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Myocardial *Mycn* is essential for mouse ventricular wall morphogenesis

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ABSTRACT

MYCN is a highly conserved transcription factor with multifaceted roles in development and disease. Mutations in *MYCN* are associated with Feingold syndrome, a developmental disorder characterized in part by congenital heart defects. Mouse models have helped elucidate MYCN functions; however its cardiac-specific roles during development remain unclear. We employed a *Cre*/*loxp* strategy to uncover the specific activities of MYCN in the developing mouse myocardium. Myocardial deletion of *Mycn* resulted in a thin-myocardial wall defect with dramatically reduced trabeculation. The mutant heart defects strongly resemble the phenotype caused by disruption of BMP10 and Neuregulin-1 (NRG1) signaling pathways, two central mediators of myocardial wall development. Our further examination showed that expression of MYCN is regulated by both BMP and NRG1 signaling. The thin-wall defect in mutant hearts is caused by a reduction in both cell proliferation and cell size. MYCN promotes cardiomyocyte proliferation through regulating expression of cell cycle regulators (including CCND1, CCND2, and ID2) and promotes cardiomyocyte growth through regulating expression of p70S6K. In addition, expression of multiple sarcomere proteins is altered in *Mycn* myocardial-inactivation embryos, indicating its essential role for proper cardiomyocyte differentiation. In summary, *Mycn* acts downstream of BMP and NRG1 cardiogenic signaling pathways to promote normal myocardial wall morphogenesis.

Introduction

The heart is the first organ to develop and function during embryogenesis. In order to sustain the growing embryo, heart muscle must rapidly expand through cardiomyocyte proliferation, growth, and differentiation (Christoffels et al., 2000; Sedmera et al., 2000). The relatively low-proliferative nonchamber myocardium along the inner curvature of the heart includes the atrioventricular canal (AVC), the outflow tract, and the conduction system (Christoffels et al., 2004; Evans et al., 2010). Atrial and ventricular chamber myocardium is characterized by highly proliferative, less differentiated cardiomyocytes (Christoffels et al., 2000, 2004; Evans et al., 2010). After rightward looping, the proliferating cardiomyocytes increase chamber mass and volume by contributing to the thickening myocardial wall and to the trabecular myocardium on the luminal side of the ventricles (Christoffels et al., 2000; Dunwoodie, 2007; Sedmera et al., 2000; Srivastava and Olson, 2000). The trabecular myocardium consists of organized layers of cardiomyocytes with muscular projections (Sedmera et al., 2000). It has multiple roles during early heart development including coordinating intraventricular conduction, enhancing contractile force to support continuing embryonic development, and facilitating nutrient/oxygen supplies to the myocardium (Dunwoodie, 2007; Sedmera et al., 2000). Later in development, the trabecular myocardium is incorporated into the compact myocardium, papillary muscles, and the interventricular septum (IVS) (Sedmera et al., 2000). Abnormalities in ventricular myocardial wall morphogenesis cause embryonic lethality in mice (Chen et al., 2004; Gassmann et al., 1995; Lai et al., 2010; Lee et al., 1995; Meyer and Birchmeier, 1995), and adult cardiomyopathies in humans (Klaassen et al., 2008; Pignatelli et al., 2003; Weiford et al., 2004; Xing et al., 2006).

MYCN is a member of the conserved MYC family of transcription factors involved in development and disease (Adhikary and Eilers, 2005; Brunner and Winter, 1991; Charron et al., 1992; Hurlin, 2005; Kenney et al., 2003, 2004; Knoepfler et al., 2002; Moens et al., 1992; Moens et al., 1993; Okubo et al., 2005; Ota et al., 2007; Sawai et al., 1993; Stanton et al., 1992; Strieder and Lutz, 2002; van Bokhoven et al., 2005). Multiple developmental signaling pathways converge on MYCN and, depending on the cellular context, MYCN can promote proliferation and growth, inhibit terminal differentiation, and regulate apoptosis (Adhikary and Eilers, 2005; Hurlin, 2005; Kenney et al., 2003, 2004; Knoepfler et al., 2002; Okubo et al., 2005; Ota et al., 2007; Strieder and Lutz, 2002). Haploinsufficiency for *MYCN* is associated with Feingold syndrome (FS, OMIM 164280),

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a developmental disorder characterized in part by congenital heart defects (CHDs) (Brunner and Winter, 1991; Büttiker et al., 2000; Celli et al., 2003; Geneviève et al., 2007; Piersall et al., 2000; van Bokhoven et al., 2005). In mice, global deletion or severe reduction of MYCN causes phenotypes that are similar to, but more severe than those associated with FS (Charron et al., 1992; Moens et al., 1992, 1993; Sawai et al., 1993; Stanton et al., 1992). Mice null for *Mycn* (Charron et al., 1992; Sawai et al., 1993; Stanton et al., 1992) or compound heterozygous for a null and a hypomorphic *Mycn* allele (Moens et al., 1993) (with MYCN protein reduced to 15% of normal) had heart defects such as delayed development with no septavalvulogenesis (Charron et al., 1992), lack of IVS formation (Sawai et al., 1993), and underdeveloped ventricular myocardial walls (Charron et al., 1992; Moens et al., 1993; Sawai et al., 1993; Stanton et al., 1992). These studies provided evidence that *Mycn* potentially has roles in several key cardiogenic processes, yet it remains unclear if the reported heart defects were caused by grossly abnormal embryo development. Moreover, global removal of *Mycn* from the developing embryo precludes investigation of its functions within specific cardiac tissues during cardiogenesis.

Our lab and others have identified *Mycn* as a transcriptional target of Bone Morphogenetic Protein (BMP) signaling in the myocardium during heart development (Cai et al., 2005; Song et al., 2007b). BMP cytokines are necessary for cardiomyocyte induction, proliferation, and survival during chamber morphogenesis (Azhar et al., 2003; Barnett and Desgrosellier, 2003; Chen et al., 2004; Délot, 2003; Délot et al., 2003; Gaussin et al., 2005, 2002; Jiao et al., 2003; Judge and Dietz, 2005; Loeys et al., 2006; McFadden and Olson, 2002; Song et al., 2007a; Srivastava, 2003; Waite and Eng, 2003; Zaffran and Frasch, 2002). BMP signaling is also important for AVC valvuloseptal development (Desgrosellier et al., 2005; Gaussin et al., 2005, 2002; Jiao et al., 2003; Ma et al., 2005; Moskowitz et al., 2011; Park et al., 2006; Rivera-Feliciano and Tabin, 2006; Song et al., 2007a, 2011; Sugi et al., 2004; Wang et al., 2005). In the current study, we tested the hypothesis that myocardial *Mycn* encodes an essential regulator of cardiomyocyte proliferation, size, survival, and differentiation, using a novel mouse model with *Mycn* specifically removed from the myocardium.

Materials and methods

Mice

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 1996). All protocols were approved by the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham. The *cTnt-Cre* (Jiao et al., 2003) and $Mycn^{loxp}$ (Knoepfler et al., 2002) transgenic mouse lines have been described previously. $Mycn^{loxp}$ mice were provided by R. Eisenman, Fred Hutchinson Cancer Research Center. Male *cTnt-Cre*;$Mycn^{loxp/+}$ mice were mated with $Mycn^{loxp/loxp}$ females to conditionally delete *Mycn* from the developing mouse myocardium. Upon *cTnt-Cre* mediated recombination, the entire coding region of *Mycn* is deleted within the myocardium between embryonic day E9.5 and E10.5. The day of the plug was considered E0.5. Embryos were dissected in PBS and processed for further experiments. Living embryos were defined by beating hearts. Controls in all experiments were *cTnt-Cre* negative littermates. Only living embryos were used for experiments.

DNA analyses and western blot

Genotyping was performed on tissue from the yolk sac or tail. Primers and PCR conditions for *cTnt-Cre*, *Mycn* and *Smad4* were described previously (Jiao et al., 2003; Knoepfler et al., 2002; Yang et al., 2002). The distribution of genotypes of living embryos was compared with the expected Mendelian ratio and a chi-square test was performed for statistical analysis, with $P < 0.05$ considered significant. Semiquantitative PCR analysis was performed on genomic DNA isolated from pooled embryonic heart ventricles and whole bodies from $Mycn^{loxp/loxp}$ and *cTnt-Cre*;$Mycn^{loxp/loxp}$ littermates. Western analysis was performed as described previously (Song et al., 2007a). Results were analyzed with ImageJ, public domain NIH Image program (developed at the U.S. National Institutes of Health and available at http://imagej.nih.gov/ij/).

Experiments of histology, immunohistochemistry, immunofluorescence, and in situ hybridization

Hematoxylin and Eosin (HE) staining, immunohistochemistry (IHC), and immunofluorescence (IF) experiments were performed as described previously (Song et al., 2007a). Slides were counterstained with Hematoxylin QS (Vector labs) and DAPI to visualize total nuclei for IHC and IF analysis, respectively. A light microscope (Zeiss AxioCam MRc) with a Zeiss Axio Imager A1 digital camera and AxioVision AC software were used for imaging. Images were processed with Adobe Photoshop. *In situ* hybridization using a *Bmp10* probe was performed as described in Chen et al. (2004).

Embryonic mouse heart cultures

E11.5 ICR hearts were isolated in PBS and placed in 1.5 mL DMEM (Invitrogen) supplemented with 1% Glutamine and 10% FBS in a 24-well plate. Embryonic hearts were treated with BMP10 (100 ng/ml, R&D), BMP inhibitor DMH1 (8 μM, Sigma), Neuregulin-1 (625 ng/ml, R&D), or ERBB2 inhibitor AG1478 (100 μM, Calbiochem) for 22 h before Western analysis.

Analysis of cardiomyocyte proliferation, apoptosis, and size

Cell proliferation experiments were performed by both phospho-Histone H3 (pH3) staining and BrdU labeling experiments. For pH3 staining, sagittal sections of paraffin-embedded embryos were stained with an antibody against pH3 (Upstate) to identify mitotic cells and with DAPI to identify nuclei (Song et al., 2007a, 2007b). Cells were counted within 4 regions of the heart: the atrial myocardium, atrioventricular canal myocardium, cushion mesenchyme, and ventricular myocardium. BrdU labeling experiments were performed as described in (Li and Rozen, 2006). Briefly, pregnant female mice (E10.5) were injected with BrdU (50 mg/kg, Invitrogen). 1.5 h afterwards, embryos were isolated and processed for wax embedding. Embryos were sagittally sectioned and stained with an antibody against BrdU (Iowa Hybridoma Bank). To visualize apoptotic cells, sagittal sections of paraffin-embedded embryos were subjected to terminal transferase-mediated dUTP-biotin nickend labeling (TUNEL) experiments with DeadEnd™ Fluorometric TUNEL System (Promega), per the manufacturer's protocol. Apoptosis was calculated as the number of positive cells divided by total cell number (Song et al., 2007a). Cells were counted within 4 regions of the heart: the atrial myocardium, atrioventricular canal myocardium, cushion mesenchymal cells, and ventricular myocardium. For both proliferation and apoptosis experiments, 3 embryos of each genotype were analyzed from three different litters, and at least three sections were analyzed for each embryo. A two-tailed, unpaired student's *t* test was used to calculate *P* values, with $P < 0.05$ considered significant.

Cardiomyocyte width was measured as previously described (Wang et al., 2010). Briefly, immunostaining experiments were

performed on cryosections of embryos (E10.5) using Wheat Germ Agglutinin Conjugate (conjugated with Oregon Green 488, Invitrogen) to outline cell membranes, an antibody against TNNT2 to distinguish cardiomyocytes, and DAPI mounting media (Vectashield) to identify nuclei. Samples were examined with a Leica SP1 confocal microscope. Images from comparable regions of the ventricle wall were analyzed for each embryo. To measure cardiomyocyte width, we identified cross sections of cardiomyocytes that cut through the nuclei and measured width as the shortest axis through the middle of the nucleus. Three embryos were analyzed for each genotype. A two-tailed, unpaired Student's *t* test was used to calculate *P* values, with $P<0.05$ considered significant. Results are expressed as mean $+/-$ standard error.

Antibodies and reagents

Antibodies for MYCN (ab16898, Western blotting (WB)), α-MHC (ab50967, WB 1:1000), β-MHC (ab11083, WB, 1:75,000), MLC1V (ab680, WB, 1:20,000), and TNNI3 (ab19615, WB, 1:2000) were from Abcam. The anti-phospho-Histone H3 antibody (06–570, IF, 1:300) was obtained from Upstate Cell Signaling Solutions. The anti-CCND1 antibody (556470, WB, 1:2500; IHC, 1:300) was purchased from BD Biosciences. Antibodies for CCND2 (sc-593, WB, 1:10,000; IHC, 1:20,000) and MLC2V (sc-34490, WB, 1:2500) were from Santa Cruz Biotechnology. Antibodies for ACTC1 (A9357, WB, 1:5000; IHC 1:3000), ACTA2 (A2547, WB, 1:10,000; IHC 1:3000), and ID2 (HPA027612, WB 1:500; IHC 1:8000) were from Sigma. Antibodies for BrdU (IF, 1:200), TNNT2 (WB, 1:2000; IF 1:200), and β-tubulin (WB, 1:50,000) were provided by the Developmental Studies Hybridoma Bank at the University of Iowa. Antibodies for p70S6K (9202, WB, 1:2000; IHC 1:200), ERRB2 (29D8, WB, 1:1000; IF, 1:200), ERRB4 (111B2, WB, 1:1000), mTOR (7C10, WB, 1:1000), and AKT (9272, WB, 1:2000) were supplied by Cell Signaling. Antibodies for ACTA1 (NBP1-35265, WB, 1:5000; IHC 1:20,000) and TNNI3 (NBP1-56641, WB, 1:10,000) were from Novus Biologicals. MLC2A antibody (WB, 1:15,000) was a gift from S. Kubalak at the University of South Carolina. Recombinant human BMP10 and NRG1-β1 were purchased from R&D Systems (377-HB). ERBB2 inhibitor, AG 1478, was from Calbiochem (658552). DMH1 was from Sigma. Fluorescent wheat germ agglutinin (WGA) conjugate Oregon Green 488 (IF, 10 μg/ml) was purchased from Invitrogen.

Results

Conditional deletion of Mycn in the developing mouse myocardium causes embryonic lethality

To investigate the specific role of myocardial *Mycn* during heart development, *Mycn* was deleted from the myocardium by crossing male *cTnt-Cre*;$Mycn^{loxp/wt}$ mice with $Mycn^{loxp/loxp}$ females (Knoepfler et al., 2002). *cTnt-Cre* efficiently deletes target genes within the cardiomyocyte lineage between embryonic day 9.5 (E9.5) and E10.5 (Chen et al., 2006; Ilagan et al., 2006; Jiao et al., 2003; Wang et al., 2001, 2000). Deletion of *Mycn* was confirmed with PCR analyses on genomic DNA from E10.5 embryos (Fig. 1A and B). The unrecombined $Mycn^{loxp}$ allele was reduced in *cTnt-Cre*;$Mycn^{loxp/loxp}$ mutant hearts to approximately 25% of the controls ($Mycn^{loxp/loxp}$). The recombined $Mycn^{loxp}$ allele was detected only in mutant hearts. MYCN reduction was confirmed at the protein level with Western blot experiments on proteins extracted from E10.5 to E11.5 ventricles (Fig. 1C).

Embryos heterozygous for myocardial *Mycn* (*cTnt-Cre*;$Mycn^{loxp/wt}$) developed normally and were viable in adulthood. *cTnt-Cre*;$Mycn^{loxp/loxp}$ mutants were recovered at the expected Mendelian frequency until E12.5, at which point they were no longer isolated alive (Fig. 1D, Supplementary Table 1). Living embryos were defined by beating hearts. At E12.5, mutants had delayed development and internal hemorrhaging (Fig. 1E). This result strongly suggests that deletion of *Mycn* from the myocardium disrupts mouse cardiogenesis, resulting in embryonic lethality.

Myocardial Mycn is necessary for ventricular wall morphogenesis

Only living embryos were used for the following experiments. To examine cardiac defects, we performed detailed histological examination of E9.5–E11.5 embryos. Mutants began to display a thin-walled ventricle phenotype at E9.5, which progressively became more pronounced until E11.5, the latest stage of survival, when mutant ventricles were extremely thin and almost completely devoid of trabeculae (Fig. 2A through F). Atrial myocardium was also noticeably thinner in mutant hearts at E10.5 and E11.5. Development of the cushions and outflow tract appeared to occur normally. Myocardial wall thickening and trabecular layer formation are necessary for proper ventricle contractility and embryo survival. Impaired myocardial wall morphogenesis in mutant hearts likely caused cardiovascular insufficiency, resulting in embryonic lethality.

Expression of MYCN is regulated by both EGF and BMP pathways

The thin myocardium in *cTnt-Cre*;$Mycn^{loxp/loxp}$ mutant ventricles resembles that of mouse models with disruption of *Bmp10* (Chen et al., 2004) and Neuregulin-1 (*Nrg-1*) (Gassmann et al., 1995; Lai et al., 2010; Lee et al., 1995; Meyer and Birchmeier, 1995) signaling pathways. Therefore, we directly tested whether expression of MYCN in embryonic hearts can be regulated by BMP and EGF pathways. Although BMP10 treatment did not increase MYCN expression, blocking BMP signaling using a BMP specific inhibitor, DMH1 (Hao et al., 2010; Wiley et al., 2011), reduced expression of MYCN in cultured embryonic hearts (Fig. 3A). This result suggests that endogenous BMP activity is required for the normal level of MYCN expression. Our data thus confirmed previous observations that myocardial *Mycn* is a transcriptional target of BMP signaling (Cai et al., 2005; Song et al., 2007b). To determine if MYCN expression can also be regulated by NRG1 signaling, we treated wild type E11.5 cultured hearts with NRG1-β1 (Carlsson et al., 2011; Clewes et al., 2011; Safa et al., 2011; Young et al., 2005) and found that MYCN protein levels were increased with NRG1-β1 stimulation (Fig. 3A). Furthermore, application of AG1478, which is a pharmacological inhibitor of the NRG1 receptor, ERRB2, (Fukazawa et al., 2003; Honjo et al., 2008; Kim et al., 2007; Rohrbach et al., 2005) to cultured E11.5 hearts reduced expression of MYCN (Fig. 3A). These results strongly suggest that *Mycn* expression is maintained in the myocardium by both BMP and NRG1 signaling pathways.

We further examined whether *Mycn* can regulate expression of BMP10 and ERBB2/4. Western analysis using proteins extracted from mutant and littermate control embryonic hearts (E11.5) showed that myocardial deletion of *Mycn* led to reduced expression of ERBB2 in embryonic hearts and has no effect on expression of BMP10 or ERBB4 (Fig. 3B). The result observed with *Bmp10* is consistent with the *in situ* hybridization experiment shown in *Supplementary* Fig. 1. Reduced expression of ERBB2 in mutant hearts was confirmed with an immunofluorescence study (Fig. 3C and D). Thus, our results suggest a reciprocal regulation between *Mycn* and *ErbB2* in embryonic hearts.

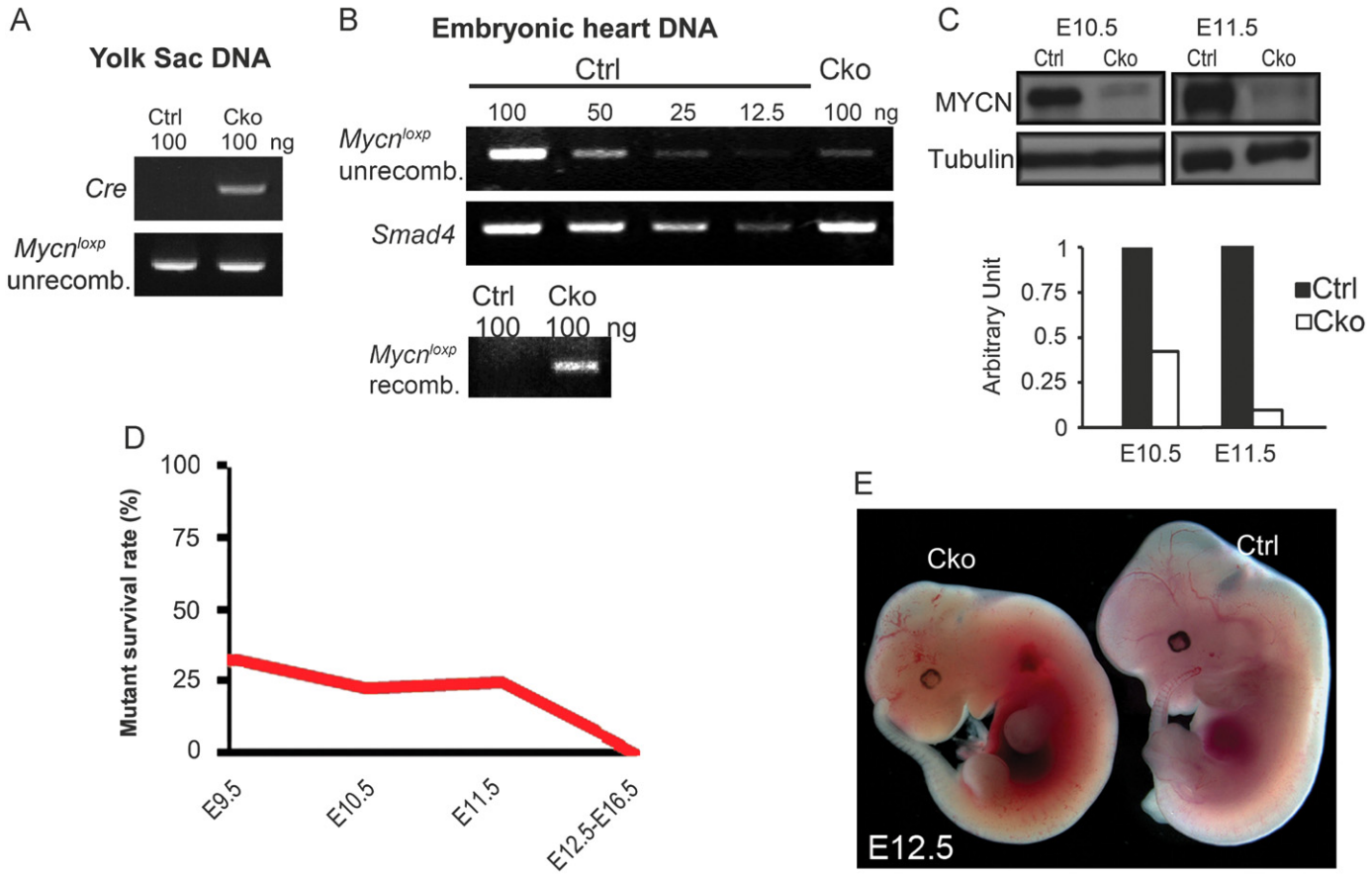

Fig. 1. Conditional deletion of *Mycn* from the myocardium causes embryonic lethality. (A) PCR analysis was performed on genomic DNA (100 ng) isolated from embryonic yolk sacs with genotyping primers for *cTnt-Cre* and the unrecombined $Mycn^{loxp}$ allele. (B) Genomic DNA from control ($Mycn^{loxp/loxp}$) and mutant (*cTnt-Cre*;$Mycn^{loxp/loxp}$) embryonic hearts was analyzed with semiquantitative PCR using primers for the unrecombined $Mycn^{loxp}$ allele (top). Lanes 1 to 4 are control samples with 100, 50, 25 and 12.5 ng of input DNA. Lane 5 is a mutant sample with 100 ng of input DNA. The unrecombined $Mycn^{loxp}$ allele was reduced in mutant hearts to approximately 25% of the control. *Smad4* primers were used as a loading control (middle). Bottom, heart DNA was analyzed with PCR using primers for the recombined $Mycn^{loxp}$ allele, which was only detected in mutant hearts. (C) Loss of MYCN in mutant ventricles was confirmed using Western blot analysis on pooled embryonic ventricle protein lysate from control and mutant embryos. Protein samples were pooled from at least 5 embryos for each experiment. β-tubulin served as the loading control. The band intensity was semi-quantified using ImageJ. (D) No living mutants were recovered after E11.5. At least 9 litters were collected at each stage. (E) At E12.5, a dead mutant embryo (left) was underdeveloped and exhibited hemorrhaging, compared to a littermate control (right). Ctrl, $Mycn^{loxp/loxp}$ controls; Cko, *cTnt-Cre*;$Mycn^{loxp/loxp}$ conditional knockout.

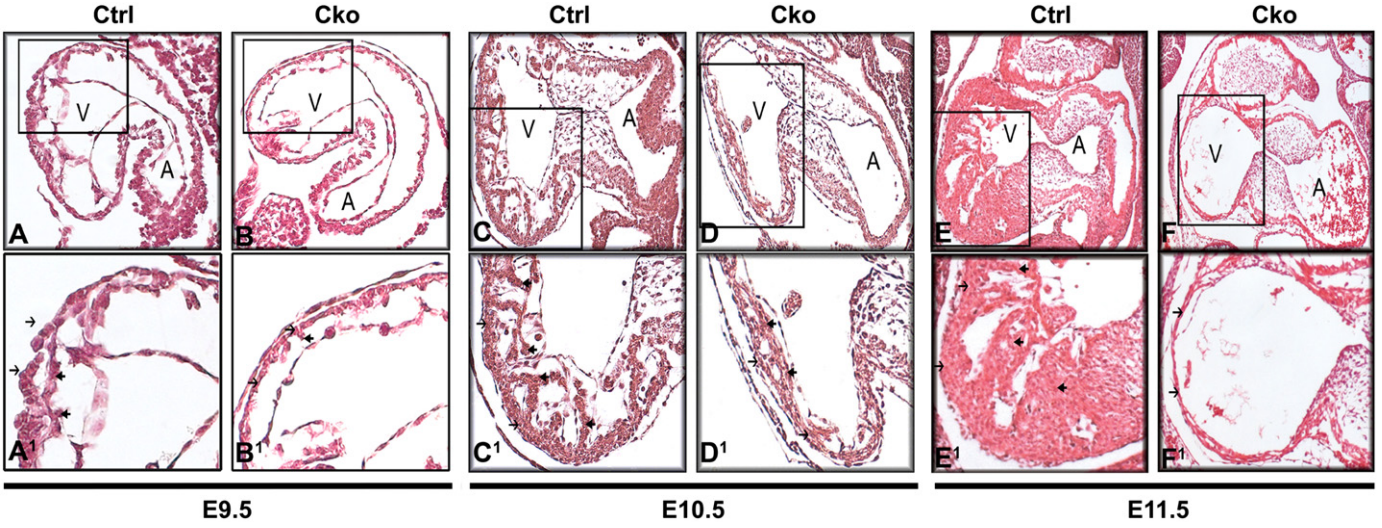

Fig. 2. Thin myocardial wall defect in *cTnt-Cre*;$Mycn^{loxp/loxp}$ embryonic hearts. (A)–(F^1), Hematoxylin/eosin-stained sagittal sections of embryos from E9.5 ((A)–(B^1)), E10.5 ((C)–(D^1)), and E11.5 ((E)–(F^1)). (A^1)–(F^1) correspond to the boxed regions of (A)–(F), respectively. Open arrows indicate ventricular myocardial wall. Closed arrows point to trabeculae. Ctrl, $Mycn^{loxp/loxp}$ controls; Cko, *cTnt-Cre*;$Mycn^{loxp/loxp}$ conditional knockout.

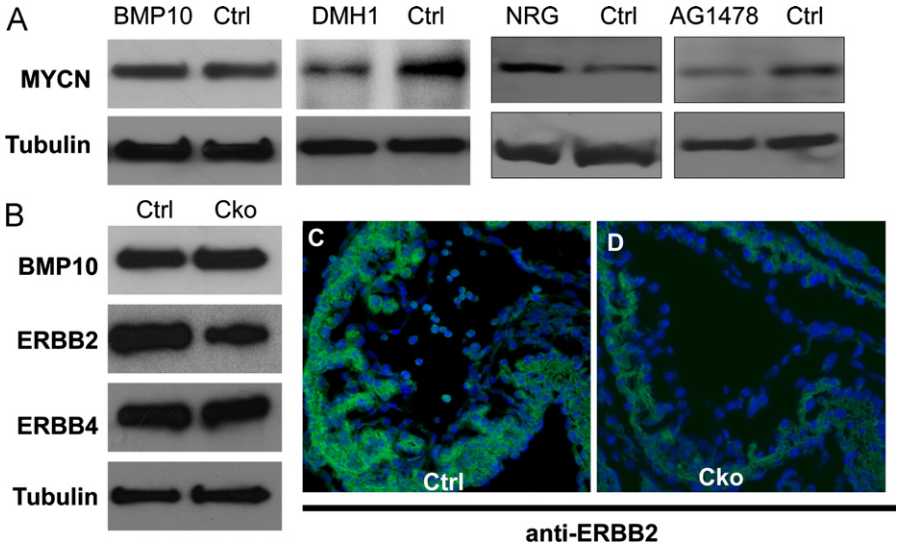

Fig. 3. Regulation of MYCN expression by BMP and EGF signaling pathways. (A) Wild type E11.5 embryonic hearts were cultured 2 h with BMP10 (100 ng/ml), BMP inhibitor DMH1 (8 uM), Neuregulin-1 (625 ng/ml), or ERBB2 inhibitor AG1478 (100 uM). Western analysis was performed using an anti-MYCN antibody. Similar results were obtained with BMP2- and BMP4-treatment (data not shown). β-tubulin served as the loading control. (B) Total proteins were isolated from E11.5 embryonic hearts followed by Western analysis using antibodies against BMP10, ERBB2 and ERBB4. Tubulin was used as the loading control. (C)and (D), Immunofluorescence staining was performed on sagittal sections of E10.5 control and mutant embryos using an anti-ERBB2 antibody (green). Total nuclei were visualized by DAPI staining. Ctrl, $Mycn^{loxp/loxp}$ controls; Cko, *cTnt-Cre*;$Mycn^{loxp/loxp}$ conditional knockout.

Reduced cardiomyocyte proliferation, but not increased cell death, contributes to hypocellular myocardial wall in mutant ventricles

MYCN regulates gene expression programs in a tissue-specific manner to control cellular processes such as proliferation, survival, growth, and differentiation (Adhikary and Eilers, 2005; Hurlin, 2005). To determine if loss of myocardial *Mycn* altered proliferation, cardiomyocyte number and proliferation rate were measured. A significant reduction in cardiomyocyte number was found in mutant ventricles at E9.5 ($P=0.001$, Fig. 4A). Cardiomyocyte proliferation was measured using immunofluorescence assays with an antibody for pH3, a marker for mitotic cells. Mutants had significantly reduced cardiomyocyte proliferation in the ventricles ($P=0.02$, Fig. 4B through D). Reduced cell proliferation in mutant ventricles was further confirmed with a BrdU labeling experiment (Supplementary Fig. 2).

To test if *Mycn* was necessary for cardiomyocyte survival during cardiogenesis, apoptosis was measured using TUNEL assays. No changes in apoptosis were detected in the mutant hearts from E9.5 to E11.5 (Supplemental Fig. 3). Therefore, decreased cardiomyocyte proliferation, but not increased apoptosis, contributed to the hypocellular myocardial wall phenotype in mutant ventricles.

Mycn is required for expression of cell cycle regulators CCND1, CCND2, and ID2 in the ventricular myocardium

MYC proteins are important activators of proliferation through their ability to upregulate cell cycle regulatory genes (Adhikary and Eilers, 2005; Hurlin, 2005). To better understand the mechanism whereby MYCN regulates cardiomyocyte proliferation during heart development, we measured the levels of MYCN targets cyclin D1 (CCND1) (Kenney et al., 2003), cyclin D2 (CCND2) (Bouchard et al., 2001, 1999), and inhibitor of DNA binding 2 (ID2) (Lasorella et al., 2002, 2000). Proteins were extracted from embryonic ventricles and analyzed with Western blot experiments. All three proteins were significantly decreased in mutant ventricles from E10.5 to E11.5 ($P<0.001$, Fig. 5A). These results were confirmed with immunohistochemistry experiments (Fig. 5B through G). These data indicate that MYCN promotes cardiomyocyte proliferation at least in part through upregulation of CCND1, CCND2, and ID2.

Mutant hearts have smaller cardiomyocytes and decreased levels of p70S6K, a regulator of cell growth

Through its regulation of the cell cycle, ribosome synthesis, and protein translation, MYCN ultimately enhances cell size (Adhikary and Eilers, 2005; Hurlin, 2005). To investigate if *Mycn* was required for cardiomyocyte growth, we measured cardiomyocyte width in cryosections of control and mutant ventricle walls at E10.5. Membranes of cardiomyocytes were labeled with anti-WGA (Condorelli et al., 2002) (green, Fig. 6A–B[1]) and cell width was measured as described in (Wang et al., 2010). The size of cardiomyocytes was significantly reduced in mutant hearts ($P<0.001$, Student's t test) as shown in Fig. 6C. Similar results were observed on paraffin sections (data not shown).

To further elucidate how MYCN promotes cardiomyocyte growth, we examined expression of ribosomal protein S6 kinase I (p70S6K), a well-known regulator of ribosome biogenesis and cell growth (Ahuja et al., 2007; Crackower et al., 2002; Fingar et al., 2002; Shima et al., 1998; Shioi et al., 2000). Western blot experiments on E10.5–E11.5 ventricle proteins revealed that mutants had a noticeable reduction of p70S6K (Fig. 7A). Expression of p70S6K upstream genes, including *Akt* and *mTOR*, was not altered by deletion of *Mycn* (Fig. 7A). Decreased expression of p70S6K was confirmed with immunohistochemistry assays in E10.5 ventricular myocardium (Fig. 7B–C[1]). These data suggest that p70S6K is a regulatory target of MYCN in controlling cardiomyocyte size.

Aberrant cardiac myofilament gene expression in Mycn-depleted ventricles

During development, MYCN is necessary for maintaining certain cell types in a proliferative, undifferentiated state (Knoepfler et al., 2002; Okubo et al., 2005). To determine if *Mycn*-depletion causes premature cardiomyocyte differentiation, we examined myofilament proteins including myosin heavy chain

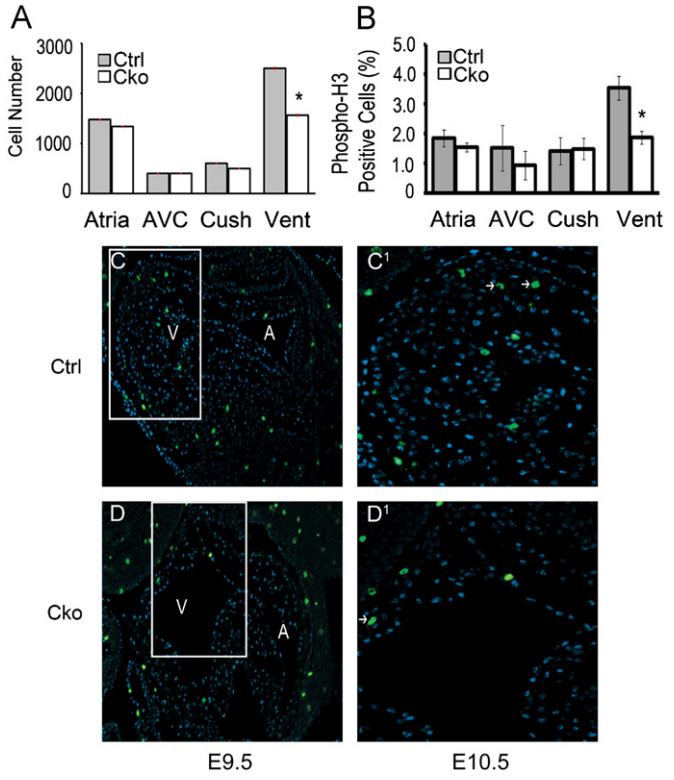

Fig. 4. Cell number and proliferation analysis of E9.5 embryonic hearts. (A), Sagittal sections of E9.5 embryos were analyzed with DAPI staining to identify nuclei. Total nuclei were counted within 4 regions of the heart: the atrial myocardium, AVC myocardium, cushion mesenchyme, and ventricular myocardium. Three embryos were analyzed from three different litters, and at least three sections were analyzed for each embryo. Mutant ventricles displayed a significant decrease in cardiomyocyte number (Student's t test, $p=0.001$). SEMs for control measurements were: Atria $+/-9.1$, AVC $+/-3$, Cushion $+/-3.4$, and Ventricle $+/-18.9$. Mutant SEMs were: Atria $+/-8.2$, AVC $+/-4.4$, Cushion $+/-5.8$, and Ventricle $+/-19.2$. (B) Cell proliferation experiments were performed on sagittal sections of E9.5 embryos using antibodies against phospho-Histone H3 (pH3) to mark mitotic cells and DAPI to identify nuclei. Cells were counted within 4 regions of the heart: the atrial myocardium, AVC myocardium, cushion mesenchyme, and ventricular myocardium. Proliferation was calculated as total pH3-positive nuclei divided by total nuclei; the result was expressed as the mean percentage of pH3-positive cells/total number of nuclei. Three embryos were analyzed from three different litters, and at least three sections were analyzed for each embryo. A significant decrease in cardiomyocyte proliferation was evident in mutant ventricles (Student's t test, $P<0.02$). Error bars represent standard deviation. (C)–(D) Representative images from pH3 immunostaining experiments on sagittal sections of E9.5 control ((C)–(C^1)) and mutant (D)–(D^1)) embryos. (C^1) and (D^1) correspond to boxed regions of (C) and (D), respectively. Arrows point to examples of cardiomyocytes positive for pH3 (green). Total nuclei were stained with DAPI (blue). Ctrl, $Mycn^{loxp/loxp}$ controls; Cko, *cTnt-Cre*;$Mycn^{loxp/loxp}$ conditional knockout; A, atria; AVC, atrioventricular canal; Cush, endocardial cushion; V or Vent, ventricle.

(MHC), actin, and troponin I (TNNI) that have unique expression patterns in embryonic, less differentiated cardiomyocytes versus adult, differentiated cardiomyocytes (Fig. 8A). For myosin heavy chain proteins, expression of the embryonic form, β-MHC (Morkin, 2000), was not changed, while expression of the adult form, α-MHC (Morkin, 2000), was reduced. For actins, no difference was observed in either embryonic forms [α-skeletal actin (α-ACTA1) (Clément et al., 2007) and α-smooth muscle actin (α-ACTA2) (Clément et al., 2007)] or the adult form [α-cardiac actin (α-ACTC1) (Clément et al., 2007)]. For troponin I, expression of the adult form [TNNI3 (Bhavsar et al., 1991; Hunkeler et al., 1991)] was increased while that of the embryonic form (TNNI2) was decreased in mutant hearts. We next analyzed the expression of a group of myofilament proteins necessary for proper cardiomyocyte structure and function. Western blot experiments showed that ~E11.0 mutant ventricles had reduced expression of myosin light chain 1V (MLC1V), MLC2V, and MLC2A. Expression of troponin T2 was not altered in mutant hearts. Our data suggest that *Mycn* is required for normal expression of multiple myofilament proteins. Abnormal expression of cardiac structural proteins may contribute to the myocardial wall defects in mutant hearts.

Discussion

The major goal of this study was to reveal the precise cardiogenic functions of *Mycn*. Haploinsufficiency for *MYCN* causes Feingold syndrome, which is characterized in part by CHDs (Brunner and Winter, 1991; Büttiker et al., 2000; Celli et al., 2003; Geneviève et al., 2007; Piersall et al., 2000; van Bokhoven et al., 2005). Previous reports have shown that global loss of MYCN (Charron et al., 1992; Sawai et al., 1993; Stanton et al., 1992) or severe reduction in MYCN (Moens et al., 1993) during mouse embryogenesis causes varying and complex CHDs. We show for the first time that depletion of *Mycn* solely in the developing myocardium leads to abnormal myocardial wall morphogenesis and embryonic lethality at midgestation. Our study thus definitively establishes that the developing myocardium requires MYCN and supports the notion that impaired activity of *Mycn* in the myocardium may contribute directly to the heart defects observed in Feingold syndrome patients.

Myocardial inactivation of *Mycn* led to embryonic lethality at E12.5, roughly the same time as global knockout mouse models (Charron et al., 1992; Sawai et al., 1993; Stanton et al., 1992) and earlier than in the hypomorphic mice (Moens et al., 1993). This result strongly suggests that defective cardiogenesis due to loss of *Mycn* within the myocardium was the primary cause of lethality in earlier models. In the present study, the mutant phenotype was restricted to the myocardial wall. Other aspects of cardiac morphogenesis such as AVC cushion development and IVS initiation occurred normally, indicating that myocardial *Mycn* is not necessary for those aspects of cardiogenesis. Interestingly, *Mycn*-null mice displayed abnormal valvuloseptal development (Charron et al., 1992). We speculate that endocardial, but not myocardial, *Mycn* is required for proper cushion morphogenesis. This idea is substantiated by a recent report showing that *Mycn* is a downstream target of BMP2-induced TBX20 regulation in chicken endocardial cushion culture systems (Shelton and Yutzey, 2007).

BMP10 (Chen et al., 2004; Grego-Bessa et al., 2007; Zhang et al., 2011) and NRG1 (Gassmann et al., 1995; Lai et al., 2010; Lee et al., 1995; Meyer and Birchmeier, 1995) are central regulators for myocardial wall development. Myocardial inactivation of *Mycn* resulted in the thin-walled defect similar to that observed in embryos with deletion of *Bmp10* (Chen et al., 2004), *Nrg1* (Lai et al., 2010), and *ErbB2/4* (Gassmann et al., 1995; Lee et al., 1995; Meyer and Birchmeier, 1995). Previous studies suggested that *Mycn* is a transcriptional target of BMP signaling during heart development (Cai et al., 2005; Song et al., 2007b). We confirmed this by showing that blocking endogenous BMP signaling using a BMP specific inhibitor reduced expression of MYCN in cultured embryonic hearts (Fig. 3). Expression of cell cycle regulators including CCND1, 2 and ID2 (Fig. 5) was reduced in *Mycn* knockout embryonic hearts, a deficiency which was also observed in *Smad4* myocardial inactivation mice (Song et al., 2007b). Our study thus provides further support for the hypothesis that MYCN acts as a critical mediator of BMP signaling during cardiogenesis.

We show for the first time that exogenous NRG1 can sufficiently upregulate MYCN expression and that blocking its receptor (ERRB2) can downregulate MYCN expression in cultured embryonic hearts (Fig. 3). The potential role of EGF signaling in regulating *Mycn*

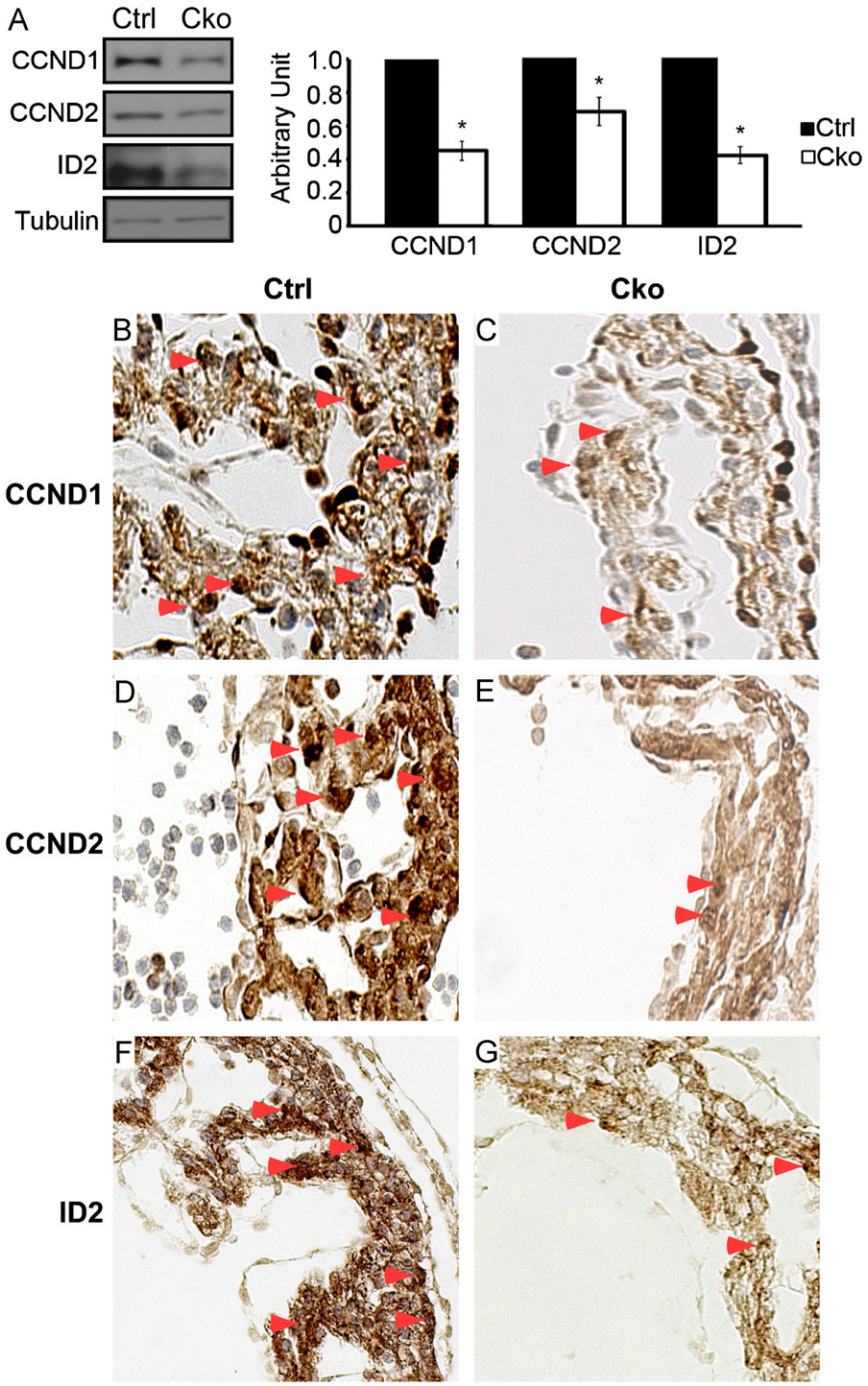

Fig. 5. Examination of MYCN targets CCND1, CCND2, and ID2. (A) Western blot experiments with proteins extracted from E10.5 to E11.5 control and mutant ventricles revealed decreased expression of cell cycle regulators. β-tubulin served as the loading control. Ventricle protein samples were pooled from at least 5 embryos for each experiment and each experiment was repeated at least 3 times. Quantification of Western blot results using ImageJ showed a significant decrease in all three proteins (Student's *t* test, $P < 0.001$). (B)–(G), Immunohistochemistry for CCND1, CCND2 and ID2 on E10.5 control ((B), (D), (F)) and mutant ((C), (E), (G)) ventricles. The overall staining intensity was reduced in mutant ventricle myocardium. In addition, the number of strongly stained cells was also reduced in mutants. Red arrowheads point to examples of strongly stained cardiomyocytes. Ctrl, $Mycn^{loxp/loxp}$ controls; Cko, *cTnt-Cre*;$Mycn^{loxp/loxp}$ conditional knockout.

expression has not been well documented in the literature. It was recently reported that EGF stimulation can recruit Sp1 to the *MYCN* promoter to activate its expression in neuroblastoma-derived cell lines (Hossain et al., 2012). We are currently investigating whether NRG1 can regulate *Mycn* transcription through a similar mechanism in developing hearts. Our study revealed further that *ErbB2* is a novel target (direct or indirect) of MYCN in embryonic hearts, suggesting a positive feedback loop between NRG1 signaling and MYCN in developing ventricles. The mutually positive regulation between NRG1 signaling and MYCN can reinforce both their activities to ensure proper proliferation/differentiation of cardiomyocytes. Collectively, our data suggest that MYCN acts as a hub for

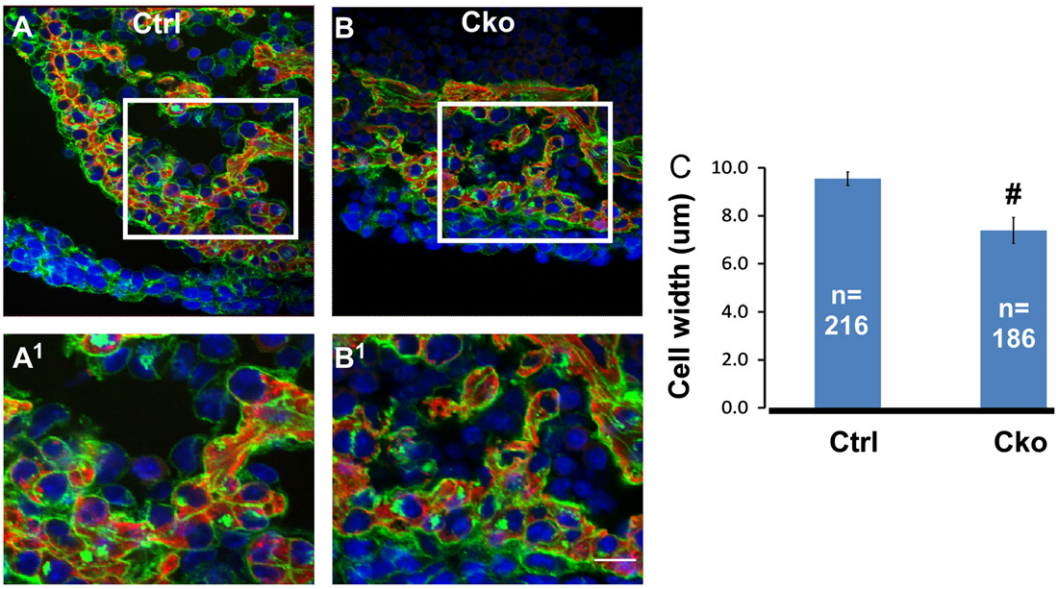

Fig. 6. Measurement of cardiomyocyte size. (A)–(B), Representative cryosections of embryonic ventricle myocardium from control ((A)–(A^1)) and mutant ((B)–(B^1)) hearts at E10.5. Cell membranes were labeled by WGA (green), cardiomyocytes were labeled with anti-TNNT2 (red), and nuclei were labeled with DAPI (blue). (C) Measurements of cardiomyocyte width were made from comparable regions of mutant and control ventricles at E10.5. Mutant cardiomyocytes were significantly smaller than controls. Cardiomyocyte width was measured as the shortest axis through the middle of the nucleus, as described in Wang et al. (2010). Three embryos were analyzed from 2 different litters, and at least 3 sections were analyzed for each embryo. Error bars represent standard error. #: Student's *t* test, $P < 0.001$. Scale bar: 20 um.

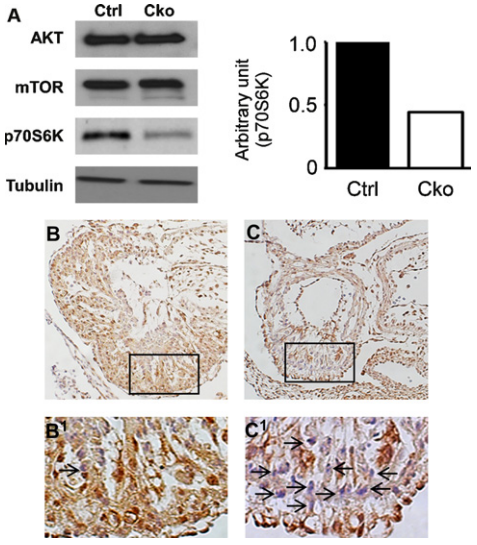

Fig. 7. Reduced expression of p70S6K in mutant myocardium. (A) Western blot assays were performed on proteins extracted from E10.5 to E11.5 embryonic heart ventricles using antibodies as indicated. Protein samples were pooled from at least 5 embryonic ventricles. β-tubulin served as the loading control. (B)–(C), Loss of p70S6K in mutant ventricles was confirmed with immunohistochemistry on E10.5 control ((B)–(B^1)) and mutant ((C)–(C^1)) ventricles. (B^1) and (C^1) correspond to the boxed regions of (B) and (C), respectively. Arrows point to examples of cardiomyocytes without p70S6K. Ctrl, $Mycn^{loxp/loxp}$ controls; Cko, *cTnt-Cre*;$Mycn^{loxp/loxp}$ conditional knockout; *V*, ventricle.

mediating both BMP and NRG1 signaling pathways to promote normal myocardial wall morphogenesis. It will be interesting in future studies to determine whether overexpression of *Mycn* in hearts can rescue the cardiac wall defects caused by disruption of *Bmp10* and/or *Nrg1*/*ErbB2*,*4*.

Cell proliferation and growth are tightly coupled during development (Ahuja et al., 2007) and yet how this is achieved in embryonic cardiomyocytes is not well understood. Since MYC proteins can promote cell growth (Adhikary and Eilers, 2005; Hurlin, 2005) we wanted to determine if loss of *Mycn* stunted cardiomyocyte growth in addition to reducing proliferation. Indeed, loss of myocardial *Mycn* caused a significant size reduction in ventricular cardiomyocytes (Fig. 6). This result is consistent with a previous study showing that ectopic expression of *c-Myc* led to hypertrophy in adult hearts (Xiao et al., 2001). The dual roles of *Mycn* provide a potential molecular mechanism for the coordinated regulation of cell proliferation and growth during heart development.

We further showed that MYCN mediates cardiomyocyte growth (at least in part) by maintaining proper expression of p70S6K within the ventricles (Fig. 7). p70S6K acts downstream of the Akt/mTOR pathway and is a well-known regulator of cell growth during development and in adult diseases such as cardiac hypertrophy (Ahuja et al., 2007; Crackower et al., 2002; Fingar et al., 2002; Pereira et al., 2009; Shima et al., 1998; Shioi et al., 2000; Takano et al., 1996). Recent studies have implicated that the Akt/mTOR/p70S6K pathway and MYC act coordinately in different cellular events including cell proliferation and metabolism (Schramm et al., 2012; Wang et al., 2012; Ward and Thompson, 2012). Our results show that MYC can regulate expression of p70S6K and thus provide clues as to how exactly MYC interacts with the Akt/mTOR/p70S6K pathway. We are currently testing whether p70S6K is a direct target of MYCN. It is noteworthy that both MYC and p70S6K have been known to regulate cardiac hypertrophy and may serve as potential therapeutic targets for cardiomyopathy (Boluyt et al., 2004; Hurlin, 2005; Kang et al., 2011; Olson et al., 2012; Wolfram et al., 2011). Our findings from embryonic hearts may also inform how their activities are regulated in postnatal hypertrophic cardiomyopathy.

MYCN has been described as a "molecular switch" (Charron et al., 1992) that serves to keep cells in a proliferative, undifferentiated state since *Mycn* is expressed in proliferative, undifferentiated cell populations (Kato et al., 1991) and conditional deletion of *Mycn* has been reported to alter differentiation of various cell types (Knoepfler et al., 2002; Okubo et al., 2005; Ota et al., 2007). We investigated the possibility that loss of myocardial *Mycn* in mutant ventricles altered normal cardiac structural gene expression patterns. Among the group of proteins that are differentially expressed in embryonic hearts vs. adult hearts, only

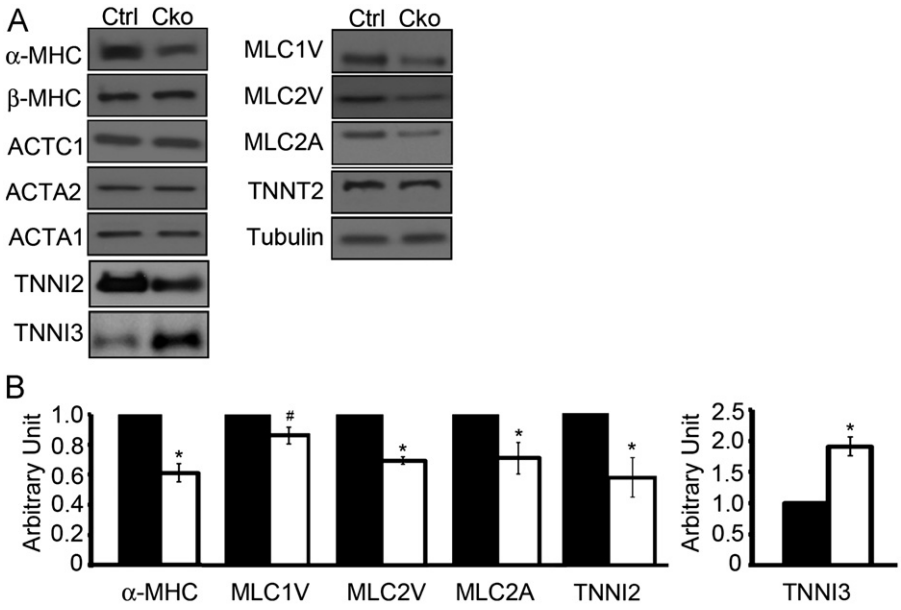

Fig. 8. Analysis of structural protein expression. (A), Expression of myofilament proteins was examined by Western blot analysis on protein extracts from ~E11.0 heart ventricles using antibodies as indicated. β-tubulin served as the loading control. Ventricle proteins were extracted and pooled from at least 5 embryos. (B) Altered expression of α-MHC, MLC1V, MLC2V, MLC2A, TNNI2, TNNI3 was semi-quantified using ImageJ. Data were averaged from three independent experiments. Black bars represent the level of control hearts, while the white bars represent that of mutant hearts. Error bars represent standard deviation. Ctrl, $Mycn^{loxp/loxp}$ controls; Cko, *cTnt-Cre*; $Mycn^{loxp/loxp}$ conditional knockout; *V*, ventricle; *: $P<0.05$ (Student's *t* test); #: $P=0.07<0.1$.

expression of troponin I showed premature shift from the embryonic form (TNNI2) to the adult form (TNNI3) (Fig. 8). Such a premature shift was not observed in myosin heavy chain proteins (α-MHC, β-MHC) or actins (α-ACTA1, α-ACTA2, α-ACTC1). Therefore, our results do not support that embryonic cardiomyocytes lacking MYCN undergo general prematuration. Despite their high structural similarities, TNNI2 and TNNI3 show different activities in regulating contraction of cardiac myofibrils (Fentzke et al., 1999; Westfall and Metzger, 2001; Westfall et al., 2001). Compared to cardiac myofilaments associated with TNNI3, those associated with TNNI2 display a higher Ca^{2+} sensitivity of tension and do not respond to cAMP-dependent protein kinase (PKA) (ibid.). Proper switching of TNNI forms during cardiomyocyte maturation is essential for normal activities of cardiac myofilaments. Ectopic myocardial expression of TNNI2 in a transgenic model or a delay in switching off TNNI2 in mXinβ knockout mice led to diastolic dysfunction (Fentzke et al., 1999; Wang et al., 2010). Thus, the premature switch of TNNI proteins may impair Ca^{2+} sensitivity of cardiac myofibrils and have a negative effect on cardiomyocyte relaxation/contraction in mutant hearts.

In addition to TNNI proteins, we observed reduced expression of α-MHC, MLC1V, MLC2A, and MLC2V in mutant ventricles. We conclude that *Mycn* is required for normal cardiomyocyte differentiation by maintaining expression of multiple sarcomere proteins during ventricular myocardial wall morphogenesis. It is unclear at present whether these genes are direct downstream targets of MYCN. We cannot fully exclude that abnormal expression of sarcomere proteins is secondary to proliferation and/or trabeculation defects in mutants. However, we feel that this is unlikely as such a phenotype has not been reported in some other mouse models with a similar thin-walled abnormality. For example, reduced expression of MLC2V and MLC2A occurred in *Nrg1* knockout hearts (Lai et al., 2010) but not in hearts lacking *Bmp10* (Chen et al., 2004).

In summary, deletion of myocardial *Mycn* resulted in hypoplastic ventricle walls with dramatically reduced trabeculation. These cardiac defects cause embryonic lethality at midgestation. *Mycn* is necessary for myocardial wall morphogenesis through its regulation of cardiomyocyte proliferation, growth, cardiac structural gene expression, and differentiation.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2012.10.005.

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