Supporting Information

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Materials and Methods

CPC Isolation and Culture. Cardiac small cells were isolated by enzymatic digestion of the mouse heart (1, 2) and plated overnight in F12K medium supplemented with 20% FBS (high-serum medium). The following day, cells were labeled with c-kit monoclonal antibody conjugated with magnetic beads (Miltenyi Biotec) and c-kit-positive CPCs were selected by using MACS Separation Columns (Miltenyi Biotec) according to the manufacturer's instructions. After selection, c-kit-positive CPCs were expanded and gradually starved to 5% FBS (low-serum medium). Before treatment with Jagged1 and γ-secretase inhibitor, cells were sorted again for c-kit to obtain an enriched population of undifferentiated c-kit-positive CPCs.

Stimulation with Jagged1 and γ-Secretase Inhibition. Petri dishes and chamber slides were precoated with 25 μg/ml human IgG1 antibody (Sigma) and subsequently treated with blocking buffer (PBS plus BSA 1%) for 1 h at room temperature. After removal of excess blocking buffer, dishes and chamber slides were coated with 6 μg/ml human Fc-Jagged1 chimera (R & D Systems) for 2 h at room temperature. γ-Secretase inhibitor XXI (Calbiochem) was dissolved in 2 mM DMSO. Enriched CPCs were plated under three different conditions: (*i*) dishes coated with human IgG1 antibody only (control); (*ii*) dishes coated with the Jagged1 ligand; and (*iii*) dishes coated with the Jagged1 ligand in the presence of γ-secretase inhibitor at a final concentration of 10 nM. Additional control samples consisted of CPCs exposed to 0.0005% DMSO only. Three time points were examined: 2, 5, and 8 days. Medium was replaced at 2 and 5 days.

FACS Analysis. c-kit-positive sorted cells were labeled (1, 3, 4) with a c-kit antibody (R & D Systems) and with Notch1 extracellular domain antibody (Upstate Biotechnology). Cells were analyzed by FACSAria (Becton Dickinson) ($n = 6$).

Immunocytochemistry. Cells were fixed with 4% paraformaldehyde and treated with 0.01% Triton X-100 for the detection of intracellular antigens ($n = 5$) (1–5). The following antibodies were used: goat polyclonal c-kit antibody (R & D Systems), prediluted mouse monoclonal antibody for the extracellular domain of Notch1 (Abcam), rabbit polyclonal antibodies for the extracellular domain of Notch2, Notch3, and Notch4 (Santa Cruz Biotechnology), rabbit polyclonal antibody specific for the intracellular domain of Notch1 (Cell Signaling), goat polyclonal anti-Nkx2.5 (Santa Cruz Biotechnology), goat polyclonal anti-GATA4 (Santa Cruz Biotechnology), rabbit polyclonal anti-Ki67 (Vector), and mouse monoclonal anti-α-sarcomeric actin (Sigma). The cell body was detected by incubation with phalloidin directly conjugated with a fluorochrome (Invitrogen).

Quantitative RT-PCR. Total RNA was extracted using the RNeasy plus mini kit (Qiagen), and RNA concentration was measured with the NanoDrop system (4, 6). Reactions were performed by using the ABI Prism 7300 Real Time PCR Systems ($n = 3–6$). After DnaseI treatment, 25 ng of total RNA per well was retrotranscribed in the presence of One Step RT-PCR Master Mix Reagent (Applied Biosystems). For Nkx2.5, cDNA was generated from 1 μg of total RNA with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The following primers and probes were designed with Integrated DNA Technologies software (IDT): Hes1 forward, 5′-CAC GAC ACC GGA CAA ACC A-3′; Hes1 reverse, 5′-GCC GGG AGC TAT CTT TCT TAA GTG-3′; Hes1 probe, 5′-/56-FAM/AGC CTA TCA TGG AGA AGA GGC GAA GGG CAA/3BHQ_1/-3′; GATA4 forward, 5′-GAA TAA ATC TAA GAC GCC AGC AGG TCC-3′; GATA4 reverse, 5′-GAC AGC TTC AGA GCA GAC AGC A-3′; GATA4 probe, 5′-/56-FAM/ATG TCC CAG ACA TTC AGT ACT GTG TCC GGC CA/3BHQ_1/-3′; GATA6 forward, 5′-AAG CGC GTG CCT TCA TCA C-3′; GATA6 reverse, 5′-GAG CCA CTG CTG TTA CCG GA-3′; GATA6 probe, 5′-/56-FAM/ATG CTG AGG GTG AGC CTG TGT GCA ATG CTT/3BHQ_1/-3′; Vezf1 forward, 5′-CCA GGG AAG CAG GTA GAG ACA C-3′; Vezf1 reverse, 5′-TTT GAC ATA GTC CCA GAC GAC ACA G-3′; Vezf1 probe, 5′-/56-FAM/AGA AAG AAA GAA GCT GCC AAC CTG TGC CAA/3BHQ_1/-3′; Nkx2.5 forward, 5′-CCC AAG TGC TCT CCT GCT TTC C-3′; Nkx2.5 reverse, 5′-ACA GCT CTT TCT TAT CCG CCC GA-3′; β-actin forward, 5′-CTG AAC CCT AAG GCC AAC CG-3′; β-actin reverse, 5′-CAG CCT GGA TGG CTA CGT AC-3′; β-actin probe, 5′-/56-FAM/TGA CCC AGA TCA TGT TTG AGA CCT TCA ACA CCC/3BHQ_1/-3′.

Immunoprecipitation and Western Blotting. Protein lysates of untreated and Jagged1-treated CPCs were obtained by using Triton lysis buffer (BP-117; Boston BioProducts) with the addition of the Protease Inhibitors Mixture (Roche) ($n = 4$). Equivalents of 20–50 μg of proteins were loaded and subjected to Western blotting with Hes1 mouse monoclonal antibody (1:2,000; BD Biosciences Pharmingen) and with N1ICD rabbit polyclonal antibody (1:2,000; Novus Biologicals). Equivalents of 200 μg of protein extracts were incubated with rabbit polyclonal anti-N1ICD antibody (Novus Biologicals) overnight at 4°C and subsequently were exposed to protein A/G agarose (Santa Cruz Biotechnology) for 3 h at 4°C. Immunoprecipitated proteins were separated on 8% SDS/PAGE and transferred onto a nitrocellulose membrane (7, 8). Blots were incubated overnight at 4°C with rabbit polyclonal RBP-Jk antibody (Santa Cruz Biotechnology). HRP-conjugated IgG were used as secondary antibodies. Proteins were detected by chemiluminescence.

Electophoretic Mobility Shift Assay. P19 cells were transfected with an expression vector for RBP-Jk, and nuclear extracts from nontransfected and transfected cells were used in this assay ($n = 4$). Single-stranded oligonucleotides containing the conserved RBP-Jk consensus site (GTGGGAA) of the mouse Nkx2.5 promoter (sense, 5′-TAGGGGTGGGAAAGTCA-3′; antisense, 5′-TGACTTCCCACCCCTA-3′) were end-labeled with T4 polynucleotide kinase (Promega) and [γ-^{32}P]ATP (PerkinElmer). After precipitation, labeled oligonucleotides were annealed and purified by precipitation (7, 9). Binding reactions (20 μl) were prepared in binding buffer (Promega) with 30 μg of nuclear extract and 4 mCi of labeled oligonucleotide probe and incubated for 20 min at room temperature. Where indicated, 4 μg of rabbit polyclonal anti-RBP-Jk antibody (Santa Cruz Biotechnology) was added to the binding reactions. For competition assays (specific and unspecific), nonlabeled wild-type probes were added at the same concentration of the labeled probe. Complexes were resolved by electrophoresis on 4% polyacrylamide gels (acrylamide/bisacrylamide at 29:1) in 0.5× TBE buffer containing 2.5% glycerol at 350 V for 40 min at room temperature (7, 9). Gels were dried and exposed to a film overnight at −80°C with intensifying screens.

ChIP. ChIP assays were performed in CPCs and P19 cells following the same protocol. CPCs and P19 cells were expanded in F12K plus 5% FBS and α-MEM plus 10% FBS, respectively, to reach ≈80–90% confluency at the time of the experiment ($n = 6$). On the day of the experiment, CPCs and P19 cells were cross-linked with 1% formaldehyde. Cell extracts were sonicated for DNA shearing (Artek Sonic Dismembrator 150), and chromatin was immunoprecipitated with RBP-Jk antibody (Santa Cruz Biotechnology). After de-cross-linking, immunoprecipitated DNA fragments were treated with RNase and Proteinase K and purified with spin columns (Qiagen). For PCR analysis, three different sets of primers were used: Nkx2.5 forward, 5′-GAGGTCACCCCAATTCTGTT-3′; Nkx2.5 reverse, 5′-GGGCTTCCAGAGTTGACAAA-3′; Hes1 forward, 5′-GCCTCTGAGCACAGAAAGGT-3′; Hes1 reverse, 5′-CGCCTCTTCTCCCTGATAGG-3′; MEF2c forward, 5′-GACCGACCTGCTTTACTTGA-3′; MEF2c reverse, 5′-TGACACACCAGGCTGTTCAAC-3′. Template (20 ng) was amplified (35 cycles). DNA amplicons corresponding to each specific target were eluted from the gel and sequenced.

Gene Reporter Activity Assay in P19 cells and CPCs. P19 cells, ≈80% confluency, were transfected with Lipofectamine transfection reagent (Invitrogen). A plasmid carrying the mouse Nkx2.5 promoter containing 10 kb of the 5′ flanking region of the gene and located upstream of the β-gal reporter gene (1 μg) was used in three different sets of cotransfections along with expression vectors carrying the mouse N1ICD, GATA4, and Smad4 coding sequences (1 μg each). The Promega pGL3-*luc* control vector (1 μg) was used to define the efficiency of transfection and to normalize the data. After transfection cell extracts were obtained and β-galactosidase and luciferase enzyme activity assays were performed (Promega) according to manufacturer instructions ($n = 4$).

Reporter gene assay was then performed in CPCs. Because Jagged1 coating of Petri dishes interfered with the detection of luciferase activity, we used non-Jagged1-stimulated CPCs at 5 days after plating. At this interval, 51 ± 11% of N1ICD-positive CPCs coexpressed Nkx2.5. Activation of Notch1 in these cells was dictated by cell-to-cell contact between Notch1-positive CPCs and adjacent CPCs that expressed Jagged1 on their membrane. A plasmid carrying the mouse Nkx2.5 promoter containing ≈3 kb (−3059) of the 5′ flanking region of the gene (10) located upstream of the luciferase reporter gene was used (wild-type promoter). Moreover, a single base mutation (11) was introduced in the RBP-Jk binding site of the Nkx2.5 promoter (mutated promoter). The mutated reporter was generated by using QuikChange Lightning Site-Directed Mutagenesis Kit (Stratagene) with mutagenic forward (5′-GGTTGTAGGGG-TGGCAAAGTCACTGATTTTGTTC-3′) and reverse (5′-AATCAGTGACTTTGCCACCCCTACAACCTTGGTG-3′) primers. The mutated nucleotide is underlined. A total of 100 ng of -3kb-Nkx2.5-luciferase reporter plasmid was amplified by PCR in the presence of 125 ng of each mutagenic primer, 1.5 μl of QuikSolution reagent, and 1 μl of QuikChange Lightning Enzyme in a total volume of 50 μl. Cycling conditions were as follows: 95°C for 2 min, followed by 18 cycles of 95°C for 20 s, 60°C for 10 s, and 68°C for 4 min. The final step was done at 68°C for 5 min. After digestion with DpnI restriction enzyme at 37°C for 5 min, 2 μl of the reaction mixture was used for transformation of XL10-Gold competent cells. Cloned plasmids were digested with AflII and NotI restriction enzymes, and the DNA fragment including the site of mutagenesis was subject to ligation with the original -3kb-Nkx2.5-luciferase reporter plasmid to replace the corresponding region. This was done to exclude that PCR errors occurred in portions of the plasmid outside of the mutated region. Finally, the mutated reporter plasmid was sequenced to confirm site-directed mutagenesis with six primers: pGL3-F, 5′- CTAACATACGCTCTCCATC-3′; F1, 5′-TGTT-GATACAGTAGTCCG-3′; F2, 5′- TCTGTCTCCAC-CAGTCTC-3′; R2, 5′-CCTTGGGTTGTCTTCAAC-3′; R3, 5′-ACGAGCCACATCCGATTC-3′; R4, 5′-CACAGATAT-GAAGGACGAG-3′. CPCs were cotransfected with 1 μg of -3kb-Nkx2.5-luciferase reporter ($n = 8$) or 1 μg of mutated reporter ($n = 9$) and 0.05 μg of pRL-CMV (Promega). The siPORT XP-1 Transfection Agent (Applied Biosystems) was used. pRL-CMV was used to normalize transfection efficiency. Luciferase activity was measured by Dual-Luciferase Reporter Assay Systems (Promega). Cells were incubated with 150 μl of 1× Passive Lysis Buffer (Promega); 50 μl of cell lysate was mixed with 50 μl of Luciferase Assay Buffer II (Promega) for measurement of firefly luciferase activity. Subsequently, 50 μl of Stop & Glo Reagent (Promega) was used to measure *Renilla* luciferase activity. The ratio of these two measurements was calculated to normalize luciferase activity.

Animals. Female C57BL/6 mice at 2 months of age ($n = 12$) were injected i.p. with γ-secretase inhibitor XXI (Calbiochem) for 3 days before myocardial infarction (2, 3). After ligation of the left coronary artery, mice were treated for 9 more days and then killed. Treated animals received 80 nM γ-secretase inhibitor per day, and at the time of myocardial infarction two intramyocardial injections each of 0.25 nM γ-secretase inhibitor were made in the region bordering the infarct. Control animals were infarcted and treated with vehicle only. In both groups of infarcted mice, BrdU was delivered i.p. twice a day for 9 days (50 mg/kg per day) after infarction; BrdU was also added to the drinking water (1 g/liter). Before they were killed, mice were anesthetized with chloral hydrate (400 mg/kg of body weight, i.p.), and a microtip pressure transducer (SPR-671; Millar Instruments) connected to a recorder (iWorx214) was advanced into the LV for the evaluation of LV pressures and LV+ and LV− dP/dt in the closed-chest preparation (2, 3, 12). Subsequently, the abdominal aorta was cannulated with a polyethylene catheter, PE-50, filled with a phosphate buffer (0.2 M, pH 7.4) and heparin (100 units/ml). In rapid succession, the heart was arrested in diastole by the injection of 0.15 ml of 100 mM $CdCl_2$ through the aortic catheter, the thorax was opened, perfusion with phosphate buffer was started, and the vena cava was cut to allow drainage of blood and perfusate. After perfusion with buffer for 2 min, the coronary vasculature was perfused for 15 min with fixative (2, 3, 12). Subsequently, the heart was excised, and weights were recorded (13).

Immunohistochemistry. Paraffin-embedded tissue sections were stained with the following antibodies (1–6): goat polyclonal anti-c-kit (R & D Systems), goat polyclonal anti-Nkx2.5 (Santa Cruz Biotechnology), prediluted mouse monoclonal antibody for the extracellular domain of Notch1 (Abcam), rabbit polyclonal anti-N1ICD (Cell Signaling). BrdU incorporation was detected with mouse monoclonal antibody (Roche). Secondary antibodies, conjugated with the appropriate fluorochrome (Jackson ImmunoResearch), were used. The concentration of primary and secondary antibodies corresponded to those indicated by the manufacturer.

Data Analysis. Data are presented as mean ± SD. Significance between two groups was determined by an unpaired two-tailed Student *t* test. For multiple comparisons, ANOVA test was used. $P < 0.05$ was considered significant (14).

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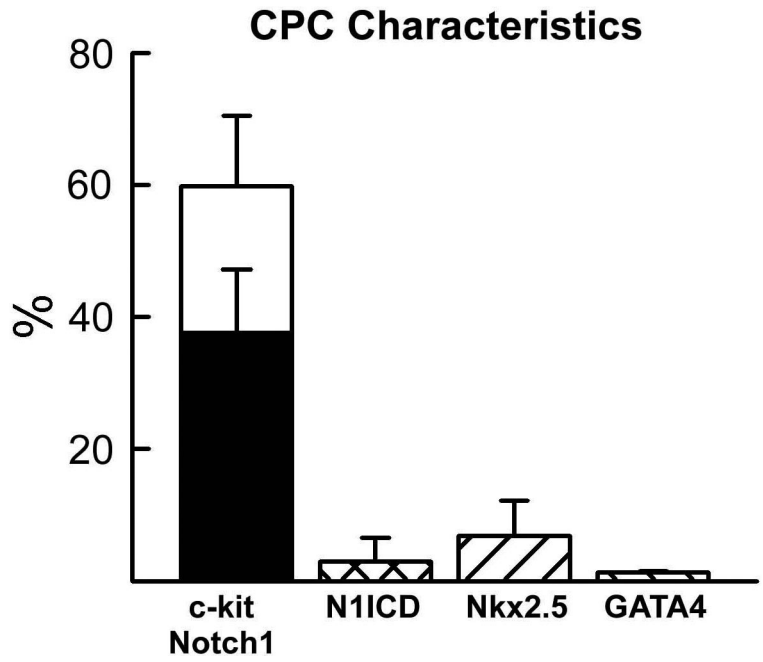

Fig. S1. CPC characteristics at baseline. Percentage of CPCs expressing c-kit, Notch1, and lineage markers.

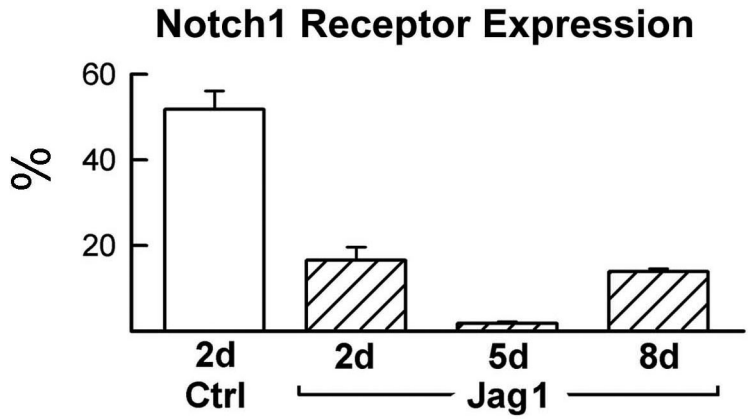

Fig. S2. Jagged1 stimulation and Notch1 receptor activation. The activation of Notch1 by Jagged1 (Jag1) at 2, 5, and 8 days (d) leads to a significant decrease in the percentage of CPCs expressing Notch1 extracellular domain. Ctrl, CPCs in the absence of Jagged1.

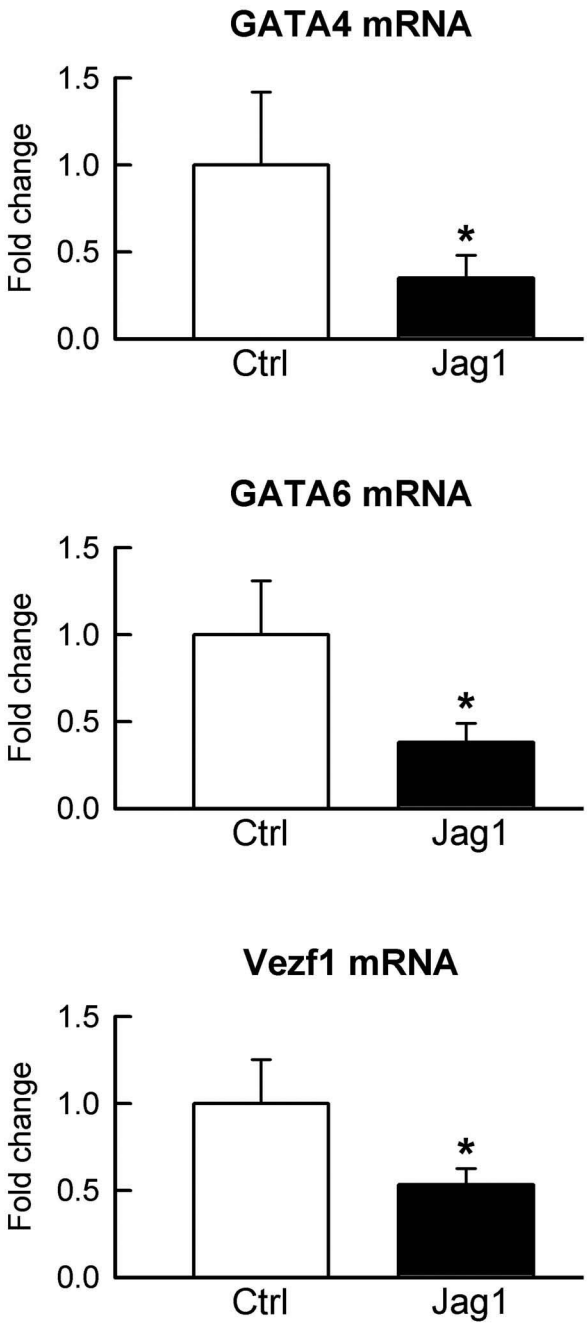

Fig. S3. Quantitative RT-PCR for GATA4, GATA6, and Vezf1. GATA4, GATA6, and Vezf1 transcripts were measured in CPCs at 8 days under control conditions (Ctrl) and in the presence of Jagged1 (Jag1). GATA4, GATA6, and Vezf1 quantity is shown in fold changes versus Ctrl. *, $P < 0.05$ vs. Ctrl.

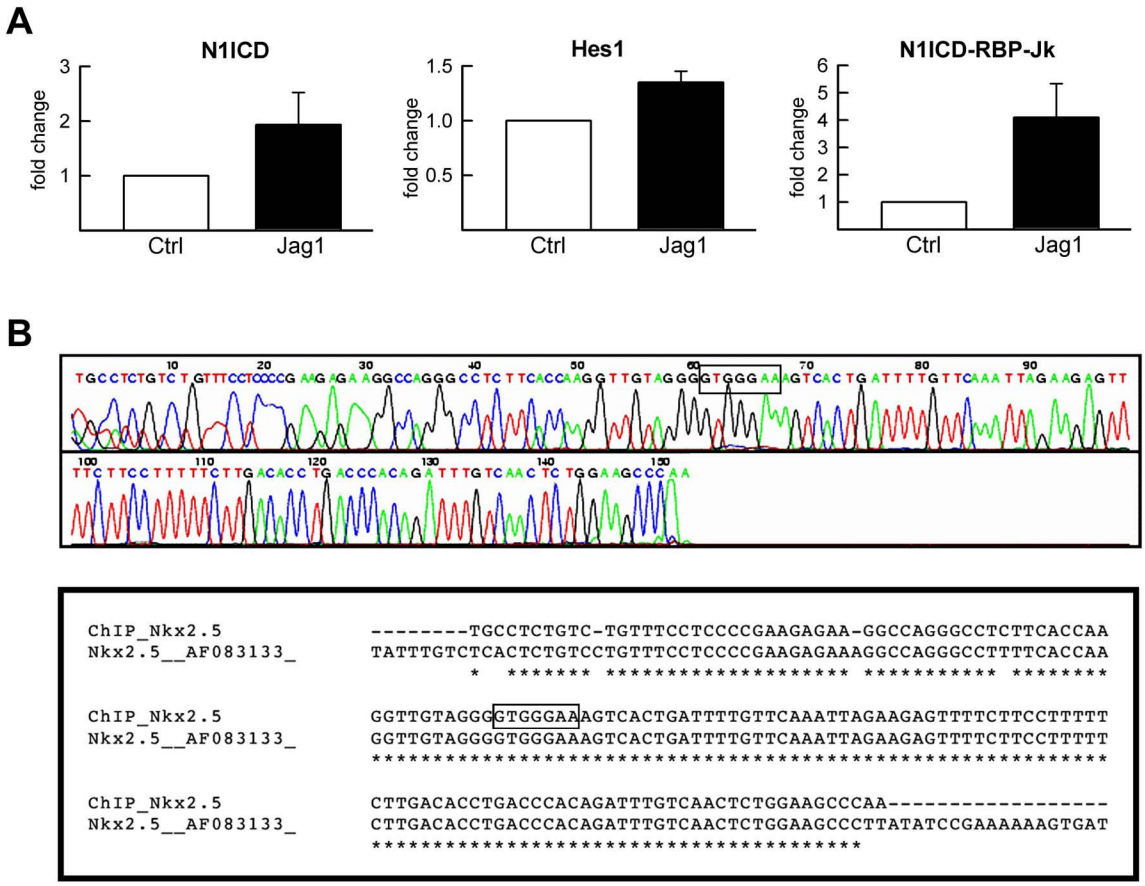

Fig. S4. Western blotting and ChIP assay. (*A*) The optical density of N1ICD, Hes1, and N1ICD-RBP-Jk in Jagged1-treated CPCs is shown as fold changes with respect to control CPCs. (*B*) Sequence of immunoprecipitated DNA fragments amplified with promoter-specific Nkx2.5 primers and sequence alignment.

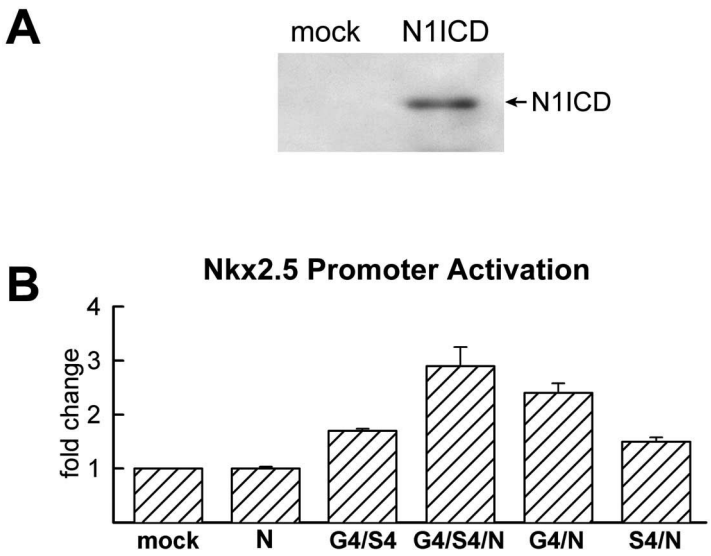

Fig. S5. Reporter gene assay in P19 cells. (*A*) Western blotting for N1ICD documenting the efficiency of transfection of P19 cells with the corresponding expression vector. (*B*) Nkx2.5 promoter transactivation was measured by β-galactosidase activity assay after transfection of P19 cells with the expression vectors carrying N1ICD (N), GATA4 (G4), and/or Smad4 (S4).

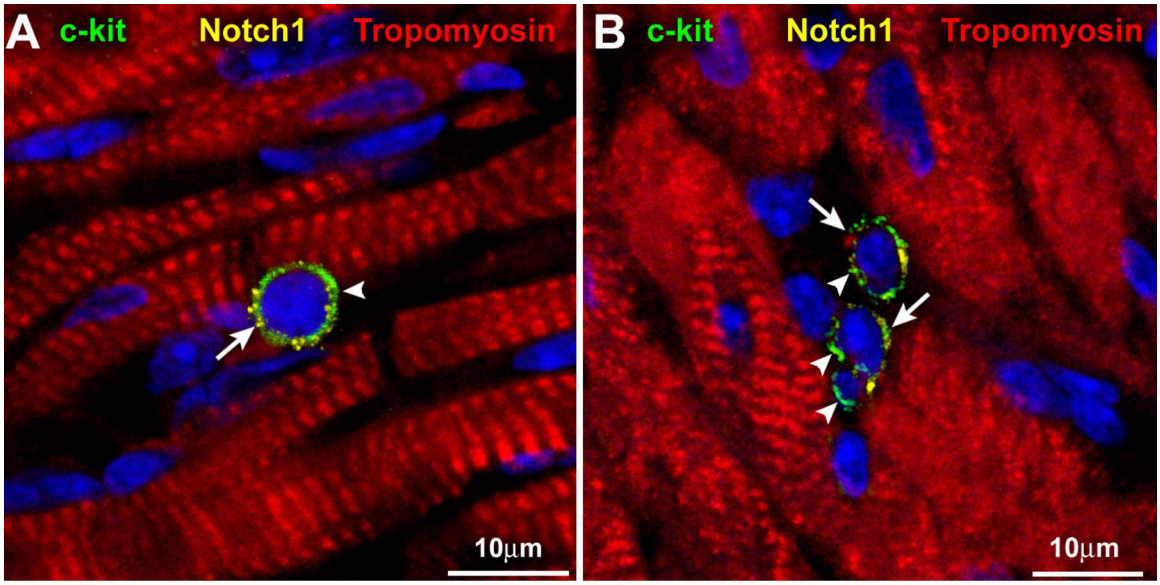

Fig. S6. CPCs and border zone. (*A* and *B*) Single (*A*) and small clusters (*B*) of CPCs (c-kit, green, arrowheads) are located in the border zone of untreated infarcts and express Notch1 receptor (yellow, arrows).

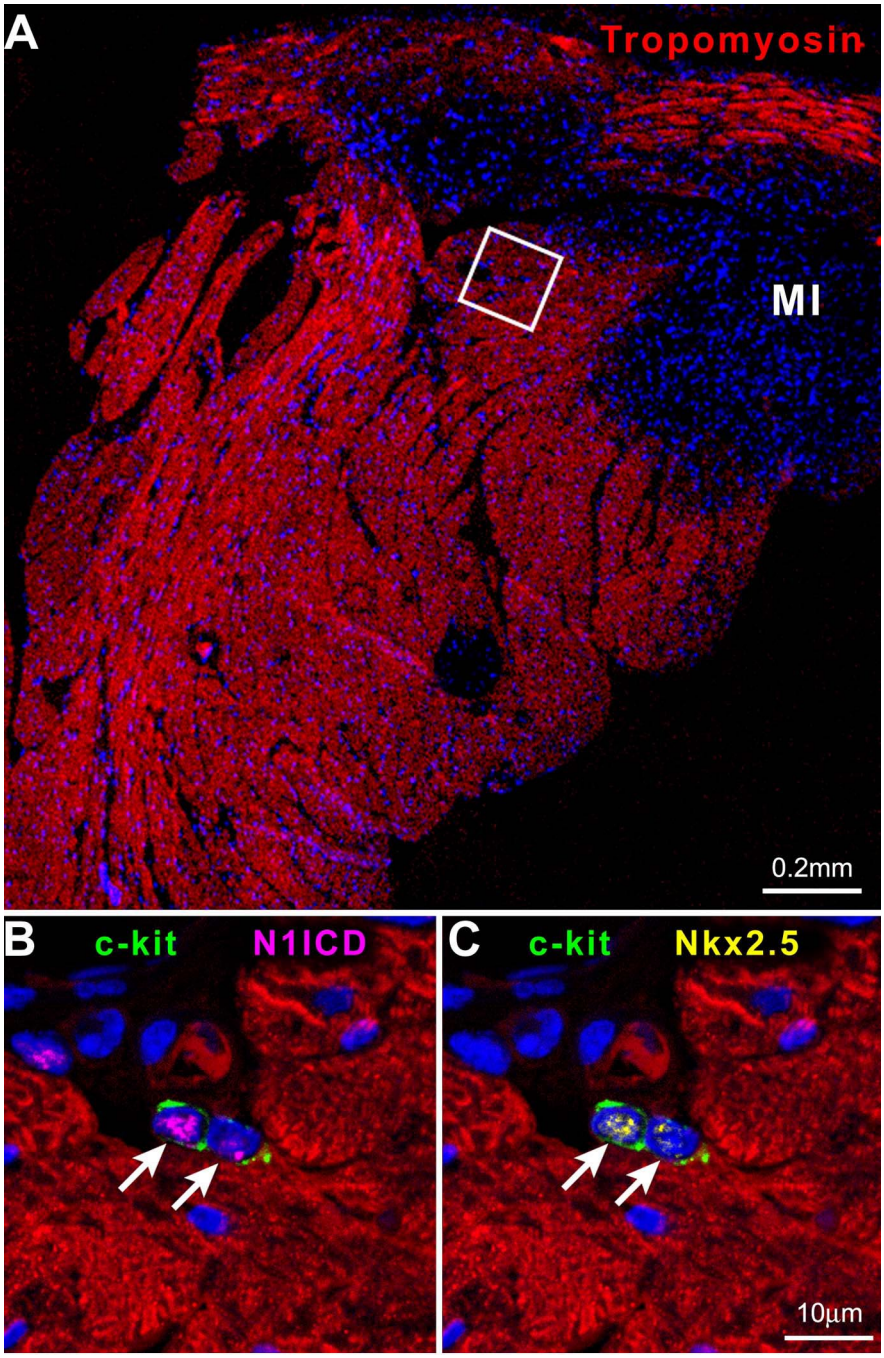

Fig. S7. Notch1 activation and Nkx2.5 expression in CPCs. Shown is the border zone of untreated infarct (*A*) in which the area included in the rectangle is shown at higher magnification in *B* and *C*. CPCs (c-kit, green, arrows) exhibit nuclear colocalization of N1ICD (*B*, magenta, arrows) and Nkx2.5 (*C*, yellow, arrows).

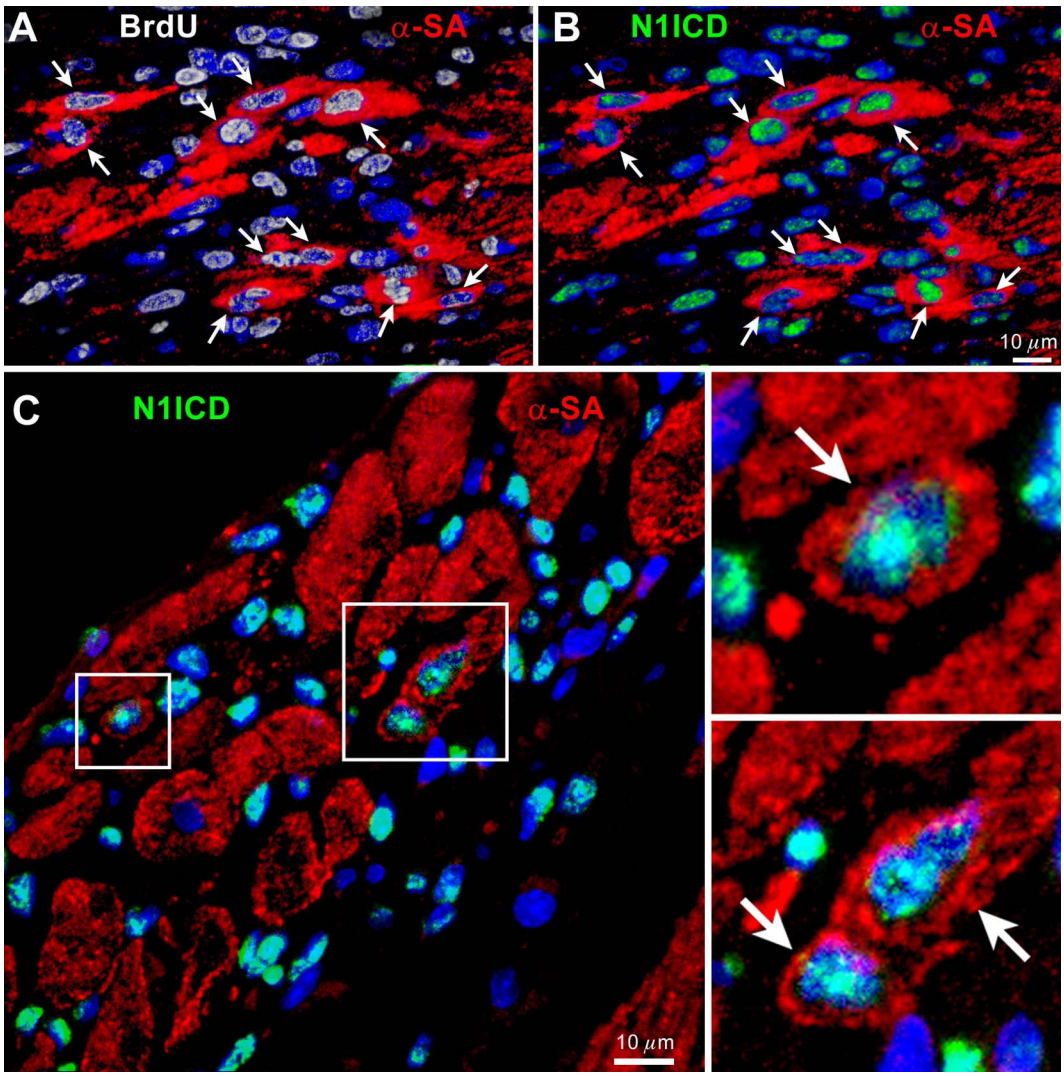

Fig. S8. Myocardial regeneration in the border zone of untreated infarcts. (*A* and *B*) BrdU-labeled (white) regenerated myocytes express N1ICD (green). (*C*) Small new myocytes (α-SA, red) are N1ICD-positive (green, arrows). The areas in the squares are shown at higher magnification in the adjacent panels.