Supporting Information

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SI Materials and Methods

Zebrafish Strains. Zebrafish embryos, larvae, and adults were produced, grown, and maintained according to standard protocols approved by the Institutional Animal Care and Use Committees of Massachusetts General Hospital. Ethical approval was obtained from the Institutional Animal Care and Use Committees of Massachusetts General Hospital.

Zebrafish from 6 to 12 mo of age were used for ventricular resection surgeries as described (1). Published strains used in this study include: wild-type AB; wild-type TuAB; *mind bomb (mib)*^{ta52b} (2); *Tg(-1.5hsp70l:Gal4-VP16)*^{kca4} (3, 4); *Tg(5xUAS-E1b:6xMYC-notch1a-intra)*^{kca3} (3, 4); *[Tg(-3.5ubi:loxP-EGFP-loxP-mCherry) III]*^{cz1701} (5); *Tg(tcf21:DsRed2)*^{pd37} (6), *Tg(kdrl:mCherry-ras)*^{s896} (7). Animal density was maintained at four fish per liter in all experiments.

Construction of Transgenic Lines. The creation of the *Tg(-14.8gata4:DsRed2)* transgene will be described in detail in another publication (K. Kikuchi and K. D. Poss). For the *Tg(hsp70l:DN-MAML-GFP)*^{fb10}, *Tg(hsp70l:DN-MAML-GFP)*^{fb11}, and *Tg(hsp70l:1xMYC-notch1a-intra)*^{fb12} strains, transgenes were engineered using Gateway technology (Life Sciences). Specifically, middle clones were constructed containing the coding sequences for either amino acids 13–74 of murine MAML1 (dominant negative mastermind-like 1; DN-MAML) fused to GFP (8) or the zebrafish Notch1a intracellular domain (NICD) fused at the N terminus to a single copy of the MYC epitope (3, 4). These middle clones were recombined with previously described 5′ (p5E-hsp70l) and 3′ (p3E-polyA) entry clones (9) and a destination vector pDest-Tol2AB2 (10) that carries a lens-specific promoter upstream of the Cerulean fluorescent protein. To generate the *Tg(kdrl:CreERT2)*^{fb13} strain, a transgene was engineered by recombination of a 5′ entry clone carrying a 6.1-kb *kdrl* promoter (11), a middle entry clone containing a *CreERT2* coding sequence amplified from *ERT2-Cre-ERT2* (12), and the 3′ entry clone p3E-polyA (9) into the destination vector pDestTol2AB2 (10). To generate stable transgenic lines, each plasmid was injected into one-cell-stage embryos together with Tol2 transposase mRNA (9).

Heat Shock Treatment of Adult Zebrafish. Adult heat shock treatments were performed similar to those previously described (13). Control (nontransgenic) siblings, *Tg(hsp70l:DN-MAML-GFP)*^{fb10}, *Tg(hsp70l:DN-MAML-GFP)*^{fb11}, *Tg(hsp70l:1xMYC-notch1a-intra)*^{fb12}, and *Tg(hsp70l:Gal4-VP16)*^{kca4}*; Tg(5xUAS-E1b:6xMYC-notch1a-intra)*^{kca3} adults were injured and allowed to recover overnight from surgery at room temperature. Animals were then exposed daily to 60-min automated elevations in temperature from 26 °C to 38 °C. Alternatively, animals were heat shocked for 60 min at 1 and 2 d postamputation (dpa) or 6 and 7 dpa before analysis at 7 dpa.

Histological Sectioning and Staining. Hearts were fixed with 4% paraformaldehyde (PFA) in PBS overnight at 4 °C, washed in PBS, and embedded with 1.2% (wt/vol) agarose in 30% (wt/vol) sucrose-PBS solution overnight at 4 °C. Cryosections (10 μm) were cut on a Leica cryostat (CM3050S), mounted on Superfrost slides, and dried overnight at room temperature. For all assays described, three sections per heart were analyzed and the total number of analyzed hearts reported in the figure legends. Acid Fuchsin-Orange G (AFOG) staining was performed as described (1).

Western Blotting. Single ventricles from adult zebrafish were isolated and suspended immediately in 60 μL of prechilled lysis buffer [50 mM Tris·HCl, pH 7.4, 50 mM NaCl, 10 mM $MgCl_2$, 1% Triton X-100, 1% deoxycholate and protease inhibitors from Thermo Scientific (cat. no. 78425)]. Ventricles were mechanically dissociated with a prechilled pestle until the tissue was visibly dissociated (~10 s), the lysates were incubated for 10 min on ice with occasional vortexing, and they were cleared by microcentrifugation for 7 min at 16,873 × *g* at 4 °C. Half of each lysate was separated on a 10% (wt/vol) SDS/PAGE gel and transferred to a PVDF membrane using standard procedures. The membrane was blocked for 1 h at room temperature in 5% (wt/vol) milk diluted in TBST. The membrane was probed overnight at 4 °C with anti-MYC (9B11, 1:500 dilution, Cell Signaling) or anti-GAPDH (6C5, 1:1,000 dilution, Abcam) primary antibodies and for 2 h at room temperature with a horse anti-mouse HRP-conjugated secondary antibody (1:2,000 dilution to detect the anti-MYC antibody, 1:3,000 to detect the anti-GAPDH antibody, Cell Signaling). Band intensities were quantified using ImageJ software (14).

In Situ Hybridization, Immunofluorescence, Imaging, and Cell Counts. For colormetric expression analyses, in situ hybridization (ISH) on 10-μm cryosections of 4% (wt/vol) PFA fixed hearts was performed using digoxygenin-labeled cRNA probes with the Tyramide Signal Amplification (TSA) plus DIG Reagent kit (PerkinElmer) according to the manufacturer's instructions. Sections were imaged using a Nikon Eclipse 80i compound microscope (Nikon) and Sony Cybershot digital camera.

For endocardial colabeling studies, in situ hybridization (ISH) on 10-μm cryosections of 4% (wt/vol) PFA fixed *Tg(kdrl:mCherry-ras)*^{s896} hearts was performed using digoxygenin-labeled cRNA probes with the Tyramide Signal Amplification (TSA) plus Fluorescein kit (PerkinElmer) according to the manufacturer's instructions followed by mCherry immunostaining. Sections were imaged using a Zeiss LSM5 Pascal Laser Scanning Microscope (Carl Zeiss MicroImaging).

Immunofluorescence was performed as described (6). Cryosections (10 μm) were immersed in precooled acetone at −20 °C for 10 min and then blocked in 0.5% goat serum. Primary and secondary antibody stainings were performed at 4 °C overnight or at room temperature for 2 h, respectively. Sections were mounted with Vectashield containing DAPI (Vector Laboratories), and imaged using a Nikon Eclipse 80i compound microscope (Nikon) and a QImaging Retiga 2000R CCD camera (QImaging). Primary antibodies used in this study include anti-mCherry (mouse, 1:200; Abcam); anti-Tropomyosin (CH1, mouse, 1:100; Developmental Studies Hybridoma Bank), anti-Mef2 (rabbit, 1:50; Santa Cruz Biotechnology), and anti-PCNA (mouse, 1:200; Sigma-Aldrich). Secondary antibodies (1:200; Life Technologies) used in this study include Alexa Fluor 488 goat anti-mouse, Alexa Fluor 488 goat anti-rabbit, Alexa Fluor 555 goat anti-mouse, and Alexa Fluor 568 goat anti-rabbit.

To calculate cardiomyocyte proliferation indices, cardiac sections were costained with antibodies against Mef2 and PCNA as described (15). The percentages of Mef2+ cardiomyocyte nuclei that were positive for PCNA were quantified from three sections and averaged to determine the proliferation index for each animal. To quantify the numbers of subepicardial DsRed2+ cells in heat shocked wild-type and *Tg(hsp70l:DN-MAML-GFP)*^{fb10} hearts, cardiac sections from 7 dpa *Tg(tcf21:DsRed2)*^{pd37} zebrafish were mounted with Vectashield containing DAPI. The numbers of subepicardial DAPI+, DsRed2+ cells were quantified in

images of three sections per heart. Four hearts were analyzed per experimental group.

Genetic Lineage Tracing. *Tg(kdrl:CreERT2)*^{fb13} adults were mated to *Tg(ubi:loxP-EGFP-loxP-mCherry)*^{cz1701} fish. At 24 h post-fertilization (hpf), their GFP+ progeny, which were hemizygous for the *Tg(ubi:loxP-EGFP-loxPmCherry)*^{cz1701} reporter transgene, were incubated in E3 containing 10 μM 4-hydroxytamoxifen (4-HT; Sigma-Aldrich) for 24 h. At 96 hpf, 4-HT–treated double transgenic embryos exhibiting red reporter fluorescence throughout the blood vessels and endocardium were raised to adulthood for genetic lineage tracing studies.

Quantitative PCR. During embryogenesis, 19-hpf control sibling, *Tg(hsp70l:DN-MAML-GFP)*^{fb10}, or *Tg(hsp70l:Gal4-VP16)*^{kca4}*; Tg(5XUAS-E1b:6XMYC-notch1a-intra)*^{kca3} embryos were heat shocked at 37 °C for 45 min and then incubated at 28 °C until 24 hpf when they were collected in TRIzol reagent (Life Technologies). Simultaneously, *mib*^{ta52b} mutant embryos were generated and collected at 24 hpf in TRIzol. During adulthood, unamputated and 7-dpa ventricles were collected in TRIzol reagent. RNA was isolated from each experimental group and cDNA libraries were generated using the Super Script III first-Strand Synthesis System according to the manufacturer's protocol (Life Technologies). Quantitative PCR was performed with the Applied Biosystems 7500 Fast Real-Time PCR System (Life Technologies), Fast SYBR Green Supermix (Bio-Rad), and the following primer pairs:

hey2F (5′-GAAGCGGAGAGGGATCATTG-3′)

hey2R (5′-CCACTGTCATCTGCAATATTTCC-3′)

her4F (5′-AGGAGAACTGAACACAAGACAC-3′)

her4R (5′-TGCTGTTGATTCGCTCTCG-3′)

β-actinF (5′-ATCTTCACTCCCCTTGTTCAC-3′)

β-actinR (5′-TCATCTCCAGCAAAACCGG-3′)

notch1aF (5′-TGTCGGACTCAAGCCTTTG-3′)

notch1aR (5′-CTGACGGTGATCCAACTGAC-3′)

notch1bF (5′-CGATGGTGTTGCTTAAGAATGG-3′)

notch1bR (5′-ATGTGGTCTGTGATTTCCCG-3′)

notch2F (5′-ATCAGTGCGAGTGTCAGAAG-3′)

notch2R (5′-CACCCTGAAAACCTGCTTTAC-3′)

notch3F (5′-ACATTACATCTGCTCCTGCC-3′)

notch3R (5′-ACCTGTACCCTCCAACTCTG-3′)

18SF (5′-TCGCTAGTTGGCATCGTTTATG-3′)

18SR (5′-CGGAGGTTCGAAGACGATCA-3′)

During embryogenesis, relative expression levels of *hey2* and *her4* in each experimental group were calculated after normalization to β-*actin* transcripts using the comparative Ct method. During adulthood, relative expression levels of *notch1a*, *notch1b*, *notch2*, and *notch3* in ventricles from each experimental group were calculated after normalization to *18S* transcripts using the comparative Ct method. In all cases, three biological replicates were analyzed in triplicate.

Statistical Analysis. *P* values were calculated using an unpaired two-tailed *t* test with unequal variance using Microsoft Excel (Microsoft).

1. Poss KD, Wilson LG, Keating MT (2002) Heart regeneration in zebrafish. *Science* 298(5601):2188–2190.
2. Jiang YJ, et al. (1996) Mutations affecting neurogenesis and brain morphology in the zebrafish, Danio rerio. *Development* 123:205–216.
3. Scheer N, Groth A, Hans S, Campos-Ortega JA (2001) An instructive function for Notch in promoting gliogenesis in the zebrafish retina. *Development* 128(7):1099–1107.
4. Scheer N, Campos-Ortega JA (1999) Use of the Gal4-UAS technique for targeted gene expression in the zebrafish. *Mech Dev* 80(2):153–158.
5. Mosimann C, et al. (2011) Ubiquitous transgene expression and Cre-based recombination driven by the ubiquitin promoter in zebrafish. *Development* 138(1):169–177.
6. Kikuchi K, et al. (2011) tcf21+ epicardial cells adopt non-myocardial fates during zebrafish heart development and regeneration. *Development* 138(14):2895–2902.
7. Chi NC, et al. (2008) Foxn4 directly regulates tbx2b expression and atrioventricular canal formation. *Genes Dev* 22(6):734–739.
8. Weng AP, et al. (2003) Growth suppression of pre-T acute lymphoblastic leukemia cells by inhibition of notch signaling. *Mol Cell Biol* 23(2):655–664.
9. Kwan KM, et al. (2007) The Tol2kit: A multisite gateway-based construction kit for Tol2 transposon transgenesis constructs. *Dev Dyn* 236(11):3088–3099.
10. Zhou Y, et al. (2011) Latent TGF-β binding protein 3 identifies a second heart field in zebrafish. *Nature* 474(7353):645–648.
11. Jin SW, Beis D, Mitchell T, Chen JN, Stainier DY (2005) Cellular and molecular analyses of vascular tube and lumen formation in zebrafish. *Development* 132(23):5199–5209.
12. Boniface EJ, Lu J, Victoroff T, Zhu M, Chen W (2009) FlEx-based transgenic reporter lines for visualization of Cre and Flp activity in live zebrafish. *Genesis* 47(7):484–491.
13. Lepilina A, et al. (2006) A dynamic epicardial injury response supports progenitor cell activity during zebrafish heart regeneration. *Cell* 127(3):607–619.
14. Abramoff MD, Magelhaes PJ, Ram SJ (2004) Image Processing with ImageJ. *Biophotonics International* 11(7):36–42.
15. Kikuchi K, et al. (2011) Retinoic acid production by endocardium and epicardium is an injury response essential for zebrafish heart regeneration. *Dev Cell* 20(3):397–404.

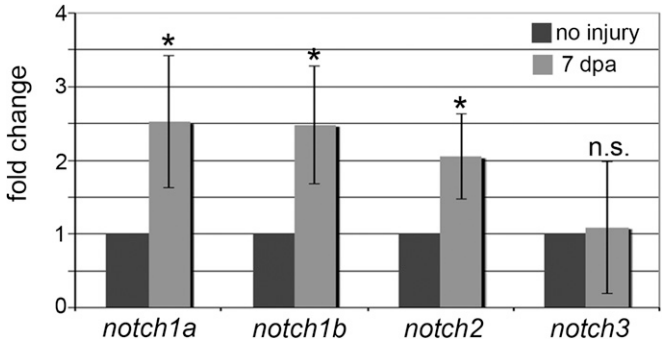

Fig. S1. Notch receptor expression is induced by cardiac injury. The graph depicts fold-changes in *notch* receptor expression at 7 dpa as determined by quantitative PCR. Mean values for three biological replicates are shown. Error bars show ± 1 SD. *$P < 0.05$; n.s., not significant.

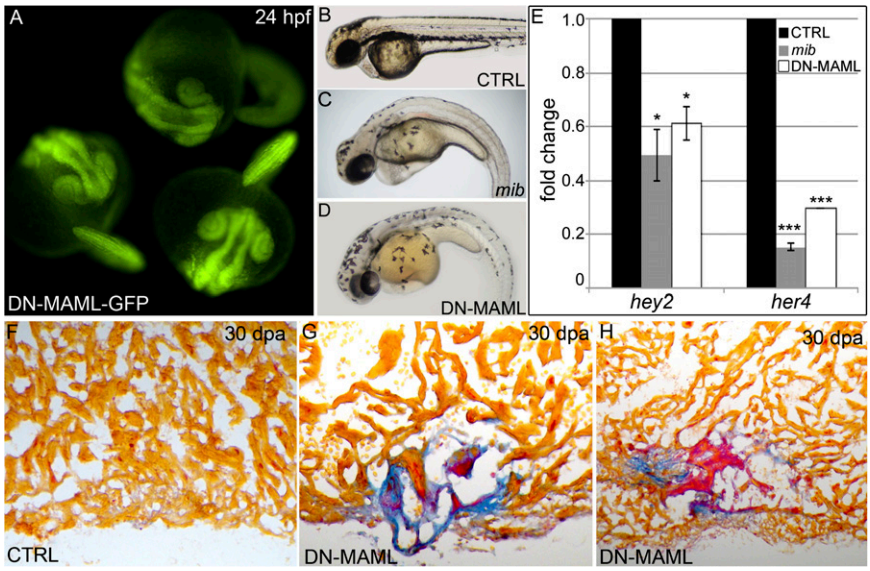

Fig. S2. Heat shock inducibility of DN-MAML-GFP, suppression of Notch signaling by DN-MAML-GFP, and cardiac regenerative deficiencies in an independent *Tg(hsp70:DN-MAML-GFP)* line. (*A*) Embryos carrying the *Tg(hsp70:DN-MAML-GFP)*^{fb10} transgene were heat shocked for 30 min at 37 °C following gastrulation (bud stage). At 24 hpf, these embryos exhibited prominent GFP fluorescence indicating DN-MAML-GFP protein expression. (*B*–*E*) Heat-shock-inducible suppression of Notch signaling in *Tg(hsp70:DN-MAML)* animals. Control (CTRL) and *Tg(hsp70:DN-MAML)* embryos were heat shocked at bud stage (*B* and *D*) or 19 hpf (*E*) and analyzed for gross morphological defects at 48 hpf (*B* and *D*) or relative expression of Notch target genes *hey2* and *her4* by qPCR at 24 hpf (*E*). *Mind bomb* (*mib*) mutant embryos, which are devoid of Notch signaling, were analyzed in parallel. Whereas control embryos were unaffected by heat shock (*B*), *mib* (*C*), and heat shocked *Tg(hsp70:DN-MAML)* (*D*) embryos displayed similar body axis curvature defects. (*E*) Graph showing the relative expression levels of *hey2* and *her4* transcripts in each experimental group. Three biological replicates were included in the qPCR analysis. Data are presented as means ± 1 SD, $*P < 0.05$; $***P < 0.001$. (*F*–*H*) AFOG staining of representative cardiac sections at 30 dpa from heat shocked control (CTRL; *F*) and transgenic adults carrying an independent insertion of the *Tg(hsp70:DN-MAML-GFP)* transgene [*Tg(hsp70:DN-MAML-GFP)*^{fb11}] (*G* and *H*). Similar to *Tg(hsp70:DN-MAML)*^{fb10} hearts, heat shocked *Tg(hsp70:DN-MAML)*^{fb11} hearts displayed prominent regenerative deficiencies as evidenced by fibrin retention (red) and collagen deposition (blue). Sections shown in *G* and *H* are from independent animals. Cardiac regeneration failed in 0 of 8 heat shocked control hearts and 5 of 5 heat shocked DN-MAML^{fb11} hearts.

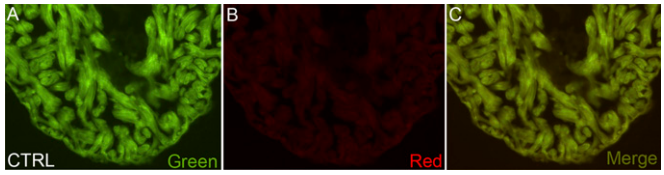

Fig. S3. Dependence of mCherry reporter fluorescence on 4-HT exposure. (*A*–*C*) Representative cardiac section from an untreated (no exposure to 4-HT) *Tg(kdrl:CreER)*; *Tg(ubi:Switch)* double transgenic adult zebrafish imaged in the green (*A*) and red (*B*) channels. Merged image shown in *C*. No mCherry reporter fluorescence was visible ($n = 4$).

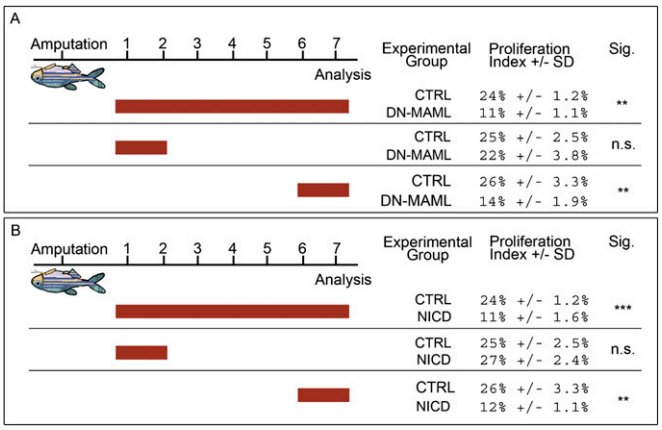

Fig. S4. Inactivation or hyperactivation of Notch signalling specifically during cardiomyocyte proliferation impairs heart regeneration. (*A*) Control and *Tg(hsp70:DN-MAML)* animals were heat shocked daily, at 1 and 2 dpa, or 6 and 7 dpa as indicated by the red bars in the schematic. Hearts were collected at 7 dpa, and cardiac sections were double immunostained for Mef2 and PCNA. The percentages of myocardial nuclei undergoing DNA replication near the wound edges were quantified as mean proliferation indices ± 1 SD. Sig., statistical significance; $^{**}P < 0.01$; n.s., not significant. At least four hearts were analyzed for each experimental group. (*B*) Control and *Tg(hsp70:NICD)* animals were heat shocked daily, on 1 and 2 dpa, or 6 and 7 dpa as indicated by the red bars in the schematic. Hearts were collected at 7 dpa, and cardiac sections were double immunostained for Mef2 and PCNA. The percentages of myocardial nuclei undergoing DNA replication near the wound edges were quantified as mean proliferation indices ± 1 SD. Sig., statistical significance, $^{**}P < 0.01$; n.s., not significant. At least four hearts were analyzed for each experimental group.

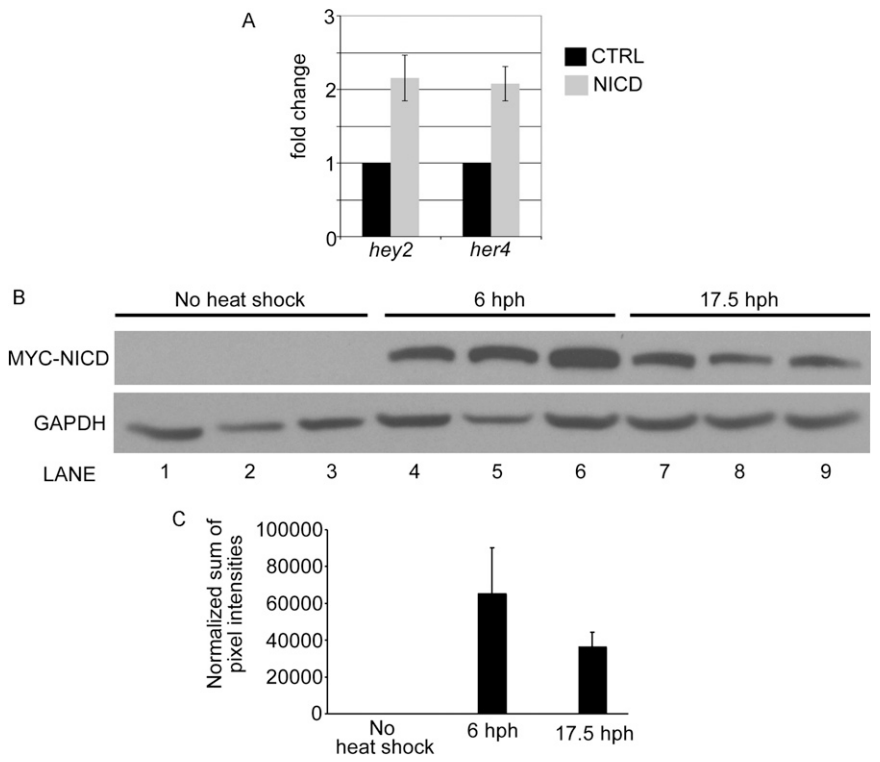

Fig. S5. Degree and reproducibility of Notch hyperactivation in heat shocked *Tg(hsp70:MYC-NICD)* and *Tg(hsp70:Gal4); Tg(UAS:MYC-NICD)* animals. (*A*) Bar graph showing mean fold increases in Notch target genes *hey2* and *her4* induced by heat shocking control (CTRL) and *Tg(hsp70:Gal4); Tg(UAS:MYC-NICD)* embryos as determined by quantitative PCR. $^{*}P < 0.05$; error bars indicate ± 1 SD. (*B*) Western blot showing MYC-NICD protein levels in individual ventricles collected from adult *Tg(hsp70:MYC-NICD)* zebrafish 6 or 17.5 h post heat shock (hph). Ventricles from *Tg(hsp70:MYC-NICD)* animals not exposed to elevated temperature were analyzed in parallel. (*C*) Bar graph showing the average intensities of MYC-NICD bands normalized to those for GAPDH from the Western blot shown in *B*. Error bars indicate ± 1 SD.

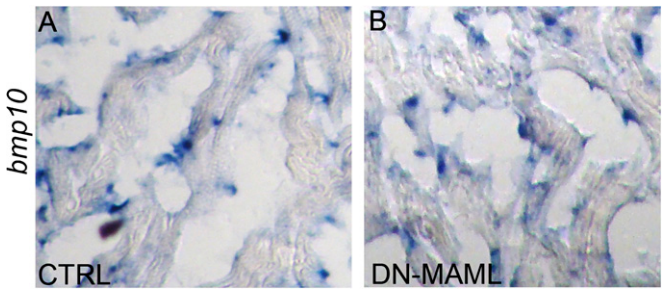

Fig. S6. *Bmp10* expression appears unchanged in Notch-suppressed hearts. (*A* and *B*) In situ hybridization showing *bmp10* transcripts in cardiac sections from control and Notch-suppressed hearts at 7 dpa. In both cases, *bmp10* transcripts localized to presumptive endocardial cells, and myocardial cells appeared devoid of *bmp10* transcripts.