Distinct Roles for Cell-Autonomous Notch Signaling in Cardiomyocytes of the Embryonic and Adult Heart

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Rationale: The Notch signaling pathway is important for cell–cell communication that controls tissue formation and homeostasis during embryonic and adult life, but the precise cell targets of Notch signaling in the mammalian heart remain poorly defined.

Objective: To investigate the functional role of Notch signaling in the cardiomyocyte compartment of the embryonic and adult heart.

Methods and Results: Here, we report that either conditional overexpression of Notch1 intracellular domain (NICD1) or selective silencing of Notch signaling in the embryonic cardiomyocyte compartment results in developmental defects and perinatal lethality. In contrast, augmentation of endogenous Notch reactivation after myocardial infarction in the adult, either by inducing cardiomyocyte-specific Notch1 transgene expression or by intramyocardial delivery of a Notch1 pseudoligand, increases survival rate, improves cardiac functional performance, and minimizes fibrosis, promoting antiapoptotic and angiogenic mechanisms.

Conclusions: These results reveal a strict requirement for cell-autonomous modulation of Notch signaling during heart morphogenesis, and illustrate how the same signaling pathway that promotes congenital heart defects when perturbed in the embryo can be therapeutically redeployed for the treatment of adult myocardial damage. (Circ Res. 2010;106:559-572.)

Key Words: Notch ■ heart ■ development ■ myocardial infarction
Notch1, Notch2, and Rbp-Jk display early embryonic lethality caused by multiple cardiac defects.9–12 The actions of Notch in heart morphogenesis are spatially and temporally diverse; although Notch signaling is required for ventricular myocardial differentiation,9,13 cardiomyocyte-specific ablation of Notch1 does not affect cardiac development in mice,14 suggesting that Notch pathway regulates cardiogenesis by signaling in nonmuscle cell types of the embryonic heart, such as the adjacent endocardium,9 as well as earlier in mesodermal development.15

Several lines of evidence also implicate Notch signaling as a potent modulator of regeneration in the adult. Studies on Xenopus tail,16 medaka fin,17 and mammalian liver18 regeneration have linked Notch signaling with tissue regeneration. In the mouse, reactivation of Notch signals in models of brain ischemia19 and skeletal muscle injury20 promotes tissue repair. In the heart, activation of the Notch pathway precedes cardiac regeneration in zebrafish, a model organism with remarkable reparative capacity.21 Endogenous Notch signaling is downregulated in the adult mammalian heart,22,23 which may underlie its poor regenerative prognosis. Indeed, genetic ablation of Notch1 gene specifically in mouse cardiomyocytes results in cardiac hypertrophy, fibrosis and compromised function under stress conditions,14 whereas injection of a viral vector expressing the Notch1 intracellular domain (NICD1) into infarcted mouse hearts improves hemodynamic function.23 In vitro, Notch activation induces cell cycle reentry in quiescent cardiomyocytes22,24 and plays an important role in cardiac cell differentiation.25,26 Taken together, these studies highlight the complexity of functions played by Notch1 signaling during heart morphogenesis and adult cardiac repair.

To characterize the role of Notch signaling specifically in the differentiated cardiomyocyte compartment of developing and adult hearts, we used conditional and inducible mouse mutagenic models. Selective inhibition of Notch signaling or overexpression of NICD1 (which signals cell autonomously) in cardiomyocytes of the embryonic heart led to morphological defects and embryonic and perinatal lethality. In contrast, supplementing the transient de novo activation of the Notch signaling pathway after cardiac infarction with sustained induction of a cardiomyocyte-restricted NICD1 transgene blunted myocardial injury by promoting cardiomyocyte survival. The therapeutic relevance of this observation is supported by direct intramyocardial delivery of a Notch1 pseudoligand, which enhanced neovascularization and antiapoptotic signals in the injured heart. We conclude that aberrant Notch signaling in differentiated cardiomyocytes perturbs normal morphogenesis of the developing heart, whereas in a regenerative context, cardiomyocyte-specific Notch signaling can be beneficially redeployed for the treatment of acquired HD.

**Methods**

All mouse procedures were approved by European Molecular Biology Laboratory Monterotondo Ethical Committee (Monterotondo, Italy) and were in accordance with national and European regulations. Experimental protocols are described in the expanded Methods section available in the Online Data Supplement at http://circres.ahajournals.org.

**Results**

**Notch1 Signaling Is Activated in Embryonic and Adult Cardiomyocytes**

Notch receptors exhibit a dynamic expression pattern during cardiac development.10,27,28 In particular, Notch1 is highly induced in the endocardium during early (embryonic day [E]8.5 to E10.5) cardiogenesis.9,29 In addition, we detected the activated form of Notch1 receptor (NICD1) in the nucleus of atrial and ventricular cardiomyocytes at later stages in the embryonic (E13.5) and neonatal (postnatal day [P]1) heart, performing double immunofluorescence staining for NICD1 and the cardiomyocyte marker α-actinin (Figure 1A and 1B).

In the adult, Notch1 activity has been documented in cardiomyocytes22,23 and cardiac precursor cells.14,30 Correspondingly, NICD1 activity was detected in the nuclei of cardiomyocytes (Figure 1C), as well as in endothelial and smooth muscle cells of the cardiac vessels (Online Figure I),

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which is in agreement with recent observations during avian coronary development.\textsuperscript{31} Thus, active Notch1 signaling persists in embryonic and postnatal hearts, leading us to study the mechanisms whereby Notch1 receptor signaling in the cardiomyocyte compartment mediates heart morphogenesis and repair.

**Selective Overexpression of NICD1 in Cardiomyocytes Results in Multiple Cardiac Defects**

To gain insight into the role of Notch1 receptor in embryonic cardiomyocytes, we bred transgenic mice carrying a single copy of an NICD1 floxed allele (CAG-CAT-NICD1)\textsuperscript{15} with αMyHC-Cre transgenic mice\textsuperscript{32} to achieve cardiomyocyte-specific NICD1 overexpression. αMyHC-Cre recombinase expression initiates at E8.5, peaks at birth and is maintained at high levels during postnatal life in both atrial and ventricular cardiomyocytes.\textsuperscript{32} Double transgenic offspring, hereafter referred to as NICD1-COE (cardiomyocyte overexpression), were born close to Mendelian frequency (20.1\% instead of 25\%) but displayed perinatal lethality caused by multiple cardiac defects. Hence, only a subgroup of NICD1-COE reached weaning (5.1\% instead of 25\%) and died around 3 to 4 months of age (Online Table I). To confirm Notch gain-of-function, we assessed the cardiac expression of the Notch targets Hey1, Hey2, and Hes1 at P1. Relative to control, NICD1-COE samples showed upregulation of the Hey1, Hey2 and Hes1 mRNA levels (Figure 2A), indicating constitutive activation of Notch signaling. As expected, NICD1 and HES1 protein levels were significantly increased in the NICD1-COE heart compared to control (Figure 2B). Additionally, double staining for α-actinin and NICD1 proved an increase in the number of NICD1 positive cardiomyocytes in the neonatal NICD1-COE heart, suggesting a cell-autonomous mode of action for NICD1 (Online Figure II, A).

To decipher the cause of perinatal lethality in NICD1-COE mice as well as the premature death of the few perinatal survivors, which reached 3 to 4 months of age, cardiac morphology was examined at various developmental stages. Whole mount analysis (Figure 2C and 2D, first image) revealed an increase in NICD1-COE heart size when compared to control sibs, with a readily enlarged right atrium and increased heart-to-body weight ratio at all stages examined (Online Figure II, B). Trichrome staining on longitudinal sections revealed thick myocardial walls in the neonatal and adult NICD1-COE heart (Figure 2C and 2D, second image), suggesting cardiomyocyte hyperplasia. Notably, NICD1-COE mice displayed a complete penetrance of ASDs at various postnatal stages (Figure 2C and 2D, third image; and data not shown), whereas combined ASD and VSD were present in 80\% of the NICD1-COE mice examined at P1 (Figure 2C, fourth image), indicating that left-to-right shunting of oxygenated and deoxygenated blood may have caused circulatory distress and perinatal lethality. NICD1-COE mice that reached adulthood also displayed ASD (Figure 2D, third and fourth images) and impaired cardiac function (Online Figure II, C), which are possible causes of premature death (3 to 4 months) in this mutant subgroup.

**NICD1-COE Animals Exhibit Cardiac Hyperplasia and Impaired Differentiation**

To assess whether excessive cardiomyocyte proliferation (cardiac hyperplasia) could be responsible for the thick myocardial walls observed in NICD1-COE mice, immunohistochemical (DAB) and double fluorescence staining for the mitotic (G2/M phase) marker phospho-Hist3 and α-actinin was performed on tissue sections from neonatal NICD1-COE samples showed upregulation of the Hey1, Hey2 and Hes1 mRNA levels (Figure 2A), indicating constitutive activation of Notch signaling. As expected, NICD1 and HES1 protein levels were significantly increased in the NICD1-COE heart compared to control (Figure 2B). Additionally, double staining for α-actinin and NICD1 proved an increase in the number of NICD1 positive cardiomyocytes in the neonatal NICD1-COE heart, suggesting a cell-autonomous mode of action for NICD1 (Online Figure II, A).

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differentiation markers. Apart from GATA binding protein 4 (Gata4) and myosin heavy chain 6 (Myh6), NICD1-COE hearts displayed increased expression levels of myosin heavy chain 7 (Myh7), atrial natriuretic peptide (Anp), brain natriuretic peptide (Bnp), and NK2 transcription factor related, locus 5 (Nkx2.5) at the neonatal stage (P1), suggesting impairment in myocardial differentiation (Figure 3C). Glucose transporter 1 (Glut1) mRNA was also upregulated in NICD1-COE hearts, indicating anabolic conditions (Figure 3C). Our data confirm that constitutive Notch1 signaling in the cardiomyocyte compartment sustains proliferation and perturbs differentiation, consistent with its role in promoting cell growth.

Silencing of Notch Signaling Perturbs Cardiac Morphogenesis
To unravel the role of Notch signaling specifically in cardiomyocytes and bypass possible Notch1-Notch2 redundancy issues, we exploited the conditional dominant negative Maml1 (Maml1DN) line previously described as a genetic tool for pan-Notch pathway inhibition in vivo, which inhibits canonical Notch signaling by all four mammalian Notch receptors. When homozygous Maml1DN/DN mice were bred with αMyHC-CRE transgensics, both αMyHC-CRE/+;Maml1DN/+ and αMyHC-CRE/+;Maml1DN/DN offspring were born at Mendelian ratios, were viable and fertile (Online Table 1). Histological analysis of neonatal αMyHC-CRE/+;
Maml1DN/DN hearts revealed occasionally thinner right ventricular (RV) walls, whereas other cardiac structures appeared normal (Figure 4A). Assessment of cardiac performance by echocardiography on adult Maml1DN/DN animals revealed no functional abnormalities (Figure 4B). These findings suggest a regional requirement for myocardial Notch signaling in RV morphogenesis; a notion further supported by a recent study where increased NICD1 activity was observed at the RV of the developing heart.29

To assess the role of Notch signaling earlier in cardiogenesis, homozygous Maml1DN/DN mice were bred with the Nkx2.5-CRE knock-in line, which allows robust expression of Maml1DN from E7.0 throughout development in all cardiac muscle cells.37 Interestingly, a small fraction of heterozygous Nkx2.5-CRE+/+;Maml1DN/DN offspring displayed embryonic lethality at E12.5 caused by abnormal atrial and ventricular development (Figure 4C). However, the majority of Nkx2.5-CRE+/+;Maml1DN/DN mutants were born and displayed perinatal lethality (Online Table I) caused by ASD, VSD and abnormal (hypoplastic) RV formation (Figure 4D). Importantly, no alive Nkx2.5-CRE+/+;Maml1DN/+ mutants were found at weaning (P21) (Online Table I). The variability in phenotypic defects observed in the Nkx2.5CRE+/+;Maml1DN/+ may be attributed to the functional competition between endogenous Maml1 protein and the Maml1DN mutant; however, these data suggest a requirement for cardiomyocyte-derived Notch signaling in early cardiac morphogenesis.

**Transient De Novo Notch Signaling Activation Following MI**

The activation of Notch signaling in response to cardiac injury was assessed in a mouse model of MI.38 NICD1
nuclear activity was detected in cardiomyocytes at the border zone (BZ) of the injury, as well as at the epicardial cell layer close to the BZ 1 week after MI (Online Figure III, A). Analysis by quantitative (q)RT-PCR of transcripts encoding Jag1, Notch1, Notch2, Notch3, and Notch4, and the Notch targets Hes1, Hey2 and Hey1 at several time points following MI revealed increased expression within the first week for all genes with the exception of Hey1 (Online Figure III, B; Online Figure IV). Concurrent upregulation of Jag1, Notch1, and Notch2 protein levels at 4 and/or 7 days following MI was observed (Online Figure III, C). Notably, activation of Notch signaling was transient following cardiac injury because mRNA levels of Notch1, Notch2, Hes1, and Hey2 reverted to normal values (sham) at 2 and 4 weeks after MI (Online Figure IV and data not shown).

Inducible Redeployment of Notch1 Signaling in Cardiomyocytes Blunts Myocardial Injury by Promoting Cell Survival

Because reactivation of Notch signaling leads to improved regeneration in mouse models of brain ischemia19 and skeletal muscle injury,20 we tested the potentially beneficial effects of sustained Notch signaling in the cardiomyocyte compartment of the infarcted heart. Tamoxifen-inducible αMyHC-mER-CRE-mER transgenics39 were bred with the CAG-CAT-NICD1 mice thereby allowing induction of cardiomyocyte-specific NICD1 overexpression. Double transgenic offspring were generated (referred to as αMyHC-mER-CRE-mER;NICD1 hereafter) and their response to MI was evaluated. In tests of recombination efficiency, αMyHC-mER-CRE-mER line crossed with the ROSA26;LacZ-loxP reporter40 progressively increased the number of recombined cardiomyocytes (X-gal–positive cells) after 1, 2, and 3 weeks of tamoxifen

![Figure 4. Silencing of Notch signaling perturbs cardiac morphogenesis. A, Trichrome staining on longitudinal tissue sections of +/++;MAML1DN/ON and αMyHCCRE/++;MAML1DN/ON hearts at P1 demonstrates thinner RV wall in the mutants (indicated by arrow). Bar, 0.5 mm. RA indicates right atrium; RV, right ventricle, LA, left atrium, LV, left ventricle. B, The cardiac function of adult (2- to 3-month-old) αMyHCCRE/++;MAML1DN/ON animals was assessed by echocardiography. Using LV trace on B-mode images, LV transversal area in systole (LVTA,s), LV transversal area in diastole (LVTA,d), and fractional shortening (FS) were calculated at the indicated time points; n=3. C, Whole-mount visualization of +/+;MAML1DN/ON and Nkx2.5CRE/++;MAML1DN/+ embryos at E12.5. Note the underdevelopment of the Nkx2.5CRE/++;MAML1DN/+ embryo at this stage and the abnormal morphology of all cardiac chambers (bottom). LA indicates left atrium; RA, right atrium. Bar, 0.1 mm. D, Trichrome staining on longitudinal cardiac sections from +/+;MAML1DN/+ and Nkx2.5CRE/+;MAML1DN/+ animals at P1. Arrows indicate abnormal RV, arrowhead shows the presence of ASD, and asterisk pinpoints a VSD in the Nkx2.5CRE/+;MAML1DN/+ neonatal heart. Representative images from 2 +/+;MAML1DN/+ hearts and three Nkx2.5CRE/+;MAML1DN/+ hearts are shown. Note that the bottom image of Nkx2.5CRE/+;MAML1DN/+ hearts demonstrates different planes of the same heart to present clearly the defects. Bar, 0.5 mm.](http://circres.ahajournals.org/Download/564_Circulation_Research_February_19,2010)
diet (Online Figure V). Similarly, NICD1 protein levels were gradually upregulated in the heart of mER-CRE-mER;NICD1 mice after 1, 2, and 3-week tamoxifen administration, confirming inducible Notch signaling activation in adult cardiomyocytes (Online Figure V). To maximize recombination and minimize the risks of adverse effects caused by prolonged NICD1 levels, we subjected control and mER-CRE-mER;NICD1 mice to MI 1 week after the tamoxifen diet initiation. Tamoxifen diet was maintained for an extra week following MI (when myocardial injury is complete); subsequently mice were transferred to normal diet and euthanized 4 weeks after MI (Figure 5A). This experimental scheme was followed because intraperitoneal administration of tamoxifen or force-feeding with tamoxifen did not lead to reproducible results in tests of gene recombination efficiency.

Under basal conditions (sham operation), overexpression of NICD1 in cardiomyocytes did not affect cardiac size (Figure 5A), animal survival (Figure 5C), and cardiac performance (Figure 5D through 5F). However, mER-CRE-mER;NICD1 mice displayed improved survival rate and cardiac function compared to controls at 1, 2, and 4 weeks following MI (Figure 5C through 5F). Moreover, the heart weight/body weight ratio was significantly downregulated in mER-CRE-mER;NICD1 mice 4 weeks after MI, indicating that adverse myocardial remodeling and dilation are curtailed in the mutants (Figure 5A). Indeed, trichrome staining confirmed reductions in replacement fibrosis and cardiac dilation in the mER-CRE-mER;NICD1 mice 4 weeks after MI (Figure 5B).

In view of the increased cardiomyocyte proliferation observed in the neonatal NICD1-COE heart, staining for the cell cycle marker Ki67 and the mitotic marker phospho-Hist3 was performed on infarcted mER-CRE-mER;NICD1 hearts. The numbers of Ki67 positive cardiomyocytes were significantly increased in the mER-CRE-mER;NICD1 heart at 2 and 28 days after infarction (Figure 6A), suggesting that Notch signaling activation induces cell cycle reentry in adult cardiomyocytes. However, no significant differences were observed in the number of phospho-Hist3 positive cardiomyocytes at 2 and 28 days following MI (Figure 6B), indicating that Notch signaling activation triggers incomplete cell cycle progression in adult cardiomyocytes. Importantly, blockade of apoptosis was implicated in Notch-1 mediated cardioprotection, as the numbers of apoptotic cells were significantly decreased in the left ventricular (LV) wall of mER-CRE-mER;NICD1 mice at 2 (Figure 6C) and 28 (Online Figure VI) days after MI, indicating that Notch1 signaling promotes cell survival in the injured heart. Additionally, TUNEL assay accompanied by costaining for α-actinin demonstrated reduced numbers of TUNEL positive cardiomyocytes in the heart of mER-CRE-mER;NICD1 animals (7.72% versus 3.25%, P<0.01) at 2 days after infarction (Figure 6D), revealing cell autonomous antiapoptotic effects of Notch activation in the cardiomyocyte compartment.

To confirm the effects of Notch signaling specifically on cardiomyocyte survival, primary cardiomyocytes were cultured at normal oxygen and glucose levels and showed a dose-dependent decrease in their survival rate following Notch pathway inhibition by 2 γ-secretase inhibitors (L-685,458 and DAPT) (Online Figure VII, A and B) at previously described noncytotoxic concentrations (2.5 to 10 μmol/L L-685,458; 10 to 75 μmol/L DAPT). Using an oxygen-glucose deprivation model (previously used in neuronal ischemia studies) to simulate cardiomyocyte ischemia in vitro, we observed statistically significant reduction in cardiomyocyte survival even when 2.5 μmol/L L-685,458 or 10 μmol/L DAPT were used under oxygen-glucose deprivation conditions (Online Figure VII, C), suggesting a prosurvival role for Notch signaling in isolated cardiomyocytes.

**Intramyoimdellar Delivery of a Notch1 Pseudoligand Favors Cardioprotection and Enhances Neovascularization**

To confirm the beneficial effects of Notch activation on cardiac recovery in a clinically relevant protocol, a Notch-1-activating antibody (Notch-1 Ab) was injected at 2 sites (left and right) of the BZ 5 minutes after MI on wild-type mice, which were followed up by echocardiography and euthanized 4 weeks later (Figure 7A). Upregulation of Hes-1 and Hey-1 mRNA levels 6 hours after Notch1 antibody injection confirmed Notch signaling activation in the heart of these mice (Figure 7B) but not in the control group (injected with an isotype-matched hamster IgG1 antibody). One week after injury, Notch-1 Ab hearts (compared to IgG1 Ab hearts) showed minimized scar tissue deposition and did not progress to dilation and failure (Figure 7C). Indeed, trichrome staining proved reduction in LV scar volume and increase in LV muscle sustenance in Notch-1 Ab hearts 4 weeks after MI (Figure 7D). Survival rate (Online Figure VIII, A) and cardiac functional performance (Figure 7E) were also significantly ameliorated in Notch-1 Ab mice 2 and 4 weeks after MI, whereas expression of heart failure markers such Myh6, Myh7, and Glut-1 was decreased in the Notch-1 Ab hearts (Figure 8A). A concomitant reduction in transcripts encoding proapoptotic protease caspase 9 (Casp3) and upregulation of the antiapoptotic gene Bcl-2 was observed in Notch-1 Ab hearts compared to IgG1 Ab controls 4 days after MI, whereas the expression of other apoptosis-related genes (Casp3, Casp6 and Bcl-XL) remained unaltered (Figure 8B).

Given the requirement of Notch signaling for angiogenesis and the observed NICD1 activity in endothelial and smooth muscle cells of the cardiac vessels (Online Figure I), we assessed whether neovascularization is promoted in the Notch1 Ab hearts. Staining for the angiogenesis marker smooth muscle α-actin (α-SMA) revealed increased number of small-diameter vessels at the BZ myocardium of Notch1 Ab hearts when compared to IgG1 Ab controls 4 weeks after infarction (Figure 8C, top), indicating enhanced neovascularization. Consistently, staining for the endothelial markers PECAM-1 and isoclectin B4 (Figure 8C, middle and bottom) demonstrated increased numbers of endothelial cells in the Notch1 Ab hearts. Moreover, we evaluated the levels of 5-bromodeoxyuridine incorporation at the coronary vessels of IgG1 Ab and Notch-1 Ab injected hearts 1 month after MI. The number of 5-bromodeoxyuridine-positive cells associated with coronary vessels (Border Zone) of Notch-1 Ab treated animals was
Figure 5. Temporally controlled Notch1 signaling activation in cardiomyocytes protects against MI. A, Experimental scheme. After 1 week of tamoxifen diet, MI was performed on adult wild-type (control) and mER-CRE-mER;NICD1 mice. Tamoxifen was administered for 1 more week; mice were euthanized 4 weeks after MI. Bottom, Heart/body weight ratio (HW/BW, g/g) of sham-operated (sham) and infarcted (MI) control and mER-CRE-mER;NICD1 mice at the indicated time points. B, Trichrome staining on cross-sections (apex-to-base) from control and mER-CRE-mER;NICD1 hearts (4 weeks after MI). Blue indicates collagen (asterisk); red, myocardium. Samples are shown in duplicates. Bar, 1 mm. n=7. Quantification of LV fibrosis provided at the bottom. C, Survival rate on control and mER-CRE-mER;NICD1 (MUT) mice at 1, 2, and 4 weeks following MI or sham operation (sham); n=20. For the control group, 7 samples were mER-CRE-mER-positive, 6 were NICD1-positive, and 7 had no transgene. D through F, Assessment of cardiac function (echocardiography) of control and mER-CRE-mER;NICD1 (MUT) mice before and after MI or sham operation (sham). Using LV trace on B-mode images, LV transversal area in systole (LVTA;s), LV transversal area in diastole (LVTA;d), and fractional shortening (FS) were calculated at the indicated time points; n=7. *P<0.05, **P<0.01, ***P<0.001 compared to control MI.
Figure 6. Notch1 signaling activation promotes cardiomyocyte survival but not proliferation in the adult heart. A, Representative confocal micrographs from control and mER-CRE-mER;NICD1 hearts (4 weeks after MI) stained for Ki67 (green; bottom), α-actinin (red), and nuclei (blue). Arrows and arrowheads indicate mononucleated and binucleated cycling cardiomyocytes, respectively. Boxed regions are shown in higher magnification at right. Quantification at 2 and 28 days after infarction is presented at the bottom. BZ indicates border zone; IZ, ischemic zone. Bar, 75 μm; n=6. B, Representative confocal micrographs from control and mER-CRE-mER;NICD1 hearts (2 days after MI) stained for phospho-Hist3 (green; bottom), α-actinin (red), and nuclei (blue). Quantification at 2 and 28 days after infarction is presented at the bottom. Bar, 50 μm; n=6. C, TUNEL assay on control and mER-CRE-mER;NICD1 hearts at 2 days after MI. Quantification of TUNEL-positive cells (green)/nuclei (blue) is provided at right. Bar, 25 μm; n=6. D, TUNEL assay accompanied by staining for α-actinin on control and mER-CRE-mER;NICD1 hearts at 2 days after MI. Quantification of TUNEL-positive cells (green)/nuclei (blue) is provided at right. Bar, 50 μm; n=6. *P<0.05, **P<0.01, ***P<0.001 compared to control. For the control group, 2 samples were mER-CRE-mER-positive, 2 were NICD1-positive, and 2 had no transgene.
increased compared to those treated with IgG1 Ab (Online Figure VIII, B), suggesting that in contrast to the mER-Cre-mER;NICD1 model, endothelial and smooth muscle cell proliferation was also triggered by Notch-1 Ab administration leading to increased neovascularization.

Selective Silencing of Notch Signaling in Adult Cardiomyocytes Results in Impaired Cardiac Function After MI

To unravel the physiological significance of Notch activation in adult cardiomyocytes after MI, control and αMyHC-CRE/+/Maml1DN/DN animals were subjected to MI and followed-up by echocardiography for 4 weeks. Importantly, the αMyHC-CRE/+/Maml1DN/DN animals exhibited reduced survival rate (Online Figure IX, A) and severely impaired cardiac function at 1, 2, and 4 weeks after infarction, as assessed by measurements for fractional shortening, LV transverse area in systole and diastole (Online Figure IX, B through D). Moreover, the heart to body weight ratio was increased in the αMyHC-CRE/+/Maml1DN/DN animals after infarction (Online Figure IX, E), which is an indication for adverse myocardial remodeling. Taken together, these results illustrate the significance of Notch activation in adult cardiomyocytes after MI and complement our inducible gain-of-function approach.

Discussion

This study establishes a cell-autonomous role for Notch signaling in cardiomyocyte proliferation and differentiation during heart development, and demonstrates how early dysregulation of Notch signaling in the mouse embryonic cardiomyocyte compartment recapitulates the pathogenesis of human congenital HD linked to Notch mutations. Conversely, in a pathological context, induction of supplemental intracellular Notch signaling in the adult myocardium improves cardiac function, reduces replacement fibrosis and increases myocyte viability, affecting survival and providing a novel paradigm for improving clinical outcome in HD.
Taken together, these observations pinpoint the cardiomyocyte as a key target of Notch action in both the embryonic and adult heart, and illustrate the critically different outcomes obtained by perturbing this signaling pathway at various life stages. Genetic manipulation of Notch signaling in the embryonic cardiomyocyte compartment led to severe cardiac malformation, including abnormal atrial and ventricular growth and septal defects, in both gain-of-function (NICD1-COE) and loss-of-function (Nkx2.5-Cre;MAML1DN) mutants. The hyperproliferative phenotype of NICD1-COE mutants specifically point to a critical role for Notch in regulating growth during normal cardiac morphogenesis, and defects caused by early disruption of the pathway in Nkx2.5-Cre;MAML1DN mutants suggest that the initial steps of heart development depend on Notch signaling in the working myocardium. However, we cannot exclude the possibility that the activation of the Nkx2.5 promoter in areas surrounding the developing heart could also contribute to the cardiac defects observed in the Nkx2.5-Cre;MAML1DN mutants. It is also tempting to speculate that Notch signaling plays a role in concert with the transcription factor Nkx2.5, a master regulator of heart development, because Nkx2.5 driven CRE expression comes to the expense of losing 1 allele of the Nkx2.5 gene in the Nkx2.5-Cre;MAML1DN mutants.

Fate-mapping studies in chick and mouse embryos highlight 2 distinct mesodermal heart fields that contribute cells to the developing heart. The first heart field contributes to the left ventricle and parts of the atria, whereas the second heart field appears later and contributes to the right ventricle, two-thirds of the atria and parts of the outflow tract. Our results indicate that second heart field–derived structures are particularly sensitive to Notch signaling, as illustrated by the cardiac defects observed in NICD1-COE hearts including enlarged right atrium and RV, ASD, and VSD and significantly increased right-sided cardiomyocyte proliferation; the ASD, VSD, and abnormal RV of the Nkx2.5-Cre;MAML1DN hearts; and the thin RV wall observed in the aMyHC-Cre;MAML1DN hearts. This interpretation is consistent with the right-sided defects previously observed in Hey2−/− and Jag1+/-;Notch2+/- hearts and with the previously reported role of Notch in left-right asymmetry determination.

The range of phenotypes (abnormal RV, ASD, and VSD) presented by NICD1-COE and Nkx2.5-Cre;MAML1DN mice
in the present study are reminiscent of heart defects seen in humans with AGS. Although human Jag1 and Notch2 mutations have linked Notch pathway with this syndrome, it is unclear whether these mutations reflect a loss or gain of function. For example, frameshift and missense mutations have been identified in AGS patients leading to a truncated or an intact protein product, respectively; raising the possibility that altered Notch signaling could be involved in the syndrome’s expressivity. Mice deficient in the Notch target gene Hey2 or haploinsufficient for Jag1 and Notch2 partially phenocopy the AGS cardiac defects, suggesting that loss-of-function mutations in the Notch pathway could be responsible for AGS. However, similar phenotypes presented here in both loss- and gain-of-function approaches rather indicate that different levels of Notch signaling specifically in cardiomyocytes underlie the cardiac manifestations of AGS and related forms of congenital HD.

Our results also indicate that Notch signaling in cardiomyocytes is not essential for later stages of cardiac formation. Indeed, later ablation of Notch signaling in cardiomyocytes using the αMyHC promoter (αMyHC-Cre;MAML1DN mice) was compatible with life, accompanied by a subtle cardiac defect (thin RV wall) but normal functional performance at the adult stage. Consistently, late deletion of the Notch1 or Notch2 receptor specifically in ventricular cardiomyocytes using the MLC2v-Cre and SM22α-Cre mouse line, respectively, did not result in cardiac pathology.14,28

Notch signaling is naturally downregulated in the adult mammalian myocardium and the transient activation of endogenous Notch signaling we observed following MI is clearly insufficient to launch an effective response to cardiac damage. The amelioration of cardiac performance in mER-CRE-mER;NICD1 mice following MI is consistent with a prosurvival cell autonomous role for Notch signaling in cardiomyocytes, supported by the significant decrease in TUNEL positive cardiomyocytes observed in infarcted mER-CRE-mER;NICD1 hearts and by the reduction in cardiomyocyte viability following Notch inhibition in our in vitro cardiac ischemia platform. This model is consistent with the observed activation of the Akt survival kinase after adenoviral delivery of NICD1 in the adult heart and the increased apoptosis (following hypertrophic stimuli) in the heart of mice lacking Notch1 specifically in cardiomyocytes.14 Notch-1 pseudoligand delivery to the infarcted heart also activated survival mechanisms such as downregulation of Casp9 and upregulation of Bcl-2 at 4 days post MI, reminiscent of the synergy between Notch1 and Bcl-2 described in the context of regeneration in fish.65

This study raises important considerations regarding possible adverse cardiac effects in clinical trials where Notch/γ-secretase inhibition has been associated with improved clinical outcome for Alzheimer’s disease and colon cancer. In contrast, we provide evidence that manipulation of Notch signaling in a temporally controlled and cell type-restricted manner could lead to new therapeutic avenues for individuals with congenital and acquired forms of HD. Moreover, augmentation of Notch signaling in the adult damaged heart, as demonstrated here with an inducible genetic strategy, clearly illustrates how redeployment of embryonic signaling could promote cardioprotection and regeneration. Finally, manipulation of Notch signaling in vivo using the Notch1 pseudoligand protocol provides a new clinically relevant avenue to exploit this signaling pathway in the therapy of post-MI patients.

Acknowledgments
We thank Dr Yumiko Saga for providing the CAG-CAT-NICD1 transgenic line through RIKEN BRC and Dr Warren Pear for the Mam1DN knockout mouse line. The Notch2 monoclonal antibody (C651.6DbHN) was obtained from the Developmental Studies Hybridoma Bank (University of Iowa). We also thank E. Slominsky and Gulia Zanin for technical assistance, E. Perlas for advice and protocols concerning the histological analysis, S. Tatti for animal care, and members of the Rosenthal laboratory and Dr Cornelius Gross for critical comments and helpful discussions.

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Disclosures

References

Novelty and Significance

What is Known?
- Depending on the context, Notch signals may promote or suppress cell proliferation, cell death, or cell differentiation.
- Congenital and acquired forms of HD in humans have been linked to Notch pathway mutations.
- Studies on various model organisms propose Notch signaling as a potent modulator of tissue regeneration.

What New Information Does This Article Contribute?
- Notch actions in the cardiomyocyte compartment regulate cardiac morphogenesis.
- Perturbation of Notch signaling in embryonic cardiomyocytes produces cardiac malformations typical of Notch-linked congenital HD in humans.
- Notch signaling can be beneficially redeployed in the adult heart to improve clinical outcome following MI.

Notch pathway mutations in humans and mice have implicated this evolutionarily conserved signaling cascade in congenital and acquired forms of heart disease (HD). Despite the well-established role for Notch signaling in intercellular communication during tissue formation and organ homeostasis, its precise cell targets in the mammalian heart remain poorly defined. Using sophisticated mouse genetics, the present study pinpoints the cardiomyocyte as a key target of Notch action in both the embryonic and adult heart, clarifying a number of previous reports lacking cell-type specificity. Briefly, selective inhibition or activation of Notch signaling pathway in cardiomyocytes of the embryonic heart led to morphological defects, embryonic and perinatal lethality. Conversely, augmentation of endogenous Notch reactivation after myocardial infarction (MI), either by Notch(ic).

References

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http://circres.ahajournals.org/content/suppl/2009/12/10/CIRCRESAHA.109.203034.DC1
SUPPLEMENTAL MATERIAL

(Contains Detailed Methods, Online Figures I – IX, and Online Tables I & II)

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DETAILED METHODS

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In vivo Notch1 activation
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Cardiac morphology and morphometric analysis
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Immunofluorescence microscopy
Immunohistochemical (DAB) staining
BrdU staining
Sample preparation and Immunoblotting
Quantitative Real Time PCR
Primary neonatal cardiomyocyte culture
Oxygen Glucose Deprivation (OGD) model and MTT viability assay
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Online Figure II Increased levels of NICD1 in cardiomyocytes lead to impaired functional performance in NICD1-COE animals.
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DETAILED METHODS

Experimental animals. The αMyHC-CRE mice\(^1\) were crossed with the conditional CAG-CAT-NICD1 transgenic mice\(^2\) in order to generate offspring that overexpress the Notch-1 receptor intracellular domain (NICD1) only in cardiomyocytes (NICD1-COE). Initially, NICD1-COE mice were generated on mixed MCH (ICR); C57BL/6 background and later on backcrossed for 8-10 generations in order to obtain the isogenic C57BL/6 background. The genetic background did not affect the phenotype penetrance of NICD1-COE offspring. The knock-in dominant negative Maml1 (Maml1\(^{DN}\)) mouse model has been generated by placing the Maml1\(^{DN}\) fragment downstream of a neomycin cassette, which is flanked by loxP sites, in the ubiquitously expressed ROSA26 locus\(^3, 4\). The Maml1\(^{DN}\) mouse line was bred with the cardiomyocyte-specific Nkx2.5-CRE\(^5\) and αMyHC-CRE\(^1\) lines in order to achieve Notch pathway silencing at early and later stages during cardiac development, respectively.

Tamoxifen-inducible αMyHC;MER-CRE-mer transgenics\(^6\) were crossed with CAG-CAT;NICD1\(^2\) mice to achieve cardiomyocyte-specific NICD1 over-expression in the adult. Mice were placed on tamoxifen-containing chow (Harlan Special Diet TD.55125) at 2-3 months of age. Genotyping was performed by PCR using genomic DNA from tail biopsies. All mouse procedures were approved by European Molecular Biology Laboratory Monterotondo Ethical Committee (Monterotondo, Italy) and were in accordance with national and European regulations.

Induction of myocardial infarction. Male C57BL6 10-12 week old mice (HARLAN) were anesthetized with isoflurane (2.5% in O\(_2\)) during the operation. The animals were orally incubated using a Harvard Apparatus ventilator. A small thoracoctomy was performed to expose the heart and after pericardial opening the left coronary artery was ligated using 8-0 ETHILON nylon suture (ETHICON) through a dissecting microscope. After the closure of the thorax and skin using 6-0 ETHILON nylon suture (ETHICON), the animals were allowed to recover in a clean temperature controlled (22\(^\circ\)C) mouse facility and sacrificed at the indicated time points.

In vivo Notch-1 activation. Five minutes after induction of myocardial infarction, 10µl of a 1:4 dilution of the Notch-1 pseudoligand (Chemicon International, MAB5414) were injected in two sites (left and right) at the border zone of the ischemic myocardium. Isotype-matched hamster IgG (AbCam, ab18426) was used at the corresponding concentration for the control group of animals.

Echocardiography analysis. Animals were anesthetized with isoflurane (1.5% in O\(_2\)) and left hemithorax was shaved. The mice were placed on a temperature-controlled pad and heart rate was continuously monitored (400-550bpm). Ultrasound transmission gel (Parker Laboratories Inc.) was used and the heart was imaged in the parasternal short-axis view. Two-dimensional B-mode images were obtained at the papillary muscle level using the Vevo 770 Ultrasound system (VisualSonics) and left ventricular transversal area in systole (LVTA; s), left ventricular transversal area in diastole (LVTA; d), and fractional shortening were calculated using the using the Vevo 770 V2.2.3 software (VisualSonics).

Cardiac morphology & morphometric analysis. Hearts were excised at the indicated developmental stages and fixed over-night at 4% paraformaldehyde. Following progressive tissue dehydration with ethanol and xylene, the heart samples were embedded in paraffin. Eight micrometer (µm) thick cross-sections were subjected to trichrome staining (Sigma-Aldrich) for nuclei, cytoplasm and collagen visualization. Images were collected using a Leica DMR microscope and a Leica DC 500 camera (Leica Microsystems). Morphometric analysis for collagen tissue deposition was performed with MetaMorph software (version 6.2r5; Universal Imaging Corporation).

TUNEL Assay. Eight µm cardiac cross-sections were deparaffinized in xylene and gradually dehydrated by immersion in ethanol solutions with different concentration (100%, 95%, 85%, 70%, 50%). Then, the
slides were placed in 0.85% NaCl for 5 min and later fixed in 4% paraformaldehyde at room temperature. The samples were permeabilized by proteinase K treatment (Promega) for 30 min and then fixed again in 4% paraformaldehyde. After an equilibration step, the 3'-OH end of DNA breaks was labeled with fluorescein-12-dUTP using a recombinant Terminal Deoxynucleotidyl Transferase (rTdT) enzyme (Promega) by incubating the slides at 37°C for 1 hour. The reaction was terminated in 2X SSC solution and after washing in PBS the slides were mounted in medium with DAPI (Vectashield). Images were obtained using the Leica TCS SP5 spectral confocal microscope system and the LAS AF software (Leica, Microsystems). Quantification of TUNEL positive cells was performed on a blind basis after acquisition of at least 4 random images from border zone (BZ) and ischemic zone myocardium (IZ) per heart at 200x magnification.

**Immunofluorescence microscopy.** Animals were sacrificed at the indicated time points and the heart was excised and then fixed over-night in 4% paraformaldehyde. Following standard dehydration procedures, the heart tissue was embedded in paraffin. Eight µm thick cross-sections were produced and deparaffinized in xylene. After immersion in ethanol solutions with different concentrations (100%, 95%, 85%, 70%, 50%), antigen unmasking was performed submerging the slides in R-buffer BG (PickCell Laboratories) in a pressure cooker. Next, samples were incubated with blocking solution (10% goat serum, 0.2% fish skin gelatin and 0.01% Tween 20). Antibodies against cleaved Notch-1 (1:100, Abcam), smooth muscle α-actin (1:200, Sigma), α-actinin (1:200, Sigma), phospho-Hist3 (1:50, Cell Signaling) and Ki67 (1:100, Dianova) were diluted in 0.2% fish skin gelatin/0.01% Tween 20 in PBS and the slides were incubated over-night at 4°C in a humidified chamber. After washing in PBS, the sections were incubated with the appropriate Cy2-, Cy3-conjugated secondary antibodies (1:500, Jackson ImmunoResearch) for 1.5 hours in the dark at room temperature. The nuclei were counterstained with DAPI and the slides were analyzed using the Leica TCS SP5 spectral confocal microscope system and the LAS AF software (Leica, Microsystems). Following acquisition of at least 10 random images per cardiac chamber per heart at 400x magnification, quantification of phospho-Hist3 or Ki67 positive cardiomyocytes was performed on a blind basis.

**Immunohistochemical (DAB) staining.** Slides were deparaffinized in xylene and rehydrated in serial ethanol concentrations. Antigen unmasking was performed in citrate buffer (10mmol/Lt sodium citrate, pH 9.0 for membrane/cytoplasmic proteins or pH 6.0 for nuclear proteins) for 30 min at 80°C. Quenching of endogenous peroxidase activity took place in 0.5% H2O2/dH2O for 10 min and then slides were blocked with 5% normal goat serum (DAKO) for 1 h at room temperature (RT). Incubation with the primary antibody against smooth muscle α-actin (1:200/Sigma), phospho-Hist3 (1:50, Cell Signaling), Pecam-1 (1:10, BD Biosciences), and Isolectin-B4 (1:50, Vector Laboratories) was done over-night (O/N) at 4°C. After washing in PBS, the slides were incubated with the appropriate secondary antibody (peroxidase conjugated) for 60 min at RT. The slides were again washed in PBS and the diaminobenzidine (DAB) chromogenic reaction (Vector Laboratories) was employed in order to visualize the staining (brown color). Then, slides were dehydrated through serial ethanol concentrations and xylene and mounted permanently. Quantification of smooth muscle α-actin positive cardiac vessels was performed on a blind basis after acquisition of at least 5 images from the border zone myocardium of each sample at 100x magnification. Quantification of Pecam-1 and Isolectin-B4 positive area was performed on a blind basis after acquisition of at least 5 images from the border zone myocardium of each sample at 200x magnification and using MetaMorph software.

**BrdU staining.** 0.1% BrdU (Sigma) was administered ad libitum in the drinking water for the first 10 days after the induction of myocardial infarction. A three-week wash-out period (normal drinking water) followed and the mice were sacrificed. Hearts were perfused and embedded in paraffin. Eight micrometer (µm) thick sections were produced and deparaffinized in xylene. Next, the slides underwent rehydration by submersion in ethanol serial solutions with different concentrations (100%, 95%, 80%, 70%, 50%) and
endogenous peroxidase activity was quenched with 0.3% H₂O₂/methanol for 30min at RT. Antigen retrieval was done using citrate buffer (10mmol/lt, pH 6.0) in a microwave. Then, the slides were allowed to cool down for 30min and later incubated with the primary antibody (mouse IgG) against BrdU in the nuclease solution (RPN20EZ, Amersham Biosciences) for 1hour in a humidified chamber at 37°C. After three washes in PBS, the secondary incubation with an anti-mouse IgG horseradish peroxidase conjugated (Jackson ImmunolohyResearch) was performed for 2hours at RT and signals were detected with the DAB chromogenic reaction (Vector Laboratories). Dedyhydration in ethanol series took place and the slides were mounted permanently. Images were collected using a Leica DMR microscope and a Leica DC 500 camera (Leica Microsystems).

Sample preparation and Immunoblotting. Whole protein extracts were obtained after homogenization (doucing) of the heart tissue in protein extraction buffer (20 mmol/lt Tris-HCl pH 8.0, 100 mmol/lt NaCl, 5 mmol/lt EGTA, 2 mmol/lt EDTA, 0.5% Triton-X, 100 mmol/lt PMSF in ETOH, 200 mmol/lt sodium orthovanadate, 1 μg/ml aprotinin) and subsequent centrifugation at maximum speed (14,000rpm) at 4°C. The supernatant was recovered and protein determination was done using Brandford method (Biorad). 40 μg of protein lysates were separated in 10% SDS polyacrylamide mini-gel (Biorad system) and transferred onto a hybrid ECL nitrocellulose membrane (Amersham). Filters were blocked with 5% milk, blotted with specific antibodies o/n at 4°C, washed with 3 times for 30min with washing buffer (TBS and 0.1% Tween-20) and blotted with specific secondary antibodies (horseradish peroxidase-conjugated, 1:5000, Amersham) with 5% milk for 1h at RT. The filter was incubated for 1 min using ECL kit (Amersham) before exposure. The following primary antibodies were used: anti-Notch1 (1:500, Sigma), anti-Notch2 (1:500, Hybridoma Bank, University of Iowa) and anti-Jag1 (1:500, Santa Cruz). Anti-α-tubulin (1:2000, SIGMA) was used for normalization. Following immunoblotting, quantification of protein levels was performed by densitometry.

Quantitative Real Time PCR. Total RNA was isolated from hearts using TRIzol (Invitrogen). RNA was treated with DNaseI enzyme (Promega) for 1h at 37°C and then cleaned by column purification (Qiagen). The RNA concentration was determined with a spectrophotometer. After RNA quality verification, 1-2μg were used to prepare cDNA (Ready-To-Go, T-Primed First-Strand Kit, Amersham Bioscience). Inventoried TaqMan FAM probes (Applied Biosystems) were used for the relative quantification of the mRNA levels of Notch-1, Notch-2, Notch-3, Notch-4, Jagged-1, Hes-1, Hey-1, Hey-2, Myh6, Myh7, Anp, Bnp, Glut-1, Acta-1, Gata-4, Nkx2.5, Casp3, Casp6, Casp9, Bcl-2, Bcl-XL genes. Taqman VIC Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) probes were used for normalization. Data analysis was performed using the ΔΔC(T) method.

Primary neonatal cardiomyocyte culture. One-day-old C57Bl6 mice were sacrificed and hearts were excised. After scalpel homogenization, ventricular cardiomyocytes were isolated following a series of collagenase/pancreatin digestions (Collagenase type II, CSL2, Worthington/ Pancreatin 4x NF, Cat N. 45720-018, GIBCO) and cells were collected by centrifugation (8,000rpm for 5min). Next, fibroblasts were removed from the culture after a 45 min pre-plating step at 37°C in complete medium [DMEM/199 medium (5/1 ratio) supplemented with 10% heat inactivated horse serum (Sigma), 5% heat inactivated fetal calf serum (Sigma), 0.025 mol/lt Hepes, 0.002 mmol/lt L-glutamine (Sigma) and 1x penicillin/streptomycin (Sigma)]. Alive cardiomyocytes were counted using Tryptan Blue solution (Sigma) and a haemacytometer. Cells were transferred on 1% gelatin (Sigma) -coated 96-well plates.

Oxygen Glucose Deprivation (OGD) model and MTT viability assay. After isolation, cardiomyocytes were plated in a 96-well plate (5x10⁴ cells/well) in complete medium for 24h at 37°C. For induction of OGD, cardiomyocytes were transferred into low-glucose serum-free medium (Sigma) containing 5 mmol/lt of 2-deoxy-D-glucose (Sigma). The cells were placed into an anaerobic chamber (95% N₂ and 5% CO₂, Diazocarb) and incubated for 48h at 37°C. This time point was chosen because the amount of
cell death was reproducible and statistically significant between normal and OGD conditions in untreated (no Notch inhibitors) cardiomyocytes. For the assessment of cell viability, 20µl of 1.25mgr/ml MTT solution (Sigma) was added to each well and the cells were incubated for 1h. Next, cells were lysed with 140µl of acidic isopropanol per well and absorbance was read at 570nm.

**Statistical analysis.** Statistical analysis was performed using the Student’s t-test (tail 2, type 2). Values were expressed as mean ± SEM and differences with p value < 0.05 were considered significant (* p < 0.05, ** p < 0.01, *** p < 0.001).

**REFERENCES**


Online Figure I Notch signaling is active in cardiomyocytes, endothelial and smooth muscle cells of the cardiac vessels during adulthood. (A) Representative confocal image from an adult (3 month-old) Wt mouse heart stained for the active form of Notch1 (green) and nuclei (blue). Arrows indicate NICD1 activity in the cardiac vessel. Bar, 25 μm; n = 3. (B) Confocal micrograph from an adult (3 month-old) Wt mouse heart stained for the active form of Notch1 (green), a smooth muscle marker (αSMA in red), and nuclei (blue). Three boxed areas are shown on the right. Arrowheads indicate NICD1 nuclear localization in the endothelial cells since non-overlapping staining with αSMA was observed. Arrows show NICD1 activity in αSMA positive cells, whereas asterisks indicate NICD1 cardiomyocyte activity. Bar 25 μm; n = 3.
Online Figure II A Increased levels of NICD1 in cardiomyocytes lead to impaired functional performance in NICD1 - COE animals. (A) Double staining for the cardiomyocyte marker α-actinin and the active form of Notch1 receptor (NICD1*). Nuclei were visualised with DAPI. Confocal micrographs from the right ventricle (upper panel) and the left ventricle (lower panel) of Control and NICD1-COE hearts demonstrate an increase in the number of cardiomyocytes that are positive for NICD1 activity (α-actinin NICD1 double positive cells) in the NICD1-COE heart. No differences in the number of NICD1 positive cardiomyocytes were observed between right (RV) and left ventricle (LV) in Control mice. Scale bar, 20 μm.
Increased levels of NICD1 in cardiomyocytes lead to impaired functional performance in NICD1 - COE animals. (B) Comparative analysis of heart-to-body weight (HW/BW) measurements at one day, one month, and three month old Control and NICD1-COE mice. (C) Cardiac function was assessed by echocardiography; fractional shortening (F.S), left ventricular transversal area in systole (LVTA; s), and left ventricular transversal area in diastole (LVTA; d) values were calculated at two months of age; n = 4 (* p < 0.05, ** p < 0.01, *** p < 0.001 compared to Wt).
Online Figure III De novo activation of Notch signaling in the infarcted heart. (A) Representative confocal micrographs of paraffin sections from adult (2 - 3 month-old) WT mouse hearts stained for the active form of Notch1, α-actinin, and nuclei (green, red, and blue, respectively, in overlays) at steady-state conditions (normal) and 1 week after sham-operation (sham) or myocardial infarction (MI). Arrows and arrowheads indicate Notch1 activity in the nuclei of cardiomyocytes and epicardial cells, respectively. Boxed regions are magnified at the bottom-left corner of each image. Bars, 75 µm, Normal, Sham, MI; 50 µm, MI; n = 2. (B) Quantification of Jagged1, Notch1, and Notch2 mRNA transcripts by TaqMan qRT-PCR at the indicated time points following MI. Fold changes are normalized to sham-operated hearts (sham). * P < 0.05, ** P < 0.01 compared to sham; n = 3 – 4. (C) Representative immunoblot for Jag1, Notch1 intracellular domain (NICD1), and Notch2 (FL = full molecule; * = NICD2) on total protein extracts coming from infarcted hearts at the indicated time points. Control = kidney protein extracts. Quantification of protein expression is shown below. * P < 0.05, ** P < 0.01, *** P < 0.001 compared to sham; n = 3.
Online Figure IV  Notch pathway activation following myocardial infarction. Quantification of Notch3 (A), Notch4 (B), Hes1 (C), Hey1 (D), and Hey2 (E) mRNA transcripts by TaqMan qRT-PCR at the indicated time points following MI. Fold changes are normalized to sham-operated hearts (sham). * P < 0.05, ** P < 0.01 compared to sham; n = 3 – 4.
Online Figure V  Characterization of the αMyHC; mER-CRE-mER transgenic line using tamoxifen diet. (A) X-gal staining on cryosections from mER-CRE-mER; ROSA26 hearts at indicated periods of tamoxifen diet. No X-gal staining was observed in the absence of tamoxifen (image at the left). Arrows show X-gal positive cardiomyocytes. Bar, 100 μm; n = 2. (B) Representative immunoblot and quantification of Notch1 (NICD1, cleaved form) protein levels in Control (Co) and mER-CRE-mER; NICD1 hearts at the indicated periods of tamoxifen diet; α-Tubulin, loading control; n=2.
Online Figure VI  Decreased numbers of apoptotic cells following Notch signaling activation in the infarcted heart. TUNEL assay on Control and mER-CRE-mER;NICD1 hearts (4 weeks after MI) at the border zone (top) and ischemic zone (bottom). Quantification of TUNEL positive cells (green)/nuclei (blue) is provided below. Bar, 75 μm; n = 7. (* P < 0.05, ** P < 0.01, *** P < 0.001 compared to Control).
Online Figure VII Notch signaling inhibition impairs cardiomyocyte survival in vitro. (A) Primary cultures of neonatal cardiomyocytes were prepared and treated with increasing concentrations of L-685, 458 (Notch pathway inhibitor; 2.5 μmol/l, 5 μmol/l, 10 μmol/l). Cell survival was assessed using the MTT assay. Representative graph of at least 2 independent experiments (* p < 0.05, *** p < 0.001 compared to control). (B) Neonatal cardiomyocytes were treated with increasing concentrations of DAPT (Notch pathway inhibitor; 10 μmol/l, 50 μmol/l, 75 μmol/l). Cell survival was assessed using the MTT assay. Representative graph of at least 2 independent experiments (*** p < 0.001 compared to control). (C) Following an oxygen-glucose-deprivation (OGD) model on primary cardiomyocytes, cell survival was assessed after treatment with 2.5 μmol/l L-685, 458 and 10 μmol/l DAPT by MTT assay. Representative graph of at least 2 independent experiments (* p < 0.05, *** p<0.001 compared to control). Notch inhibitors were diluted in DMSO before addition to the medium and equal volumes of DMSO were added in the control cardiomyocyte culture.
Online Figure VIII. Survival rate and proliferation analysis on IgG1 and Notch-1 Ab treated hearts. (A) Survival rate on sham-operated (sham) or infarcted (MI) IgG1 Ab and Notch1 Ab mice at 2 and 4 weeks following operation; n = 40. (B) BrdU staining on IgG1 and Notch1 ab treated hearts 30 days following myocardial infarction. Numbers (1–6) indicate vessel position. Fewer BrdU positive cells associated with coronary vessels are observed in the border zone of IgG1 ab hearts (A') when compared to Notch1 ab hearts (B'). Scale bar, 50 μm.
Online Figure IX Selective silencing of Notch signaling in adult cardiomyocytes results in impaired cardiac function after myocardial infarction. (A) Survival rate of Control and αMyHCCRE/+;MAML1DN/DN mice at 1, 2, and 4 weeks following MI or sham-operation (sham) n = 12. Six animals carrying the αMyHCCRE transgene and six +/+;MAML1DN/DN animals were grouped together and considered Controls. (B-D) Assessment of cardiac function (echocardiography) of Control and αMyHCCRE/+;MAML1DN/DN mice before and after MI or sham-operation (sham). Using left ventricular trace on B-mode images, left ventricular transversal area in systole (LVTA;s), left ventricular transversal area in diastole (LVTA;d), and fractional shortening (F.S) were calculated at the indicated time points; n = 6 (* p<0.05, ** p<0.01, *** p<0.001 compared to Control MI). (E) Heart/Body Weight ratio (HW/BW, g/g) of sham-operated (sham) and infarcted (MI) Control and αMyHCCRE/+;MAML1DN/DN mice at 4 weeks after MI.
## Online Table I. Survival of Notch loss- and gain-of-function mutants.

<table>
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<tr>
<th>Parents</th>
<th>Offspring - Mendelian frequency (%)</th>
<th>Offspring at P1 (%)</th>
<th>Offspring at P21 (%)</th>
</tr>
</thead>
<tbody>
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<td><strong>♂</strong> +/+; Maml1\textsuperscript{DN/DN}</td>
<td>+/+; Maml1\textsuperscript{DN/+} (50%)</td>
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<tr>
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<tr>
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<tr>
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<tr>
<td></td>
<td>αMyHCCRE/+; +/+ (25%)</td>
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<td>40 (34.1%)</td>
</tr>
<tr>
<td><strong>♀</strong> +/+; NICD1/+</td>
<td>+/+; NICD1/+ (25%)</td>
<td>34 (24.2%)</td>
<td>39 (33.3%)</td>
</tr>
<tr>
<td></td>
<td>αMyHCCRE/+; NICD1/+ \textsuperscript{A} (25%)</td>
<td>28 (20.1%)</td>
<td>6 (5.1%)</td>
</tr>
</tbody>
</table>

A: NICD1-COE offspring are referred to as αMyHCCRE/+; NICD1/+.
**Online Table II.** Inventoried TaqMan assays for the indicated genes.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Assay part number</th>
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<tbody>
<tr>
<td>Anp</td>
<td>Mm01255747_g1</td>
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<tr>
<td>Bnp</td>
<td>Mm00435304_g1</td>
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<tr>
<td>Myh6</td>
<td>Mm00440354_m1</td>
</tr>
<tr>
<td>Myh7</td>
<td>Mm00600555_m1</td>
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<tr>
<td>Glut1</td>
<td>Mm00441473_m1</td>
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<tr>
<td>Acta1</td>
<td>Mm00808218_g1</td>
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<td>Nkx2.5</td>
<td>Mm00657783_m1</td>
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<td>Gata-4</td>
<td>Mm00484689_m1</td>
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<tr>
<td>Notch1</td>
<td>Mm00435245_m1</td>
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<td>Notch2</td>
<td>Mm00803077_m1</td>
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<tr>
<td>Notch3</td>
<td>Mm00435270_m1</td>
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<td>Notch4</td>
<td>Mm00440525_m1</td>
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<td>Hey1</td>
<td>Mm00468865_m1</td>
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<td>Hey2</td>
<td>Mm00469280_m1</td>
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<td>Hes1</td>
<td>Mm01342805_m1</td>
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<td>Casp3</td>
<td>Mm01195085_m1</td>
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<td>Casp6</td>
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<td>Casp9</td>
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<td>Bcl-2</td>
<td>Mm00477631_m1</td>
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<td>Bcl-XL</td>
<td>Mm00437783_m1</td>
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<td>Gapdh</td>
<td>4352339E</td>
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