Notch signaling regulates cardiomyocyte proliferation during zebrafish heart regeneration

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The human heart's failure to replace ischemia-damaged myocardium with regenerated muscle contributes significantly to the worldwide morbidity and mortality associated with coronary artery disease. Remarkably, certain vertebrate species, including the zebrafish, achieve complete regeneration of amputated or injured myocardium through the proliferation of spared cardiomyocytes. Nonetheless, the genetic and cellular determinants of natural cardiac regeneration remain incompletely characterized. Here, we report that cardiac regeneration in zebrafish relies on Notch signaling. Following amputation of the zebrafish ventricular apex, Notch receptor expression becomes activated specifically in the endocardium and epicardium, but not the myocardium. Using a dominant negative approach, we discovered that suppression of Notch signaling profoundly impairs cardiac regeneration and induces scar formation at the amputation site. We ruled out defects in endocardial activation, epicardial activation, and dedifferentiation of compact myocardial cells as causative for the regenerative failure. Furthermore, coronary endothelial tubes, which we lineage traced from preexisting endothelium in wild-type hearts, formed in the wound despite the myocardial regenerative failure. Quantification of myocardial proliferation in Notch-suppressed hearts revealed a significant decrease in cycling cardiomyocytes, an observation consistent with a noncell autonomous requirement for Notch signaling in cardiomyocyte proliferation. Unexpectedly, hyperactivation of Notch signaling also suppressed cardiomyocyte proliferation and heart regeneration. Taken together, our data uncover the exquisite sensitivity of regenerative cardiomyocyte proliferation to perturbations in Notch signaling.

myocardial infarction | model organism

When blood flow to a segment of the human heart becomes acutely interrupted, the hypoxic muscle suffers irreparable damage termed an acute myocardial infarction (1). An important public health concern, myocardial infarctions cause significant morbidity and mortality worldwide (2). As one of the least regenerative organs in the human body, the heart replaces the infarcted myocardium with noncontractile scar tissue instead of new muscle. As a result, the spared myocardium carries an increased hemodynamic burden that often leads to adverse ventricular remodeling and congestive heart failure. Therefore, the development of novel therapeutic strategies to stimulate human cardiac regeneration remains a top priority.

Unlike mammals, adult zebrafish completely regenerate their hearts following amputation, cryoinjury, hypoxia/reoxygenation injury, or genetic ablation of cardiomyocytes (3–11). Lineage tracing studies have demonstrated that new cardiomyocytes arise by proliferation of partially dedifferentiated cardiomyocytes spared from injury (12, 13). Specifically, cardiomyocytes in the compact muscular layer proximal to the wound break down their sarcomeres, activate *gata4* regulatory sequences, and initiate cell cycling with cytokinesis (12, 13). Interestingly, 1-d-old neonatal mice also achieve heart regeneration through cardiomyocyte proliferation following amputation injury (14). However, this endogenous regenerative potential is dampened by neonatal day 7 as

cardiomyocytes exit the cell cycle through the up-regulation of *meis1* and *miR-15* (15, 16). Ultimately, the natural capacity for cardiac regeneration exhibited by adult zebrafish and neonatal mice suggests that the human heart could be stimulated to regenerate if the cellular and genetic determinants of cardiomyocyte proliferation were fully elucidated.

Although adult mammalian cardiomyocytes have long been considered quiescent, recent stable isotope labeling studies in mice and humans have uncovered bona fide cardiomyocyte cell division during adult life (17–19). Moreover, following experimental myocardial infarction in mice, ~3% of cardiomyocytes in the peri-infarct region arise through cardiomyocyte proliferation (19). These data demonstrate that, although insufficient to restore heart function, cardiomyocyte renewal does occur in the adult mammalian heart. These observations highlight the potential of augmenting endogenous cardiomyocyte proliferation as an effective treatment for myocardial infarction.

The Notch signaling pathway plays fundamental roles in myriad developmental and regenerative processes (20). A previous study reported that partial amputation of the zebrafish ventricle stimulates expression of the Notch signaling components *deltaC* and *notch1b* (8), but the functional significance of this observation remains unexplored. Here, we report that amputation injury stimulates expression of three Notch receptors specifically in the endocardium and epicardium. Furthermore, Notch pathway suppression impaired the regeneration of new muscle and induced scar formation at the site of injury. Activation of the endocardium, epicardium, and *gata4*

Significance

Heart failure is a term used to describe the heart's inability to pump sufficient oxygen-rich blood throughout the body. This condition is commonly caused by the loss of heart muscle cells termed cardiomyocytes that results from a heart attack. As one of the least regenerative organs in the human body, the heart fails to regenerate damaged cardiomyocytes, forms a noncontractile scar instead, and progressively fails. Unlike mammals, adult zebrafish robustly regenerate their hearts following injury through division of uninjured cardiomyocytes, thus providing an opportunity to dissect innate cardiac regenerative mechanisms. Here, we provide evidence that Notch signaling is required for cardiomyocyte division and heart regeneration in zebrafish and, therefore, highlight a genetic determinant of natural heart renewal.

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cis-regulatory elements in compact myocardium were grossly unaffected by Notch suppression, but cardiomyocyte proliferation was significantly impaired. Unexpectedly, ubiquitous Notch pathway activation also inhibited cardiomyocyte proliferation and cardiac regeneration. These studies demonstrate that cardiomyocyte proliferative renewal is exquisitely sensitive to perturbations in Notch signaling.

Results

Endocardial and Epicardial Cells Up-Regulate Notch Receptors Following Ventricular Apex Amputation. The zebrafish genome encodes four Notch receptors including *notch1a*, *notch1b*, *notch2*, and *notch3*. We evaluated their expression patterns in uninjured hearts and injured hearts at 7 d postamputation (dpa) by section in situ hybridization. We observed *notch1a* and, to a lesser extent, *notch1b* expression in the endocardium of uninjured hearts (Fig. 1 *A* and *E*). *Notch 2* and *notch3* transcripts were sparsely distributed in the endocardium before injury (Fig. 1 *I* and *M*). In the uninjured epicardium, we found evidence of scattered *notch1a*, *notch2*, and *notch3* expression (Fig. 1 *A*, *I*, and *M*). Following amputation injury, *notch1a*, *notch1b*, and *notch2* transcripts were prominently up-regulated in the endocardium, with the highest levels of expression residing proximal to the wound (Fig. 1 *B*, *C*, *F*, *G*, *J*, and *K*). *Notch1a* and *notch2* were also strongly up-regulated in the epicardium covering the wound (Fig. 1 *B* and *J*). *Notch3* expression was qualitatively unaffected by injury (Fig. 1 *M–O*). Myocardial cells appeared devoid of all four Notch receptors both before and after injury (Fig. 1 *A–C*, *E–G*, *I–K*, and *M–O*). Colocalization studies with endocardial fluorescence driven by the *kdrl:mCherry* transgene confirmed endocardial expression of all four Notch receptors after injury and the absence of expression in the intervening myocardium
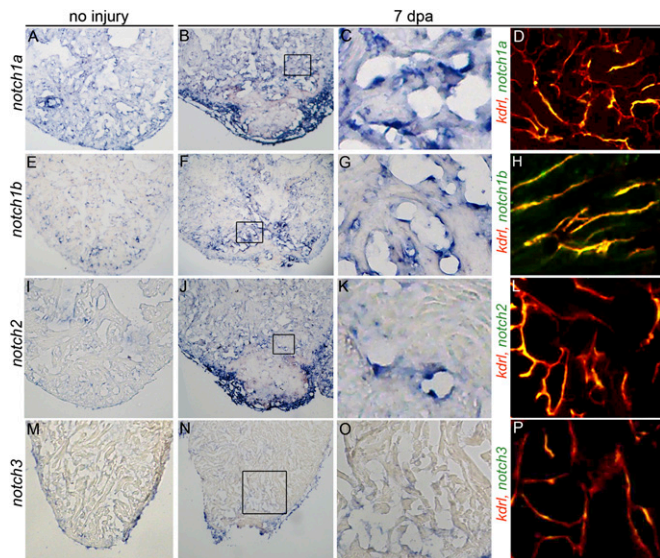
Fig. 1. Ventricular apex amputation induces up-regulation of Notch receptor expression in endocardial and epicardial cells. Representative histological sections of uninjured (*A*, *E*, *I*, and *M*) and injured hearts (*B*, *F*, *J*, and *N*) at 7 d postamputation (dpa) showing up-regulation of three Notch receptors in the endocardium and/or epicardium following amputation injury by colorimetric in situ hybridization analyses. Boxed regions of *B*, *F*, *J*, and *N* are shown at higher magnifications in *C*, *G*, *K*, and *O*. (*D*, *H*, *L*, and *P*) Representative cardiac sections from *Tg(kdrl:mCherry)* zebrafish processed for fluorescent in situ hybridization to visualize expression of the indicated Notch receptors and mCherry immunohistochemistry to visualize endocardial cells. *Notch1a*, *notch1b*, *notch2*, and *notch3* transcripts colocalized with endocardial cells without expression in the intervening myocardium. (*M* and *N*) *Notch3* transcripts were unchanged by injury. Consistent staining patterns were observed in eight hearts per experimental group for the colorimetric analyses and in two hearts for the fluorescent analyses.

(Fig. 1 *D*, *H*, *L*, and *P*). Lastly, quantitative PCR analysis corroborated our conclusions from the in situ hybridization studies (Fig. S1). Together, these data uncover injury-dependent up-regulation of three Notch receptors specifically in the endocardium and epicardium of the zebrafish ventricle.

Notch Signaling Is Required for Zebrafish Heart Regeneration. Tissue-specific expression of multiple Notch receptors following injury suggests that functional redundancy might exist to ensure that Notch signaling becomes activated during the regenerative response. To determine whether Notch pathway activation is required for cardiac regeneration, we exploited a previously validated experimental strategy for pan-Notch pathway inhibition that circumvents potential redundancy between the four receptors. Specifically, we isolated a transgenic zebrafish strain, *Tg(hsp70: DN-MAML)*, which expresses a dominant negative (DN) isoform of the murine mastermind-like (MAML) protein fused to GFP (21) under transcriptional control of the zebrafish heat shock promoter. Whereas wild-type MAML proteins recruit essential cofactors to the Notch transcriptional complex, the N-terminally truncated DN-MAML protein incorporates into the complex but lacks recruitment activity, thereby rendering the complex inert (21).

Heat shocking *Tg(hsp70:DN-MAML)* animals during embryogenesis resulted in GFP+ embryos (Fig. S2*A*) that appeared morphologically similar to those devoid of Notch signaling caused by the *mind bomb* (*mib*) mutation (Fig. S2 *B–D*) (22, 23). Furthermore, quantitative PCR analyses revealed that two previously identified Notch targets, *hey2* and *her4* (24–26), are similarly down-regulated in *mib* and heat shocked *Tg(hsp70:DN-MAML)* embryos (Fig. S2*E*). These data validate the *Tg(hsp70:DN-MAML)* strain as an inducible model of ubiquitous Notch inhibition.

To learn whether cardiac regeneration relies on Notch signaling, we performed ventricular apex amputation surgeries on control (nontransgenic) and *Tg(hsp70:DN-MAML)* zebrafish, heat shocked the animals daily for 30 d, and analyzed cardiac sections for evidence of impaired regeneration. Sections were stained with Acid Fuschin-Orange G (AFOG) to visualize cardiomyocytes (brown), fibrin (red), and collagen (blue) or immunostained with an antibody that recognizes the myocardial protein tropomyosin (TPM). Control animals achieved robust cardiac regeneration as evidenced by new myocardial tissue at the ventricular apex (Fig. 2 *A* and *C*). By contrast, *Tg(hsp70:DN-MAML)* hearts failed to regenerate myocardium, instead retaining fibrin and depositing collagen at the site of amputation (Fig. 2 *B* and *D*). A similar regenerative failure was observed in a second strain of *Tg(hsp70:DN-MAML)* zebrafish (Fig. S2 *F–H*). Taken together, these data demonstrate that suppression of Notch signaling causes a regenerative failure in amputated zebrafish hearts.

Notch Signaling Is Dispensable for Epicardial and Endocardial Activation. To identify potential cellular mechanisms underlying the observed regenerative failure, we analyzed the epicardium and endocardium of Notch-suppressed hearts for characteristic cellular behaviors associated with successful regeneration. As sites of injury-dependent Notch receptor expression, these tissues were of particular interest. Following ventricular amputation, epicardial cells undergo an epithelial-to-mesenchymal transition to produce epicardial derived cells (EPDCs) that invade the regenerate between 7 and 30 dpa (27–30), ultimately becoming perivascular smooth muscle cells that support the regenerating coronary network (27, 30). We monitored EPDC production using a transgenic reporter strain, *Tg(tcf21:DsRed2)*, that expresses DsRed2 in Tcf21+ epicardial cells and EPDCs (27). At 7 dpa, both control and *Tg(hsp70:DN-MAML)* hearts expressed DsRed2 robustly in epicardial and subepicardial cells away from the wound (Fig. 3 *A* and *B*), reflective of normal tissue architecture. Quantification of DsRed2+ EPDC numbers revealed no significant difference between experimental groups (Fig. 3*C*). Nonetheless, we could not rule out the possibility that Notch signaling is required for organ-wide epicardial activation characterized by injury-induced

Fig. 2. Inhibition of Notch signaling following amputation injury prevents cardiac regeneration. (*A–D*) Inhibition of Notch signaling impairs cardiac regeneration. Representative cardiac sections from heat shocked control (*A* and *C*) and *Tg(hsp70:DN-MAML)* (*B* and *D*) animals 30 dpa evaluated by AFOG staining (*A* and *B*) or immunofluorescence (*C* and *D*) for the myocardial marker TPM. Whereas heat shocked control animals robustly regenerated myocardium (asterisks in *A* and *C*), *Tg(hsp70:DN-MAML)* hearts failed to regenerate new myocardium (arrowheads in *B* and *D*) instead showing evidence of residual fibrin (red in *B*) and collagen deposition (blue in *B*). Cardiac regeneration failed in 0 of 24 heat shocked control animals and 34 of 35 heat shocked *Tg(hsp70:DN-MAML)* animals.

expression of *raldh2* (27, 29). Therefore, we examined *raldh2* expression by section in situ hybridization at 7 dpa in control and Notch-inhibited hearts and witnessed roughly equivalent epicardial transcript levels both away from and covering the wound (Fig. 3 *D* and *E*). From these findings, we conclude that inhibition of Notch signaling does not interfere with epicardial activation.

Previous studies demonstrated that ventricular apex amputation also stimulates organ-wide activation of endocardial cells characterized by up-regulation of *raldh2* transcripts and a shift from a flattened to a rounded morphology (31). Within 1 dpa, endocardial cells away from the wound revert to a preinjury phenotype, but endocardial cells at the wound site remain activated at 7 dpa. Both control and *Tg(hsp70:DN-MAML)* hearts expressed high levels of *raldh2* at the wound edge (Fig. 3 *D* and *E*) in flat and rounded endocardial cells (Fig. 3 *F* and *G*). Therefore, inhibition of Notch signaling does not impair endocardial activation.

Evidence for Coronary Endothelial Regeneration in Notch-Deficient Hearts. Following injury, coronary arteries regenerate to perfuse the new myocardium (29). Using a transgenic reporter that expresses red fluorescence in endocardial and coronary endothelial cells, *Tg(kdrl:mCherry)* (32), we tested the hypothesis that regeneration of coronary endothelium requires Notch signaling. To that end, we performed apex amputation surgeries on control and *Tg(hsp70:DN-MAML)* hearts, exposed the animals to daily heat shock treatments, and analyzed red reporter fluorescence in cardiac sections at 30 dpa. Control hearts exhibited a regenerated ventricular apex consisting of new endocardium and coronary vessels (Fig. 4*A*). Interestingly, although *Tg(hsp70:DN-MAML)* hearts lacked regenerated myocardium, they exhibited mCherry+ endothelial tubes in the wound area (Fig. 4*B*), demonstrating that Notch signaling is dispensable for initiating coronary artery regeneration.

Regenerated Coronary Endothelium Derives from Preexisting Endothelium or Endocardium. Although endothelial tubes inhabited the wound area of injured *Tg(hsp70:DN-MAML)* hearts, it was unclear from our section analysis whether these nascent coronary vessels connected to the preexisting coronary network. To date, the cellular source of new coronary endothelium during zebrafish heart regeneration remains undefined. It is possible that new coronary endothelial cells differentiate de novo from progenitor cells through vasculogenesis. Alternatively, they might derive from preexisting endothelial cells by angiogenesis.

To test the hypothesis that regenerated coronary endothelium derives, at least in part, from preexisting endothelial cells, we engineered transgenic zebrafish, *Tg(kdrl:CreER)*, that express a 4-hydroxy-tamoxifen (4-HT)–inducible Cre recombinase under transcriptional control of the endothelial specific *kdrl* promoter. Second, we obtained a previously described ubiquitous reporter strain, *Tg(ubi:Switch)*, in which Cre-expressing cells and all of their progeny become permanently labeled with mCherry fluorescence (33). We created double transgenic *Tg(kdrl:CreER); Tg(ubi:Switch)* embryos and confirmed that 4-HT treatment between 24 and 48 h postfertilization (hpf) induced reporter recombination specifically in endothelial cells during development (Fig. 5 *A–D*).

Fig. 3. Notch signaling is dispensable for injury-induced epicardial and endocardial activation. (*A* and *B*) Representative cardiac sections from 7dpa heat shocked control (CTRL) (*A*) and *Tg(hsp70:DN-MAML)* (*B*) animals carrying the *Tg(tcf21:DsRed2)* transgene. In regions away from the wound, DsRed2+ epicardial cells (arrows) and EPDCs (asterisks) were grossly normal in Notch suppressed hearts. Seven of seven hearts in each experimental group expressed DsRed2 in epicardium and EPDCs. (*C*) Graph representing the average numbers of subepicardial DsRed2+ cells quantified in three ventricular sections from four hearts per experimental group. n.s., not significant. (*D–G*) Representative cardiac sections from heat shocked control (*D*) and *Tg(hsp70:DN-MAML)* (*E*) animals at 7 dpa stained for *raldh2* transcripts by in situ hybridization. In both experimental groups, prominent *raldh2* expression was observed in epicardial cells covering the wound (black arrowheads in *D* and *E*), away from the wound (open arrowheads in *D* and *E*), and in endocardial cells near the wound edge (black arrows in *D* and *E*). Higher magnifications of endocardial *raldh2* expression are shown in *F* and *G*. Endocardial cells near the wound displayed both flattened (black arrowheads in *F* and *G*) and rounded morphologies (open arrows in *F* and *G*). Four of four hearts in each experimental group expressed endocardial and epicardial *raldh2* transcripts.

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Fig. 4. Evidence for regeneration of coronary endothelium in Notch-inhibited hearts. (*A* and *B*) Representative cardiac sections from heat shocked control (CTRL) (*A*) and *Tg(hsp70:DN-MAML)* (*B*) animals carrying the *Tg(kdrl:mCherry)* endothelial reporter at 30 dpa. (*Insets*) Higher magnification views of boxed regions in *A* and *B*. Whereas control animals regenerated mCherry+ endocardium and coronary vessels, *Tg(hsp70:DN-MAML)* hearts harbored mCherry+ endothelial tubes in the nonregenerated region of the heart. The myocardium is autofluorescent in *A* and *B*. Fourteen of 14 control and 11 of 11 *Tg(hsp70:DN-MAML)* hearts demonstrated these phenotypes.

Control and 4-HT–treated embryos were grown to adulthood and analyzed for mCherry expression in the heart. Importantly, double transgenic adults never exposed to 4-HT did not exhibit mCherry reporter fluorescence demonstrating that this transgene combination is not leaky (Fig. S3 *A–C*). Conversely, we witnessed widespread mCherry expression throughout the endocardium (Fig. 5 *E* and *F*) and coronary endothelium (Fig. 5*G*) of 4-HT–treated animals indicating that a high percentage of these adult tissues derive from *kdrl*+ cells in the 24- to 48-hpf embryo. Next, we performed amputation surgeries on labeled adults and evaluated the color of nascent coronary endothelium with red or green vessels indicating an endothelial or nonendothelial source, respectively. At 14 dpa, we witnessed mCherry+ endothelial cells in the regenerating coronary network (Fig. 5 *H* and *I*), demonstrating that new coronary endothelium derives, at least partially, from preexisting endothelium perhaps including endocardium.

Notch Signaling Is Essential for Cardiomyocyte Proliferation. Although Notch signaling is required for heart regeneration and Notch receptor expression increases by 7 dpa in the endocardium and epicardium, no cellular phenotypes were detected in either tissue of Notch-inhibited hearts. Therefore, we turned our attention to the myocardium. Regenerated cardiomyocytes arise by dedifferentiation and proliferation of spared cardiomyocytes within the compact layer (12, 13). Thus, we evaluated *Tg(hsp70: DN-MAML)* hearts for defects in cardiomyocyte dedifferentiation and proliferation following injury. First, we used a new transgenic strain, *Tg(gata4:DsRed2)*, that expresses DsRed2 fluorescent protein within dedifferentiated cardiomyocytes (12). In both control and *Tg(hsp70:DN-MAML)* hearts, *DsRed2*+ cells were observed in compact myocardium contiguous to and within the regenerate (Fig. 6 *A* and *B*), indicating that Notch signaling is dispensable for cardiomyocyte dedifferentiation during zebrafish heart regeneration.

Next, we compared myocardial proliferation between control and *Tg(hsp70:DN-MAML)* ventricles at 7 and 14 dpa by quantifying the number of nuclei double positive for the myocardial marker Mef2 and the DNA replication marker proliferating cell nuclear antigen (PCNA) (Fig. 6 *C–F*). Control hearts exhibited proliferation indices between 20% and 25% at 7 and 14 dpa (Fig. 6 *G* and *H*). By contrast, *Tg(hsp70:DN-MAML)* hearts harbored significantly fewer proliferating cardiomyocytes as reflected in proliferation indices at 53% and 40% of control at the same time points (Fig. 6 *G* and *H*). These data reveal that Notch signaling is essential for stimulating cardiomyocyte proliferation following injury.

We refined the time frame following injury during which Notch signaling is required for cardiomyocyte proliferation. Whereas Notch suppression before the initiation of myocyte division, on 1 and 2 dpa (12), did not significantly reduce the proliferation index (Fig. S4*A*), Notch suppression during cardiomyocyte proliferation,

on 6 and 7 dpa (12), significantly decreased the proliferation index (Fig. S4*A*). These observations demonstrate that Notch signaling is required coincident with cardiomyocyte renewal.

Hyperactivation of Notch Signaling Blocks Zebrafish Heart Regeneration. Because Notch signaling is required for cardiac regeneration in zebrafish, we hypothesized that hyperactivation of Notch signaling might accelerate the regenerative process. To test this hypothesis, we used a previously described GAL4/UAS-based double transgenic zebrafish strain for heat shock activation of ligand-independent Notch signaling following cardiac injury (34, 35). Heat shocking these animals caused a twofold induction of Notch target genes *hey2* and *her4* during embryogenesis, demonstrating that the magnitude of Notch hyperactivation is twice that of endogenous levels (Fig. S5*A*). We performed cardiac amputation surgeries on control and double transgenic *Tg(hsp70: Gal4); Tg(UAS:NICD)* zebrafish, heat shocked the animals daily for 30 d, and assessed myocardial regeneration in cardiac sections. Unexpectedly, hyperactivation of Notch signaling did not accelerate heart regeneration, but instead resulted in fibrin retention and collagen deposition as revealed by AFOG staining (Fig. 7 *A* and *B*). Consistent with this observation, immunostaining with anti-TPM antibodies revealed large myocardial deficits within the ventricular wall of *Tg(hsp70:Gal4); Tg(UAS:NICD)* hearts at 30 dpa (Fig. 7 *C* and *D*). Using an alternative transgenic line, *Tg (hsp70:NICD)*, in which NICD levels are directly controlled by the *hsp70* promoter in a reproducible manner (Fig. S5 *B* and *C*), we witnessed a quantitatively similar decrease in the cardiomyocyte proliferation indices between Notch-suppressed and Notch-activated hearts with the latter showing a 47% reduction at both 7 and 14 dpa compared with controls (Fig. 7 *E–I*). Notch hyperactivation
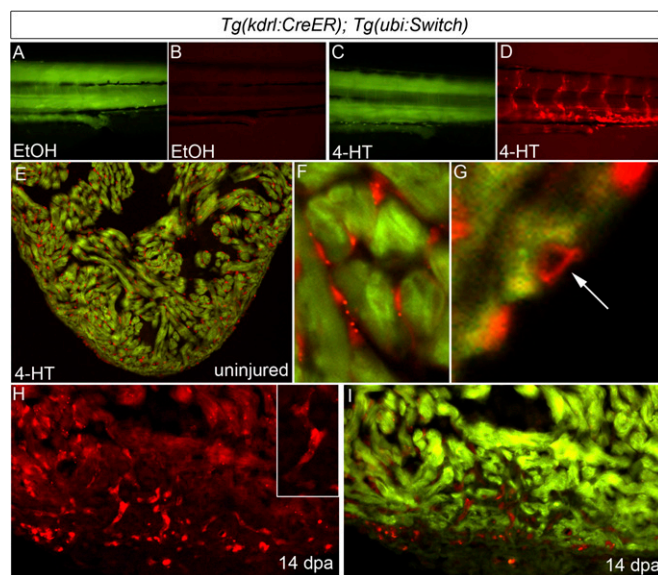
Fig. 5. Regenerated coronary endothelium derives from preexisting endothelial cells during zebrafish heart regeneration. (*A–D*) Permanent labeling of endothelial cells during zebrafish embryogenesis. Tails of 96-hpf *Tg(kdrl: CreER); Tg(ubi:Switch)* embryos treated between 24 and 48 hpf with EtOH (*A* and *B*) or 4-HT (*C* and *D*) and imaged in the green (*A* and *C*) and red (*B* and *D*) channels. All 4-HT–treated embryos examined expressed mCherry in endothelial cells (*n* > 100), whereas EtOH-treated embryos failed to report red fluorescence (*n* > 100). (*E–G*) The adult endocardium and coronary endothelium displayed widespread and permanent mCherry labeling following 4-HT treatment during embryonic stages (*n* = 3/3). Representative cardiac section (*E*) and close up views of mCherry-labeled endocardium (*F*) and coronary endothelium (*G*). (*H* and *I*) Representative cardiac section from a regenerating *Tg(kdrl:CreER); Tg(ubi:Switch)* animal with mCherry-labeled endocardial and endothelial cells at 14 dpa. Numerous mCherry+ endothelial tubes (*Inset*) populated the regenerating myocardium. (*n* = 3/3.)
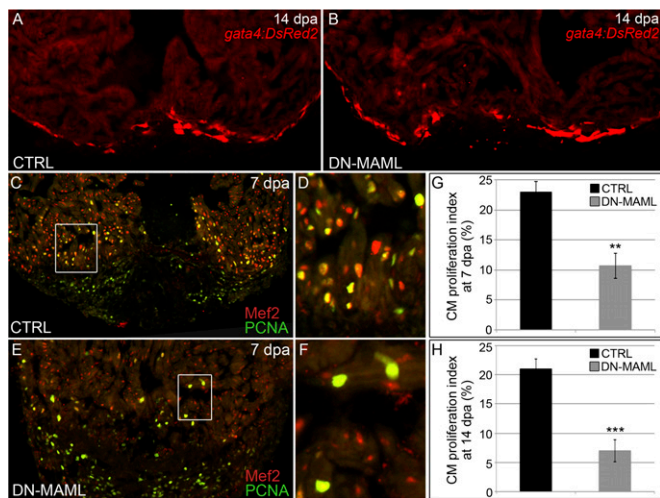
Fig. 6. Notch signaling is required for cardiomyocyte proliferation during zebrafish heart regeneration. (*A* and *B*) Representative cardiac sections from heat shocked control (CTRL) (*A*) and *Tg(hsp70:DN-MAML)* (*B*) animals carrying the *Tg(gata4:DsRed2)* transgene at 14 dpa. DsRed2 expression was visible in the compact myocardium of both control and *Tg(hsp70:DN-MAML)* hearts (*n* = 12/12 in each group). (*C–F*) Representative cardiac sections from heat shocked control (*C* and *D*) and *Tg(hsp70:DN-MAML)* (*E* and *F*) animals at 7 dpa. Sections were double immunostained to identify cardiomyocyte nuclei (Mef2+) and nuclei undergoing DNA replication (PCNA+). Boxed regions in *C* and *E* are shown at higher zoom in *D* and *F*. The percentages of myocardial nuclei undergoing DNA replication near the wound edges were quantified at 7 (*G*) and 14 (*H*) dpa and reported as mean proliferation indices ± 1 SD, **$P < 0.01$ and ***$P < 0.001$. In the control group, seven and eight hearts were analyzed at 7 and 14 dpa, respectively. In the *Tg(hsp70:DN-MAML)* group, three and four hearts were analyzed at 7 and 14 dpa, respectively.

specifically during cardiomyocyte proliferation impaired heart regeneration (Fig. S4*B*). Collectively, our data reveal the exquisite sensitivity of zebrafish heart regeneration to alterations in Notch signaling.

Discussion

We explored the functional role of Notch signaling during zebrafish heart regeneration. We learned that injured zebrafish hearts induce the expression of three Notch receptors tissue-specifically in the endocardium and epicardium, but not the myocardium. Using a dominant negative approach, we inhibited Notch signaling during the regenerative window and witnessed a significant defect in myocardial regeneration accompanied by scar formation at the amputation site. Through a systematic evaluation of the stereotypical cellular behaviors associated with cardiac regeneration, we learned that Notch signaling is grossly dispensable for endocardial and epicardial activation, coronary endothelial regeneration, and dedifferentiation of compact myocardial cells. However, Notch suppression caused significant deficits in cardiomyocyte proliferation thereby revealing the cellular requirement for Notch signaling during adult cardiac regeneration. Interestingly, a recent study implicated Notch signaling in reconstitution of the zebrafish larval ventricular myocardium following injury (36). Thus, the requirement for Notch signaling in cardiac regeneration appears to exist throughout the lifespan of zebrafish.

Although we observed injury-dependent Notch receptor up-regulation in the endocardium and epicardium, the Notch suppression phenotype we documented resided in the myocardium. These data point to a noncell autonomous role for Notch signaling in the regulation of zebrafish cardiomyocyte proliferation and support a model in which Notch signaling stimulates production of an endocardial and/or epicardial paracrine signal that permits or instructs myocardial cell division.

A recent study identified retinoic acid (RA) as a permissive signal for cardiomyocyte proliferation during zebrafish heart regeneration (31). In the current study, we learned that transcripts encoding Raldh2, the biosynthetic enzyme for RA, is grossly unaffected by Notch suppression, suggesting that other signals mediate Notch-dependent cardiomyocyte proliferation during regeneration. Additional candidate signals include ligands for *TGFβ*, *sonic hedgehog*, and *igf* ligands, all of which are induced by injury specifically in the endocardium and/or epicardium (37, 38). Furthermore, inhibition of these pathways with inducible transgenes and/or small molecules impairs cardiomyocyte proliferation. Current efforts are underway to evaluate Notch-suppressed hearts for perturbations in the expression of these ligands.

An additional candidate factor comes from the established noncell autonomous role of endocardial Notch signaling in cardiomyocyte proliferation during mouse embryonic ventricular trabeculation (39). In this setting, an unidentified Notch-dependent ligand produced by the endocardium stimulates myocardial cells to secrete the proliferation signal Bmp10. We localized *bmp10* transcripts to the endocardial compartment of amputated zebrafish ventricles with qualitatively equivalent levels being observed between control and Notch-deficient hearts (Fig. S6). Ultimately, identifying the downstream targets of Notch signaling that stimulate regenerative cardiomyocyte proliferation remain an active area of investigation.

In higher vertebrates, including humans, up-regulation of Notch signaling in both myocyte and/or nonmyocyte lineages of the heart appears to be a general response to ischemic injury (40–43) and cardiomyopathies (44, 45). Within the myocyte lineage, the expression of Notch signaling components is cardioprotective, as myocardial-specific inhibition of Notch signaling worsened
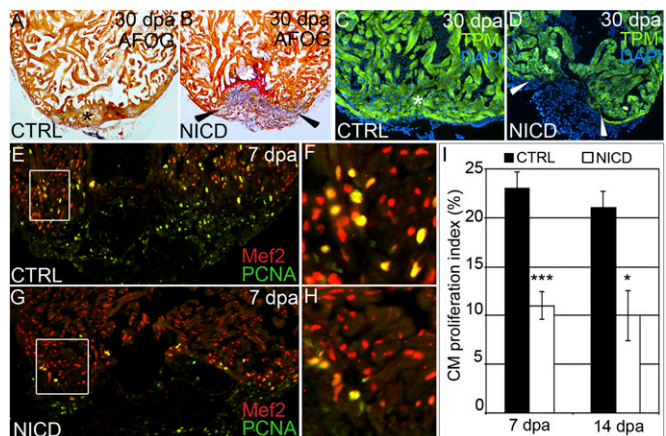
Fig. 7. Hyperactivation of Notch signaling impairs cardiomyocyte proliferation and heart regeneration. (*A–D*) Representative cardiac sections from heat shocked control (CTRL) (*A* and *C*) and *Tg(hsp70:Gal4)*; *Tg(UAS: NICD)* (*B* and *D*) animals at 30 dpa stained with AFOG (*A* and *B*) or an antibody recognizing the myocardial marker TPM (*C* and *D*). Whereas heat shocked CTRL animals regenerated myocardium (asterisks in *A* and *C*), *Tg (hsp70:Gal4)*; *Tg(UAS:NICD)* hearts exhibited impaired regeneration (arrowheads in *B* and *D*), residual fibrin (red in *B*), and collagen deposition (blue in *B*). In the CTRL group, >25 hearts were analyzed and all showed significant myocardial regeneration. In the *Tg(hsp70:Gal4)*; *Tg(UAS:NICD)* group, 18 hearts were analyzed and 14 showed a significant myocardial deficit, residual fibrin, and collagen deposition. (*E–H*) Representative cardiac sections of heat shocked CTRL (*E* and *F*) and *Tg(hsp70:NICD)* (*G* and *H*) animals at 7 dpa double stained with antibodies that recognize cardiomyocyte nuclei (Mef2+) and nuclei undergoing DNA replication (PCNA+). Boxed regions in *E* and *G* are shown at higher zoom in *F* and *H*. (*I*) The percentages of cardiomyocyte nuclei undergoing DNA replication were quantified along the wound edge at 7 and 14 dpa and expressed as mean proliferation indices ± 1 SD, ***$P < 0.001$; *$P < 0.05$. In the control group, seven and eight hearts were analyzed at 7 and 14 dpa, respectively. In the *Tg(hsp70:NICD)* group, three and four hearts were analyzed at 7 and 14 dpa, respectively.

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cardiac function and animal survival (41, 44). Intriguingly, amputated zebrafish hearts up-regulate Notch receptors exclusively in nonmyocyte lineages, highlighting species- and/or injury-model–specific differences. In the mouse epicardium, Notch-expressing cells are amplified and increasingly adopt a fibroblast cell fate following injury (43). Although these data suggest that the role of Notch signaling in the mouse is to aid in fibrosis repair, they also reveal a conserved propensity for Notch-expressing cells to become active following injury.

Although hyperactivated Notch signaling generally improves the responses of higher vertebrates to cardiac injury (40, 41) and disease (44, 45), ubiquitous Notch hyperactivation is detrimental to zebrafish heart regeneration. While the underlying mechanism remains unclear, hyperstimulation of Notch signaling in the endocardium and/or epicardium might compromise production of the hypothesized proliferation signal similar to Notch suppression.

Equally plausible is that aberrant activation of Notch signaling in the myocardium is incompatible with cardiomyocyte proliferation. Although induction of Notch signaling in immature mammalian cardiomyocytes stimulates proliferation in vitro (46, 47) and in vivo (41, 48), Notch activation in mature cardiomyocytes fails to stimulate proliferation (41) and causes cell cycle arrest

(46). Ultimately, longer-term experiments designed to activate Notch signaling tissue-specifically in myocardium, endocardium, and epicardium will be necessary to determine the mechanism underlying the regenerative block we observed in *Tg(hsp70:NICD)* ventricles.

Materials and Methods

Detailed materials and methods are available in *SI Materials and Methods*. Adult heat shock treatments, histological sectioning, in situ hybridization, and immunohistochemistry were performed similar to those described (7, 29). Cardiomyocyte proliferation indices were determined as described (31).

P values were calculated using an unpaired two-tailed t test with unequal variance using Microsoft Excel.

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