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Short Communication

Ctnna3 Deficiency Promotes Heart Regeneration by Enhancing Cardiomyocyte Proliferation in Neonatal Mice

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Abstract

Background: Heart regeneration requires renewal of lost cardiomyocytes. However, the mammalian heart loses its proliferative capacity soon after birth, and the molecular signaling underlying the loss of cardiac proliferation postnatally is not fully understood. **Purpose**: This study aimed to investigate the role of Catenin alpha 3 (*Ctnna3*), coding for alpha T catenin (αT-catenin) protein in regulating cardiomyocyte proliferation and heart regeneration during the neonatal period. **Methods**: Here we report that ablation of *Ctnna3* and highly expressed in hearts, accelerated heart regeneration following heart apex resection in neonatal mice. **Results**: Our results show that *Ctnna3* deficiency enhances cardiomyocyte proliferation in hearts from postnatal day 7 (P7) mice by upregulating Yes-associated protein (Yap) expression. **Conclusion**: Our study demonstrates that *Ctnna3* deficiency is sufficient to promote heart regeneration and cardiomyocyte proliferation in neonatal mice and indicates that functional interference of α-catenins might help to stimulate myocardial regeneration after injury.

Keywords: *Ctnna3*; α-catenins; cardiomyocyte proliferation; heart regeneration; Yap

1. Introduction

Heart failure, caused by the loss of cardiomyocytes during injury and disease, is a leading cause of morbidity and mortality in humans [1]. The adult mammalian heart has a limited ability to regenerate cardiomyocytes, which is insufficient to restore contractile function after injury. In contrast, the neonatal mammalian heart possesses a remarkable capacity for cardiac regeneration, but this ability is lost by the age of 7 days, coinciding with the onset of cardiomyocyte proliferative arrest [2–4]. Various experimental strategies have been investigated to restore functional myocardium for repairing the injured heart. These are: (a) cell therapy using embryonic stem cells, induced pluripotent stem cells (iPS) and cardiac progenitor cells [5]; (b) reprogramming of nonmyocytes, e.g., cardiac fibroblasts, to a cardiac cell fate (cardiomyocytes) using cardiogenic genes and small molecules; (c) re-activation of cardiomyocyte mitosis in the adult heart [6–9]. Genetic fate-mapping experiments in the neonatal mice [4] and adult zebrafish [10,11] indicate that the regenerated cardiomyocytes mainly arise from reactivation of preexisting cardiomyocytes, rather than from undifferentiated stem or progenitor cells. Thus, identifying the genes and thoroughly understanding the mechanisms underlying that regulate cardiomyocyte proliferation and regeneration could provide critical insights for developing new therapeutic strategies for heart failure.

α-catenins play fundamental roles in cadherin-mediated cell-cell adhesion and actin dynamics [12–14], playing significant roles in cell differentiation, proliferation, and regeneration [12,14,15]. There are three subtypes: αE-catenin, αN-catenin, and αT-catenin, encoded by the genes Catenin alpha 1 (*Ctnna1*), *Ctnna2* and *Ctnna3*, respectively [14–16]. While they share amino acid similarities, they have distinct tissue distributions: αE-catenin acts as a tumor suppressor with a broad expression profile [14]. αN-catenin is restricted to neuronal tissues [17] and αT-catenin is expressed primarily in the heart and testis [16]. Although cardiac-specific deletion of *Ctnna1* resulted in progressive dilated cardiomyopathy and susceptibility to wall rupture in adult mice [18] and *Ctnna3* deficient mice displayed ventricular arrhythmia and dilated cardiomyopathy after acute ischemia [19], enhanced cardiomyocyte proliferation occurs in mice lacking both *Ctnna1* and *Ctnna3* [20]. Furthermore, mutations in *CTNNA3* were identified in patients with arrhythmogenic right ventricular cardiomyopathy [21], but the specific roles of αE-catenin and αT-catenin in heart regeneration and cardiomyocyte proliferation remain unclear.

Here we present experimental evidence indicating that deficiency of *Ctnna3* is sufficient to promote heart regeneration following heart apex resection in neonatal mice. Our study revealed that cardiomyocyte proliferation increased in neonatal *Ctnna3* deficient mice, probably due to the up-regulation of Yes-associated protein (Yap) expression. These results suggest that αT-catenin does contribute to regulation of heart regeneration and cardiomyocyte proliferation in neonatal mice.

2. Materials and Methods

2.1 Animals

The *Ctnna3*$^{-/-}$ mice (a kind gift from Dr. Radice's lab at Thomas Jefferson University, Philadelphia, PA, USA) have been characterized previously [19]. *Ctnna3*$^{-/-}$ (C57/129) mice were crossed to wildtype (WT) Friend Virus B-type (FVB) mice to generate *Ctnna3*$^{+/-}$ mice. These mice were maintained and raised on a C57/129/FVB genetic background in a specific pathogen-free facility. In this study, we analyzed: (1) apex resection experiments at 7 days post-resection (dpr) (WT, n = 5; *Ctnna3*$^{-/-}$, n = 5) and 14 dpr (WT, n = 3; *Ctnna3*$^{-/-}$, n = 3); (2) proliferation at (postnatal day 1) P1/P3/P7/P14: WT (n = 5), *Ctnna3*$^{-/-}$ (n = 5) per time point; (3) cardiomyocyte cultures (WT, n = 9; *Ctnna3*$^{-/-}$, n = 12); and (4) cardiac β-catenin/Yap expression by western blot (WT, n = 3; *Ctnna3*$^{-/-}$, n = 3). Experiments were performed in accordance with "the National Institutes of Health guide for the care and use of laboratory animals" and approved by the Institutional Animal Care and Use Committee (IACUC) of the Institute of Developmental Biology and Molecular Medicine, Fudan University, Shanghai, China.

Neonatal mice (<7 days old) for terminal procedures received 5% isoflurane (until loss of toe-pinch reflex, typically <2 mins) followed by cervical dislocation (5%, #792632, Sigma-Aldrich, St. Louis, MO, USA) while adult mice were euthanized via sodium pentobarbital overdose (150 mg/kg, IP, P3761, MilliporeSigma, St. Louis, MO, USA) followed by bilateral thoracotomy to confirm death.

2.2 Isolation and Culture of Neonatal Mouse Cardiomyocytes

Cardiomyocytes from neonatal mice were isolated as previously described [20]. Briefly, hearts from neonatal mice at P1 were disassociated with collagenase II (1 mg/mL, #C6885, Sigma-Aldrich, St. Louis, MO, USA). The disassociated cells were plated in a 10 cm plate for 2 hours with DMEM/F12 medium (#11330032, Gibco, Carlsbad, CA, USA), and the supernatant was collected and cells were re-plated on laminin-coated glass coverslips in 12-well plates at 3 $\times$ 10^5 cells per well. On the following day, the proliferating cells were labeled by 5-ethynyl-2′-deoxyuridine (EdU) in fresh medium for 24 hours and detected using Cell-light EdU Apollo 643 *In Vitro* Kit (50 mg/kg, #C10310–2, RiboBio, Guangzhou, Guangdong, China) following the manufacturers' instructions. All cell lines were validated by STR profiling and tested negative for mycoplasma.

2.3 Neonatal Mouse Apical Resection

Neonatal mouse apical resection of hearts from P1 pups was performed as described previously [22]. Neonatal mice for apex resection surgeries, pups were anesthetized by hypothermia (3–5 min on ice) and maintained on a cooling plate during the procedure. Postoperative pups were revived on a 37 °C warming pad for 10 min before dam reunion.

2.4 Histological and Immunofluorescent Analyses

Tissue processing, frozen sections and immunofluorescent microscopic analysis were performed as previously described [23]. Briefly, for frozen sections, mouse hearts were dissected out, fixed with 4% paraformaldehyde (PFA) (#P6148, Sigma-Aldrich, St. Louis, MO, USA) in phosphate-buffered saline (PBS) (#10010023, Thermo Fisher Scientific, Waltham, MA, USA) overnight, dehydrated with 30% sucrose (#S7903, MilliporeSigma, St. Louis, MO, USA) at 4 °C for 3 days and then embedded in optimal cutting temperature (OCT) (#4583, Sakura Finetek, Torrance, CA, USA). Sections were collected at 10 µm.

For histological analysis, mouse hearts were paraffinized and were sectioned at 4 µm followed by hematoxylin/eosin (H&E) staining or Masson's trichrome (MT) staining (#G1340, Solarbio, Beijing, China) as previously described [24]. For quantification of cardiac fibrosis, images of the MT stained sections were captured with Leica Aperio VERSA microscope (Model Aperio VERSA 8, Leica Biosystems, Wetzlar, Germany) and the area of fibrosis in heart apex was determined using Visiopharm software (version 2018.4, Visiopharm, Horsholm, Denmark).

For immunofluorescent analysis, the sections were stained with antibodies against the following proteins: sarcomeric α-actinin (1:200, #A7732, MilliporeSigma, St. Louis, MO, USA); Phospho-Histone H3 (PH3) (1:500, #53348, Cell Signaling Technology, Danvers, MA, USA) and proliferating cell nuclear antigen (PCNA) (1:1000, #2586, Cell Signaling Technology), Anti-rabbit IgG (H+L), Alexa Fluor 488-conjugated (1:500, #4412, Cell Signaling Technology, Danvers, MA, USA), Anti-mouse IgG (H+L), Alexa Fluor 594-conjugated (1:500, #8890, Cell Signaling Technology, Danvers, MA, USA), Cardiomyocyte proliferation was quantified by counting PH3/α-actinin + (mitotic) nuclei cells, while general proliferation was assessed via or EdU+ or PCNA+ cell density, and Fluorescence micrographs were acquired using a Zeiss LSM710 confocal microscope (Carl Zeiss Microscopy, Oberkochen, Germany).

2.5 Western Blot

Standard Western blot protocol was followed. Protein extracts were prepared with radioimmunoprecipitation as-

say (RIPA) buffer and then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The antibodies against the following proteins were used in our study: αT-catenin (1:1000, #13974-1-AP, Proteintech, Rosemont, IL, USA), β-catenin (1:2000, #51067-2-AP, Proteintech, Rosemont, IL, USA), β-actin (1:1000, #A1978, Sigma-Aldrich, St. Louis, MO, USA) and Yap (1:1000, #14074T, Cell Signaling, Danvers, MA, USA), Anti-rabbit IgG-HRP (1:3000, #7074, Cell Signaling, Danvers, MA, USA), Anti-mouse IgG-HRP (1:3000, #7076, Cell Signaling, Danvers, MA, USA). The protein bands were detected with an enhanced chemiluminescence (ECL) substrate (#32106, Thermo Scientific, Waltham, MA, USA) Western Blot Analysis System. The images were obtained by Tanon-5200 (Tanon Science, Shanghai, China), and the density of bands was determined with Image J 1.54d (NIH, Bethesda, MD, USA).

2.6 Statistical Analysis

Statistical analysis was performed using unpaired Student's *t*-tests in GraphPad Prism (v9.0, GraphPad Software, San Diego, CA, USA). Data are presented as mean $\pm$ SD. A p value < 0.05 was considered statistically significant.

3. Results

3.1 Loss of Ctnna3 Accelerates Heart Regeneration in Neonatal Mice After Heart Apex Resection

Although the mammalian adult heart is generally considered nonregenerative, neonatal mouse hearts have a genuine capacity to regenerate following apex resection [1,4]. To evaluate whether *Ctnna3* affects heart regeneration, we performed surgical apical resection of hearts (5%~10% of the ventricular myocardium) of WT and *Ctnna3*$^{-/-}$ neonatal mice at P1 and harvested hearts at 7 and 14 dpr (Fig. 1A) for histological analysis and immunofluorescence staining with antibody against PCNA, separately. To systematically evaluate regeneration dynamics, we analyzed proliferation markers at 7 dpr when cardiomyocyte proliferation peaks [22], and fibrosis at 14 dpr when scar resolution indicates complete regeneration [4] (Fig. 1B–F). This staggered analysis captures both the active proliferative phase (PCNA/PH3/EdU) and ultimate regenerative outcome (fibrosis reduction), providing complementary evidence of enhanced repair in *Ctnna3*$^{-/-}$ mice. The results revealed that, as reported by Porrello *et al*. [25], the resection plane was characterized by progressive regeneration of the apex with some restoration of the resected myocardium within 14 days (Fig. 1B). The MT staining showed that the accumulation of fibrotic tissue (blue staining in Fig. 1C) in hearts from *Ctnna3*$^{-/-}$ mice at 14 dpr dramatically decreased compared with WT mice (Fig. 1C,D). The number of PCNA-positive cells in the border zone of regenerated hearts of *Ctnna3*$^{-/-}$ neonatal mice at 7 dpr was significantly higher than the counterpart in hearts of WT neonatal mice (Fig. 1E,F). As heart regeneration is thought to occur primarily through cardiomyocyte proliferation [10,11], our results suggest that loss of *Ctnna3* may enhance heart regeneration by promoting cardiomyocyte proliferation in neonatal mice.

3.2 Ctnna3 Deficiency Promotes Cell Proliferation in Neonatal Mouse Hearts

Since cardiomyocytes in neonatal mouse heart retain active proliferation before P7 [3], we tested whether *Ctnna3* deficiency promoted cardiomyocyte proliferation in neonatal mice. The ventricles of WT and *Ctnna3*$^{-/-}$ mice at P1, P3, P7 were sectioned and stained separately with antibodies against PH3 (a marker of mitosis) [26], and Sarcomeric α-actinin (a specific marker for α-skeletal and α-cardiac muscle actinins) [27]. Quantification of PH3-positive cardiomyocytes revealed that cardiomyocyte proliferation was strikingly increased in the ventricles of *Ctnna3*$^{-/-}$ neonatal mice at P3 and P7 (Fig. 2A,B), but not at P14 compared to WT counterparts.

To further verify the enhanced cell proliferation in *Ctnna3*$^{-/-}$ neonatal mice, the proliferating cells in *Ctnna3*$^{-/-}$ and WT neonatal mice at P7 were *in vivo* EdU-pulse labeled and detected by EdU staining (see Materials and Methods). The results showed that the number of EdU-positive cells was significantly increased in ventricles and atriums of *Ctnna3*$^{-/-}$ neonatal mice compared to those in control littermates (Fig. 2C–F). These results demonstrate that *Ctnna3* deficiency promotes cell proliferation in neonatal mouse hearts.

3.3 Ctnna3 Deficiency Promotes Proliferation of Primary Cardiomyocytes

Since heart is mainly composed of cardiomyocytes, in addition to several other types of cells, such as cardiac fibroblasts, endothelial cells, and smooth muscle cells [28], we hypothesized that *Ctnna3* deficiency promoted the proliferation of cardiomyocytes. To test this hypothesis, primary cardiomyocytes were isolated from P5 WT and *Ctnna3*$^{-/-}$ mouse hearts and pulse-labelled with EdU *in vitro* followed by immunostaining with antibody against sarcomeric α-actinin and EdU stainng. Our study revealed that a significant increase of the proportion of the EdU-positive cardiomyocytes (proliferating cardiomyocytes) from *Ctnna3*$^{-/-}$ neonatal mice compared to that from WT mice at P5 (Fig. 3A,B). These results demonstrate that the loss of *Ctnna3* promotes proliferation of cardiomyocytes in neonatal mice.

3.4 Loss of Ctnna3 Enhances Yap Expression

α-catenins directly bind to both α-catenin and actin filaments, thereby coupling stable actin filaments to the cadherin adhesion molecules [12,13,17]. α-catenin is also a well-documented positive regulator for cell proliferation. To study the mechanism(s) by which αT-catenin deficiency promotes cell proliferation, the expression level of β-catenin in hearts from *Ctnna3*$^{-/-}$ and WT mice at P7

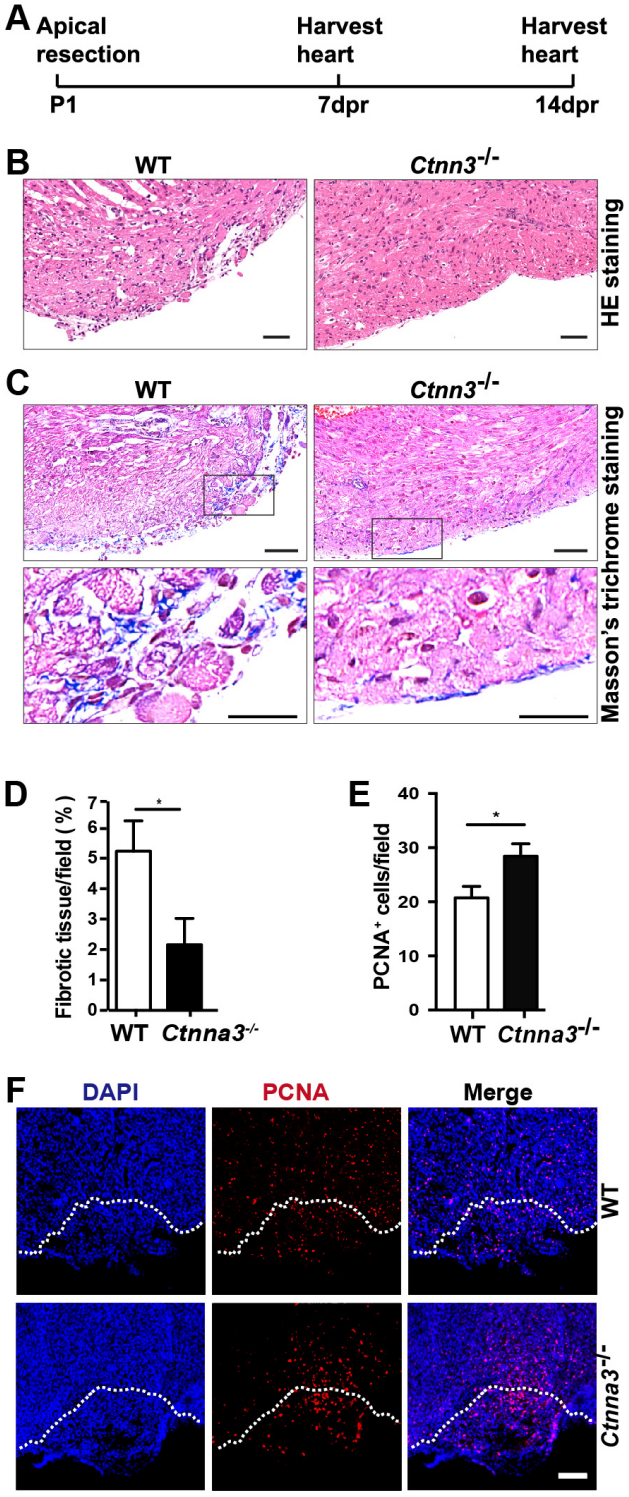

Fig. 1. *Ctnna3* deficiency promotes heart regeneration in neonatal mice after heart apical resection. (A) The scheme of the open thoracotomy experiment. (B,C) Representative images of paraffin heart sections from WT and *Ctnna3*$^{-/-}$ mice at 14 dpr stained with HE (B) or MT (C). Scale bar = 50 μm. Low panels in (C) are enlarged views of the cropped regions in up panels in (C). (D,E) Statistics analysis of the area of fibrotic tissue per field in C (WT, n = 3; *Ctnna3*$^{-/-}$, n = 3) and PCNA-positive cells per field at apex in (F) (WT, n = 5; *Ctnna3*$^{-/-}$, n = 5). (F) Representative fluorescent images of frozen sections of mouse hearts at 7 dpr stained with anti-PCNA. White dash lines mark the resection boundaries. Scale bar = 50 μm. Values in D and E represent the means ± SD. *, $p < 0.05$. *Ctnna3*, Catenin alpha 3; P1, postnatal day 1; dpr, day(s) post-resection; WT, wildtype; HE, hematoxylin and eosin; MT, Masson's trichrome; PCNA, proliferating cell nuclear antigen; DAPI, 4′,6-Diamidino-2-Phenylindole.

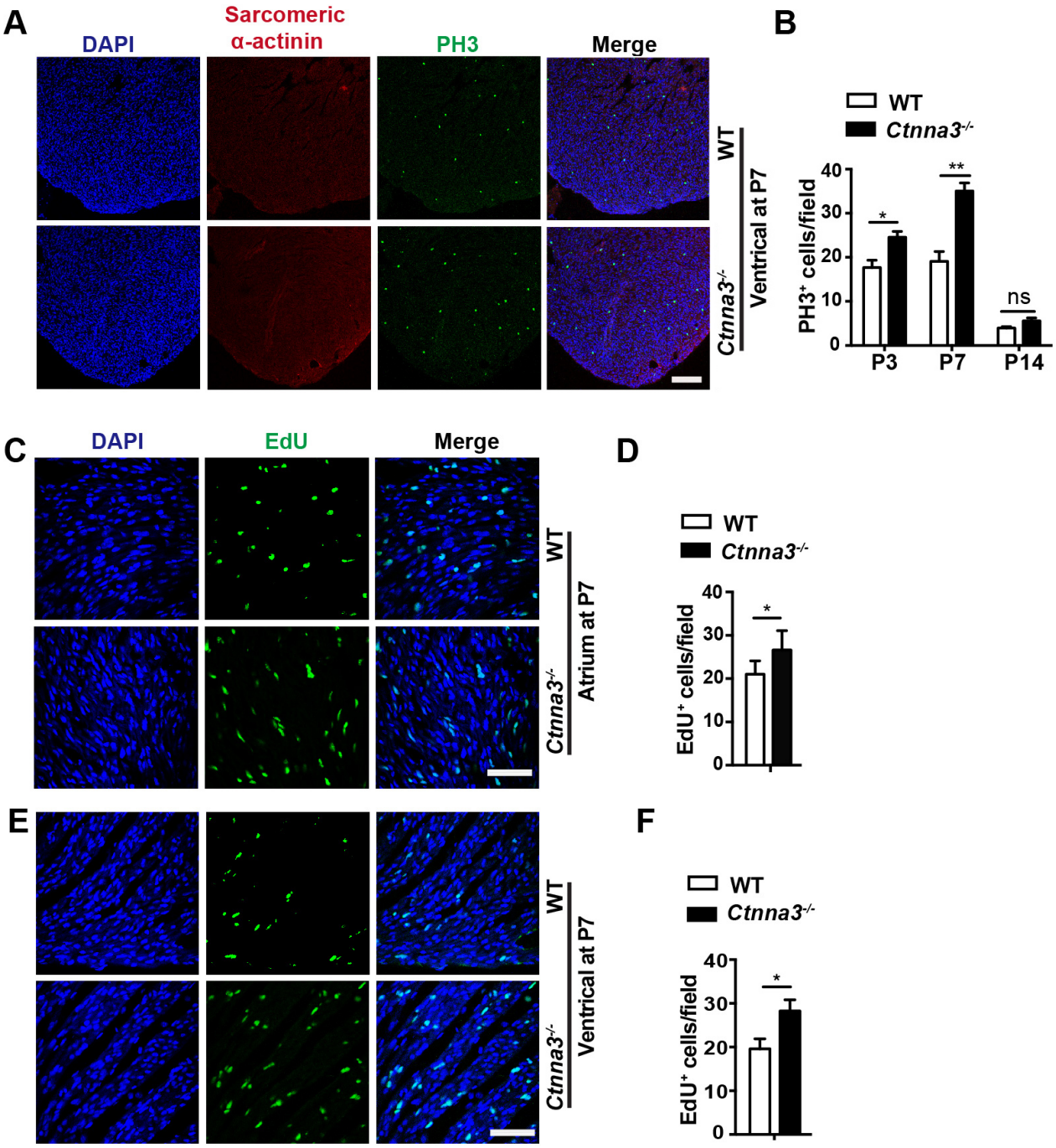

Fig. 2. Enhanced cell proliferation in hearts of *Ctnna3*$^{-/-}$ neonatal mice. (A) Representative florescent images of mouse heart sections from P7 WT and *Ctnna3*$^{-/-}$ mice co-stained with anti-PH3 (for prophase mitotic cells) and anti-Sacrometric-α-actinin (for Cardiomyocytes). Scale bar = 200 µm. (B) Statistics analysis of PH3-positive cells per field (WT, n = 5; *Ctnna3*$^{-/-}$, n = 5). (C,E) Representative fluorescent images of the sections of atrium (C) and ventricle (E) from P7 WT and *Ctnna3*$^{-/-}$ neonatal mice pulse-labelled with EdU. Scale bar = 50 µm. (D,F) Statistics analysis of EdU-positive cells per field (WT, n = 5; *Ctnna3*$^{-/-}$, n = 5) in (C,E). Values in (B,D,F) represent the means ± SD. **, $p < 0.01$; *, $p < 0.05$; ns, not significant. EdU, 5-ethynyl-2′-deoxyuridine.

was evaluated by Western blot. The result showed that the protein level of β-catenin was not significantly changed between WT and *Ctnna3*$^{-/-}$ hearts (Fig. 3C,D).

The Hippo pathway is a key regulatory signaling pathway for heart development and organ size [29]. Thus, we inspected whether loss of *Ctnna3* only had any effect on Yap expression in the neonatal heart. Our study revealed that the protein level of Yap significantly increased in hearts from P7 *Ctnna3*$^{-/-}$ mice compared to that in control mouse hearts (Fig. 3C,D). This result suggests that *Ctnna3* deficiency may promote neonatal cardiomyocyte proliferation by enhancing Yap expression.

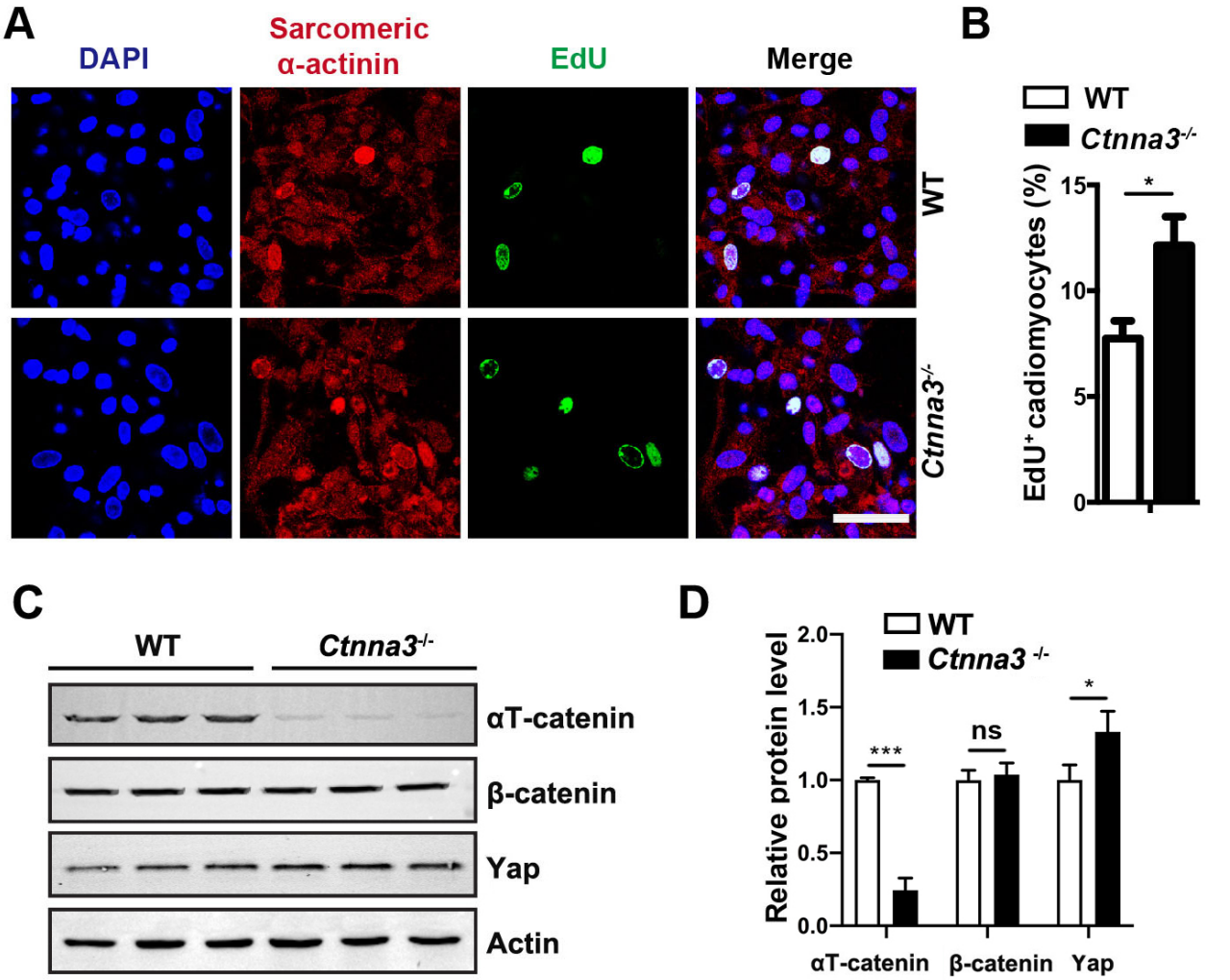

Fig. 3. *Ctnna3* deficiency promotes proliferation of primary cardiomyocytes and up regulates Yap expression. (A) Representative fluorescent images of cultured primary mouse cardiomyocytes from neonatal WT and *Ctnna3*$^{-/-}$ mice. The cultured cells were pulse-labeled with EdU for 24 hours followed by fluorescent staining. Scale bar = 100 µm. (B) Statistics analysis of EdU-positive cardiomyocytes per field in (A). (WT, n = 9; *Ctnna3*$^{-/-}$, n = 12). (C,D) Western blot analysis (C) and quantification (D) of β-catenin and Yap protein expression in hearts from WT and *Ctnna3* deficient mice. (n = 3 for WT and *Ctnna3*$^{-/-}$, respectively). Values in (B,D) represent the means ± SD. ***, $p < 0.001$; *, $p < 0.05$; ns, not significant. Yap, Yes-associated protein.

4. Discussion

In this study, we investigated the role of *Ctnna3* deficiency in enhancing cardiac regeneration and cardiomyocyte proliferation in neonatal mice. Our findings suggest that the absence of *Ctnna3* plays a significant role in promoting cardiac repair processes, potentially through the modulation of key signaling pathways.

4.1 Mechanisms of Ctnna3 Deficiency

Our results indicate a significant upregulation of Yap protein levels in *Ctnna3*-deficient hearts, implicating the Hippo pathway as a key regulatory mechanism [29]. The Hippo pathway is crucial for heart development and organ size control, and the observed increase in Yap suggests that *Ctnna3* may normally act to suppress Yap expression or activity [30–33]. This upregulation of Yap in the absence of *Ctnna3* may enhance cardiomyocyte proliferation by activating growth-promoting signals, which are critical during neonatal heart regeneration.

4.2 Limitations of Current Experiments

Several limitations should be considered. Firstly, this study utilized neonatal mouse models, which may not fully replicate the complexity of human cardiac biology and regeneration [34,35]. The inherently higher regenerative capacity of neonatal mice may amplify the effects observed in *Ctnna3*-deficient models and future work should include transcriptomic analyses but emphasize that our combinatorial approach (histology + biochemistry) is established in regeneration studies, long-term effects and external factors (genetics, environment) were not studied [35–37].

4.3 Prospects for Future Clinical Applications

Despite these limitations, our findings open promising avenues for future research and potential therapeutic applications. Future studies should aim to develop gene therapy or pharmacological approaches to modulate *Ctnna3* activity [38]. Personalized medicine approaches, tailoring interventions based on genetic backgrounds and specific cardiac conditions, could complement these efforts [39]. Integration with existing cardiac therapies, such as stem cell treatments or biomaterials, may provide synergistic benefits, enhancing overall cardiac repair efficacy [40,41].

5. Conclusion

In conclusion, we demonstrate that *Ctnna3* deficiency alone enhances neonatal heart regeneration through Yap-mediated cardiomyocyte proliferation. These findings identify αT-catenin as a potential therapeutic target for cardiac repair, though future studies must validate its role in adult models.

Availability of Data and Materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

SZ and WD performed experiments and wrote the manuscript; JL provided *Ctnna3*$^{-/-}$ mice and expert guidance in the experimental design of transgenic mouse studies; WT and HW designed the study and revised the manuscript; ZC analyzed data and edited the discussion. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the Institute of Developmental Biology and Molecular Medicine, Fudan University (Approval No. 201902065S), in accordance with NIH guidelines. We confirm the authenticity of this statement.

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Conflict of Interest

The authors declare no conflict of interest.

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