Numb family proteins are essential for cardiac morphogenesis and progenitor differentiation

Chen Zhao1,*, Hua Guo1,*, Jingjing Li1, Thomas Myint1, William Pittman1, Le Yang1, Weimin Zhong2, Robert J. Schwartz3, John J. Schwarz1, Harold A. Singer1, Michelle D. Tallquist4 and Mingfu Wu1,‡

ABSTRACT
Numb family proteins (NFPs), including Numb and numb-like (Numbl), are cell fate determinants for multiple progenitor cell types. Their functions in cardiac progenitor differentiation and cardiac morphogenesis are unknown. To avoid early embryonic lethality and study NFP function in later cardiac development, Numb and Numbl were deleted specifically in heart to generate myocardial double-knockout (MDKO) mice. MDKOs were embryonic lethal and displayed a variety of defects in cardiac progenitor differentiation, cardiomyocyte proliferation, outflow tract (OFT) and atrioventricular septation, and OFT alignment. By ablating NFPs in different cardiac populations followed by lineage tracing, we determined that NFPs in the second heart field (SHF) are required for OFT and atrioventricular septation and OFT alignment. MDKOs displayed an SHF progenitor cell differentiation defect, as revealed by a variety of methods including mRNA deep sequencing. Numb regulated cardiac progenitor cell differentiation in an endocytosis-dependent manner. Studies including the use of a transgenic Notch reporter line showed that Notch signaling was upregulated in the MDKO. Suppression of Notch1 signaling in MDKOs rescued defects in p57 expression, proliferation and trabecular thickness. Further studies showed that Numb inhibits Notch1 signaling by promoting the degradation of the Notch1 intracellular domain in cardiomyocytes. This study reveals that NFPs regulate trabecular thickness by inhibiting Notch1 signaling, control cardiac morphogenesis in a Notch1-independent manner, and regulate cardiac progenitor cell differentiation in an endocytosis-dependent manner. The function of NFPs in cardiac progenitor differentiation and cardiac morphogenesis suggests that NFPs might be potential therapeutic candidates for cardiac regeneration and congenital heart diseases.

KEY WORDS: Numb family proteins, Cardiac progenitor cell, Cardiac morphogenesis, Mouse

INTRODUCTION
Congenital heart defects (CHDs) occur in ~1% of live births (Hoffman and Kaplan, 2002), yet knowledge of the underlying genetic causes of the specific structural defects involved is still limited (Olson, 2006; Bruneau, 2008). Moreover, the lack of understanding of how cardiac progenitor cells renew and differentiate in vivo has impeded progress toward treating cardiovascular disease by cardiac regeneration using endogenous cardiac progenitors.

Drosophila Numb, an intracellular adaptor protein, was the first molecule discovered to influence cell fate by its asymmetric segregation during cell division (Rhyu et al., 1994) and by inhibiting Notch signaling (Uemura et al., 1989; Frise et al., 1996; Sapa and Doe, 1996; Petersen et al., 2002). In mice, Numb has two homologs, Numb and numb-like (Numbl), collectively known as Numb family proteins (NFPs). NFPs are nearly ubiquitously expressed during embryogenesis (Zhong et al., 1997) and are known to function as cell fate determinants by maintaining neural stem cell fate and regulating its differentiation (Verdi et al., 1996; Petersen et al., 2002; Li et al., 2003; Petersen et al., 2004). NFPs are also involved in the specification and differentiation of hematopoietic stem cells (Wu et al., 2007), muscle satellite cells (Conboy and Rando, 2002), cancer stem cells (Ito et al., 2010) and hemangioblasts (Cheng et al., 2008). Recently, additional mechanisms of Numb signaling at the molecular level have been revealed. Numb functions as a component of the adherens junction to regulate cell adhesion and migration (Rasin et al., 2007; Wang et al., 2009; Wu et al., 2010), and is involved in the ubiquitylation of p53 (Trp53) (Colaluca et al., 2008) and Gli1 (Di Marcotullio et al., 2006) to regulate cancer initiation. Numb has also been reported to complex with β-catenin (Rasin et al., 2007; Wu et al., 2010; Kwon et al., 2011) to regulate Wnt signaling. Additionally, Numb interacts with integrin β subunits (Caldenerwood et al., 2003) and promotes their endocytosis for directional cell migration (Nishimura and Kaibuchi, 2007).

Cardiac development is a spatiotemporally regulated multistep morphogenetic process that depends on the addition of progenitor cells from four different sources, including cells from the first heart field and second heart field (FHF and SHF), cells derived from cardiac neural crest cells (CNCCs) and cells derived from the pro-epicardial organ (Kelly et al., 2001; Mjaatvedt et al., 2001; Waldo et al., 2001; Verzi et al., 2005; Vincent and Buckingham, 2010). The SHF progenitor cells migrate to the pre-existing scaffold of the linear heart tube and contribute to the right ventricle, outflow tract (OFT) myocardium and to some endocardium at embryonic day (E) 8.5-10.25 (Kelly and Buckingham, 2002; Buckingham et al., 2005; Verzi et al., 2005; Ward et al., 2005). Perturbation of SHF deployment and progenitor differentiation leads to a spectrum of CHDs (Kelly, 2012) and is responsible for the majority of CHDs (Buckingham et al., 2005; Bruneau, 2008). The posterior SHF contributes to the dorsal mesenchymal protrusion (DMP), an essential structure for chamber septation (Snarr et al., 2007a). Abnormal differentiation and development of the posterior SHF has been associated with cardiac morphogenesis defects, such as atrial septal defect (ASD) and atrioventricular septal defect (AVSD) (Briggs et al., 2012). However, the molecules and the mechanisms that regulate posterior SHF development are not entirely clear.
Knowledge of NFP function in the mammalian heart is limited and the specific role of NFPs in cardiac development is still unclear. In Drosophila, Numb is implicated in controlling myogenic cell fate by interfering with Notch signaling (Han and Bodmer, 2003). In zebrafish, Numb is required for heart left-right asymmetric morphogenesis via regulation of Notch signaling (Niikura et al., 2006). In mice, Numb is reportedly expressed in adult cardiac progenitor cells and is asymmetrically distributed during progenitor asymmetric division (Cottage et al., 2010; Mishra et al., 2011). Additionally, we have shown that NFPs are essential for epicardial cells entering into myocardium (Wu et al., 2010). Very recent work has shown that NFPs regulate myocardial compaction by inhibiting Notch2 (Yang et al., 2012).

We previously reported that conditional deletion of NFPs in epicardial cells caused epicardial cell epithelial-mesenchymal transition defects (Wu et al., 2010). In this study, we deleted NFPs via Nkx2.5Cre/+ to generate Numb and Numbl myocardial double-knockout (MDKO) mice, and found that NFPs are required for myocardial trabeculation, cardiac progenitor cell differentiation, cardiomyocyte proliferation, OFT and atrioventricular septation and OFT alignment. Deletion of NFPs in the SHF recapitulated the morphogenetic defects of the MDKO. Further biochemical and genetic studies revealed that Notch1 signaling is upregulated in the myocardium of MDKOs and suppression of Notch1 signaling partially rescued the defects of trabeculation and proliferation in MDKOs. In summary, NFPs inhibit Notch1 to regulate cardiomyocyte proliferation and trabecular thickness, and mediate cardiac progenitor cell differentiation in an endocytosis-dependent, Notch1-independent manner.

RESULTS

NFPs are essential for cardiac development and morphogenesis

Numb and Numbl were deleted in mouse heart by Nkx2.5Cre/+, which is active in myocardium (Moses et al., 2001) and some endocardial cells based on the Rosa26-mTmG (mTmG) reporter line (supplementary material Fig. S1A-C, Movie 1) (Muzumdar et al., 2007). We refer to these mice as Numb and Numbl myocardial double knockout (MDKO). Loss of expression of Numb and Numbl in E9.5-13.5 MDKO ventricles was 70-90% as determined by Q-PCR (Fig. 1A) and western blot (Fig. 1B). Immunofluorescence (IF) staining confirmed the deletion of Numb in the myocardium of the MDKO (Wu et al., 2010). Very recent work has shown that NFPs regulate myocardial compaction by inhibiting Notch2 (Yang et al., 2012).

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and Numbl function redundantly during cardiac morphogenesis. MDKO embryos were indistinguishable from their littermates until E12.5, at which point they began to show edema that became more severe at E13.5 (supplementary material Fig. S1E,F).

MDKO hearts displayed a variety of morphogenetic defects. By E11.5, MDKO heart size was larger than that of the control and the OFT of MDKO hearts exhibited abnormal alignment with the right ventricle (supplementary material Fig. S1G). At E12.5, the aortic and pulmonary artery in the distal OFT (Webb et al., 2003) were separated in control but not in MDKO hearts, indicating an OFT septation defect (Fig. 1E, Table 2). At E13.5, the aorta and pulmonary artery in MDKO hearts were finally separated, but displayed an alignment defect with the aortic and pulmonary valves being in the same plane (Fig. 1F). The alignment defect at E14.5 in MDKO was visible by gross morphology as both aortic and pulmonary arteries extended from the compact zone, in control and MDKO embryos, in a parallel pattern (supplementary material Fig. S1H), which resulted in a double outlet right ventricle (DORV) (Fig. 1G, Table 2). MDKO hearts also displayed an AVSD, permitting both atrial and ventricular shunting (Fig. 1H, Table 2).

**NFPs regulate ventricular trabeculation in a cardiomyocyte-autonomous manner**

We then examined the formation of trabeculae, which are sheet-like structures extending from the compact zone, in control and MDKO hearts. E10.5 MDKO; mTmG and Nkx2.5<sup>Cre<sup>−/−</sup>; mTmG embryos, in which Nkx2.5-expressing cells were GFP positive, were stained for PECAM (Pecam1) to identify endocardial/endothelial cells. When whole hearts were three-dimensionally (3D) imaged by confocal microscopy we found that control trabecula had a diameter of one or two cells, whereas the MDKO trabecula had a diameter of two or three cells at E10.5 (Fig. 2A,B; supplementary material Movie 2, Fig. S3). The number of trabeculae and the thickness of the trabeculae and the compact zone were quantified. Beginning at E10.5, MDKO hearts displayed fewer trabeculae per unit length but thicker trabeculae compared with controls (Fig. 2C-E). The thickness of the compact zone was not significantly different between control and MDKO from E10.5 to E13.5, whereas at E14.5 the compact zone of MDKO was thinner than that of the control (Fig. 2C-E), indicating a non-compaction defect, consistent with a previous report (Yang et al., 2012). Dual-fluorescence staining of heart sections with MF20 (which recognizes myosin II heavy chain) and PECAM antibodies (Chen et al., 2009) showed that endocardial cells could invade the cardiac jelly and surround the myocardium in both MDKO and control (Fig. 2A-D), indicating that the trabeculation defect was not due to a defect in endocardial cell invasion.

The variety of defects in the MDKO might be caused by the loss of NFP function in different cardiac cell types. To determine the population that is responsible for these morphogenetic defects, SM22-Cre, Tie2-Cre, Wnt1-Cre, WT1<sub>CReERT2</sub> and Me2c-Cre were applied to delete NFPs in cardiomyocytes, endothelial cells, CNCCs, epicardial cells and cells derived from the SHF, respectively. The deletion of NFPs in cardiomyocytes via SM22-Cre, which is expressed in cardiomyocytes at the early stage of heart development in addition to its expression in smooth muscle cells (Li et al., 1996; Umans et al., 2007; Stankunas et al., 2008), resulted in trabeculation defects with thicker trabeculae and a decreased number of trabeculae per unit length at E14.5 (supplementary material Fig. S2A,B), whereas NFP deletion mediated by Tie2-Cre, Wnt1-Cre or WT1<sub>CReERT2</sub> did not display any trabeculation defects (supplementary material Fig. S2A-D; data not shown), indicating that NFPs regulate trabeculation in a cardiomyocyte-autonomous manner.

**NFPs in the SHF are required for OFT and atrioventricular septation**

We further determined that NFP deletion mediated by SM22-Cre, Tie2-Cre and WT1<sub>CReERT2</sub> in cardiomyocytes, endothelial/endothelial cells and epicardial cells, respectively, did not display any defects in morphogenesis (supplementary material Fig. S2A; data not shown), indicating that the function of NFPs in differentiated cardiomyocytes, endothelial cells or epicardial cells does not contribute to cardiac morphogenesis. Wnt1-Cre-mediated NFP deletion in CNCCs caused cranial facial defects (supplementary material Fig. S2C), but no obvious cardiac morphogenesis defect (supplementary material Fig. S2D), indicating that NFP function in...
CNCCs does not contribute to cardiac morphogenesis. Instead, Mef2c-Cre-mediated deletion in the SHF recapitulated the OFT septation defect and alignment defect in six out of ten E13.5 mutant hearts (supplementary material Fig. S2E), AVSD in eight out of ten mutant hearts (Fig. 3A) and trabeculation defect in the right ventricle in four out of four mutant hearts (data not shown). Mef2c-Cre is not active in the endocardial cells of the atrioventricular cushion (AVC) (Fig. 3B), indicating that the AVSD is not caused by the lack of NFPs in endocardial cells of the AVC, which is consistent with the observation that Tie2-Cre-mediated NFP deletion did not cause any defects.

Both Nkx2.5Cre+/--mediated and Mef2c-Cre-mediated mutants lacked the DMP (Fig. 1H, Fig. 3A), which is required for atrioventricular septation (Snarr et al., 2007b; Hoffmann et al., 2009). Using lineage tracing and 3D imaging, we observed that cells derived from the SHF migrated to the AVC and formed the DMP in the control, but fewer (data not shown) or no cells (Fig. 3C,D; supplementary material Movies 4, 5) were observed in the DMP in the knockout, indicating that NFPs are required for the migration of cells in the posterior SHF to form the DMP and the atrioventricular septum. In control hearts, Mef2c-Cre-labeled cardiomyocytes displayed myocardial spikes protruding towards the cushion in the OFT region (Fig. 3E), indicating a polarized migration toward the cushion. In the mutants, most of the NFP-null cells lacked myocardial spikes and fewer cells invaded the OFT cushion (Fig. 3E).

**MDKOs display differentiation defects**

Whole-mount in situ hybridization of E9.5-12.5 embryos with probes of the heart chamber markers ANP (Nppa – Mouse Genome Informatics) and Hand1 demonstrated that their expression patterns in the ventricles did not differ between control and MDKO hearts (supplementary material Fig. S3A), indicating that there are no chamber specification defects. We then screened for genome-wide transcriptional differences between control and MDKO E10.5 ventricles via mRNA deep sequencing (supplementary material Table S1). There were 340 genes with an expression ratio of MDKO to control that was higher than 1.7 (P<0.05) and 474 genes with a ratio of MDKO to control that was lower than 0.6 (P<0.05) (supplementary material Table S2). Of the 814 dysregulated genes, 42 have been reported to be involved in heart morphogenesis and heart diseases (Fig. 4A). Notably, genes that are involved in the differentiation of progenitors in the SHF, such as Isl1, Shox2, Fgfr8, Hoxa3 and Tbx1, were expressed at significantly higher levels in MDKO than in control (Fig. 4A). The transcriptional differences were consistent with the Q-PCR results (Fig. 4B). The expression of Isl1 (Cai et al., 2003) was consistently upregulated 2- to 4-fold in E9.5-11.5 MDKO hearts (Fig. 4C). The transcription of Isl1 in OFT, where the Isl1-expressing cells accumulate, was ~30-fold higher than in the ventricles (Fig. 4D). Isl1 expression in the OFT of the MDKO is about double that in the OFT of the control, similar to its upregulation in the ventricles (Fig. 4D). Isl1 transcription, as assessed by in situ hybridization, did not show any obvious expansion from right to left ventricle in the MDKO (data not shown). Instead, we observed more Isl1-expressing cells in the OFT of the MDKO based on IF staining (Fig. 4E).

Cardiomyocyte differentiation is a sequential process and to further define the differentiation defect we examined the expression of genes that encode cardiac differentiation regulators and structural proteins in E12.5 and E13.5 hearts by Q-PCR. Irx3, Irx4, Irx5 and Myh6 were significantly downregulated in the MDKO, whereas Myh11 and Bmp10 were upregulated and Myh7, Myl2 and Tmnt2 were not significantly different from the control (Fig. 4F). The ratio of Mhc7:Mhc6 after being normalized to cyclophilin A (peptidylprolyl isomerase A) was ~2-fold greater in the MDKO than in the control at both E12.5 and E13.5, indicating a cardiomyocyte differentiation defect. Transcriptional patterns of Irx3 and Irx4 were investigated by in situ hybridization. Irx3 was uniformly expressed...
in the trabeculae of the control heart, whereas the MDKO pattern was less uniform and there was no Irx3 expression in some trabeculae (Fig. 4G). Irx4, which specifies cardiomyocytes in ventricles (Bao et al., 1999; Bruneau et al., 2001), was expressed in the compact and trabecular zones in controls, whereas in MDKOs the compact zone exhibited weaker or no expression of Irx4 (Fig. 4G). We found that 88% of the trabeculae in the control displayed organized sarcomere arrays at E12.5 (n=4 hearts), whereas in the MDKO only 15% of the trabeculae displayed sarcomeric arrays (n=3 hearts) (Fig. 4H). In summary, MDKO hearts displayed defects in the differentiation of cardiac progenitors and in the maturation of sarcomeres.

**Numb regulates cardiac progenitor differentiation through its function in endocytosis**

Cardiomyocyte differentiation in cultured mouse embryoid bodies (EBs) recapitulates the genetic network that regulates this process in vivo (Smith, 2001; Mummery et al., 2002). To study NFP regulation of cardiac progenitor differentiation, we took advantage of a pluripotent P19 embryonal carcinoma cell line (P19Cl6-GFP) (McBurney and Rogers, 1982; Habara-Ohkubo, 1996), in which GFP is under the transcriptional control of the rat Mlc-2v promoter and therefore functions as a marker for differentiated cardiomyocytes (Moore et al., 2004). Adenovirus-mediated expression of Numb (p65 isoform) in the EB increased the number of GFP+ cells (Fig. 5A-C) and the number of beating cultures (data not shown), suggesting that Numb overexpression promotes cardiomyocyte differentiation. We examined the expression of genes that are involved in cardiac progenitor differentiation and those that encode structural proteins. Nkx2.5, Gata6, ANP, Irx3, Myh7 and Myh6 were all upregulated 2- to 4-fold (Fig. 5D), whereas progenitor cell markers Isl1 and Gata4 were significantly downregulated (Fig. 5D).

Numb interacts with endocytic adapters such as α-adaptin and Eps15 via the NPF and DPF motifs in the C-terminus (Salcini et al., 1997; Santolini et al., 2000) to selectively promote clathrin-dependent endocytosis of transmembrane proteins including Notch1 and the EGF receptor (Traub, 2003; Sorkin, 2004). To determine if Numb regulates cardiomyocyte differentiation via endocytosis, EB cultures were treated with Bafilomycin A1 (BafA1), an inhibitor of endocytosis. BafA1 abolished the Numb-induced increase in cardiac progenitor differentiation (Fig. 5E). When the α-adaptin or Eps15 binding sites (NPF and DPF, respectively) of Numb were mutated to generate NbNPFm and NbDPFm they failed to promote differentiation, as evidenced by fewer GFP+ cells and no upregulation of Nkx2.5, Gata6 and Myh7 (Fig. 5F,G). We then examined whether Numb can normalize Isl1 expression in E9.5 MDKO hearts using an ex vivo culture system. Ad-Numb, but not Ad-NbDPFm, reduced Isl1, Fgf8 and Shox2 expression significantly in the MDKO (Fig. 5H). These results demonstrate that NFPs...
regulate cardiac progenitor differentiation in an endocytosis-dependent manner.

Both CBF1-dependent and -independent Notch signaling are upregulated in MDKO hearts

Numb negatively regulates Notch1 signaling, and the MDKO phenotype is comparable to that of transgenic mice expressing activated Notch1 in cardiogenic mesoderm (Watanabe et al., 2006), which led us to investigate Notch1 signaling in MDKO hearts. We examined the level of Notch1 intracellular domain (NICD1), the activated form of the Notch1 receptor, in control and MDKO hearts. MDKO hearts displayed higher levels of NICD1 protein (Fig. 6A). IF staining showed that endocardial cells were positive for NICD1 (Fig. 6B; supplementary material Fig. S4A), consistent with a previous report (Grego-Bessa et al., 2007), whereas in MDKOs NICD1 staining was also observed in some cardiomyocyte nuclei at
E9.5 (Fig. 6B) and in all nuclei at E12.5 (supplementary material Fig. S4A).

To determine whether upregulation of Notch signaling in MDKO hearts is dependent on CBF1/canonical Notch signaling, the transgenic Notch reporter (TNR) line (Duncan et al., 2005), in which GFP expression is under the control of CBF1 (Rbpj – Mouse Genome Informatics), a downstream target of Notch signaling, was used to generate the MDKO; TNR strain. GFP intensity was stronger in MDKO; TNR hearts than in the control (supplementary material Fig. S4B). Western blot indicated that GFP levels were higher in eight out of nine MDKO; TNR hearts than in littermate controls (Fig. 6C), indicating upregulated canonical Notch signaling in MDKO hearts. We observed GFP in some cardiomyocytes as well as endocardial cells in MDKO; TNR hearts at E9.5 (Fig. 6D).

However, at E12.5, although all cardiomyocytes expressed NICD1 (supplementary material Fig. S4A), not all of them were TNR/GFP+ (supplementary material Fig. S4C). The discrepancy is due to TNR incompletely representing all NICD1+ cells, indicating that NFPs regulate both canonical and non-canonical – or CBF1-dependent and -independent – Notch signaling (Hellström et al., 2007; Mizutani et al., 2007; Sanalkumar et al., 2010).

Expression levels of Notch target genes, including Hey1, Hey2, Heyl and Hes1, were used to assess the activation of Notch signaling in MDKO hearts. Q-PCR showed that Hey1 and Hes1 expression levels were slightly higher in MDKO hearts than controls at certain ages (supplementary material Fig. S4D). To confirm that Notch signaling was upregulated in NFP-null cardiomyocytes, we used a lentivirus RBP-Jk luciferase reporter

Fig. 5. Numb regulates cardiac progenitor differentiation via endocytosis. (A-C) Numb overexpression in EB culture promotes the differentiation of P19Cl-6GFP cells, in which GFP is under the control of rat Mlc-2v, as evidenced by increased numbers of GFP+ cells compared with β-gal-overexpression EB cultures. The percentage of GFP+ cells was quantified by flow cytometry. FSC, forward scatter channel.

(D) Numb overexpression EB cultures showed higher expression of Nkx2.5, Gata6, ANP (Nppa), Irx3, Myh7 and Myh6, but lower expression of Isl1 and Gata4. (E) The endocytosis inhibitor BafA1 abolished Numb overexpression-induced EB culture differentiation. (F,G) Endocytosis-defective Numb variants: both NbDPFm and NbNPFm failed to enhance cardiomyocyte differentiation and induce Nkx2.5, Gata6 and Myh7 expression using the EB culture system. (H) Using an ex vivo culture system, Numb, but not NbDPFm and control virus GFP, could rescue the expression of Isl1, Fgf8 and Shox2 in E9.5 MDKO hearts. Isl1 and Numb expression in the control and virus-infected MDKO hearts was examined by Q-PCR. Isl1 expression in GFP-infected wild-type hearts was set as 1. Error bars indicate s.d.
to measure Notch signaling activation in cardiomyocytes that were isolated from control or αMHC-Cre-mediated cardiomyocyte-specific NFP knockout hearts. The NFP knockout showed higher Notch signaling activation, indicating upregulated Notch signaling in cardiomyocytes (Fig. 6E).

To determine whether upregulated Notch1 signaling was responsible for the defects in morphogenesis in MDKO hearts, Notch1 signaling was suppressed by deleting one of the Notch1 alleles (Garg et al., 2005) in the MDKO to generate MDKO; Ntfl/+. This resulted in reduced NICD1 staining intensity (supplementary material Fig. S4A). However, this reduction did not rescue the OFT septation defect or A VSD (data not shown). Instead, Notch1 suppression did reduce the thickness of the trabeculae, although it did not increase the number of trabeculae per unit length (Fig. 6F; supplementary material Fig. S5A,B). This suggests that NFPs regulate trabecular thickness through Notch1 signaling.

**NFPs regulate cardiomyocyte proliferation through inhibition of Notch1 signaling**

To examine if the thicker trabeculae and the larger size of MDKO hearts are due to an increase in cell proliferation, the cardiomyocyte proliferation rate was assessed by BrdU pulse labeling (Fig. 7A,B). In the compact zone, the percentage of BrdU-positive cardiomyocytes in the MDKO was significantly higher than in the control from E9.5 to E12.5 (Fig. 7C), consistent with the increased heart size in MDKOs. This result was also apparent specifically in the trabecular zone, where there was a 3-fold increase in the percentage of BrdU-positive cells in the MDKO compared with the control at E12.5 (Fig. 7D), consistent with the thicker trabeculae in MDKOs.

We examined the mRNA deep sequencing data and found, among the known cell cycle regulators, that only p57 (Cdkn1c or p57kip2), a cyclin-dependent kinase inhibitor, was significantly different. Expression levels of cyclin D1 (Ccd1), Cdkn1a (p21) and p57 were further examined by Q-PCR and only p57 was significantly different from the control in E9.5-13.5 MDKO hearts (Fig. 7E). We then examined whether reduced p57 expression in MDKOs was caused by increased Notch1 signaling. MDKO; Nt+/fl mice showed increased p57 expression compared with the MDKO (Fig. 7F). Consistent with the higher expression of p57 and the thinner trabeculae, cardiomyocyte proliferation in the trabeculae of MDKO; Nt+/fl mice was significantly reduced compared with the MDKO (Fig. 7G). To further study how NFPs regulate p57, embryonic cardiomyocytes from control, Numbflox/flox; Numbl flox/flox or Notch1flox/flox; Numbflox/flox; Numblflox/flox were cultured and treated with Ade-Cre to induce recombination. We found that p57 was downregulated in Cre+; Numbflox/flox; Numblflox/flox cardiomyocytes, but was upregulated in Cre+; Notch1flox/flox; Numbflox/flox; Numblflox/flox cardiomyocytes (Fig. 7H-I), indicating that downregulation of p57 in NFP-null cardiomyocytes is Notch1 dependent.
To study how NFPs regulate Notch1 signaling, we first determined whether Notch1 and Numb interact in the heart. Both Numb and Notch1 antibodies could pull down Numb based on co-immunoprecipitation using whole heart lysates (Fig. 7J) or endocardial cell-specific NFP-null heart lysate (Wu et al., 2012) (supplementary material Fig. S6A), indicating that Numb interacts with Notch1 in the mouse heart. To determine how Numb inhibits Notch1 signaling, Numb was overexpressed in cultured embryonic cardiomyocytes. E12.5 mouse brain and Nbfl/fl; Nlfl/fl; Notch1fl/fl cardiomyocytes treated with pAd-Cre were used as positive and negative control, respectively. *A weak band in the positive control, DAPT and GFP lanes that is non-specific. **The Numb antibody can detect all four Numb isoforms. (L) Quantification of NICD1 in different treatments from three independent experiments. Error bars indicate s.d. Scale bar: 50 μm.

Fig. 7. NFPs regulate cardiomyocytes proliferation via Notch1. (A,B) BrdU pulse labeling indicated that cardiomyocytes in the MDKO proliferated at a higher rate than those in the control. Cardiomyocytes were identified by collagen IV, a marker for the basement membrane. (C,D) MDKO displayed higher proliferation rates of cardiomyocytes in the compact zone (C) and in the trabecular zone (D) at various ages. (E) Transcription of the cell cycle inhibitor p57 (p57kip2) was significantly reduced in the MDKO. (F) p57 expression was partially rescued in MDKO; Ntfl/+. The expression of Numb (Nb), Numb (Nl) and Notch1 (N1) in MDKO; N1fl/+ was comparable to their expression in MDKO. (H,I) p57 expression was downregulated in Nbfl/fl; Nlfl/fl cardiomyocytes but upregulated in Nbfl/fl; Nlfl/fl; Notch1fl/fl after Cre-mediated recombination for 72 hours. (J) Numb and Notch1 antibody can immunoprecipitate Numb using P4 heart lysates. IP, immunoprecipitate; WB, western blot.
cardiomyocytes. Western blot indicated that Numb:GFP overexpression reduced the level of NICD1. However, overexpressing GFP or the endocytosis-defective NbDFF-α::GFP did not reduce the level of NICD1 (Fig. 7K,L), indicating that Numb promotes NICD1 degradation via endocytosis.

DISCUSSION
NFPs play multiple roles during cardiac development and their disruption results in multiple CHDs
The defects in trabeculation, proliferation, progenitor differentiation, aorta and atrioventricular septation and OFT alignment in MDKO hearts, together with the recent report that NFPs inhibit Notch2 to regulate compaction (Yang et al., 2012), suggest that NFPs are essential for cardiac development and morphogenesis. These findings extend our previous analysis which found that NFPs are essential for epicardial development (Wu et al., 2010). Our present analysis using NFP knockout models mediated by TIE2-Cre, Wnt1-Cre, SM22-Cre, WT1CreERT2, Mef2C-Cre and Nkx2.5CreT2 suggests that the key functions of NFPs in cardiac development are stage- and cell type-specific. NFP knockouts mediated by TIE2-Cre, WT1CreERT2 and Wnt1-Cre did not display any obvious cardiac morphogenetic defects (supplementary material Fig. S2), indicating that NFP functions in endocardial cells, epicardial cells and CNCCs are not essential for heart morphogenesis. SM22-Cre-mediated NFP knockout in cardiomyocytes partially phenocopied the MDKO, with disrupted trabeculation, but did not display differentiation and morphogenetic defects (supplementary material Fig. S2). Mef2C-Cre-mediated NFP knockouts recapitulated the morphogenesis defects of the MDKO, indicating that NFPs in the SHF are essential for cardiac morphogenesis (Fig. 3; supplementary material Fig. S2). The difference in phenotypes between SM22-Cre-mediated and Nkx2.5CreT2-mediated mutants might be due to the different timing and cell types in which NFPs were deleted. Nkx2.5CreT2 was active in cardiomyocytes derived from the FHF and SHF, some endocardial cells of the OFT and AVC (supplementary material Fig. S1A,B), and very few endocardial cells of the ventricles (supplementary material Fig. S1C, Movie 1) and was active beginning at E7.5 based on Nkx2.5CreT2; mTmG. By contrast, SM22-Cre is active in some cardiomyocytes in the E8.5 heart tube, but not in all cardiomyocytes until E9.0, a stage at which cardiac progenitor cells have already initiated the differentiation process.

However, the expression of Nkx2.5CreT2 in the endocardial cells appears not to be the cause of the AVSD and OFT septation in the MDKO, as TIE2-Cre-mediated NFP deletion did not cause any defect. Furthermore, NFP deletion via Mef2C-Cre, which is not active in the endocardial cells of the AVC (Fig. 3B), caused the AVSD, indicating that NFP absence in endocardial cells of the AVC is not responsible for the AVSD in the mutants. Instead, the morphogenesis defects in MDKOs might be caused by the SHF progenitor cell differentiation defect, as evidenced by the upregulation of Isl1, Fgf8, Hoxa3 and Tbx1 in MDKOs. This is consistent with previous reports that down- or upregulation of Fgf8/Fgf10 (Kelly et al., 2001; Ilagan et al., 2006; Park et al., 2006), Isl1 (Ai et al., 2007; Kwon et al., 2007; Lin et al., 2007; Ueno et al., 2007) and Tbx1 (Vitelli et al., 2002) interferes with SHF progenitor cell differentiation and subsequently causes cardiac morphogenetic defects or CHDs (Buckingham et al., 2005; Black, 2007; Rochais et al., 2009). Further studies showed that NFP-null cells fail to form myocardial spikes that orient perpendicularly to the heart wall (Fig. 3E) and fail to migrate into the OFT cushion, which might result in the OFT septation defect.

NFPs regulate ventricular trabeculation and compaction
Ventricular trabeculation and compaction are two related but different biological processes during cardiac morphogenesis. Trabeculation initiates at E9.0-9.5 and myocardial compaction occurs at E14.5 (Samsa et al., 2013; Zhang et al., 2013). Lack of trabeculation causes embryonic lethality in mice and excess trabeculation causes cardiomyopathy and heart failure in humans (Jenni et al., 1999; Weiford et al., 2004; Breckenridge et al., 2007). Despite the fundamental nature of these morphogenetic processes, the molecular and cellular mechanisms controlling trabeculation and compaction are not fully understood. Pathways that regulate trabeculation include the Notch1 and Notch2 signaling pathways. Global or endothelial-specific Notch1 deletion causes ventricular hypoplasia and trabeculation defects (Grego-Bessa et al., 2007). Notch1 activation in early cardiac cell lineages by Mesp1-Cre-mediated overexpression of NICD1 leads to several cardiac morphogenetic defects, including abnormal ventricular morphology similar to ventricular non-compaction (Watanabe et al., 2006). However, NICD1 overexpression in cardiomyocytes at a later stage via Mlc-2v-Cre does not cause obvious trabecular morphogenesis (Croquelois et al., 2008). The discrepancy could be caused by Notch1 activation at different developmental stages or in different cell populations.

Notch2 intracellular domain (N2ICD) is detected throughout the myocardium before E11.5, but at a later stage Notch2 activity is specifically downregulated in the compact zone and is restricted in trabecular myocardium during ventricular compaction (Yang et al., 2012). Notch2 global knockout displays ventricular hypoplasia (McCright et al., 2001). Notch2 deletion in heart via SM22-Cre displays cyanosis at birth due to narrow arteries, but whether or not the knockout displays a trabeculation defect was not reported (Varadkar et al., 2008). However, loss-of-function analysis for Notch1 or Notch2 in cardiomyocytes cannot determine whether they are involved in trabecular morphogenesis, as all four Notch receptors are expressed at relatively high levels in the heart (supplementary material Fig. S6B) and the deletion of one Notch receptor in cardiomyocytes may be compensated by the function of another, although they might not be expressed in cardiomyocytes under normal conditions. Yang et al. (Yang et al., 2012) reported that N2ICD continues to be present in the compact zone at E12.5 and E13.5, which was not observed in the control, and we observed that NICD1 is present in cardiomyocytes in both the compact and trabecular zones of the MDKO, indicating that NFPs regulate trabecular morphogenesis via inhibition of both Notch1 and Notch2.

Trabecular morphogenesis is a multistep process that includes, but is not limited to, trabecular initiation, proliferation/growth, differentiation and compaction. Notch1 and Notch2 might regulate different steps of trabecular morphogenesis. Genetic epistasis studies can determine whether Notch receptors function downstream of NFPs in the regulation of a particular trabecular morphogenesis step. MDKOs displayed lower p57 expression, higher proliferation rates and thicker trabeculae. When one of the Notch1 alleles was deleted in the MDKO, p57 expression and proliferation were partially rescued (Fig. 7F,G) and the thickness of the trabeculae was reduced compared with that of the MDKO (Fig. 6F). However, Notch1 deletion did not rescue the defects in trabecular initiation and non-compaction (supplementary material Fig. S5A,B; data not shown). This indicates that NFPs inhibit Notch1 to regulate cardiomyocyte proliferation and trabecular growth/thickness, but not trabecular initiation and compaction. NICD2 overexpression in cardiomyocytes mediated by αMHC-Cre results in hypertrabeculation and non-compaction, indicating that Notch2 is involved in compaction and
that NFPs might inhibit Notch2 to regulate compaction (Yang et al., 2012). However, many questions regarding the regulation of trabecular morphogenesis by Notch1 and Notch2 remain. Notch2 activity is restricted to trabeculae, where cells are more differentiated, more mature and less proliferative compared with cardiomyocytes in the compact zone, raising a number of questions for future research, such as whether Notch2 inhibits the proliferation of cardiomyocytes and promotes their differentiation to regulate compaction, and what the unique Notch2 downstream targets regulating compaction are. The observation that both Notch1 deletion (Grego-Bessa et al., 2007) and overexpression (Venkatesh et al., 2008) in endocardial cells reduced trabeculation indicates that further work is needed to reveal how Notch1 regulates trabeculation.

**NFPs regulate Notch1 signaling during cardiac development**

One of the well-known pathways that NFPs regulate is Notch1 signaling. It has been shown that Numb inhibits Notch1 signaling in *Drosophila* and mammalian cells (Guo et al., 1996; Spana and Doe, 1996; Zhong et al., 1996; McGill and McGlade, 2003; Cheng et al., 2008; McGill et al., 2009; Beres et al., 2011). However, the Numb-Notch1 relationship during vertebrate embryogenesis remains an enigma (Zhong et al., 1996; Petersen et al., 2006). Although the phenotype of the NFP global knockout is similar to that of Notch1 pathway disrupted mutants, leading to the hypothesis that Numb and Notch1 signaling are associated, Notch1 targets were not upregulated in the NFP global knockout during mammalian development, as would be predicted if Numb inhibits Notch1 signaling (Petersen et al., 2006). The exact relationship between Numb and Notch1 during embryogenesis is therefore unclear. Genetic tools to test the interaction between Numb and Notch1 should be applied to reveal the relationship. We used a transgenic Notch reporter line and other assays to clearly show that canonical Notch signaling is upregulated in MDKO hearts. Significantly, deletion of one *Notch1* allele partially rescued the MDKO phenotype, indicating the expression of *p57*, cardiomyocyte proliferation and trabecular thickness (Fig. 6). This indicates that NFPs regulate trabecular proliferation/growth and thickness via Notch1 signaling.

Notch1 signaling is local and only cells that are adjacent to the ligand will be activated. *Notch1* transcription was highest in endocardial cells and weak in cardiomyocytes. It has been proposed that jagged 1 in cardiomyocytes can activate Notch1 in endocardial cells, so NICD1 is usually detected in endocardial cells (Grego-Bessa et al., 2007). The activated Notch1 signaling in endocardial cells then can enhance cardiomyocyte proliferation by inhibiting *p57* through Bmp10 in the myocardium (Chen et al., 2004; Grego-Bessa et al., 2007; Chen et al., 2013; Luxán et al., 2013). In MDKO hearts, the *Notch1* transcriptional level did not differ from that of the control based on mRNA sequencing and Q-PCR (data not shown), and NICD1 was detected in both endocardial cells and cardiomyocytes, indicating that *Notch1* is expressed in cardiomyocytes and that NFPs are required to inhibit NICD1 in a post-transcriptional manner, consistent with their functions as endocytic proteins. Furthermore, *Nkx2.5*+/−-mediated *Notch1*βC expression deletion reduced NICD1 to ~46% of control levels based on western blot using lysates of E12.5 ventricles (supplementary material Fig. S6C,D), and presumably the remaining Notch1 protein was contributed by endocardial and epicardial cells. This suggests that Notch1 is expressed in cardiomyocytes at E12.5, considering that *Nkx2.5*+/− is active in all cardiomyocytes and in very few endocardial cells in the ventricles. This is consistent with a previous report that NICD1 is detected in cardiomyocytes in E13.5 and older hearts (Kratsios et al., 2010). We propose that Notch1 in cardiomyocytes is activated by Delta4 (delta-like 4) in the endocardial cells and/or jagged 1 in cardiomyocytes to produce NICD1, which is degraded by NFP-mediated endocytosis and is not detectable in cardiomyocytes in the control hearts. In the cardiomyocytes of MDKO hearts, NFP-mediated NICD1 degradation is abolished, which results in NICD1 accumulation to a detectable level. This NICD1 accumulation in cardiomyocytes activates Notch1-related signaling, including the inhibition of *p57* expression to promote cell proliferation in a myocardium-autonomous manner in the MDKO (Fig. 8A).

**NFPs regulate Notch1-independent signaling pathways**

NFPs play a variety of roles during normal and disease conditions in a cell context-dependent manner (Gulino et al., 2010). Numb is an endocytic protein (Santolini et al., 2000), and NFPs execute their functions mainly through their involvement in endocytosis and interaction with other proteins. Numb functions as a component of the adherens junction to regulate cell adhesion and migration in neural cells (Nishimura and Kaibuchi, 2007). Numb also interacts with Notch and Wnt signaling to specify the hemangioblast (Colaluca et al., 2008; Wu et al., 2010), regulates cancer initiation by controlling the stability of p53 (Colaluca et al., 2008) and Gli1 (Di Marcotullio et al., 2006), regulates neuroblast specification in *Drosophila* SOP cells by inhibiting Notch signaling (Guo et al., 1996), and regulates axon growth in neural cells (Nishimura and Kaibuchi, 2007). Numb also interacts with Notch and Wnt signaling to specify the hemangioblast to the primitive erythroid lineage (Cheng et al., 2008). Of all the pathways with which NFPs interact, the best-studied is the Notch1 pathway. However, suppression of Notch1 did not rescue all the defects of MDKOs, indicating that NFPs function upstream of Notch1-independent signaling pathways to regulate cardiac morphogenesis and cardiac progenitor differentiation.

To reveal the mechanisms that cause the defects in morphogenesis and progenitor differentiation, we examined mRNA deep sequence data. Of the 814 dysregulated genes in the MDKO, 36.9% encode membrane-associated proteins, 30.4% encode glycoproteins and 26.8% encode signal peptides (supplementary material Fig. S7A), indicating that NFPs are involved in multiple signaling pathways, which is consistent with previous findings that Numb is an adaptor protein involved in the endocytosis of multiple proteins (Santolini et al., 2008).
et al., 2000; Krieger et al., 2013). Indeed, it is possible that NFPs regulate cardiac morphogenesis and progenitor differentiation through the endocytosis of multiple proteins. Analysis of transgenic lines expressing endocytosis-defective Numb might help to determine how NFPs regulate cardiac morphogenesis and progenitor differentiation.

MATERIALS AND METHODS

**Mice**

Mouse strains *Numbfl/fl, Numbfl/fl* (Zilian et al., 2001; Wilson et al., 2007), transgenic Notch reporter (TNR) (Duncan et al., 2005), Tie2-Cre (Kisanuki et al., 2001), Rosa26-mR despite (mTmG) (Muzumdar et al., 2007), SM22-Cre (Holtwick et al., 2002; Boucher et al., 2003), CMV-Cre (Schwenk et al., 1995), WT1-Cre (Danielian et al., 1998), WT1CreERT2 (Zhou et al., 2008), aMHC-Cre (Oka et al., 2006) and NotchI (Yang et al., 2004) were purchased from Jackson Lab. Dr Robert Schwartz provided *Nkh2.5Cre* (Moses et al., 2001) mice. Dr Brian Black provided *Mef2-Cre* (Verzi et al., 2005). *Nkh2.5Cre*; *Numbfl/fl* or *Nkh2.5Cre*; *Numbfl/fl* males were mated to *Numbfl/fl*; *Numblfl/fl*, designated as MDKO; their sibling embryos without the Cre allele are designated as controls. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at Albany Medical College and performed according to the NIH Guide for the Care and Use of Laboratory Animals.

**Paraffin and frozen section immunohistochemistry**

Immunohistochemistry, immunofluorescence (IF) and Hematoxylin and Eosin (H&E) staining were performed as described (Lechler and Fuchs, 2005; Mellgren et al., 2008). Primary antibodies were: BrdU (1:50; Becton Dickinson, 347583), collagen IV (1:1000; Chemicon, AB756P), β-tubulin (1:1000; BD Pharmingen, 556321), active caspase 3 (1:1000; Cell Signaling, 9661S), WT1 (1:100, DAKO, M3561; or 1:50, Santa Cruz Biotechnology, sc192), PECAM (1:50; BD Pharmingen, 550274), MF20 [1:100; Cell Signaling, 4147S], Notch1 (1:1000; Cell Signaling, 4380s), NICD1 (1:500; Santa Cruz Biotechnology, sc-6014), GFP (1:10,000; Abcam, ab290) and Gapdh (1:1000; Santa Cruz Biotechnology, sc-25778).

**RNA isolation and quantitative PCR (Q-PCR)**

For Q-PCR analysis, total RNA was isolated from three ventricles for each experiment using Trizol and the RNeasy Micro Kit (Qiagen). The experiments were repeated at least three times for each age. At least 300 ng total RNA was used for reverse transcription (iScript cDNA Synthesis Kit, Bio-Rad). Gene expression was analyzed using standard Q-PCR methods with iTAQ SYBR Green Master Mix on a CFX96 instrument (Bio-Rad). Each sample was run in triplicate and normalized to cyclophilin A. Primer sequences are listed in supplementary material Table S3. Transcription of *Isl1* and *Hey1* was confirmed using two sets of primers.

**In situ hybridization**

Digoxigenin-labeled RNA probes were made as described (Xu et al., 2009). *In situ* hybridization of whole-mount mouse embryos and paraffin sections was as described (Xu et al., 2011). Briefly, after fixation, embryos were treated with 10 μg/ml proteinase K, refixed in a 0.2% glutaraldehyde/4% paraformaldehyde (PFA) solution, and prehybridized at 65°C for 1 hour. The samples were then transferred into hybridization mix containing 0.5 μg/ml digoxigenin-labeled probes. Post-hybridization washes and antibody incubation were as described (Xu et al., 2009). Color development employed BM Purple solution (Roche).

**Imaging**

The following systems were used: for confocal imaging, Zeiss LSM510 META and Zeiss 510 META NLO; for color imaging, Zeiss Observer Z1 with a Hamamatsu ORCA-ER camera; for fluorescence imaging, Zeiss Observer Z1 with an AxioCam MRm camera. 3D images were produced by Zeiss 510 META NLO with 0.9 μm per section and reconstructed with Imaris software (Bitplane).

**Cardiomyocyte proliferation assay by BrdU pulse labeling**

Pregnant females were intraperitoneally injected with BrdU for 1 hour before embryo harvesting. The proliferation rate was assessed by the percentage of BrdU-positive cells among total cells as described (Wu et al., 2010). Collagen IV was used to stain the basement membrane to distinguish cardiomyocytes from non-cardiomyocytes.

**Western blot and co-immunoprecipitation (Co-IP)**

Lyssates from E12.5 or E13.5 ventricles of each genotype were processed for western blot as described (Mellgren et al., 2008). Lyssates from postnatal day (P) 4 hearts were used for Co-IP according to the protocol of uMACS Protein A/G Microbeads (Miltenyi Biotec). Antibodies used for western blots and Co-IP included Numb (1:1000; Cell Signaling, 2756s), NICD1 (1:500; Cell Signaling, 4147s), Notch1 (1:1000; Cell Signaling, 4380s), Notch1 (1:500; Santa Cruz Biotechnology, sc-6014), GFP (1:10,000; Abcam, ab290) and Gapdh (1:1000; Santa Cruz Biotechnology, sc-25778).

**Trabecular density and thickness quantification and whole-mount IF staining**

One in every four sections from E10.5-13.5 hearts was stained with PECAM and MF20 antibodies. At least six sections from the medial part of the heart were quantified for the number of trabeculae per unit length and for the thickness of trabeculae or compact zone in both left and right ventricles. Trabecular thickness was defined as the average thickness of six different spots in left and right ventricle in each section. The data presented in figures include both left and right ventricles.
Embryoid body (EB) culture and analysis
EB culture was as described (Moore et al., 2004). Briefly, 800 P19Cl6-GFP cells in 20 μl medium (passage number less than 20) were used for each hanging drop culture. Forty-eight hours after hanging drop formation, adenovirus at a multiplicity of infection (MOI) of 100, DAPT at a final concentration of 10 μM, or BafA1 at a final concentration of 0.1 μM, or an equal amount of vehicle (DMSO) mixed in medium was added to the hanging drop without disturbing the cell aggregates. The hanging drops were allowed to grow for another 4 days. Then, the EB or cell aggregates were transferred to culture plates to grow for another 5 days. On day 6, the EB cultures were harvested and dissociated into single cells for flow cytometry analysis or RNA isolation. The percentage of GFP+ cells was analyzed by FACScan (Becton Dickinson).

Site-directed mutagenesis
Numb mutant variants including NumbΔPFAQ (NumbΔPFAQ) and NumbΔNPF (NumbΔNPF), in which DPF and NPF were mutated to AAF and AAF, respectively, were generated by site-directed mutagenesis following protocols provided with the Phusion Site-Directed Mutagenesis Kit (Thermo Scientific). Primers used for NumbΔPFAQ and NumbΔNPF generation are (5′-3′, forward and reserve): NumbΔPFAQ CCAGGGGACCTGCCCA-GTGTCGTCGCTGCCACAGGGCCAGTGACTACAGA and AGGGAGGCTTCTGATGAGCCGAACTGCCGTTTCGCAGTGCCTTGTCTCTCTCTCAGTACAGA and CTGCTTTGCCGTTGCTTTCTCTCAGTCCCGGC.

Ex vivo culture system
The ex vivo culture protocol was developed in Dr Brian Black’s laboratory. Briefly, embryos lacking the head and tail with the heart exposed to medium were cultured in medium containing Ad-Nb, Ad-GFP or Ad-NbΔPFAQ for 48 hours. Then, the heart was used for Q-PCR analysis.

Statistics
Data are shown as mean ± s.d. An unpaired two-tailed Student’s t-test was used for statistical comparison. P<0.05 was considered statistically significant.

Lenti RBP-Jk luciferase reporter assay
The Lenti RBP-Jk reporter is a preparation of ready-to-transduce lentiviral particles for monitoring the activity of Notch signaling pathways in virtually any mammalian cell type (Qiagen, CLS-014L). Cardiomyocytes from E16.5-18.5 control or aMHC-Cre-mediated NKF knockout hearts were cultured in 48-well plates for 4 days. Then, the cells were infected with Lenti RBP-Jk luciferase and Lenti renilla luciferase, which functions as a control. Seventy-two hours after infection, the lysate was used to measure luciferase activity according to the supplied protocol.

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