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SEMA6D Regulates Perinatal Cardiomyocyte Proliferation and Maturation in Mice

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Abstract

Cardiomyocytes undergo dramatic changes during the fetal to neonatal transition stage to adapt to the new environment. The molecular and genetic mechanisms regulating these changes remain elusive. In this study, we showed *Sema6D* as a novel signaling molecule regulating perinatal cardiomyocyte proliferation and maturation. SEMA6D is a member of the Semaphorin family of signaling molecules. To reveal its function during cardiogenesis, we specifically inactivated *Sema6D* in embryonic cardiomyocytes using a conditional gene deletion approach. All mutant animals showed hypoplastic myocardial walls in neonatal hearts due to reduced cell proliferation. We further revealed that expression of MYCN and its downstream cell cycle regulators is impaired in late fetal hearts in which *Sema6D* is deleted, suggesting that SEMA6D acts through MYCN to regulate cardiomyocyte proliferation. In early postnatal mutant hearts, expression of adult forms of sarcomeric proteins is increased, while expression of embryonic forms is decreased. These data collectively suggest that SEMA6D is required to maintain late fetal/early neonatal cardiomyocytes at a proliferative and less mature status. Deletion of *Sema6D* in cardiomyocytes led to reduced

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proliferation and accelerated maturation. We further examined the consequence of these defects through echocardiographic analysis. Embryonic heart deletion of *Sema6D* significantly impaired the cardiac contraction of male adult hearts, while having a minor effect on female mutant hearts, suggesting that the effect of *Sema6D*-deletion in adult hearts is sex dependent.

Keywords

Sema6D; cardiogenesis; Semaphorin signaling; conditional knockout; cardiomyocyte proliferation; cardiomyocyte maturation; heart development; *Sema6D*; perinatal cardiomyocyte proliferation; perinatal cardiomyocyte maturation

Introduction

Congenital heart diseases (CHDs) remain the leading cause of infant morbidity and mortality in developed countries, affecting ~1% of newborns (Benjamin et al., 2018; Clark et al., 2006; Hoffman, 1995; Hoffman and Kaplan, 2002; Onuzo, 2006). Moreover, CHDs are also risk factors for devastating diseases in the cardiovascular and other systems, such as heart failure, arrhythmia, sudden death, lung dysfunction and Alzheimer's disease (Bagge et al., 2018; Cohen et al., 2013; Gaeta et al., 2016; Lui et al., 2017; Verheugt et al., 2010; Zomer et al., 2011). An enhanced understanding of the molecular and genetic mechanisms controlling normal cardiogenesis is highly significant for both basic and clinical research because it will provide crucial clues for the development of novel clinical strategies to treat CHDs.

Neonatal cardiomyocytes undergo dramatic adaptation changes. Most neonatal mouse cardiomyocytes stop cell proliferation and withdraw from the cell cycle within 7–14 days after birth (Alkass et al., 2015; Cui et al., 2018; Pasumarthi and Field, 2002). At the same time, cardiomyocytes start to express adult forms of sarcomeric proteins and expression of embryonic forms is reduced (Bhavsar et al., 1991; Clement et al., 2007; Hunkeler et al., 1991; Morkin, 2000). Such sarcomeric isoform switching is essential for the normal activity of mature hearts (*ibid*). In addition, the primary source for ATP generation is switched from glycolysis in fetal hearts to fatty acid β-oxidation in postnatal hearts (Lopaschuk and Jaswal, 2010; Porter et al., 2011). How these dramatic changes are precisely controlled in fetal/neonatal hearts remains elusive. Such knowledge can be particularly important for cardiomyocyte regenerative medicine. A current major barrier in regenerating functional cardiomyocytes is that the cardiomyocytes induced from various types of stem cells remain largely immature and cannot undergo the normal molecular/ morphological switches from the embryonic form to the mature form (Cui et al., 2018; Eschenhagen et al., 2017; Machiraju and Greenway, 2019). Our current study reveals a new role for Semaphorin signaling as a regulator of perinatal cardiomyocyte proliferation and maturation.

SEMA6D is a member of the Semaphorin signaling family and acts both locally through cell-cell interactions and at a long distance through secretion of its ectodomain (Peng et al., 2016; Toyofuku et al., 2004a; Toyofuku et al., 2004b). We previously showed that endocardial expression of SEMA6D is essential for the initiation of mesenchyme formation in the atrioventricular canal region of mouse embryonic hearts (Peng et al., 2016; Sun et al.,

2018). In this study, we focused on its role in cardiomyocytes. A previous study in chicken suggested that SEMA6D regulates myocardial wall compaction (Toyofuku et al., 2004b); however the function of *Sema6D* in mammalian cardiomyocytes has not been reported in the literature. In our current research we applied a conditional gene inactivation approach to specifically delete *Sema6D* in developing cardiomyocytes. No major morphological defects were observed in the myocardial wall of mutant mice, suggesting differential roles of SEMA6D in avian and mammalian hearts. We show for the first time that SEMA6D is essential for normal cardiomyocyte proliferation and maturation in late fetal and early newborn hearts. Our results shed light on the complex regulatory mechanisms underlying perinatal cardiomyocyte development.

Materials and Methods

1. Mouse mating and tissue treatment

This study conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication no. 85–23, revised 2011). All protocols were approved by the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham. Euthanasia of mice was achieved through inhalation of CO_2 followed by cervical dislocation. Generation of *Sema6D*$^{loxp/loxp}$ mice and *cTnt-Cre* mice were described previously (Jiao et al., 2003; Peng et al., 2016). We crossed male *cTnt-Cre;Sema6D*$^{loxp/+}$ mice with female *Sema6D*$^{loxp/loxp}$ mice to obtain mutant (*cTnt-Cre;Sema6D*$^{loxp/loxp}$) and control (*Sema6D*$^{loxp/loxp}$ or *Sema6D*$^{loxp/+}$) animals at various stages. PCR Genotyping was performed as described previously (Jiao et al., 2003; Peng et al., 2016). To prepare paraffin blocks, whole embryos, embryonic hearts or postnatal hearts with desired genotypes were isolated at proper stages, fixed with 4% paraformaldehyde, dehydrated, and embedded in wax. When needed, these wax blocks were sectioned at 10um. Sections were stained with Hematoxylin and Eosin (HE) for morphological examination or were immunostained with proper antibodies. If necessary, the body weight, heart weight and tibial bone length were measured before tissue treatment.

2. Western and immunostaining analyses

Western and immunostaining analyses were performed as described previously (Liu et al., 2014; Peng et al., 2014; Peng et al., 2017). For Western analysis, protein samples were prepared in 1.5x Laemmli buffer and separated via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were then transferred to a PVDF membrane (Biorad), blocked with 5% BSA and incubated with a primary antibody at 4°C overnight. The next day, the membrane was incubated with a Horseradish Peroxidase conjugated secondary antibody and the signals were detected using the ECL kit (Amersham). The signal intensity was quantified using the Li-COR Odyssey system following the manufacturer's instructions. For immunofluorescence analysis, embryo sections were dewaxed, rehydrated and incubated with a primary antibody at 4°C overnight. The next day, slides were incubated with an Alexa 488 (green) or Alexa 594 (red) conjugated secondary antibody. Samples were then stained with DAPI to visualize the nuclei followed by a brief treatment with TrueBlack Lipofuscin Autofluorescence Quencher (Biotium). Samples were observed under a Zeiss fluorescent microscope. The CellC Cell Counting program (https://sites.google.com/site/

cellcsoftware/) was used to count cells after images were taken. The primary antibodies used in this study were purchased from Abcam (MYCN #ab16898, α-MHC #ab50967, β-MHC #ab11083, MLC1V #ab680), BD Biosciences (cyclin D1 #556470), Cell Signaling (cleaved Caspase3, #9661), Iowa Hybridoma Bank (cardiac MHC MF20, Tubulin E7), Millipore (phospho-Histone H3, #06–570), Novus Biologicals (ACTA1 #NBP1–35265, TNNI3 #NBP1–56641), Santa Cruz ((cyclin D2 #sc593, SEMA6D # sc-393258,TNNI1 #sc-393330,), and Sigma (ACTC1 #A9357, ACTA2 #A2547, ID2 #HPA027612). The MLC2A antibody was provided by Dr. S. Kubalak at the University of South Carolina.

3. Culture of cardiomyocytes and EDU-labeling

Preparation of the SEMA6D conditional medium was described previously (Peng et al., 2016). Briefly, the plasmid expressing the ectodomain of human SEMA6D was transfected into 293T cells using Lipofectamine2000 (Life Technologies). The next day, the cells were washed with PBS and incubated with DMEM containing 1% FBS for 24 hours. The medium was then collected as conditioned medium. The medium isolated from cells transfected with the empty vector was included as the negative control. Cardiomyocytes from wild type E16.5 embryos were isolated using the neonatal heart dissociation kit (Miltenyi Biotec 130–098-373) following the instructions provided by the kit. Isolated cardiomyocytes were grown in DMEM (with 10% FBS, 1x glutamine, and 1x Penicillin Streptomycin) for 48 hours at 37°C in a cell culture incubator (5% CO_2). The day before EDU labeling, cells were starved overnight (1% FBS). The next day they were treated with conditioned medium harboring the ectodomain of SEMA6D or control medium. EDU was added to culture medium at a final concentration of 10uM and incubated with cardiomyocytes for 1 hour. The EDU signaling was detected using the Click-iT EdU Imaging Kit (ThermoFisher, C10337) following the manufacturer's instructions. To distinguish cardiomyocytes from other cell types, samples were co-stained with cardiac MHC (MF20).

4. Echocardiographic analysis

Heart function and morphometry of control and mutant mice (12-week old) were assessed by echocardiography. Parasternal long and short axis views were used to collect M-mode and 2-dimensional images on the VisualSonics Vevo770 high resolution ultrasound system (Fujifilm VisualSonics) using the 30 MHz RMV707B scanhead in mice under isoflurane anesthesia (1–2% inhaled isoflurane in 100% O2) with body temperature maintained at 37°C and heart rate of 450 ± 50 bpm. Left ventricle (LV) end-diastolic and end-systolic chamber volumes (EDV and ESV, respectively) were determined by the area-length method from parasternal long axis images. LV function was measured as ejection fraction, calculated using [(EDV-ESV)/EDV]*100.

Results and discussion

1. Deletion of *Sema6D* in embryonic heart led to hypoplastic myocardial wall in fetal hearts

To reveal the potential function of *Sema6D* during mammalian cardiogenesis, we specifically deleted *Sema6D* from developing cardiomyocytes. We crossed *cTnt-Cre;Sema6D*$^{loxp/+}$ male mice with *Sema6D*$^{loxp/loxp}$ female mice to acquire mutant (*cTnt-*

Cre;Sema6D$^{loxp/loxp}$) and control (*Sema6D*$^{loxp/loxp}$ or *Sema6D*$^{loxp/+}$) animals. Our immunostaining analysis showed that expression of *Sema6D* was efficiently inactivated in the myocardium of mutant embryos at E10.5 (Fig. 1A). Expression of SEMA6D in the atrioventricular cushion region was not altered, supporting the cardiomyocyte-specificity of the *cTnt-Cre*. Our histologic examination did not reveal overt morphologic defects in mutant hearts at E18.5 (Fig. 1B). At P0, mutant hearts displayed the hypoplastic myocardial wall defect in both ventricles (Fig. 1C), suggesting the requirement of *Sema6D* for proper heart growth at the perinatal stage. The weight of mutant hearts was also significantly reduced, which correlates with results observed from HE staining of P0 heart sections. The mutant mice were born following the Mendelian ratio (41/157), suggesting that myocardial-deletion of *Sema6D* does not lead to embryonic lethality.

A published chicken study suggested that SEMA6D regulates myocardial wall compaction (Toyofuku et al., 2004b); however, we did not observe any compaction defect in mutant mice. This result suggests that SEMA6D may have differential roles in avian and mammalian hearts. Alternatively, other semaphorin signaling pathways may compensate for the loss of function of SEMA6D in hearts of mutant mice.

2. SEMA6D acts through MYCN to promote cardiomyocyte proliferation in embryonic hearts

To understand the cellular basis of the hypoplastic myocardial wall defect in mutants, we examined cell proliferation and apoptosis. No reduced cell proliferation defect was observed in mutant hearts at E16.5 (Fig. S1). At E17.5, we observed significantly reduced cell proliferation in the myocardial wall and ventricular septum of mutant hearts (Fig. 2A), suggesting that SEMA6D promotes cardiomyocyte proliferation at late fetal stages. No increase in cell death was observed in mutant hearts (Fig. S2). To further confirm the direct role of SEMA6D in cardiomyocytes, we cultured cardiomyocytes isolated from E16.5 hearts, treated them with conditioned medium containing the ectodomain of SEMA6D (Peng et al., 2016) and examined their cell proliferation through EDU labeling. As shown in Fig. 2B, exogenous SEMA6D significantly increased cell proliferation in cultured fetal cardiomyocytes.

We previously showed that *Mycn* encodes a critical transcription factor to promote embryonic cardiomyocyte proliferation (Harmelink et al., 2013), and this result was confirmed by a recent study (Munoz-Martin et al., 2019). Our result in Fig. 2C shows that expression of MYCN and its known downstream cell cycle regulatory proteins, including Cyclin D1, D2 and ID2 were all significantly decreased in mutant hearts at E17.5. Our results therefore suggest that SEMA6D acts through MYCN to promote fetal cardiomyocyte proliferation. This is the first study linking Semaphorin signaling to MYCN-mediated transcription.

3. Deletion of *Sema6D* led to neonatal cardiac defects

Our results in Fig. 3A show that cardiomyocyte proliferation was significantly reduced in mutant hearts at P1 after birth. We measured the thickness of the myocardial wall and found that the wall of the left ventricle (LV) in mutant hearts was significantly thinner than that of

the control one at P6 (Fig. 3B). The ratio of heart weight to whole body weight was also significantly reduced in mutants (Fig. 3B), confirming our histological examination. At the neonatal stage, the majority of cardiomyocytes start to withdraw from the cell cycle and this is accompanied by maturation (Alkass et al., 2015; Cui et al., 2018; Pasumarthi and Field, 2002). In Fig. 3C, we tested the impact of *Sema6D*-deletion on cardiomyocyte maturation at the neonatal stage by examining expression of multiple cardiac sarcomeric proteins that are known to undergo isoform switching from embryonic forms to adult forms during maturation. We found that in mutant hearts at P1, expression of mature sarcomeric isoforms, including ACTC1, TNNI3 and α-MHC (Bhavsar et al., 1991; Clement et al., 2007; Hunkeler et al., 1991; Morkin, 2000) was all increased, whereas expression of their corresponding embryonic isoforms, including ACTA1, ACTA2, TNNI1 and β-MHC was all decreased. Therefore, our data collectively suggest that SEMA6D expressed in cardiomyocytes is required for maintaining neonatal cardiomyocytes at the immature and proliferative state. *Sema6D*-deletion led to reduced cell proliferation and accelerated maturation in neonatal hearts.

4. Deletion of *Sema6D* in embryonic heart led to reduced heart function in adult mice in a sex-dependent manner

All mutant mice examined survived to >6-months old, allowing us to examine the effect of myocardial deletion of *Sema6D* on adult heart activity. In Fig. 4A, we examined the morphology of mutant and control hearts at 3-months old. Histological examination revealed the size of mutant adult hearts was reduced compared to littermate control mice. The heart weight before and after normalization against tibial bone length was significantly reduced in both male and female mutant mice (3-months old). In Fig. 4B and 4C, we further examined the potential changes in anatomical and functional cardiac function in these mice using echocardiography. Morphometric examination confirmed the reduced heart size in both male and female mutant mice. The ejection fraction and stroke volume were both significantly reduced in male mutant mice. To our surprise, despite a reduced heart size in female mutant mice, ejection fraction of female mutant hearts was not significantly altered compared to control female mice. Therefore, embryonic heart deletion of *Sema6D* selectively impairs the heart activities of male mutant mice. The reduced heart activity in adult mutant mice could be due to the combined defects observed in fetal and postnatal hearts.

In summary, our study reveals *Sema6D* as a novel signaling molecule regulating perinatal cardiomyocyte maturation and proliferation in mammals. It acts, at least partially, through upregulating expression of *Mycn* and its downstream cell cycle regulators during embryonic heart development. Further studies are warranted to address whether such a mechanism also functions in cardiomyocytes derived from embryonic stem cells or induced Pluripotent Stem cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- *Sema6D* is required for normal proliferation of fetal and neonatal cardiomyocytes.
- *Sema6D* regulates expression of *Mycn*.
- *Sema6D* is required for prevention of precocious maturation of neonatal cardiomyocytes.
- Embryonic heart deletion of *Sema6D* significantly impairs heart contraction of male adult mice but not female adult mice.

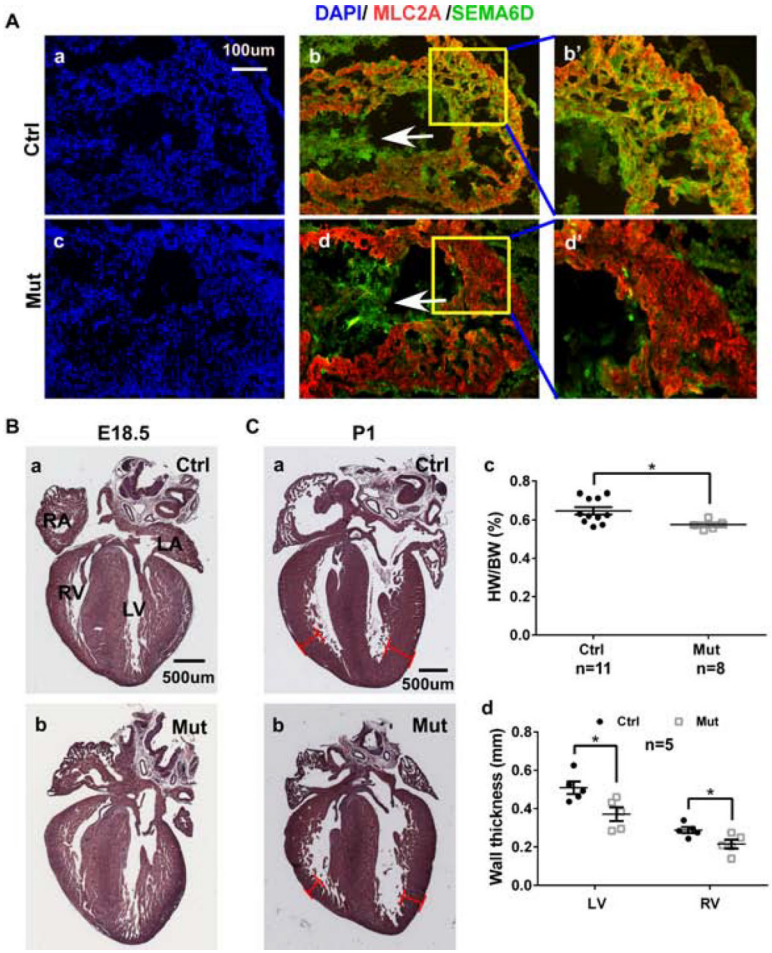

Fig. 1. Cardiomyocyte deletion of *Sema6D* in embryos led to a hypocellular myocardial wall defect.

(A) A mutant (*cTnt-cre;Sema6D*$^{loxp/loxp}$) embryo and its litter-mate control embryo (*Sema6D*$^{loxp/loxp}$) at E10.5 were sagittally sectioned and immunostained with an antibody against SEMA6D (green) and MLC2a (red, a myocardial marker). Total nuclei were visualized with DAPI staining (blue). The arrows indicate expression of SEMA6D at the atrioventricular (AV) cushions. The SEMA6D signal in mutant hearts was reduced in cardiomyocytes, while it remained unchanged in AV cushions. **(B)** Sections of E18.5 control and mutant embryos were HE stained. No overt morphological defects were observed in mutants. **(C)** At P1, the thickness of the myocardial wall of mutant hearts was significantly decreased compared to that of control hearts. The heart weight to body weight ratio was also significantly decreased. Error bars show the standard error of the mean (SEM). LV: left ventricle; RV: right ventricle. *: $p<0.05$, unpaired Student's t test.

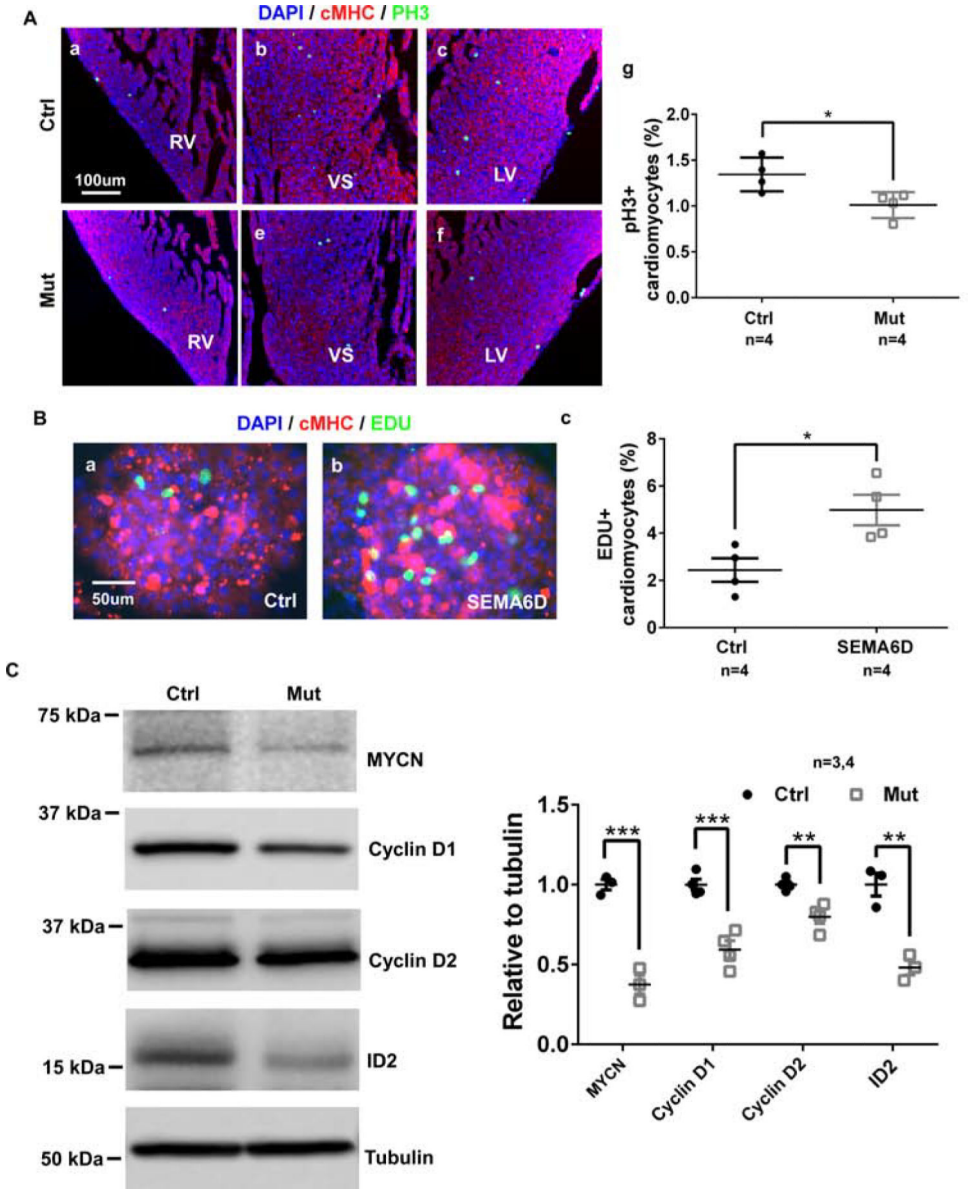

Fig. 2. Mutant hearts show decreased cell proliferation starting at E17.5.

(A) Sections of control and mutant hearts at E17.5 were immunostained with antibodies against phosphor-histone H3 (pH3, green) and cardiac myosin heavy chain (cMHC, red). **(B)** Cardiomyocytes were isolated from wild type hearts at E16.5 and cultured with conditioned medium containing the ectodomain of SEMA6D or control medium. EDU labeling (green) was performed to detect proliferating cells. Cultures were stained with an antibody against cMHC (red) and DAPI (blue). **(C)** Equal amounts of protein lysate from E17.5 control and mutant hearts were subjected to western analysis using the indicated antibodies. Tubulin was used as the loading control. Quantification of signal intensity was performed using the Li-COR Odyssey system. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$, unpaired Student's t test. Error bars represent SEM.

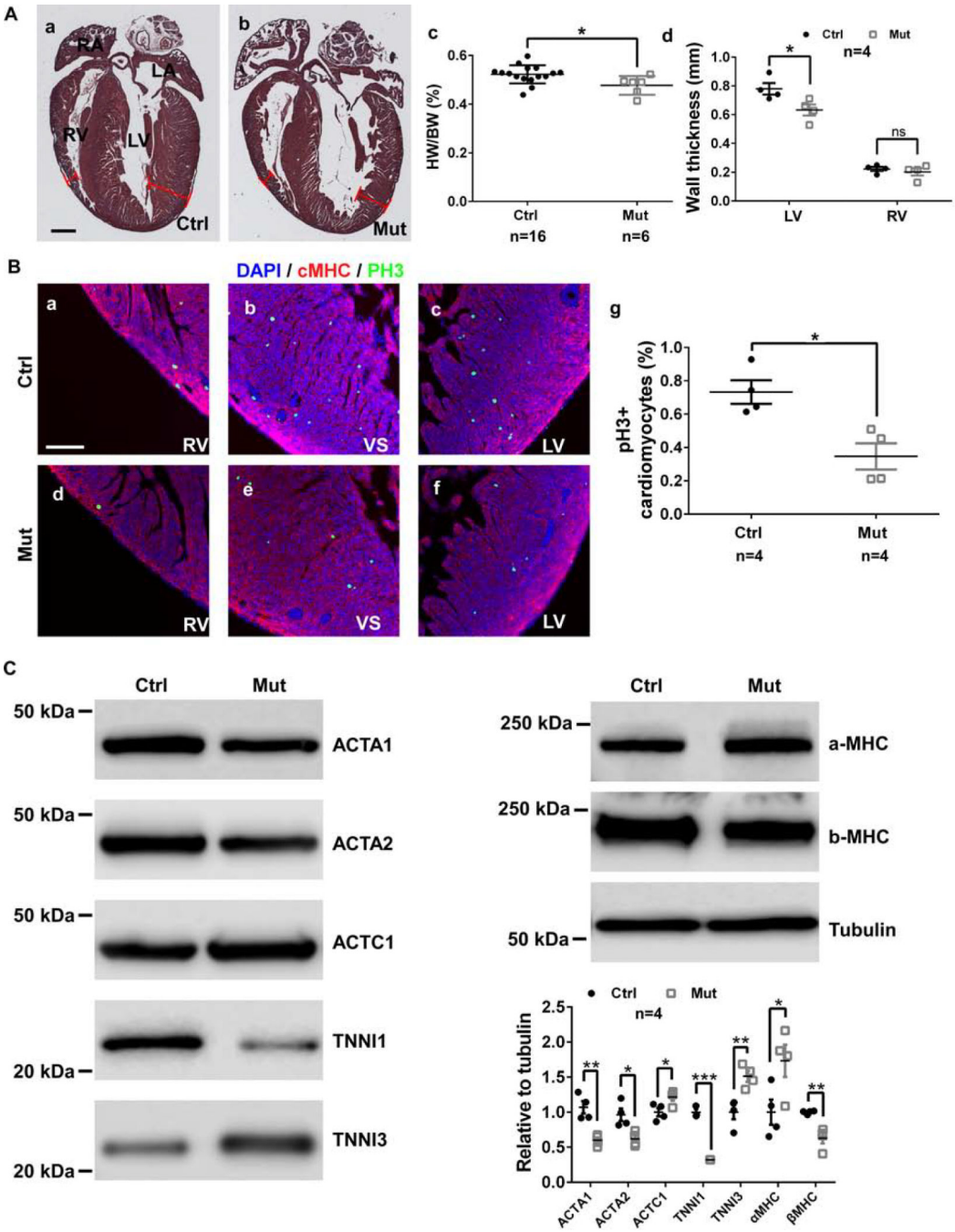

Fig. 3. Mutant neonatal hearts displayed reduced cell proliferation and accelerated maturation.

(A) Sections of control and mutant hearts at P1 were immunostained with antibodies against phospho-histone H3 (pH3, green) and cardiac MHC (cMHC, red). Total nuclei were visualized with DAPI staining (blue). **(B)** HE staining of sections of mutant and control hearts at P6. The thickness of the LV was significantly reduced compared to controls. The ratio of heart weight (HW) to body weight (BW) was also significantly decreased in mutant hearts. **(C)** To evaluate cardiomyocyte maturation, equal amounts of protein lysate from control and mutant hearts at P1 were subjected to western analysis using indicated

antibodies. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$, unpaired Student's t test. Error bars represent SEM.

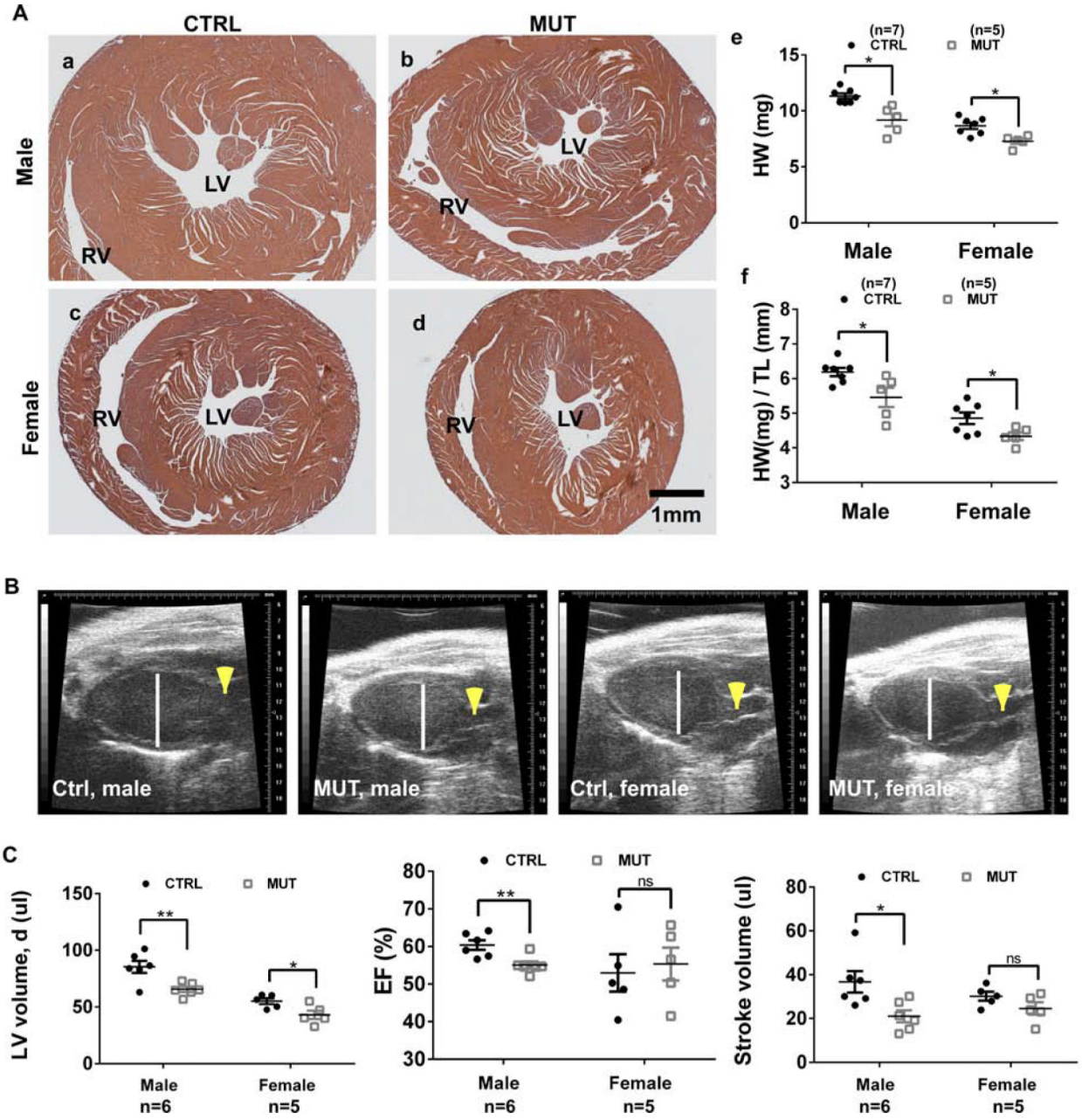

Fig. 4. Embryonic heart deletion of *Sema6D* impairs heart growth and function in adult male mice.

(A) HE staining of cross sections of 3-months old male and female mice. Gross heart weight and heart weight after normalization against tibial bone length were both significantly reduced in mutants. **(B)** Heart function and morphometry were measured by echocardiography in 3-month old male and female control (*Sema6D*$^{loxp/loxp}$) and mutant (*cTnt-Cre;Sema6D*$^{loxp/loxp}$) mice. Representative long axis LV views of male and female hearts at end diastole. Line denotes left ventricle (LV) end diastolic diameter and arrows denote aorta. **(C)** Summary echocardiographic data of LV volume (μl), ejection fraction (EF,

%) and stroke volume (μl). *: $p<0.05$; **: $p<0.01$; unpaired Student's t test. Error bars represent SEM.