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Alpha-Catenins Control Cardiomyocyte Proliferation by Regulating Yap Activity

Jifen Li[1], **Erhe Gao**[1,*], **Alexia Vite**[1], **Roslyn Yi**[1], **Ludovic Gomez**[1,4], **Steven Goossens**[2,3], **Frans van Roy**[2,3], and **Glenn L. Radice**[1]

[1]Center for Translational Medicine, Department of Medicine, Thomas Jefferson University, Philadelphia, PA

[2]Department of Biomedical Molecular Biology, Ghent University, B-9052 Ghent, Belgium

[3]Inflammation Research Center, Flanders Institute for Biotechnology (VIB), B-9052 Ghent, Belgium

[4]INSERM UMR-1060, Laboratoire CarMeN, Université Lyon 1, Faculté de médecine, Rockefeller et Charles Merieux Lyon-Sud, Lyon, F-69003, France

Abstract

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 co-activator 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 (α-cat DKO) mice.

Methods and Results—We used two 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-cat DKO hearts. Fetal genes were increased in the α-cat DKO hearts indicating a less mature cardiac gene expression profile. Knockdown (KD) of α-catenins in neonatal rat cardiomyocytes also resulted in increased proliferation, which could be blocked by KD 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 following myocardial infarction.

Conclusions—These studies demonstrate that α-catenins are critical regulators of Yap, a transcriptional co-activator essential for cardiomyocyte proliferation. Furthermore, we provide

Address correspondence to: Dr. Glenn Radice, Center for Translational Medicine, Department of Medicine, Sidney Kimmel Medical College, Thomas Jefferson University, Suite 543E Jefferson Alumni Hall, 1020 Locust St., Philadelphia, PA19107, glenn.radice@jefferson.edu.
*Current address: Center for Translational Medicine, Temple University School of Medicine, Philadelphia, PA19140.

DISCLOSURES
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proof-of-concept that inhibiting α-catenins might be a useful strategy to promote myocardial regeneration following injury.

Keywords

αE-catenin; αT-catenin; cardiac regeneration; cell cycle progression; cytokinesis; animal model; cadherin; cardiac myocyte; myocardial infarction; Yap

INTRODUCTION

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 de-differentiation 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.

α-catenins are adherens junction proteins that link the cytoplasmic domain of cadherin to the actin cytoskeleton and act as mechanosensors[6]. Recent studies suggest that α-catenins have more complex and diverse functions than previously appreciated. αE-catenin has been implicated in sensing cell density and restricting basal cell proliferation in epidermis[7] and neural progenitor cells[8]. Loss of αE-catenin triggers severe epidermal hyperproliferation and tumors in mice[7, 9]. Two α-catenins are expressed in the heart: the ubiquitously expressed αE-catenin and the largely cardiac-restricted αT-catenin. Both proteins contain vinculin homology domains and share 57% overall amino acid identity[10]. Importantly, both α-catenins function as part of the N-cadherin adhesion complex at the cardiac intercalated disc (ID)[10]. The N-terminal domain of α-catenin binds β-catenin while its C-terminal domain interacts with F-actin facilitating linkage of N-cadherin with the actin cytoskeleton. Additionally, 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. αT-catenin is unique in that it can bind to the desmosomal protein, plakophilin-2 (PKP-2) whereas αE-catenin lacks the PKP-2 binding domain[11]. Deletion of either *αE-catenin* (*Ctnna1*) or *αT-catenin* (*Ctnna3*) in the murine heart leads to progressive cardiomyopathy[12, 13], consistent with their role in mechanical junctions. Of note, mutations in human *αT-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 TEAD transcriptional factors 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 (Salv) 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 mice[18] and/or constitutive activation of Yap[19, 20] results in overgrowth of the heart. Conversely, ablation of Yap during cardiac development results in a hypoplastic heart and embryonic lethality[19, 20]. Additionally, disruption of the Hippo pathway or constitutively active Yap is sufficient to enhance cardiac regeneration in mice following injury[21–23]. Conversely, Yap haploin sufficiency leads to decrease cardiomyocyte proliferation and worse cardiac function following myocardial infarction compared to wild-type 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 due to the ability of α-catenins to prevent the transcriptional co-activator 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 following myocardial infarction.

METHODS

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

αMHC/Cre; αE-catenin$^{fl/fl}$; αT-catenin$^{fl/fl}$ animals (DKO) and αMHC/MerCreMer; αE-catenin$^{fl/fl}$; αT-catenin$^{fl/fl}$ animals (IN-DKO) were generated by standard breeding protocols. The mice were maintained on a mixed 129 Sv-C57BL/6J genetic background. Adult IN-DKO mice were injected intraperitoneally once a day for 5 consecutive days with 80 mg tamoxifen (Sigma) per kg body weight.

A detailed Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

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 (fl), αT-catenin flox, and αMHC/Cre mice to generate cardiac-specific αE- and αT-catenin double knockout (DKO) mice. Littermates lacking the Cre transgene (i.e. αE-cat$^{fl/fl}$; αT-cat$^{fl/fl}$) served as wild-type (WT) controls. The DKO animals were born in the expected Mendelian frequency, indicating that depletion of both α-catenins using αMHC/Cre is compatible with cardiac development during the perinatal period. Normally co-localized with N-cadherin, both α-catenins were lost from the ID in the DKO heart (Fig. 1A). Western blot analysis showed significant reduction of αE- and αT-catenin proteins in the DKO heart compared with WT control (Fig. 1B), indicating efficient depletion of both α-catenins. Heart weight/body weight (HW/BW) ratios were determined at selected ages beginning at 6 weeks (Fig. 1C). The difference between the HW/BW ratios of DKO and control littermates 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 (Fig. 1D, left panels). However, atria were often enlarged. The DKO hearts displayed fibrosis with age, as revealed by Masson trichrome staining (Fig. 1D, right panels). Terminal

deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) analysis did not detect a change in cardiomyocyte apoptosis in the DKO compared to WT hearts (data not shown). An increase in nuclear density was observed in the H&E-stained DKO heart sections (Fig. 1D), hence we counted myocyte cells in ventricular myocardium stained with anti-desmin antibody to identify cardiomyocytes and with DAPI 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 (Fig. 1E). Further, 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 due to increased fibrosis and less efficient perfusion of the adult DKO heart compared to 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 to WT (3.07×10^5, $n=20$) P7 hearts (Fig. 1H). No significant differences in myocyte numbers were observed at P4. Interestingly, myocyte cross-sectional areas were reduced in the adult DKO hearts (−17%, $n=5$, $p<0.05$) (Fig. 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 (Fig. 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 if loss of α-catenins affected cardiomyocyte morphology, we examined isolated adult cardiomyocytes from DKO and WT hearts (Fig. 2A). WT myocytes showed their characteristic rod shape with step-like structures at their termini representing the ID. 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 (Fig. 2B). WT myocytes displayed a typical sharp linear pattern of N-cadherin expression at the termini. In comparison, DKO myocytes showed variable diffuse N-cadherin expression at the ID as well as cytoplasmic staining not observed in WT. The cadherin-binding protein, β-catenin, showed a similar expression pattern as N-cadherin in the DKO hearts (data not shown). Total protein levels of N-cadherin, β-catenin and p120 did not significantly change in the absence of α-catenins (Fig. 2C). Consistent with the immunostaining data, cellular fractionation demonstrated increased N-cadherin in the cytoplasm of the DKO hearts (Fig. 2D). These data suggest that loss of the cytoskeletal proteins, α-catenins, destabilizes N-cadherin at the ID leading to its aberrant distribution in the myocyte.

α-catenins inhibit cardiomyocyte proliferation

To examine cardiomyocyte proliferation, we analyzed DNA synthesis in DKO hearts by BrdU incorporation (Fig. 3A). The percentage of BrdU-positive cardiomyocytes in DKO hearts (1.51% ±0.22%) was two-fold higher than in WT control hearts (0.66% ± 0.11%; n=3, $p<0.05$) at two months of age. We next used an anti-phosphohistone-3 (H3P) antibody to examine cardiomyocyte mitosis (Fig. 3B). The mitotic index (ratio of H3P-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 increase 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 Salv 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, $p<0.001$) (Fig. 3C, E). Moreover, we observed increased Proliferating Cell Nuclear Antigen (PCNA) staining in the nuclei of DKO cardiomyocytes and confirmed this PCNA increase by Western analysis of total heart lysates (+234% of WT, n=5, $p<0.01$) (Fig. 3D, E). Expression of cell cycle genes was examined in WT and DKO hearts by quantitative reverse transcription polymerase chain reaction (qRT-PCR). *Aurka*, *Cdc20*, *Cnnb1*, and *Cdc5l* were all increased in the DKO hearts (Fig. 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 one week after birth that continued to at least 16 weeks of age (Fig. 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 co-activator of the Hippo signaling pathway, and that this increased nuclear localization is associated with increased proliferation in the skin[7, 25]. To determine if Yap was affected in our α-cat DKO model, we performed immunohistochemistry with anti-Yap antibody on hearts from 2-month old α-cat DKO mice. Yap-positive cardiomyocytes were rarely seen in the WT heart (Fig. 4A). In contrast, DKO heart displayed a significant nuclear localization of Yap (Fig. 4A). Western blot analysis demonstrated upregulation of both total and phosphorylated Yap (S127) in α-cat DKO hearts (Fig. 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 qRT-PCR (data not shown), indicating that posttranscriptional 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 (Fig. 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. 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 (α-cat KD) was performed using siRNA in primary rat neonatal cardiomyocytes (Fig. 5A). We observed enhanced BrdU uptake and increased PH3 expression in the α-cat KD cells, consistent with the proliferative phenotype observed *in vivo* (Fig. 5B, C). Furthermore, cells in late anaphase and cytokinesis, as identified by Aurora-B staining of central mitotic spindles and midbodies, were increased in α-cat KD cells (Fig. 5D). To determine whether Yap was responsible for the increased proliferation in the α-cat KD myocytes, siRNA against Yap was used to generate triple KD cells (Fig. 6A). We observed decreased BrdU incorporation after Yap KD in the α-cat KD cells demonstrating that Yap is required for the proliferative phenotype (Fig. 6 B, C).

α-catenins regulate post-MI remodeling

To determine whether depletion of α-catenins had a similar effect in the adult heart, we utilized 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 H3P staining, and survived to at least one 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 (LAD)[26] in 2-month-old αMHC-MerCreMer; αE-cat$^{fl/fl}$; αT-cat$^{fl/fl}$ mice 10 days after inducing deletion of the genes with tamoxifen. We refer to this experimental protocol as a prevention study (Fig. 7A), since α-catenins were depleted at the time of the myocardial infarction (MI) (Fig. 7B). Myocardial function was determined by serial echocardiography (Fig. 7C). Nine weeks after MI, dimensions of the left ventricle (LV) decreased in IN-DKO animals whereas control animals had sustained LV 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 (Fig. 7C and Online Table I). Furthermore, following isoproterenol administration hemodynamic measurements 12 weeks after MI showed improved LV contractility (as measured by +dP/dt) and LV relaxation (as measured by −dP/dt) in IN-DKO hearts compared with WT controls (Fig. 7D and 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 (Fig. 7E). Measurement of infarct size and fibrosis area showed that the injured area was smaller in the IN-DKO than in WT controls (Fig. 7F).

Furthermore, the anterior wall was thicker consistent with the improved cardiac function in the IN-DKO compared to WT (Fig. 7F). Cardiomyocyte cross-sectional area was smaller, consistent with attenuation of cardiomyocyte hypertrophy in the IN-DKO mice (Fig. 7F). To assess potential differences in the initial injury, hearts were stained with 2,3,5-triphenyltetrazolium chloride (TTC) 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 following injury, we performed immunofluorescent staining with anti-Yap antibody on IN-DKO hearts one week after MI. Yap-positive cardiomyocyte nuclei were identified by staining with anti-α-cardiac actin antibody and counted in different areas: border zone (BZ), infarct zone (IZ) and remote area (RM). Yap-positive cardiomyocytes were rarely seen in the RM of either IN-DKO or WT controls (Fig. 8A). In contrast, the numbers of Yap-positive nuclei in the IN-DKO heart were significantly increased in both BZ (+2.5-fold vs WT, n=4, $p<0.05$) and IZ (+2.3-fold vs WT, n=4, $p<0.05$). Analysis of DNA synthesis by BrdU incorporation and mitosis by H3P immunostaining confirmed enhanced proliferation of cardiomyocytes in IN-DKO hearts one week after MI (Fig. 8B, C). 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 (Fig. 8D). Collectively, these data indicate that loss of α-catenins enhances Yap accumulation in the nucleus, where it promotes cardiac regeneration following injury.

DISCUSSION

The transcriptional coactivator Yap, a downstream effector of the Hippo signaling pathway, was recently shown to be necessary and sufficient for cardiomyocyte proliferation[19, 20]. Moreover, cardiac-specific expression of activated Yap (e.g. αMHC-YapS112A) that cannot be phosphorylated by inhibitory upstream kinases in the Hippo pathway enhances cardiac regeneration post-MI[22, 23]. 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 prior to injury (IN-DKO model) led to improved cardiac function compared to control littermates. A two-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 following MI. Additionally, 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-catenins normally inhibit cardiomyocytes in the adult heart from entering the cell cycle and undergoing cytokinesis following injury.

The structural integrity of the heart is dependent on mechanical junctions located at the end-to-end connections between cardiomyocytes called the intercalated disc (ID). The cell adhesion molecule N-cadherin is required to maintain ID structure[27]. 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[28]. 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-cat DKO might reflect cardiac remodeling due to weakened intercellular adhesion and

contractility in the mutant hearts. We speculate that α-cat DKO mice utilize 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 very 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 mid-gestation (unpublished data, G. Radice). The cause of this embryonic lethality is under investigation. 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 (e.g. *Myh7*) as early as one week of age in the DKO hearts suggest that perturbation of N-cadherin/catenin complex and cytoskeleton during the postnatal period allows cardiomyocytes to remain in a less mature proliferative state. On the other hand, if α-catenins are depleted in the adult heart using a tamoxifen inducible αMHC-MerCreMer (i.e. 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]. In another study, αE-catenin was reported to be preferentially downregulated in both the remote and infarct area of human hearts[33]. Interestingly, we did observe improved contractility after MI in the IN-DKO compared to 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 de-differentiation 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 Salv) in the developing heart by using Nkx2.5/Cre, induced Yap nuclear localization, cardiomyocyte proliferation, and a 2.5-fold increase in heart size[18]. In the conditional SalvKO 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 cat 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[34].

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, 9]. 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]. Since 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-cat 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 an αE-catenin/14-3-3/Yap complex that protected Yap from 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 co-activator Yap and cardiomyocyte proliferation. Importantly, depletion of α-catenins in adult animals led to improved cardiac function following MI. Hence, functional interference with α-catenins represents a novel mechanism for enhancing signaling pathways beneficial in cardiac repair.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard Abbreviations and Acronyms

α-cat	α-catenin
BrdU	5-Bromo-2′-deoxyuridine
DKO	double knockout

fl	flox
ID	intercalated disc
IN-DKO	inducible double knockout
KD	knockdown
LV	left ventricle
MHC	myosin heavy chain
MI	myocardial infarction
NRCM	neonatal rat cardiomyocytes
P	postnatal day
Yap	Yes-associated protein

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Novelty and Significance

What Is Known?

- Accumulating evidence suggests that re-activation of local existing cardiomyocytes can improve function of a failing heart.
- The transcriptional co-activator Yes-associated protein (Yap) is necessary and sufficient for cardiomyocyte proliferation.
- Heart muscle utilizes two intercalated disc proteins, αE-catenin and αT-catenin, the cytoskeletal network inside the cell.
- In many tissues, engagement of the cadherin/catenin complex at points of cell-cell contact provides critical signals to inhibit cell growth.
- 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.
- Alpha-catenin-deficient cardiomyocytes express fetal genes, accumulate Yap 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 lead to translocation of Yap 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.

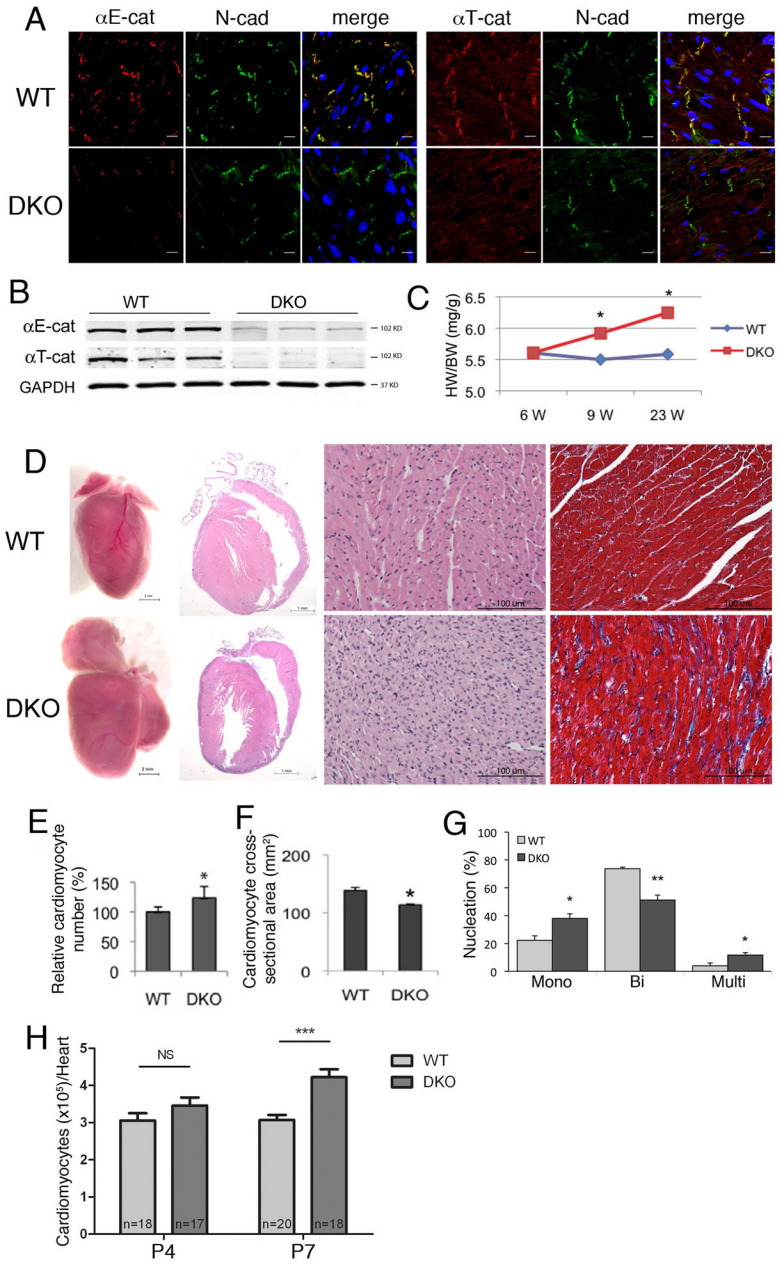

Figure 1. Loss of α-catenins causes an increased number of myocytes in α-cat DKO hearts

(A) Hearts from WT and DKO animals 2 months of age were immunostained for αE-cat, αT-cat, and N-cad. Bar, 20 μm. (B) Western blot analysis of αE-cat and αT-cat expression in whole heart lysates confirm ablation of both α-catenins in the DKO hearts. (C) HW/BW ratio increased with age in DKO animals. (D) Whole mount images of hearts from 6-week old animals, H&E stained heart sections from 2 month old animals, and Masson trichrome stained heart sections from 5 month old animals. (E) Quantification of number of cardiomyocytes per section in WT and DKO. (F) Myocyte cross-sectional area was measured at the level of the nucleus in cross-sectioned H&E stained myocytes. (G) Quantification of the percentage of mono-/bi-/multi-nucleated cardiomyocytes (WT, n=305;

DKO, n=333) isolated from adult hearts. (H) Cardiomyocyte number calculated from dissociated postnatal day (P) P4 and P7 hearts.* $p<0.05$; ** $p<0.01$; *** $p<0.001$.

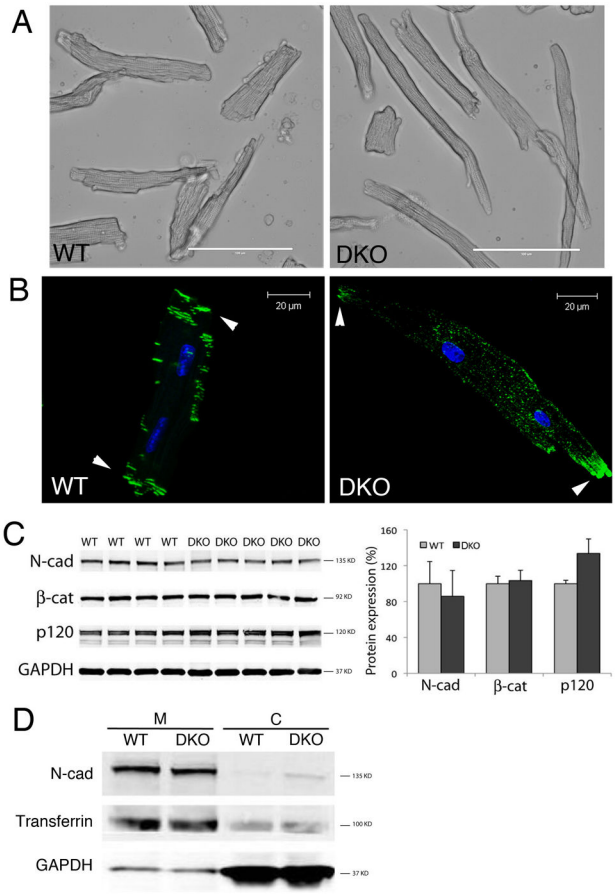

Figure 2. Aberrant N-cadherin localization in α-cat DKO hearts

(A) Representative bright field image of isolated adult cardiomyocytes from WT and DKO mice. Scale bar: 100 μm. (B) Isolated cardiomyocytes were immunostained for N-cadherin (arrowheads indicate the termini of single cardiomyocytes). To better visualize the cellular distribution of N-cadherin, a 3D reconstruction was generated from a series of confocal optical sections. (C) Western blot analysis and quantification of N-cad, β-cat, and p120 expression in whole heart lysates.(D) Western blot analysis of membrane (M) and cytoplasmic (C) fractions from WT and DKO hearts. Transferrin receptor and GAPDH serve as controls for enrichment of membrane and cytoplasmic protein fractions, respectively.

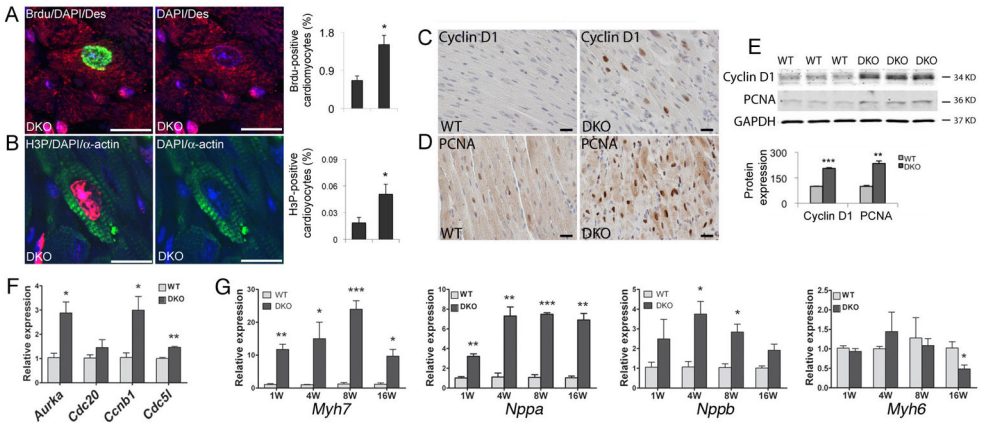

Figure 3. Increased cardiomyocyte proliferation in α-cat DKO hearts

(A) Hearts from WT and DKO animals 2 months of age were labeled with BrdU for 5 days, and DNA synthesis was determined by immunofluorescence microscopy, using anti-BrdU antibody to detect cycling cells, anti-desmin antibody to identify cardiomyocytes, and DAPI to highlight nuclei.(B) Mitosis (prophase) was visualized with an antibody specific for phosphorylated histone H3 antibody (H3P). Co-staining was with anti-α-cardiac actin and with DAPI to highlight nuclei. Cyclin D1 (C) and PCNA (D) nuclear expression was determined by immunohistochemical staining of the myocardium.(E) Cyclin D1 and PCNA expression levels were also determined by Western blot on whole heart lysates.(F) Cell cycle gene expression assessed by qRT-PCR in 1 month old WT and DKO hearts (n=3).(G) qRT-PCR measurement of fetal genes in WT and DKO hearts (n=3) at 1, 4, 8, and 16 weeks of age. Bars, 20 μm. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

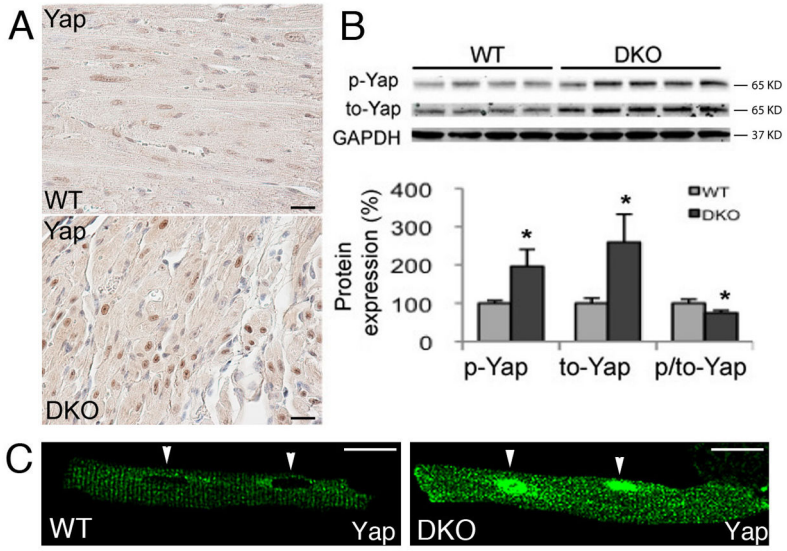

Figure 4. Loss of α-catenins leads to an increase in nuclear Yap

(A) Increased nuclear Yap in the DKO compared to WT myocardium as determined by immunohistochemistry.(B) Western blot analysis showed increased phospho- (p) and total (to) Yap levels in DKO heart lysates. (C) Isolated cardiomyocytes were immunostained for Yap and visualized with a confocal microscope (arrowheads indicate nuclei). Note the increased nuclear and cytoplasmic Yap in the DKO cell. Bars, 20 μm. *, $p<0.05$.

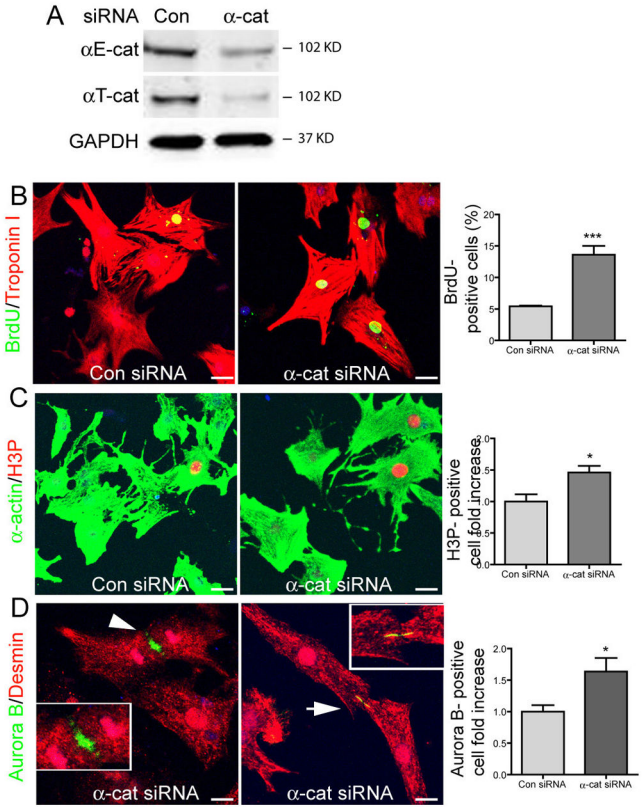

Figure 5. Increased proliferation in neonatal rat cardiomyocytes after knockdown of αE- and αT-catenin

(A) Western blot analysis of cardiomyocyte lysates 48 hr after treatment with siRNA to knock down both αE- and αT-catenins (α-cat) or with control siRNA (Con). (B) BrdU incorporation detected by anti-BrdU antibody (green), troponin I (red) and DAPI (blue). (C) Cells were stained for phosphorylated histone H3 (red), α-cardiac actin (green) and DAPI (blue). (D) Two representative images are shown of α-cat KD cells undergoing cytokinesis (arrowheads). Cells were stained for Aurora-B (green), desmin (red) and DAPI (blue). Insets show higher magnification of the Aurora-B positive cells. At least ten randomly selected fields were quantified in each experiment. Bar, 20 μm. *, $p<0.05$; ***, $p<0.001$.

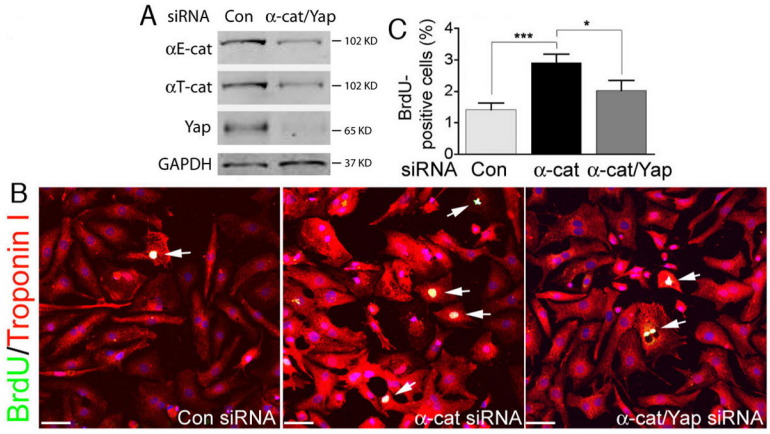

Figure 6. Yap KD suppressed cardiomyocyte proliferation induced by KD of αE-/αT-catenin in neonatal rat cardiomyocytes (NRCM)

(A) Western blot analysis of cardiomyocyte lysates 48 h after treatment with siRNA to simultaneously knock down αE-, αT-catenins, and Yap (α-cat/Yap) or with control siRNA (Con) in NRCM. (B) Representative images are shown with BrdU (green), troponin I (red) and DAPI (blue) staining of NRCM after KD with con, α-cat, or α-cat/Yap siRNA. Arrows indicate BrdU-positive cardiomyocytes. (C) BrdU incorporation was counted in 20 randomly selected fields. Bar, 20μm. ***, $p<0.001$; *, $p<0.05$.

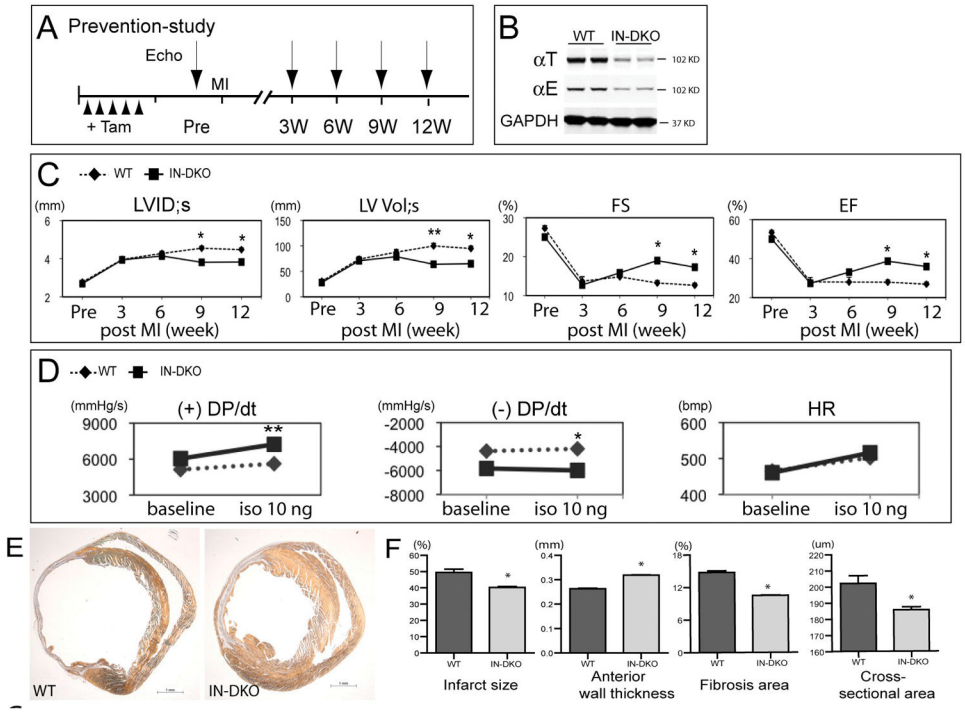

Figure 7. Improved cardiac function in α-cat IN-DKO after myocardial infarction (MI)

(A) Experimental protocol showing the prevention study design using tamoxifen (Tam)-inducible (IN) α-cat DKO mice. Pre, baseline echo before MI. (B) Western blot showing αE- and αT-catenin protein levels in hearts around the time of the MI (~ 2 weeks post Tam). (C) Serial echocardiographic measurements of left ventricular intermediate dimension (LVID), left ventricular volume (LVvol), fraction shortening (FS) and ejection fraction (EF). (D) Hemodynamic measurements in the presence of isoproterenol (iso) 12 weeks after MI: left ventricular maximum positive (+dP/dt) and negative (−dP/dt) pressure development were all improved in α-cat DKO mice. HR, heart rate. (E) Representative heart sections at the level of papillary muscle after staining with acid fuchsin-orange G (AFOG) for scar tissue (blue) and muscle tissue (orange brown). (F) Quantification of infarct scar size, anterior wall thickness, fibrosis area and cross-sectional area of cardiomyocytes in the remote area 12 weeks post MI.

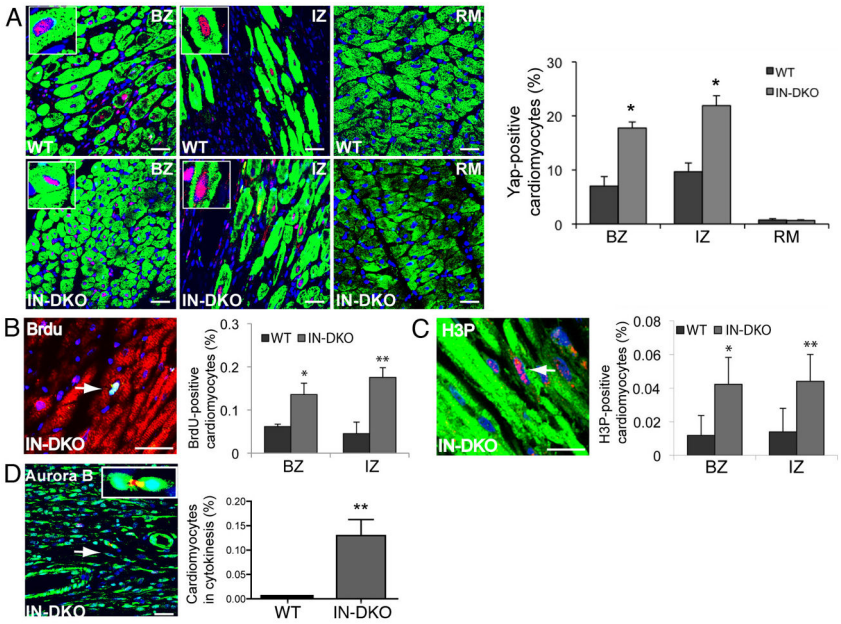

Figure 8. Increased nuclear Yap and proliferation in the α-cat IN-DKO after myocardial infarction (MI)

(A) Representative immunofluorescent staining with Yap antibody on ischemic heart tissue 1 week post-MI. Note nuclear localization of Yap (red) in both border zone (BZ) and infarct zone (IZ) in α cardiac actin-positive cardiomyocytes (green). RM, remote area. Higher magnifications of Yap-positive nuclei are shown in the insets. Right panel: quantification of Yap-positive cardiomyocytes after MI.(B) Beginning 1 day post MI, hearts were labeled with BrdU for 5 consecutive days, and DNA synthesis was determined by immunofluorescence microscopy, using anti-BrdU antibody to detect cycling cells, anti-desmin antibody to identify cardiomyocytes, and DAPI to highlight nuclei. Right panel: quantification of BrdU-positive cardiomyocytes one week post-MI. (C) Mitosis was visualized with an antibody specific for phosphorylated histone H3 antibody (H3P). Co-staining was with anti-α-cardiac actin and with DAPI to highlight nuclei. Right panel: quantification of H3P-positive cardiomyocytes one week post-MI.(D) Central mitotic spindles (arrow) and midbodies visualized in cardiomyocytes 1 week post-MI by co-staining for Aurora-B (red) and α-cardiac actin (green). Higher magnification of a dividing cardiomyocyte is shown in the inset. DAPI (blue) highlights the nuclei. Right panel: quantification of cardiomyocytes in cytokinesis (Aurora-B positive) after MI. Bars, 20 μm. *, $p<0.05$; **, $p<0.01$.