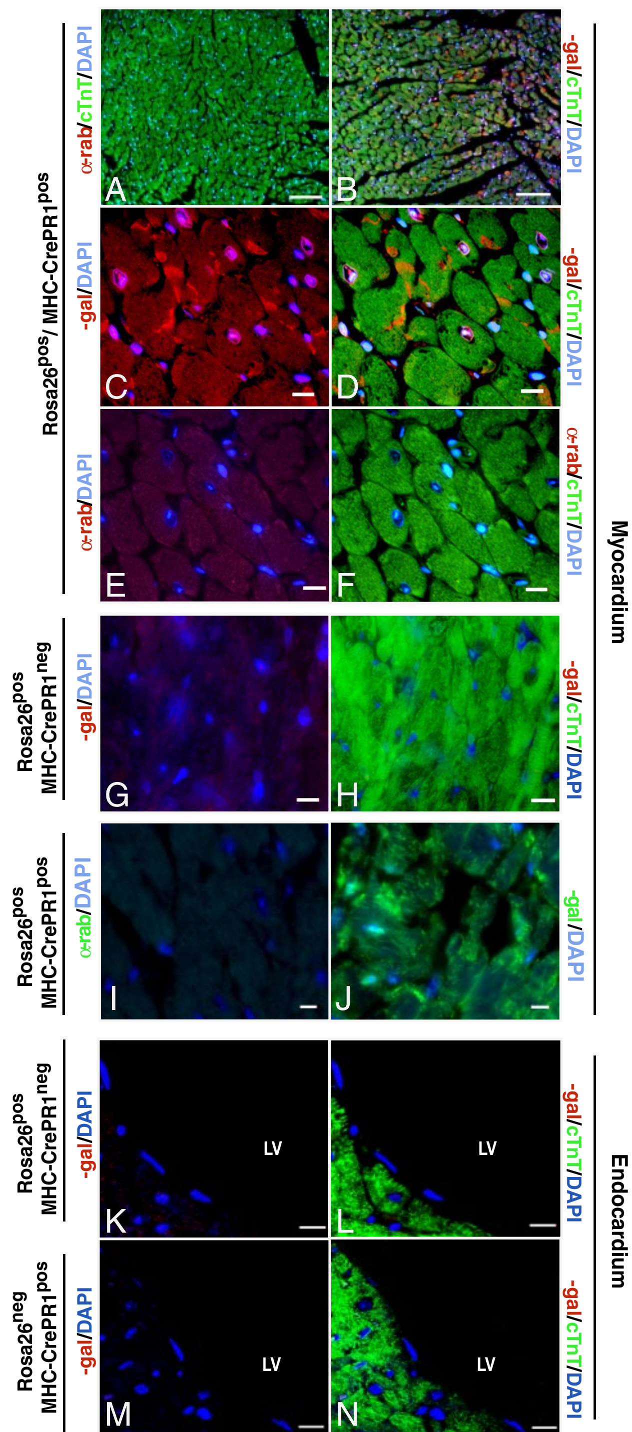


Fig. S9. Controls for β-galactosidase immunofluorescence detection in heart sections. (*A* and *B*) Myocardial β-gal/cTnT coexpression in LacZ$^{\alpha MHC\text{-}Cre}$ mice detected in nuclei (magenta) and cytoplasm (*B*, orange) and respective control β-gal antibody (*A*). (*C* and *D*) Myocardial β-gal/cTnT coexpression in LacZ$^{\alpha MHC\text{-}Cre}$ mice at higher magnification shows the specific β-gal detection (*C*, red) and the overlapped cTnT expression (*D*, magenta and orange). No detection was observed when slides were incubated with an IgG anti-rabbit Alexa 594 used as control (*E* and *F*). (*G* and *H*) No detection of myocardial β-gal expression in LacZ$^{\alpha MHC\text{-}Cre}$ $_{neg}$ mice used as negative controls. In *H* only cTnT expression is shown (control for all of the β-gal staining). (*I* and *J*) Myocardial β-gal expression in LacZ$^{\alpha MHC\text{-}Cre}$ mice using an IgG anti-rabbit Alexa 488 as a secondary antibody. (*K*–*N*) Endocardial β-gal/cTnT costaining in LacZ$^{\alpha MHC\text{-}Cre\ neg}$ (*K* and *L*) and α*MHC*-Cre mice (ROSA26 negative) (*M* and *N*) show no detection of β-gal expression. Controls for Fig. 3 *D*–*G*. LV, left ventricle. Scale bar for *A* and *B*, 100 μm and for *C*–*N*, 10 μm.

Fig. S10. (*A*) β-Gal^{pos}/cTnT^{neg}; β-gal^{pos}/Sca-1^{pos}; Sca-1^{pos}/cTnT^{neg}; and Sca-1^{pos}/GATA4^{pos} cardiac progenitor cells were detected by immunofluorescence in remote myocardium and scar area 2 weeks after infarct, both in β-*cat*$^{\Delta ex3-6/lacZ}$ and control mice without major differences (representative pictures are shown). No significant difference in tissue distribution is detected. (*B*) Proliferation of GATA4^{pos} cells 2 weeks after myocardial infarct. Semiquantification of *in vivo* labeled BrdU^{pos}/GATA4^{pos} cells matched to DAPI^{pos} cell number shows no significant difference of these cells in β-*cat*$^{\Delta ex3-6}$ mice compared to control littermates either in the scar or in the remote zone. LV, left ventricle; MI, myocardial infarct.

1. van Rooij E, *et al.* (2004) MCIP1 overexpression suppresses left ventricular remodeling and sustains cardiac function after myocardial infarction. *Circ Res* 94:18e–26.
2. Freund C, *et al.* (2005) Requirement of nuclear factor-kappaB in angiotensin II- and isoproterenol-induced cardiac hypertrophy in vivo. *Circulation* 111:2319–2325.
3. Opitz CF, Finn PV, Pfeffer MA, Mitchell GF, Pfeffer JM (1998) Effects of reperfusion on arrhythmias and death after coronary artery occlusion in the rat: Increased electrical stability independent of myocardial salvage. *J Am Coll Cardiol* 32:261–267.
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Table S1. Echocardiography M-mode measurements of β-cat^{Dex3-6} and respective control littermates before and 2 and 4 weeks after LAD ligation

	Before MI		2 weeks post-MI		4 weeks post-MI	
	CT	β-cat$^{\Delta ex3-6}$	CT	β-cat$^{\Delta ex3-6}$	CT	β-cat$^{\Delta ex3-6}$
IVS (mm)	0.73 ± 0.06	0.71 ± 0.07	1.01 ± 0.08	0.92 ± 0.06	0.90 ± 0.06	0.97 ± 0.07
LVPW (mm)	0.75 ± 0.04	0.72 ± 0.02	0.75 ± 0.04	0.71 ± 0.02	0.75 ± 0.05	0.71 ± 0.03
LVD (mm)	3.75 ± 0.15	3.50 ± 0.09	5.00 ± 0.10	4.80 ± 0.02	5.11 ± 0.21	5.00 ± 0.19
FS (%)	37.51 ± 1.6	40.88 ± 1.4	26.33 ± 2.6	30.33 ± 1.80	24.01 ± 1.9	30.23 ± 1.6***
HW/BW (%)					6.1 ± 0.42	5.3 ± 0.21*

Diastolic measurements (mm) of septum, posterior wall, and LV diameter by M-mode echocardiograms from both control and mutant mice before and 2 and 4 weeks after coronary ligation. IVS, interventricular septum; LVD, left ventricular diameter; LVPW, left ventricular posterior wall; FS, fractional shortening (%); HW/BW, heart weight to body weight ratio; MI, myocardial infarct. **, $P < 0.01$.