Alpha-Catenins Control Cardiomyocyte Proliferation by Regulating Yap Activity

Jifen Li, Erhe Gao, Alexia Vite, Roslyn Yi, Ludovic Gomez, Steven Goossens, Frans van Roy, Glenn L. Radice

Rationale: Shortly after birth, muscle cells of the mammalian heart lose their ability to divide. Thus, they are unable to effectively replace dying cells in the injured heart. The recent discovery that the transcriptional coactivator Yes-associated protein (Yap) is necessary and sufficient for cardiomyocyte proliferation has gained considerable attention. However, the upstream regulators and signaling pathways that control Yap activity in the heart are poorly understood.

Objective: To investigate the role of α-catenins in the heart using cardiac-specific αE- and αT-catenin double knockout mice.

Methods and Results: We used 2 cardiac-specific Cre transgenes to delete both αE-catenin (Ctnna1) and αT-catenin (Ctnna3) genes either in the perinatal or in the adult heart. Perinatal depletion of α-catenins increased cardiomyocyte number in the postnatal heart. Increased nuclear Yap and the cell cycle regulator cyclin D1 accompanied cardiomyocyte proliferation in the α-catenin double knockout hearts. Fetal genes were increased in the α-catenin double knockout hearts indicating a less mature cardiac gene expression profile. Knockdown of α-catenins in neonatal rat cardiomyocytes also resulted in increased proliferation, which could be blocked by knockdown of Yap. Finally, inactivation of α-catenins in the adult heart using an inducible Cre led to increased nuclear Yap and cardiomyocyte proliferation and improved contractility after myocardial infarction.

Conclusions: These studies demonstrate that α-catenins are critical regulators of Yap, a transcriptional coactivator essential for cardiomyocyte proliferation. Furthermore, we provide proof of concept that inhibiting α-catenins might be a useful strategy to promote myocardial regeneration after injury. (Circ Res. 2015;116:70-79. DOI: 10.1161/CIRCRESAHA.116.304472.)

Key Words: α-catenin ■ cytokinesis ■ models, animal ■ myocardial infarction ■ myocytes, cardiac ■ Yap protein, mouse

Damage to the mammalian heart is considered irreversible because cardiac progenitor cells and differentiated cardiomyocytes cannot effectively repopulate the injured myocardium. However, recent studies suggest cardiac repair is possible by dedifferentiation and proliferation of adult cardiomyocytes.1–5 Hence, understanding the molecular mechanisms governing cardiomyocyte cell cycle re-entry, karyokinesis and cytokinesis will be critical for developing strategies to promote cardiac regeneration.
F-actin facilitating linkage of N-cadherin with the actin cytoskeleton. In addition, α-catenins are capable of interacting with a myriad of actin-binding proteins either directly or indirectly and thus regulates actin dynamics at the sarcolemma. α-Catenin is unique in that it can bind to the desmosomal protein, plakophilin-2, whereas ε-catenin lacks the plakophilin-2 binding domain.11 Deletion of either ε-Catenin (Ctnna1) or α-Catenin (Ctnna3) in the murine heart leads to progressive cardiomyopathy,12,13 consistent with their role in mechanical junctions. Of note, mutations in human α-Catenin (CTNNA3) gene were recently identified in patients with arrhythmogenic cardiomyopathy.14

First discovered in Drosophila, the Hippo signaling pathway has emerged recently as a conserved mechanism that restricts organ size by limiting cell proliferation and promoting apoptosis.15,16 The core Hippo pathway consists of a cascade that signals from the kinase Mst1/2 (Hippo in flies) to the kinase Lats1/2 (Warts in flies) to limit the activity of the Yes-associated protein (Yap; Yorki in flies), a transcriptional coactivator that binds to the TEA domain-containing transcriptional factors (TEAD) to induce expression of cell cycle regulators and other target genes. Hippo signal transduction requires the subcellular compartmentalization of the kinases at the plasma membrane, which is dependent on interactions between Mst1/2 and Lats1/2 with the scaffold proteins Salvador and Merlin (also known as NF2), respectively.17 Phosphorylation of Yap (at site S127) by Lats1/2 causes cytoplasmic retention of Yap and thus inhibits its ability to induce transcription of target genes. Loss of Hippo signaling in mice18 and constitutive activation of Yap19,20 results in overgrowth of the heart. Conversely, ablation of Yap during cardiac development results in a hypoplastic heart and embryonic lethality.19,20 In addition, disruption of the Hippo pathway or constitutively active Yap is sufficient to enhance cardiac regeneration in mice after injury.21-23 Conversely, Yap haploinsufficiency leads to decrease cardiomyocyte proliferation and worse cardiac function after myocardial infarction (MI) compared with wild-type (WT) mice.24 Taken together, these studies demonstrate that Yap is a central player in myocardial cell growth control in both normal and pathological conditions.

In this study, we demonstrate for the first time that interfering with α-catenin function in the heart promotes cardiomyocyte proliferation. This phenotype is because of the ability of α-catenins to prevent the transcriptional coactivator Yap from entering the nucleus and activating genes critical for cell cycle progression. Moreover, we show that depletion of α-catenins can improve contractile function after MI.

Methods

Generation of Cardiac-Specific αE- and αT-Catenin Double Knockout Mice

α-Myosin heavy chain [MHC]/Cre; αE-cateninfloxflox; αT-cateninfloxflox animals (double knockout [DKO]) and αMHC/MerCreMer; αE-cateninfloxflox; αT-cateninfloxflox animals (inducible DKO [IN-DKO]) were generated by standard breeding protocols. The mice were maintained on a mixed 129Sv-C57BL/6J genetic background. Adult IN-DKO mice were injected intraperitoneally once a day for 5 consecutive days with 80 mg tamoxifen (Sigma) per kilogram body weight.

A detailed Methods section is available in the Online Data Supplement.

Results

Deletion of Both αE- and αT-Catenins in the Heart

To investigate the function of α-catenins in the postnatal heart, we bred αE-catenin flox, αT-catenin flox, and αMHC/Cre mice to generate cardiac-specific αE- and αT-catenin DKO mice. Littermates lacking the Cre transgene (ie, αE-catfloxflox; αT-catfloxflox) served as WT controls. The DKO animals were born in the expected Mendelian frequency, indicating that deletion of both α-catenins using αMHC/Cre is compatible with cardiac development during the perinatal period. Normally colocalized with N-cadherin, both α-catenins were lost from the ID in the DKO heart (Figure 1A). Western blot analysis showed significant reduction of αE- and αT-catenin proteins in the DKO heart compared with WT control (Figure 1B), indicating efficient depletion of both α-catenins. Heart weight/body weight ratios were determined at selected ages beginning at 6 weeks (Figure 1C). The difference between the heart weight/body weight ratios of DKO and control littersmates increased with age, with statistical significance noted at or after 9 weeks of age (n=6 for each group; P<0.05). Overall, DKO heart size appeared normal at 6 weeks of age (Figure 1D, left). However, atria were often enlarged. The DKO hearts displayed fibrosis with age, as revealed by Masson trichrome staining (Figure 1D, right). Terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling analysis did not detect a change in cardiomyocyte apoptosis in the DKO compared with WT hearts (data not shown). An increase in nuclear density was observed in the hematoxylin and eosin–stained DKO heart sections (Figure 1D), hence we counted myocyte cells in ventricular myocardium stained with anti-desmin antibody to identify cardiomyocytes and with 4’,6-diamidino-2-phenylindole to highlight nuclei. Compared with the WT control, the relative cardiomyocyte number was increased by 23% (n=4; P<0.05) in the DKO heart (Figure 1E). Furthermore, myocytes were isolated from DKO and WT hearts and counted. Despite multiple attempts we were not able to obtain completely dissociated myocytes from 2-month-old DKO hearts. In contrast, enzymatic digestion of WT hearts successfully dispersed the adult myocytes. The inability to completely digest the mutant heart might be because of increased fibrosis and less efficient perfusion of the adult DKO heart compared with WT. To avoid
these potential problems, hearts were isolated from postnatal day 4 (P4) and P7 pups. At these earlier time points, both DKO and WT hearts yielded single cells after enzymatic digestion. Consistent with the above-mentioned in situ data on adult mice, an increase in total cardiomyocytes was observed in the DKO (4.22 × 10^5; n=18) compared with WT (3.07 × 10^5, n=20) P7 hearts (Figure 1H). No significant differences in myocyte numbers were observed at P4. Interestingly, myocyte cross-sectional areas were reduced in the adult DKO hearts (−17%; n=5; P < 0.05; Figure 1F). Moreover, the increased number of adult cardiomyocytes was accompanied by an increase in the percentage of mononucleated cardiomyocytes and a decrease in the percentage of binucleated cardiomyocytes (Figure 1G).

At the same time, there was an increase in the percentage of multinucleated cardiomyocytes suggesting a small population of DKO myocytes continue to undergo DNA synthesis and karyokinesis without cell division. Taken together, these data indicate that loss of α-catenins caused an increase in cardiomyocyte number in the DKO mice.

α-catenins are part of the N-cadherin/catenin cell adhesion complex located at the IDs of cardiomyocytes. To determine whether the loss of α-catenins affected cardiomyocyte morphology, we examined isolated adult cardiomyocytes from DKO and WT hearts (Figure 2A). WT myocytes showed their characteristic rod shape with step-like structures at their termini. In contrast, DKO myocytes often appeared more elongated with round protruding structures at their termini. To gain insight into N-cadherin expression in the DKO myocytes, cells and their IDs were reconstructed from a series of optical sections (Figure 2B). WT myocytes displayed a typical sharp linear pattern of N-cadherin expression at the ID. In contrast, DKO myocytes showed variable diffuse N-cadherin expression at the ID as well as cytoplasmic staining not observed in WT. The cadherin-binding protein,
The mitotic index (ratio of anti–phosphohistone-3-positive cardiomyocyte nuclei to total cardiomyocyte nuclei) increased 2.5-fold in DKO hearts (0.051%±0.011% in DKO versus 0.018%±0.006% in WT; n=4; P<0.05). These data indicate that depletion of αE- and αT-catenin in the postnatal period increases cardiomyocyte proliferation in the adult heart.

Cyclin D1 is a well-known cell cycle regulator and also a transcriptional target of the Hippo signaling pathway. In mouse Salvador mutant hearts, cardiomyocyte proliferation is accompanied by increased cyclin D1.18 Nuclear and total protein expression levels of cyclin D1 were significantly increased in the DKO hearts (+205% of WT; n=4; P<0.001; Figure 3C and 3E). Moreover, we observed increased proliferating cell nuclear antigen staining in the nuclei of DKO cardiomyocytes and confirmed this proliferating cell nuclear antigen increase by Western analysis of total heart lysates (+234% of WT; n=5; P<0.01; Figures 3D and 3E). Expression of cell cycle genes was examined in WT and DKO hearts by quantitative reverse transcription polymerase chain reaction. Aurka, Cdc20, Cmm1, and Cdc5l were all increased in the DKO hearts (Figure 3F) consistent with increased cell cycle activity in the absence of α-catenins. Interestingly, we observed an increase in expression of fetal genes Myh7, Nppa, and Nppb in the DKO hearts as early as 1 week after birth that continued to ≥16 weeks of age (Figure 3G). Expression of adult myosin isoform Myh6 did not change significantly until 16 weeks of age when it decreased. Overall, these data indicate that loss of the cytoskeletal proteins, αE- and αT-catenin, results in less mature cardiomyocytes that continue to proliferate into the adult period.

**α-Catenins Regulate Yap Nuclear Localization**

It has been reported that keratinocytes lacking αE-catenin display a striking upregulation of nuclear localization of Yap, a transcriptional coactivator of the Hippo signaling pathway, and that this increased nuclear localization is associated with increased proliferation in the skin.7,25 To determine whether Yap was affected in our α-catenin DKO model, we performed immunohistochemistry with anti-Yap antibody on hearts from 2-month-old α-catenin DKO mice. Yap-positive cardiomyocytes were rarely seen in the WT heart (Figure 4A). In contrast, DKO heart displayed a significant nuclear localization of Yap (Figure 4A). Western blot analysis demonstrated upregulation of both total and phosphorylated Yap (S127) in α-catenin DKO hearts (Figure 4B). Moreover, the ratio of phosphorylated Yap (S127) to total Yap was decreased in the DKO hearts, consistent with an increase of dephosphorylated Yap leading to its nuclear accumulation. There was no change in Yap mRNA levels as assessed by quantitative reverse transcription polymerase chain reaction (data not shown), indicating that post-transcriptional regulation was responsible for the upregulation of Yap in the DKO hearts. To examine the cellular distribution of Yap, isolated adult cardiomyocytes were immunostained for Yap (Figure 4C). WT cardiomyocytes exhibited low levels of Yap in the cytoplasm with no nuclear staining. In contrast, Yap was observed at high levels in the nucleus and cytoplasm of the DKO cardiomyocytes. (phosphorylated histone H3 antibody) antibody to examine cardiomyocyte mitosis (Figure 3B). The mitotic index (ratio of anti–phosphohistone-3-positive cardiomyocyte nuclei to total cardiomyocyte nuclei) increased 2.5-fold in DKO hearts (0.051%±0.011% in DKO versus 0.018%±0.006% in WT; n=4; P<0.05). These data indicate that depletion of αE- and αT-catenin in the postnatal period increases cardiomyocyte proliferation in the adult heart.

To examine cardiomyocyte proliferation, we analyzed DNA synthesis in DKO hearts by 5-bromo-2′-deoxyuridine (BrdU) incorporation (Figure 3A). The percentage of BrdU-positive cardiomyocytes in DKO hearts (1.51%±0.22%) was 2-fold higher than in WT control hearts (0.66%±0.11%; n=3, P<0.05) at 2 months of age. We next used an anti–phosphohistone-3

**α-Catenins Inhibit Cardiomyocyte Proliferation**

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These data indicate an overall dysregulation of Yap in the absence of α-catenins.

To further examine the effects of α-catenin depletion on cardiomyocyte proliferation, combined αE- and αT-catenin knockdown was performed using small interfering RNA in primary rat neonatal cardiomyocytes (Figure 5A). We observed enhanced BrdU uptake and increased phosphorylated histone H3 antibody expression in the α-catenin knockdown cells, consistent with the proliferative phenotype observed in vivo (Figure 5B and 5C). Furthermore, cells in late anaphase and cytokinesis, as identified by Aurora-B staining of central mitotic spindles and midbodies, were increased in α-catenin knockdown cells (Figure 5D). To determine whether Yap was responsible for the increased proliferation in the α-catenin knockdown myocytes, small interfering RNA against Yap was used to generate triple knockdown cells (Figure 6A). We observed decreased BrdU incorporation after Yap knockdown in the α-catenin knockdown cells demonstrating that Yap is required for the proliferative phenotype (Figure 6B and 6C).

α-Catenins Regulate Post–MI Remodeling

To determine whether depletion of α-catenins had a similar effect in the adult heart, we used a tamoxifen-inducible (IN) αMHC/MerCreMer transgene to delete the floxed αE- and αT-catenin alleles. We refer to this model as IN-DKO. Interestingly, IN-DKO animals exhibited normal cardiac histology, no increase in proliferation as determined by phosphorylated histone H3 antibody staining, and survived for ≥1 year of age (Online Figure I). Moreover, depletion of α-catenins in the adult heart did not cause an increase in nuclear Yap (data not shown).

To determine whether depletion of α-catenins improves cardiac function after injury, we permanently ligated the left anterior descending coronary artery in 2-month-old αMHC-MerCreMer; αE-cateninflx/flx; αT-cateninflx/flx mice 10 days after inducing deletion of the genes with tamoxifen. We refer to this experimental protocol as a prevention study (Figure 7A) because α-catenins were depleted at the time of the MI (Figure 7B). Myocardial function was determined by serial echocardiography (Figure 7C). Nine weeks after MI, dimensions of the left ventricle decreased in IN-DKO cells, consistent with the proliferative phenotype observed in vivo (Figure 5B and 5C). Furthermore, cells in late anaphase and cytokinesis, as identified by Aurora-B staining of central mitotic spindles and midbodies, were increased in α-catenin knockdown cells (Figure 5D). To determine whether Yap was responsible for the increased proliferation in the α-catenin knockdown myocytes, small interfering RNA against Yap was used to generate triple knockdown cells (Figure 6A). We observed decreased BrdU incorporation after Yap knockdown in the α-catenin knockdown cells demonstrating that Yap is required for the proliferative phenotype (Figure 6B and 6C).
animals, whereas control animals had sustained left ventricle dilatation, indicating that deletion of α-catenins had a positive effect on post-MI remodeling. Loss of α-catenins induced an improvement of myocardial function after MI, as determined by an increase in fraction shortening and ejection fraction (Figure 7C; Online Table I). Furthermore, after isoproterenol administration hemodynamic measurements 12 weeks after MI showed improved left ventricle contractility (as measured by +dP/dt) and left ventricle relaxation (as measured by −dP/dt) in IN-DKO hearts compared with WT controls (Figure 7D; Online Table II). Detailed histological analysis at the level of the papillary muscle of the hearts 12 weeks after MI indicated improved cardiac remodeling in the IN-DKO hearts (Figure 7E). Measurement of infarct size and fibrosis area showed that the injured area was smaller in the IN-DKO than in WT controls (Figure 7F).

Furthermore, the anterior wall was thicker consistent with the improved cardiac function in the IN-DKO compared with WT (Figure 7F). Cardiomyocyte cross-sectional area was smaller, consistent with attenuation of cardiomyocyte hypertrophy in the IN-DKO mice (Figure 7F). To assess potential differences in the initial injury, hearts were stained with 2,3,5-triphenyltetrazolium chloride to visualize the ischemic zone 1 day after MI. Equivalent areas of ischemia were observed among the WT and IN-DKO hearts (Online Figure II).

To examine Yap expression after injury, we performed immunofluorescent staining with anti-Yap antibody on IN-DKO hearts 1 week after MI. Yap-positive cardiomyocyte nuclei were identified by staining with anti–α-cardiac actin antibody and counted in different areas: border zone, infarct zone, and remote area. Yap-positive cardiomyocytes were rarely seen in the remote area of either IN-DKO or WT controls (Figure 8A). In contrast, the numbers of Yap-positive nuclei in the IN-DKO heart were significantly increased in both border zone (+2.5-fold versus WT; n = 4; P<0.05) and infarct zone (+2.3-fold versus WT; n = 4; P<0.05). Analysis of DNA synthesis by BrdU incorporation and mitosis by phosphorylated histone H3 antibody immunostaining confirmed enhanced proliferation of cardiomyocytes in IN-DKO hearts 1 week after MI (Figure 8B and 8C). Next, we examined the IN-DKO hearts after injury for cardiomyocyte cytokinesis. One week after MI, cardiomyocytes with Aurora-B–positive central mitotic spindles or midbodies were more prevalent in IN-DKO hearts than in WT controls (Figure 8D). Collectively, these data indicate that the loss of α-catenins enhances Yap accumulation in the nucleus, where it promotes cardiac regeneration after injury.

**Discussion**

The transcriptional coactivator Yap, a downstream effector of the Hippo signaling pathway, was shown recently to be necessary and
sufficient for cardiomyocyte proliferation. Moreover, cardi-ac-specific expression of activated Yap (eg, αMHC-YapS112A) that cannot be phosphorylated by inhibitory upstream kinases in the Hippo pathway enhances cardiac regeneration post MI. However, the upstream regulators that control Yap activity in the heart remain largely unknown. In the present study, we demonstrate that depletion of αE- and αT-catenin in the postnatal heart leads to nuclear accumulation of Yap and induction of cardiomyocyte proliferation. Importantly, deletion of both α-catenin genes in adult heart before injury (IN-DKO model) led to improved cardiac function compared with control littermates. A 2-fold increase in nuclear Yap in cardiomyocytes of the injured IN-DKO relative to WT hearts suggests that Yap transcriptional activity likely contributes to the enhanced cardiomyocyte proliferation after MI. In addition, cardiomyocyte cytokinesis, as determined by Aurora-B kinase localization to the midbody, was increased in the injured area of the IN-DKO hearts. Taken together, the IN-DKO data indicate that α-catenin normally inhibit cardiomyocytes in the adult heart from entering the cell cycle and undergoing cytokinesis after injury.

The structural integrity of the heart is dependent on mechanical junctions located at the end-to-end connections between cardiomyocytes called the ID. The cell adhesion molecule N-cadherin is required to maintain ID structure. We previously reported that simultaneous depletion of the N-cadherin–binding partners, β-catenin and plakoglobin, in the adult heart results in loss of N-cadherin, disassembly of the ID, and sudden cardiac death. In contrast, here we show that in the absence of the cytoskeletal linker proteins αE- and αT-catenin the majority of N-cadherin remains at the myocyte termini; however, it is not properly organized at the ID. The interstitial fibrosis observed in the α-catenin DKO might reflect cardiac remodeling because of weakened intercellular adhesion and contractility in the mutant hearts. We speculate that α-catenin DKO mice use an alternative cytoskeletal protein(s) to mediate linkage between the adhesion complex and the cytoskeleton. Further studies will be necessary to determine how altering the cytoskeletal linkage affects mechanotransduction via the modified α-catenin–deficient N-cadherin adhesion complex.

The cardiac phenotype is much dependent on the timing of the deletion of αE- and αT-catenin genes. Deletion of the floxed α-catenin genes using the troponin T (Tnnt2) promoter to express Cre recombinase during early cardiac development resulted in embryonic lethality around midgestation (G. Radice, unpublished data). Here, we show that depletion of both αE- and αT-catenins in the perinatal period using αMHC-Cre is compatible with postnatal survival; however, the DKO animals exhibit a proliferative phenotype. During the later fetal and
early postnatal period the cardiomyocyte elongates, myofibrils align, and maturation occurs resulting in a large rod-shaped cardiomyocyte. During this morphological progression the N-cadherin/catenin complex, initially distributed all along the cell borders, becomes restricted to the polarized ends of the cell to form the mature ID.29 Interestingly, the redistribution of the N-cadherin/catenin complex to the ID coincides with cell cycle withdrawal and differentiation of the cardiomyocyte during the postnatal period,30,31 suggesting a role for N-cadherin–mediated adhesion in myocardial growth control. The increased expression of fetal genes (eg, Myh7) as early as 1 week of age in the DKO hearts suggests that perturbation of N-cadherin/catenin complex and cytoskeleton during the postnatal period allows cardiomyocytes to remain in a less mature proliferative state. However, if α-catenins are depleted in the adult heart using a tamoxifen-inducible αMHC-MerCreMer (ie, IN-DKO model) we do not observe the proliferative phenotype. The presence of an established mature ID structure in the adult heart may explain why ablation of both α-catenins at that time is not sufficient to elicit the proliferation phenotype. It was reported that altered N-cadherin expression and ID remodeling occurs in the border zone of infarcted rat hearts.32 Interestingly, we did observe improved contractility after MI in the IN-DKO compared with WT suggesting that altering the N-cadherin cytoskeletal linkage in the setting of ischemic injury promotes cardiomyocyte regeneration. Alterations in sarcomeric proteins and cytoskeleton in injured heart suggest that dedifferentiation of adult cardiomyocytes requires a transient reorganization of the cytoskeleton. It will be of interest to examine the relationship between cytoskeletal remodeling and Yap accumulation in the nucleus of the IN-DKO myocytes after injury.

The Hippo kinase cascade is a potent regulator of cell proliferation and organ size.15,16 When the Hippo pathway is active, Yap is phosphorylated, which prevents its nuclear translocation. Ablating the expression of Hippo pathway components (including Mst1/2, Lats 2, and Salvador) in the developing heart using Nkx2.5/Cre induced Yap nuclear localization, cardiomyocyte proliferation, and a 2.5-fold increase in heart size.18 In the conditional Salvador KO model, phospho-Yap was significantly reduced but total Yap protein was unaffected. In contrast, we found an overall increase in both phospho- and total Yap in the α-catenin DKO hearts, suggesting a general loss of Yap regulation. Interestingly, it was recently reported in both human and animal models of arrhythmogenic...
cardiomyopathy that disruption of the ID structure via mutations in desmosomal genes activates the Hippo signaling pathway leading to enhanced adipogenesis.4

How α-catenins regulate Yap in the heart is unknown. Deletion of the αE-catenin gene in skin leads to epidermal hyperproliferation and tumors in mice.7,8 In epithelial cells, αE-catenin can interact with the tumor suppressor NF2/Merlin,35 an upstream component of the Hippo pathway. Furthermore, 14-3-3, a phospho-serine/threonine binding protein, interacts with αE-catenin to form a tripartite complex with Yap, thereby preventing its nuclear accumulation.25 Because inhibiting the Hippo pathway in the heart primarily affects Yap phosphorylation, whereas α-catenins regulate overall Yap levels, we speculate that loss of the α-catenin-NF2/Merlin interaction would not be sufficient by itself to explain the α-catenin DKO phenotype. Interestingly, inactivation of Mst1/2 in the skin did not result in Yap activation and hyperplasia.25 In that case, Yap activity was regulated by the phosphatase PP2A. In addition to interactions with the scaffold proteins, NF2 and 14-3-3, α-catenins regulate actin dynamics. The F-actin cytoskeleton is a major regulator of Yap, mediating signals triggered by substrate stiffness, cell density, and cell detachment, as well as signaling from G-protein-coupled receptors.36 Additional studies are necessary to determine the relative contribution of each of these pathways as well as other potential signaling pathways in the regulation of Yap by α-catenins.

In conclusion, our study provides in vivo evidence for a unique role for α-catenins in the regulation of the transcriptional coactivator Yap and cardiomyocyte proliferation. Importantly, depletion of α-catenins in adult animals led to improved cardiac function after MI. Hence, functional interference with α-catenins represents a novel mechanism for enhancing signaling pathways beneficial in cardiac repair.

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Disclosures
None.

References
Whether interfering with the N-cadherin/catenin complex is sufficient to stimulate heart muscle growth is unknown.

What New Information Does This Article Contribute?

- Simultaneous depletion of both αE- and αT-catenin in the perinatal heart leads to a less stable, poorly organized N-cadherin/catenin junctional complex.
- α-catenin-deficient cardiomyocytes express fetal genes, accumulate Yes-associated protein in the nucleus, and continue to proliferate in the adult heart.
- Deletion of the αE- and αT-catenin genes in the adult heart when the intercalated disc is already formed does not stimulate cardiomyocyte proliferation.
- Inhibition of α-catenins in adult hearts subjected to ischemic injury induces cardiomyocyte regeneration and improves heart function.

In the course of our investigation of the N-cadherin/catenin complex in the heart, we discovered an unexpected link between cell–cell adhesion and heart regeneration. We found that the specialized molecular coupling between adult cardiomyocytes provides a negative signal for cell division. Further investigation is warranted to understand how altering the cell adhesion complex and the herewith associated cytoskeleton leads to translocation of Yes-associated protein from the cytosol into the nucleus where it can activate genes critical for cardiomyocyte proliferation. Functional interference with α-catenins in the heart may represent a novel mechanism for enhancing signaling pathways beneficial in cardiac repair.
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Supplemental Material

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Material and Methods - continued

Generation of cardiac-specific αE- and αT-catenin double knockout (DKO) mice.

Previously generated αT-catenin(fl/fl) mice\textsuperscript{1} were mated with αE-catenin(fl/fl) mice\textsuperscript{2} obtained from Jackson Laboratories, Bar Harbor, ME. αMHC/Cre\textsuperscript{3} and αMHC/MerCreMer\textsuperscript{4} mice were bred to produce αMHC/Cre; αE-catenin(fl/fl); αT-catenin(fl/fl) animals (DKO) or αMHC/MerCreMer; αE-catenin(fl/fl); αT-catenin(fl/fl) animals (IN-DKO). The floxed mice were genotyped by PCR. The αE-catenin floxed mice were genotyped using the following primer pair: IMR2: 5’ – CAT TTC TGT CAC CCC CAA AGA CAC – 3’; IMR3: 5’ – GCA AAA TGA TC C AGC GTC CTG GG – 3’. The WT and floxed PCR products correspond to 100 and 350 bp, respectively. The following primer pair was used to distinguish the WT and floxed αT-catenin alleles: αT-FloxF: 5’- CCA CCC CTG ATA TGA CCT GTA G -3’; αT-FloxR: 5’- TCC CCA GGA ATC AAG TCG TT -3’. The WT and floxed PCR products correspond to 287 and 366 bp, respectively. All animal experiments were performed in accordance with the guidelines of the IACUC of Thomas Jefferson University.

Histological analysis. Hearts were isolated and fixed in 4% formaldehyde freshly prepared from paraformaldehyde (PFA), dehydrated, and embedded in paraffin. Global heart architecture was determined from longitudinal 6-μm deparaffinized sections stained with Hematoxylin and Eosin (H&E). Cross-sectional cell area measurements were obtained at the level of the nucleus in cross-sectioned, H&E-stained myocytes. Surface areas were quantified using NIS-Elements D
software (Nikon). A minimum of 100 myocytes from five different animals was quantified for each experimental group. Fibrosis was detected with Masson’s Trichrome or acid fuchsin-orangene G (AFOG) staining. The infarction size, anterior wall thickness, and percent fibrosis were measured on the scanned images of the tissue sections stained with AFOG using ImageJ software (NIH). The ratio of scar length to left ventricular circumferences of the endocardium and epicardium was determined and expressed as a percentage of infarct size (% of LV) which was calculated as (infarct length/total LV length)×100. Anterior wall thickness was measured at three different points in infarction area of anterior wall perpendicularly and average was taken. Transverse fibrosis and total left ventricular areas were measured and expressed as percent fibrosis. To visualize the ischemic area 24 hrs post-MI, heart sections were stained with 2,3,5-triphenyltetrazolium chloride (TTC, Sigma) as described previously. The TTC-stained area, and TTC-negative staining area (infarct myocardium) were measured using ImageJ software (NIH). Myocardial infarct size was expressed as a percentage of the total LV area.

**Neonatal mouse ventricular cardiomyocyte cultures.** Cardiomyocytes were isolated from WT and DKO 4-day and 7-day old mice using the Neomyts isolation kit (Cellutron Life Technology, Baltimore, MD), as previously described. Briefly, ventricles were isolated and incubated separately in 2ml eppendorf tubes containing 150µl of the digestion buffer D2. After 10 min at 37°C under agitation, ventricles were roughly triturated and isolated cells were removed and placed in 1ml of the D3 buffer to inhibit the enzyme activity. Ventricles were resuspended in D2 buffer and tubes placed at 37°C for another 10 min. This procedure was repeated until complete digestion of the heart. At the end of the digestion, isolated cells in D3 buffer were centrifuged 1.5 min at 1200 rpm and the pellet was resuspended in 1ml of medium (10% Horse serum, 1%
Pen/Strep in DMEM, low glucose, pyruvate, HEPES (Life Technologies, Grand Island, NY)).
Cells were filtered in 100µm nylon cell strainer (BD Falcon, Franklin Lakes, NJ). To enrich for cardiomyocytes, cells were preplated for 2 hours at 37°C. Non-adherent cells were removed and counted using a hemocytometer. Experiments were blinded as genotypes were determined after counting cells from individual animals.

**Adult cardiomyocytes isolation and immunostaining.** Adult WT and DKO mice were pre-medicated with heparin (100 USP units/mouse) administered intraperitoneally. Anesthesia was induced with sodium nembutal (70 mg/kg). A thoracotomy was performed and the heart collected. Adult ventricular cardiomyocytes were isolated using enzymatic digestion according to previously described procedures\textsuperscript{10}. Briefly, hearts were removed, cannulated and perfused with collagenase type II (Worthington Biochemical Corp., Lakewood, NJ) to dissociate ventricular myocytes.

After isolation, cardiomyocytes were directly fixed for 10 min in 4% paraformaldehyde at RT. Cells were permeabilized in 0.1% Triton X-100 and blocked in T-BSA (0.01% Triton X-100 in BSA 5%). Cells were incubated with the primary antibodies: N-cadherin (BD Biosciences) or YAP (4912, Cell Signaling Technology, Inc. Danvers, MA), diluted in T-BSA at RT for 2 hours. After washing in PBS, cells were incubated with the secondary antibody (Alexa Fluor® 555 Goat Anti-Rabbit IgG, Life Technologies, Grand Island, NY) for 1 hour. Finally, cells were washed in PBS and mounted into ProLong® Gold Antifade Reagent with DAPI (Life Technologies, Grand Island, NY). A series of optical sections were collected at 0.2µm intervals moving progressively across the cell using a Zeiss LSM 510 META Confocal Microscope System (Carl Zeiss, Maple Grove, MN). For 3D-reconstruction, images were elaborated by IMAGEJ 1.43 software.
Neonatal rat ventricular cardiomyocyte cultures, siRNA-mediated knockdown and BrdU labeling. Neonatal rat ventricular myocytes (NRVMs) were isolated from 1- to 2-day old Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) and cultured as previously described\(^ {11} \). NRVMs were cultured in Ham’s F-10 supplemented with penicillin/streptomycin (100 U/ml), 10% horse serum and 5% fetal bovine serum at 37°C in a 5% CO2 humidified atmosphere. The isolated myocytes were plated onto Lab-Tek™ Chamber Slides (Nunc) pre-coated with laminin (40μg/ml). NRVMs were transfected (HiPerFect, Qiagen) with αT-catenin RNAi (S168107, Invitrogen), αE-catenin RNAi (SI01495690, Qiagen), Yap1 RNAi (D-100439-01-0005, Thermo) oligos for 48h in 1% FBS Dulbecco’s modified Eagle’s medium (DMEM).

For assessing cardiomyocyte proliferation after RNAi treatment, cells were incubated with 10μM BrdU (BrdU Labeling and Detection Kit I, Roche, Indianapolis, IN) for 24 hours before fixation. NRVMs were double-stained by anti-BrdU and troponin I antibody and assessed by indirect immunofluorescence. As a negative control, cells were transfected with scrambled RNAi (1027280, Qiagen).

Immunofluorescent and immunohistochemistry staining. Hearts were isolated and fixed in 4% formaldehyde freshly prepared from paraformaldehyde, dehydrated, and embedded in paraffin. Heart sections (6 μm) were cut, mounted, dewaxed in xylene, rehydrated through an ethanol series, and then treated with 1X Antigen Unmasking Solution (Vector Laboratories, Burlingame, CA) in a microwave oven (350 watts) for 10 minutes to unmask the epitope. NRVMs were fixed in 4% PFA at room temperature for 15 min and permeabilized with 0.1% Triton X-100 in PBS. After blocking with 5% nonfat milk/PBS for 30 minutes, sections were
incubated at 4°C for 16 hours with primary antibodies diluted in 5% nonfat milk/PBS.

Alternatively, hearts were embedded in OCT, freshly frozen in isopentane/dry ice bath, and sectioned (5 µm) with a cryotome. Sections were fixed with 4% paraformaldehyde for 10 min, followed by 100% methanol for 10 min at -20°C. Tissues were permeabilized with 0.1% Triton X-100 in PBS, then blocked 30 min with 5% BSA and 0.01% Triton X-100 in PBS, and finally incubated with primary antibodies. Primary antibodies used were: αE-catenin (Invitrogen), αT-catenin (Proteintech), N-cadherin (BD Biosciences), desmin (Sigma), α-cardiac actin (Sigma), phospho-H3 (H3P) (Millipore), troponin I (Cell Signaling), Aurora-B kinase (BD Biosciences), Yap (Cell Signaling). After washing in PBS, sections were incubated with Alexa Fluor 488 or 555-conjugated goat anti-mouse, or rabbit antibody (Invitrogen) for 1 hour at room temperature.

The sections were washed in PBS, then either stained with Draq5 (Cell Signaling) for 10 min, mounted with Immuno-Mount (Thermo) for imaging with Olympus FV500 Confocal Microscope System; or directly mounted with Prolong Gold Antifade reagent containing DAPI (Invitrogen) for imaging with a Zeiss LSM 510 META Confocal Microscope System.

To assess DNA synthesis in vivo, BrdU was administered to animals as described previously. Briefly, 0.1 ml BrdU (5 mg/ml) per 25g animal was administered by intraperitoneal injection twice a day for 5 consecutive days prior to tissue collection. After BrdU treatment, hearts were collected, fixed, and stained with anti-mouse BrdU antibody.

For immunohistochemical analysis, paraffin sections were prepared as described above. After 1× Antigen Unmasking Solution (Vector Laboratories) treatment, sections were incubated for 30 min in 0.3% H₂O₂ in methanol. Sections were blocked for 30 min with diluted normal blocking serum and incubated overnight with the following primary antibodies: PCNA (Invitrogen), cyclin D1 (Santa Cruz), and Yap (Cell Signaling). Sections were incubated with
anti-mouse EnVision+ System horseradish peroxidase-labeled polymer (Dako Cytomation) for 30 min at room temperature. After washing, sections were developed in peroxidase substrate (3,3′-diaminobenzidine; Vector), counterstained with hematoxylin (Gill's Formula; Vector), cleared, and mounted. Samples were analyzed under a Nikon ECLIPSE 80i microscope.

**Western blotting.** Hearts tissue were homogenized in a modified RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1mM EDTA pH 8.0, 1% NP-40, 0.5% Na deoxycholate, 0.1% SDS) containing protease inhibitors and phosphatase inhibitor cocktails I and II (Roche Diagnostics) and centrifuged at 12,000 g for 15 min. Primary antibodies used were: αE-catenin (Invitrogen), αT-catenin, N-cadherin (BD Biosciences), β-catenin (BD Biosciences), p120 (Invitrogen), transferrin (Invitrogen), Yap (Cell Signaling), pS127 YAP (Cell Signaling), PCNA (Invitrogen), cyclin D1 (Santa Cruz). For normalization of signals, blots were also analyzed with anti-GAPDH antibody (6C5, RDI), followed by IRDye 680 or IRDye 800CW conjugated secondary antibody (LI-COR). Membranes were imaged with Odyssey Infrared Imaging System (LI-COR), and quantitative densitometric analysis was performed with Odyssey version 1.2 infrared imaging software.

**Preparation of subcellular fractions.** For protein fractionation, heart tissues were lysed by mechanical disruption in cold hypotonic buffer (10mM HEPES at pH 7.4, 1mMEDTA, and protease inhibitors (Sigma) and phosphatase inhibitor cocktails II and III (Roche Diagnostics). Homogenates were centrifuged at 1,000 g at 4 °C for 10 min to obtain supernatants. The supernatants were further ultracentrifuged at 100,000 g at 4 °C for 1 hour. After ultracentrifuge, the supernatants were collected and corresponded to the cytosolic fraction (C). After washing the
pellet with hypotonic buffer by ultracentrifuge at 100,000 g at 4 °C for 30 min, the membrane fraction was extracted with membrane extraction buffer (MEB; 50 mM Tris at pH 7.4, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, and protease inhibitors and phosphatase inhibitor cocktails II and III) by ultracentrifuge at 100,000 g at 4°C for 1 hour. This supernatant corresponded to the Triton X-100 soluble membrane fraction. The final pellet was extracted with modified RIPA buffer and centrifuged at 13,800 g for 10 min; this supernatant corresponded to the Triton X-100 insoluble membrane fraction (M). Equal quantities of protein were separated by SDS-PAGE, transferred to nitrocellulose membrane, and probed with antibodies described as above.

**Echocardiographic and hemodynamic measurements.** Transthoracic two-dimensional echocardiography (TTE) was performed with a Vevo 770 high-resolution imaging system equipped with a 30-MHz probe (Visual Sonics, Toronto, Canada) in anesthetized (2% inhaled isoflurane) WT and DKO mice. LV Wall thickness and dimension were measured from M-mode images at the plane bisecting the papillary muscles. Values were obtained by averaging 3 consecutive cardiac cycles, and ventricular fractional shortening and ejection fraction were calculated. Hemodynamic analysis included heart rate; maximal left ventricular systolic pressure, end-diastolic pressure, and maximal and minimal first derivatives of left ventricular pressure were performed as described previously.

**RNA extraction and quantitative RT-PCR.** Total mRNA was isolated from hearts with TRIzol (Life Technologies) and concentrated with the RNeasy minikit (Qiagen, Hilden, Germany). Contamination of RNA by genomic DNA was eliminated by DNase I treatment (Life Technologies) prior to the synthesis of cDNA from 1 µg of total mRNA with oligo[dT]12-18
priming and the Thermoscript RT-PCR system (Life Technologies). Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed using the MX3005P System (Agilent Technologies, CA) and the SYBR Green PCR Master Mix (Life Technologies) according to the manufacturer’s instructions. Specific primers designed for RT-PCR are listed in Online Table III. For each qRT-PCR a standard curve for each gene assay was used to account for the difference of PCR efficiencies between each assay reaction. For each target gene, raw data were normalized to Gapdh mRNA level.

Surgical induction of myocardial ischemia injury. Mice at 10 weeks of age were used for myocardial infarction (MI) experiments in which left anterior descending (LAD) coronary artery ligation was carried out as described previously.

Statistics. Differences between data groups were evaluated for significance using Student’s t-test of unpaired data (significance level set at P<0.05). All experiments were repeated at least three times and the data are presented as mean ± SEM unless noted otherwise.

References


Online Table I. Echocardiographic analysis of WT and IN-DKO mice at 12 weeks post-MI

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WT</th>
<th>IN-DKO</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>9</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>HR (bmp)</td>
<td>471 ± 2.32</td>
<td>458 ± 2.27</td>
<td>0.32</td>
</tr>
<tr>
<td>LVID;d (mm)</td>
<td>5.10 ± 0.08</td>
<td>4.62 ± 0.04</td>
<td>0.08</td>
</tr>
<tr>
<td>LVID;s (mm)</td>
<td>4.47 ± 0.09</td>
<td>3.83 ± 0.04</td>
<td>0.02</td>
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<tr>
<td>LV Vol;d (mm)</td>
<td>127.19 ± 4.87</td>
<td>100.33 ± 1.73</td>
<td>0.07</td>
</tr>
<tr>
<td>LV Vol;s (mm)</td>
<td>94.77 ± 4.49</td>
<td>64.89 ± 1.30</td>
<td>0.02</td>
</tr>
<tr>
<td>FS (%)</td>
<td>12.66 ± 0.53</td>
<td>17.30 ± 0.30</td>
<td>0.03</td>
</tr>
<tr>
<td>EF (%)</td>
<td>26.87 ± 1.11</td>
<td>35.90 ± 0.57</td>
<td>0.03</td>
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</tbody>
</table>

HR, heart rate; LVID;d, left ventricle diastolic internal dimension; LVID;s, left ventricle systolic internal dimension; LV Vol;d, left ventricle diastolic volume; LV Vol;s, left ventricle systolic volume; FS, left ventricle fraction shortening; EF, left ventricle ejection fraction. Data are presented as mean ± SEM. p < 0.05 considered significant.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>WT</th>
<th>IN-DKO</th>
<th>p value</th>
</tr>
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<tbody>
<tr>
<td>n</td>
<td>6</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>baseline</td>
<td></td>
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<tr>
<td>HR (bmp)</td>
<td>465.06 ± 6.97</td>
<td>460.37 ± 4.48</td>
<td>0.45</td>
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<tr>
<td>(+) DP/dt (mmHg/s)</td>
<td>5128.23 ± 164.49</td>
<td>6044.98 ± 109.28</td>
<td>0.08</td>
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<tr>
<td>(-) DP/dt (mmHg/s)</td>
<td>-4383.30 ± 242.44</td>
<td>-5830.35 ± 171.71</td>
<td>0.09</td>
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<tr>
<td>LVSP (mmHg)</td>
<td>95.63 ± 7.11</td>
<td>112.80 ± 16.90</td>
<td>0.60</td>
</tr>
<tr>
<td>LVEDP (mmHg)</td>
<td>10.25 ± 0.97</td>
<td>11.87 ± 1.11</td>
<td>0.48</td>
</tr>
<tr>
<td>Iso 10 ng</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>HR (bmp)</td>
<td>502.38 ± 2.95</td>
<td>515.95 ± 4.33</td>
<td>0.62</td>
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<tr>
<td>(+) DP/dt (mmHg/s)</td>
<td>5616.59 ± 189.52</td>
<td>7237.19 ± 110.17</td>
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<td>(-) DP/dt (mmHg/s)</td>
<td>-4192.89 ± 213.70</td>
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<tr>
<td>LVSP (mmHg)</td>
<td>100.95 ± 8.51</td>
<td>95.34 ± 4.84</td>
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</tr>
<tr>
<td>LVEDP (mmHg)</td>
<td>8.11 ± 1.17</td>
<td>9.98 ± 1.39</td>
<td>0.51</td>
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</tbody>
</table>

HR, heart rate; (+) DP/dt, maximal values of the instantaneous first derivative of left ventricular pressure; (-) DP/dt, minimum values of the instantaneous first derivative of left ventricular pressure; LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure. The parameters were recorded at baseline and after administration of the β-adrenergic receptor agonist, isoproterenol (Iso, 10 ng) as described in the method. Data are presented as mean ± SEM. p < 0.05 considered significant.
### Online Table III. Primers used for qRT-PCR

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<tr>
<th>Gene</th>
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<th>Sequence Reverse (5’&gt;3’)</th>
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</thead>
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<td>Nppa</td>
<td>CAAGAACCTGCTAGACCACC</td>
<td>AGCTGTTCAGGACTAGTCC</td>
</tr>
<tr>
<td>Nppb</td>
<td>GCG GCATGGATCTCCTGAAGG</td>
<td>CCCAGGCAAGATCAGAAACTG</td>
</tr>
<tr>
<td>Myh6</td>
<td>CCAATGAGTACCCGCTGAA</td>
<td>ACAGTCATGCGGGGATG</td>
</tr>
<tr>
<td>Myh7</td>
<td>ATGTGCCCGGACCTTGAGA</td>
<td>CCTCGGGTTAGCTGAGAGATCA</td>
</tr>
<tr>
<td>Aurka</td>
<td>GGGTGGTCGGTGCAATGCTCCA</td>
<td>GCCTCGAAAGGAGGCATCCTCCACTA</td>
</tr>
<tr>
<td>Cdc5l</td>
<td>GCAAGTTGTGCGAGACTCCA</td>
<td>TTCTACCTGGGTCGTGCA</td>
</tr>
<tr>
<td>Ccnb1</td>
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<td>GTCAGCCCATCATCTTGCG</td>
</tr>
<tr>
<td>Cdc20</td>
<td>TTCGTGTTCGAGAGCCATTG</td>
<td>ACCTTGGAAACTAGATTTGCCAG</td>
</tr>
<tr>
<td>Yap</td>
<td>GACCCCTCGTGTGCTGCA</td>
<td>ATTTGTTCTCGAATTCTGAGAC</td>
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</table>
Online Figure I. **Normal histology and lack of proliferative phenotype in uninjured α-cat IN-DKO hearts.** (A, C) Representative images of H&E-stained hearts 11 months post tamoxifen administration. Uninjured IN-DKO hearts exhibited normal morphology and histology. (B) No difference in HW/BW ratio between WT and IN-DKO mice. (D) Masson trichrome-stained heart sections did not detect fibrosis in the IN-DKO hearts. (E) Representative immunofluorescent staining of H3P (red) and α-actin (green) in heart sections. No difference in H3P staining was observed between WT and IN-DKO mice. H3P-positive nonmuscle cells serve as a positive control (arrowheads).
Online Figure II. No difference in LV infarct size 24 hrs post MI. (A) Representative images of 2,3,5-triphenyltetrazolium chloride (TTC)-stained WT and IN-DKO hearts 24 hrs post MI. The ischemic area is outlined with stippled line. (B) Quantification of the ischemic area in WT (n=5) and IN-DKO (n=8) hearts. The infarct size was expressed as the percentage of infarct area over total LV area. NS, p = 0.87; Bar, 0.1 mm.