Epigenetic Modification at Notch Responsive Promoters Blunts Efficacy of Inducing Notch Pathway Reactivation After Myocardial Infarction

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Rationale: The Notch pathway plays a key role in stimulating mammalian cardiomyocyte proliferation during development and in the early postnatal life; in adult zebrafish, reactivation of this pathway is also essential to drive cardiac regeneration after injury.

Objective: We wanted to assess efficacy of Notch pathway stimulation in neonatal and adult hearts as a means to induce cardiac regeneration after myocardial infarction in mice.

Methods and Results: In early postnatal life, cardiomyocyte exit from the cell cycle was paralleled by decreased Notch signaling and the establishment of a repressive chromatin environment at Notch-responsive genes, characterized by recruitment of the polycomb group enhancer of zeste homolog 2 methyltransferase and the acquisition of the histone 3 Lysine 27 trimethylation histone mark, as detected by chromatin immunoprecipitation. Forced Notch pathway activation by adenoassociated virus gene transfer of activated Notch1 or its ligand Jagged1 expanded the proliferative capacity of neonatal cardiomyocytes; this correlated with increased transcription of Notch target genes and maintenance of an open chromatin conformation at their promoters. The same adenoassociated virus vectors, however, were largely ineffective in stimulating cardiac repair after myocardial infarction in adult mice, despite optimal and long-lasting transgene expression. Analysis of Notch-responsive promoters in adult cardiomyocytes showed marks of repressed chromatin and irreversible Cpg DNA methylation. Induction of adult cardiomyocyte re-entry into the cell cycle with microRNAs was independent from Notch pathway reactivation.

Conclusions: Notch pathway activation is crucial in regulating cardiomyocyte proliferation during the early postnatal life, but it is largely ineffective in driving cardiac regeneration in adults, because of permanent epigenetic modification at Notch-responsive promoters. (Circ Res. 2014;115:636-649.)

Key Words: chromatin ■ dependovirus ■ developmental biology ■ DNA methylation ■ myocardial infarction

There is a pressing need to develop novel therapeutic approaches aimed at generating new contractile tissue in patients with myocardial damage. Although there is evidence that, after cardiac injury, a partial attempt at cardiomyocyte regeneration takes place in the damaged areas, myocardial repair typically occurs by scarring. A powerful way to boost cardiomyocyte generation in damaged, adult hearts would be to reinitiate or to enhance the developmental program that sustains robust cardiomyocytes in the fetus contain activated Notch1 in their nuclei, and this factor continues to act as a key, positive regulator during the short proliferative window in the early postnatal life.7

Physiological activation of Notch signaling requires cell-cell contact and occurs through binding of one of the 4 Notch receptors (Notch 1–4) to one of their ligands (Delta and Jagged in vertebrates), followed by proteolytic release of the intracellular domain of Notch (Notch-ICD) and its translocation into the nucleus.11 Once inside the nucleus, the Notch-ICD interacts with the recombinant signal binding protein for immunoglobulin kappa J region (RBP-Jk) transcription regulator, and, together with various chromatin modifiers, triggers activation of a series of cellular genes, which include Hey1, Hey2, Hes1,5,12 CyclinD1,8,13 and Notch1 itself.14

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Many studies have provided evidence that activation of Notch signaling exerts a beneficial effect after heart injury. Transgenic mice overexpressing the Notch1-ICD or its Jagged1 ligand in cardiomyocytes show better response after myocardial damage. Similar findings were also observed when the Notch pathway was activated in the adult mouse myocardium by the hepatocyte growth factor or high mobility group box 1 (HMGBl). Notch1-activated cells were also found enriched in the epicardium in response to myocardial infarction (MI) and aortic banding. Finally, administration of bone morphogenetic protein 10, which is also controlled by Notch, improves cardiac repair after infarction.

In the adult zebrafish heart, which is capable of fully regenerating the heart after myocardial damage, the regenerative process is sustained by the reactivation of the Notch pathway in the proliferating cardiomyocytes. In mammals, an attempt at cardiac regeneration also takes place after MI, as indicated by the presence of a small number of proliferating cardiomyocytes at high efficiency and to drive transgene expression in a persistent manner. The primers used are listed in Online Table III.

On the basis of these observations, we wanted to test whether the regenerative capacity of the adult mouse heart might be stimulated by forcing reactivation of the Notch pathway after MI. We took advantage of the possibility to deliver the activated Notch1-ICD or the Jagged1 ligand to the heart using viral vectors based on the adenoassociated virus (AAV), which possess the exquisite capacity to transduce cardiomyocytes at high efficiency and to drive transgene expression in a persistent manner.

Here, we show that neonatal cardiomyocytes transduced with the AAV vectors expressing the 2 factors prolong their proliferative potential in culture and that this is paralleled by the preservation of an open chromatin conformation at the Notch1 target genes, characterized by histone 3 lysine 4 trimethylation. Activation of the Notch pathway, however, is largely ineffecual after MI in adult hearts, where the same gene promoters are embedded in a closed chromatin conformation characterized by the presence of permanent, repressive chromatin marks, including histone 3 lysine 27 trimethylation (H3K27me3) and the polycomb group (PcG) complex repressive methylase enhancer of zeste homolog 2 (Ezh2). Furthermore, in adult cardiomyocytes, the DNA sequence of these promoters shows CpG methylation, a mark of irreversible transcriptional repression. We conclude that the induction of adult cardiomyocyte proliferation through Notch pathway stimulation is not a suitable strategy to induce a regenerative response in adult mouse hearts.

**Methods**

An expanded Methods section is available in the Online Data Supplement.

**Animals**

Animal care and treatment were according to institutional guidelines and in compliance with national and international laws and policies (European Economic Community Council Directive 86/609, OJL 358, December 12, 1987).

**Culture of Rat Cardiomyocytes**

Culture of ventricular cardiomyocytes from neonatal Wistar rats was performed as previously described. Ventricular cardiomyocytes from adult mice were isolated from Langendorff-perfused hearts of female Wistar rats (2-months old).

**Chromatin Immunoprecipitation**

Chromatin immunoprecipitation was performed as described, with modification detailed in the Methods in the Online Data Supplement. The primers used are listed in Online Table III.

**Immunofluorescence and 5-Bromo-2′-Deoxyuridine Detection**

Immunofluorescence staining was performed on cultured cardiomyocytes fixed in 4% PFA, as previously described.

**MI and Echocardiography Analysis**

MI was produced in adult female CD1 mice (8–12 weeks old), by permanent left anterior descending coronary artery ligation. AAV transduction was performed with a single injection of recombinant AAV vectors, at a dose of 1×10^11 viral genomes per animal, into the myocardium bordering the infarct zone. Evaluation of cardiac function was achieved using a Visual Sonics Vevo 770 Ultrasound (Visual Sonics) equipped with a 30-MHz linear array transducer.

**AAV Vector Production and Transduction**

The AAV vectors used in this study were generated by the AAV Vector Unit at International Centre for Genetic Engineering and Biotechnology Trieste (http://www.icgeb.org/avu-core-facility.html) according to established procedures, as detailed in the Methods in the Online Data Supplement. All vectors are based on the AAV2 genome, and the transgenes are driven by the cytomegalovirus immediate early promoter.

Neonatal cardiomyocytes were transduced contextually to plating with at multiplicity of infection=1×10^5 vg per cell. Adult cardiomyocytes were transduced the day after plating at multiplicity of infection=1×10^4 vg per cell. To evaluate AAV persistence and expression in vivo, animals were injected intraperitoneally with AAVs (2.25×10^10 vg/g body weight). Heart samples were collected at 7, 14, 30, and 60 days after injection.

**Bisulfite Sequencing**

Genomic DNA (1 μg) extracted from cardiomyocytes in culture was treated for DNA conversion with sodium bisulfite using EpItect Bisulfite Kit (Qiagen) according to manufacturer’s instruction. Promoter sequences were analyzed with using Methyl Primer Express Software v1.0 (Applied Biosystems) to predict CpG islands. Primer sequences were designed using the same software and are listed in Online Table IV. Amplified fragments were separated on 2% agarose gel, visualized with ethidium bromide staining, and then purified from gel using Wizard SV Gel and Polymerase Chain Reaction Clean-up system kit (Promega). The purified DNA was cloned into TOPO T/A cloning kit (Invitrogen). Eight randomly picked clones were sequenced and analyzed using BiQ analyzer.
Statistical Analysis
All data are presented as mean±SEM. Statistical analysis was performed using Prism Software (GraphPad), using 1-way ANOVA followed by Bonferroni post hoc test for the comparison of ≥3 groups.

Results
Decrease of Cardiomyocyte Proliferation After Birth Coincides With Markedly Reduced Notch Signaling
The extent of neonatal rat cardiomyocytes proliferation, as measured by 5-bromo-2′-deoxyuridine (BrdU) incorporation, progressively declined after birth, as shown in Figure 1A by comparing cardiomyocytes at day 3 versus day 7 after isolation (P<0.01), consistent with previous results.1 Virtually no BrdU incorporation was detectable in cardiomyocytes from adult (2-months old) rats. In the same samples, the levels of the Notch1 mRNA were markedly decreased at day 7 when compared with that at day-3 cardiomyocytes, reaching levels similar to those found in adult cells (P<0.01). The mRNA levels for the Notch1 target genes Hes1, Hey1, Hey2,5 and the proliferation-associated gene Cyclin D18 showed a parallel decline for both day 7 and adult cardiomyocytes when compared with day-3 cells (Figure 1B).

Loss of Cardiomyocyte Proliferation Parallels the Establishment of Repressive Chromatin at Notch-Responsive Genes
To understand the molecular correlates accompanying the loss of Notch signaling, we analyzed chromatin at the promoters of the Notch-responsive genes Hes1, Hey1, Hey2, Notch1, and Cyclin D1 in day-3 (proliferating), day-7 (quiescent), and adult cardiomyocytes; the GAPDH promoter served as a control. For each of these genes, sets of primers were designed and validated, mapping in correspondence of the gene transcription start sites (TSSs; Figure 1C). Chromatin immunoprecipitation experiments were performed by immuno-precipitating chromatin using antibodies against 2 marks correlating with active chromatin (acetylated and lysine 4-trimethylation, respectively) and 2 marks of transcriptionally repressed chromatin. The last 2 modifications were related to transcriptional suppression by the PcG complex of proteins and included H3K27me3 and the presence of the Ezh2 methylase enzyme itself.

When cells actively proliferated (day 3), the Notch-responsive gene promoters were marked by the active chromatin marks H3panAc and histone 3 lysine 4 trimethylation. On neonatal day 7 and adult cardiomyocytes, no changes were detected at the GAPDH promoter (Figure 1D). On the contrary, on day-7 and in adult cardiomyocytes, the Notch target gene promoters were marked by the repressive H3K27me3 modification and were occupied by Ezh2 (Figure 1E).

Increased Ezh2 occupancy at the Notch-responsive promoters was unrelated to changes in Ezh2 expression levels (Online Figure 1A).

We conclude that a shift from active to repressive chromatin occurs at the Notch-related promoters concomitant with the cells exiting the proliferative phase.

Activation of the Notch Pathway by AAV-Mediated Gene Transfer Promotes Neonatal Rat Cardiomyocyte Proliferation
Given the correlation between Notch1 levels and the extent of cardiomyocyte proliferation, and considering the established role of Notch1 in driving proliferation of these cells during the last phase of cardiac development,2,6 we wondered whether the forced activation of the Notch pathway might extend the proliferative window of neonatal cardiomyocytes. We took advantage of the properties of AAV serotype 6 (AAV6) vectors to transduce these cells at high efficiency26 (Online Figure IIA and IIB). We obtained 2 AAV6 vectors, one coding for the constitutively active N1ICD (AAV6-N1ICD) and the other for the soluble form of the ligand Jagged1 (AAV6-sJ1). The latter is secreted by the transduced cells and activates Notch signaling in a contact-independent manner, therefore, acting as a soluble cytokine3−6; Figure 2A. Neonatal rat cardiomyocytes were transduced after isolation at birth (multiplicity of infection=1×104 vg per cell) with either of the 2 vectors; in both cases, transgene expression was sustained at both days 3 and 7 (Figure 2B). Exogenously introduced N1ICD interacted with cellular RBP-Jκ protein (Online Figure III).

Transduction markedly increased the number of proliferating cardiomyocytes, as evaluated by pulsed the cardiomyocyte cultures with BrdU for 24 hours before assessing the number of BrdU-positive nuclei by immunofluorescence (Figure 2C for representative images), as well as by evaluating positivity for histone H3 serine 10 phosphorylation (a marker of mitosis; Online Figure IVA and IVB) and for localization of Aurora B kinase in midbodies (showing cytokinesis; Online Figure IVC and IVD). In the case of AAV6-N1ICD–transduced cardiomyocytes, the fraction of α-actinin+, BrdU+ cells was 14.5% at day 3 and 18.7% at day 7, to be compared with 9.4% and 4.1% in cells transduced with a control AAV6 vector (P<0.05 and P<0.01, respectively). Instead, proliferation did not increase at day 3 in the cultures transduced with AAV6-sJ1; however, it was remarkably higher than control at day 7 (15.7% proliferating cells; P<0.01; Figure 2D). These different kinetics most likely reflect the requirement of sJ1 to accumulate in the extracellular environment in order for the soluble ligand to cluster and activate the Notch pathway.3,36

Activity of AAV6-sJ1, but not that of AAV6-N1ICD, was dependent on endogenous Notch1 receptor because it was inhibited by DAPT (N-[N-((3,5-difluorophenacetyl)-L-alanyl)S-phenylglycine t-butyl ester) treatment (Online Figure V).

In the same transduced cultures, we also assessed the levels of the mRNAs for the endogenous Notch1 gene and its targets. These were found to parallel the extent of cardiomyocyte proliferation, reaching statistical significance for N1ICD already at day 3 for some targets, and for all targets and both vectors at day 7 (Figure 2E). Activation of the Notch pathway by AAV6-N1ICD at days 3 and 7 and AAV6-sJ1 at day 7 also resulted in the increase of endogenous Notch1 expression, consistent with the existence of a positive feedback loop.14,15

We further analyzed whether the chromatin environment at the Notch-responsive promoters was influenced by constitutive
Figure 1. Suppression of Notch pathway on cardiomyocyte terminal differentiation. A, Quantification of cardiomyocyte proliferation level, assessed as the percentage of 5-bromo-2′-deoxyuridine (BrdU+), α-actinin+ cells, of neonatal rat cardiomyocytes at days 3 and 7 after isolation and adult rat cardiomyocytes. Shown are the mean±SEM of ≥3 independent experiments. **P<0.01. B, Transcription levels of Notch1 and its target genes in neonatal rat cardiomyocytes at days 3 and 7 after plating, and in adult cardiomyocytes. Data are expressed to cellular hypoxanthine-guanine phosphoribosyltransferase mRNA levels. Shown are the mean±SEM of ≥3 independent experiments. *P<0.05; **P<0.01. C, Localization of primer sets used for chromatin immunoprecipitation (ChIP) experiments in Notch1 and its indicated target gene promoters. D and E, ChIP analyses of Notch1 and target gene promoters on days 3, 7, and adult cardiomyocytes using antibodies against active chromatin marks (recognizing acetylated histone 3 [H3panAc] and recognizing trimethylated histone 3 lysine 4 [H3K4me3]); D) and repressive chromatin marks (recognizing trimethylated histone 3 lysine 27 [H3K27me3] and enhancer of zeste homolog 2 [Ezh2]); E). Shown are the means±SEM of ≥3 independent experiments. *P<0.05; **P<0.01 vs day 3. IP indicates immunoprecipitated; and TSS, transcription start site.
Notch signaling stimulation. Chromatin immunoprecipitation experiments were performed on neonatal rat cardiomyocytes infected at the day of isolation with either AAV6-N1ICD or AAV6-sJ1. Cells were kept in culture for 3 or 7 days, to evaluate the chromatin profile in the same settings in which gene expression and proliferation levels were previously analyzed.
In cells transduced with AAV6-N1ICD, day-7 chromatin was significantly more acetylated than in control-treated cardiomyocytes at the analyzed Notch1 responsive genes (Figure 3A). Instead, the amount of chromatin immunoprecipitated with the anti-H3K27me3 and the anti-Ezh2 antibodies increased in control conditions at day 7, whereas it remained stable in the cells transduced with AAV6-N1ICD (Figure 3B and 3C respectively). A similar pattern was also observed in cells transduced with the AAV6-sJ1 vector. The levels of Ezh2 were unchanged in cells transduced with the vectors (Online Figure IB).

To confirm the role of PcG–induced chromatin modification in Notch pathway silencing further, we treated cardiomyocytes with the Ezh2 inhibitor N-[(1,2-dihydro-4,6-dimethyl-2-oxo-3-pyridinyl)methyl]-3-methyl-1-[(1S)-1-methylpropyl]-6-[6-(1-piperazinyl)-3-pyridinyl]-1H-indole-4-carboxamide (GSK126). These experiments were performed in 5 conditions (Figure 4Aa–e). The drug had no significant effect on...
cardiomyocyte proliferation, as assessed by BrdU incorporation, at day 3, when spontaneous proliferation still occurs (a versus b). However, when proliferation was analyzed at day 7, after the drug was administered from day 5, proliferation was induced at a level comparable with that of AAV6-N1ICD (c versus d; 5.2% versus 16.3%; P<0.01). Even more important, when GSK126 treatment had started at day 3, Notch pathway activation through N1ICD was able to increase the extent of proliferation further (e; 22.2% BrdU+ cardiomyocytes; P<0.05% compared with untreated). Efficiency of GSK126 treatment in inhibiting Ezh2 was confirmed by analyzing the levels of H3K27me3 (Figure 4B and 4C). These experiments show that Ezh2 inhibition and Notch signaling have a synergistic effect in promoting cardiomyocyte proliferation.

Collectively, these results indicate that stimulation of the Notch pathway by gene transfer can significantly expand the proliferative capacity of neonatal cardiomyocytes, maintaining an open chromatin conformation at the Notch-responsive genes and thus sustaining high levels of transcription.

### AAV-Mediated Notch Pathway Activation Is Ineffective After MI in Adult Mice

Next, we wanted to assess whether the vectors expressing activated Notch1 and Jagged1 might also improve the outcome of MI by promoting tissue regeneration in vivo. After permanent ligation of the left anterior descending coronary artery in adult CD1 mice, AAV serotype 9 vectors encoding N1ICD or sJ1 were injected in the peri-infarct zone along with an AAV9 vector containing an empty multiple cloning site as a control (n=12 per group). AAV9 gene delivery in vivo was effective, as concluded by quantitative polymerase chain reaction analysis for vector DNA at days 7, 14, 30, and 60 after transduction (n=3 per group per time; Figure 5A) and in agreement with published observations. 

In particular, the number of vector genomes in the heart was the highest at 7 days after transduction, decreased at day 14 and remained stable afterward, consistent with our previous results. Transgene expression paralleled the levels of transduction and remained relatively high for the whole duration of the experiment (Figure 5B and 5C for mRNA and protein levels, respectively). Assessment of cardiac function was monitored by echocardiography ≤2 months after MI and AAV9 vector delivery. Contrary to our expectations, mice transduced with either vector displayed no significantly better cardiac performance than those injected with the control AAV9 vector. Figure 5D to 5H reports left ventricular (LV) ejection fraction (Figure 5D), LV fractional shortening (Figure 5E), LV anterior wall thickening (Figure 5F), LV systolic (Figure 5G), and LV diastolic (Figure 5H) anterior wall thickness (LV anterior wall thickening-systolic and LV anterior wall thickening-diastolic) for the 3 groups of animals, at days 7, 14, 30, and 60 after MI (P=ns at all time points for either group versus control). The absence of a significant regenerative response in the injected animals was confirmed by the lack of scar size reduction at anatomic and histological examination (representative Masson trichrome staining and quantification of the infarct size are provided in Figure 5I and 5J, respectively).

### Forced Activation of the Notch Pathway Does Not Induce Adult Cardiomyocyte Proliferation

To understand the cause underlying the lack of Notch1 effect after MI in vivo, we tested the effect of Notch1 activation in adult rat cardiomyocytes. Similar to neonatal cells, adult cardiomyocytes were also effectively transduced by AAV serotype 6 (>40% of transduced cardiomyocytes in either serum-free conditions or the presence of 5% fetal bovine serum, which is required for adult cardiomyocyte proliferation; Online Figure IIC for representative figures and Online Figure IID for quantification).

We evaluated the effect of AA-V6-N1ICD and AA-V6-sJ1 on the proliferation of these cells, and compared these results with those obtained after transduction with 2 other AAV6 vectors, containing the precursor genes for miR-199a and miR-590, 2 of the miRNAs that we recently showed to activate neonatal and adult cardiomyocyte proliferation, both in vitro and in vivo. At day 9 after transduction, no significant proliferation (as evaluated by BrdU incorporation) was observed in the cardiomyocytes transduced with AA-V6-N1ICD or AA-V6-sJ1, whereas 5.7% and 16.2% of BrdU-positive cells were found in the cultures transduced with AA-V6-miR199a or AA-V6-miR590, respectively (representative images and quantification are shown in Figure 6A and 6B, respectively). Absence of proliferation in the cardiomyocytes transduced with either of the 2 Notch pathway vectors was also confirmed by the lack of positivity to the Ki-67 and phospho-histone H3 proliferation markers (not shown).

These results clearly indicate that forcing activation of the Notch1 pathway by AAV-mediated gene transfer is ineffective in driving proliferation of adult cardiomyocytes, in contrast to the effect of other treatments, such as the investigated miRNAs. We also noticed that the levels of Notch1 itself and of its target genes Hes1, Hey1, Hey2, and Cyclin D1 did not significantly increase in the cells treated with AA-V6-miR199a or AA-V6-miR590, indicating that the molecular route through which these miRNAs function does not involve reactivation of the Notch pathway (Figure 6C).

### Notch Target Genes Are Modified by CpG Methylation in Adult Cardiomyocytes

We wanted to understand the molecular reason why gene transfer did not reactivate Notch signaling in adult cardiomyocytes. Because promoter methylation is commonly associated with permanent and irreversible transcriptional repression, we analyzed the DNA methylation status of Notch1 and its target genes Hes1, Hey1, Hey2, and Cyclin D1. In particular, we identified the CpG-rich areas in the genomic regions in correspondence of the gene TSSs. Then, by bisulfite sequencing, we compared the levels of methylation at 2 selected segments inside these areas, one upstream and another downstream the TSS, in day-3 and day-7 neonatal and in adult cardiomyocytes.

At day 3, the extent of DNA methylation at the Notch1 gene was relatively low (2.5% of the CpG dinucleotides analyzed upstream the TSS and 1.8% downstream the TSS). These levels markedly increased in day-7 cells (20.0% and 4.9%, respectively) and in adult cardiomyocytes (36.2% and 12.9%, respectively; P<0.01 for both primer sets, adult versus day 3; Figure 7A). Analogous results were obtained by analyzing the Hes1 gene. At day 3, 2.7% of the CpGs in
Figure 5. Stimulation of the Notch pathway fails to improve myocardial function and induce repair after myocardial infarction.

A–C, Quantification of viral genomes (A), levels of mRNA transgene expression (B), and of protein transgene expression (C) in mouse hearts at different times after intracardial injection of adenoassociated virus–Notch 1 intracellular domain (AAV9-N1ICD), AAV9-soluble Jagged1 (sJ1) or AAV9-Control, the last containing an empty multiple cloning site (n=3 per group). D–H, Evaluation of cardiac function at different times after myocardial infarction (MI) in mice transduced with AAV6-N1ICD, AAV6-sJ1, or AAV6-Control. The analyzed parameters are left ventricular ejection fraction (LVEF; D), LV fractional shortening (LVFS; E), LV anterior wall thickening (LVAW; F), systolic anterior wall thickness (G), and diastolic wall thickness (H). n=10 to 12 per group; P=n.s. Dashed lines indicate the average value of noninfarcted animals. I and J, Representative images of Masson trichrome staining (I) and quantification of infarct size (J) at 7 and 60 days after MI in adult mice injected with AAV9-N1ICD, AAV9-sJ1, and AAV9-Control. Scale bar, 1 mm. HPRT indicates hypoxanthine-guanine phosphoribosyltransferase.
the analyzed regions upstream the TSS scored positive for methylation and 2.3% in the region downstream the TSS. These levels rose to 4.2% and 8.6% at day 7 and to 15.3% and 22.7%, respectively, in adult cardiomyocytes (P<0.01 for both primer sets, adult versus day-3 cells; Figure 7B). Analogous results were obtained for Hey1 and Hey2, even if less pronounced, overall showing increased methylation in day-7 and adult cardiomyocytes compared with day-3 cells (Figure 7C and 7D, respectively). Of potential interest, the Cyclin D1 promoter, however, showed a different pattern, with scattered methylation also occurring in day-3 samples in the more upstream GC-rich region (2.5 kb upstream the TSS), not correlated with transcriptional silencing, whereas the CpG island close to the TSS (explored by primer set D) remained unmethylated (Figure 7E). This result is in keeping with the requirement for Cyclin D1 re-expression when proliferation of adult cardiomyocytes is triggered by other stimuli, including miRNAs.30

Analysis of the TSS regions of GAPDH, a housekeeping gene, and glial fibrillary acidic protein, a glial gene silenced in cardiomyocytes, served as negative and positive controls for methylation, respectively (Figure 7F and 7G, respectively).

To confirm that DNA methylation at the Notch target gene promoters effectively represses transcription, we generated plasmid constructs in which the Notch1, Hes1, Hey1, and Hey2 gene promoters were cloned upstream of the luciferase reporter gene and submitted these constructs to in vitro methylation using the SssI CpG methyltransferase and the methyl donor S-adenosyl-methionine, followed by transfection of neonatal rat cardiomyocytes. We found that in vitro methylation markedly blunted luciferase activity at both days 3 and 7 (Online Figure VIA and VIB, respectively). Efficiency of methylation was assessed by comparing DNA sensitivity with that of the MspI/HpaII restriction enzyme isoschizomers (Online Figure VIC); samples incubated without S-adenosyl-methionine served as a control.

Consistent with the repressive role of Notch gene promoter methylation, treatment with 5-Aza-2′-deoxycytidine (AzaC), a nucleotide analog that prevents DNA methylation, significantly increased neonatal cardiomyocytes proliferation (4.2% Brdu+ cardiomyocytes versus 15.0% when AzaC was administered between days 1 and 3, and proliferation was analyzed at day 7; P<0.01; Figure 8A). The drug markedly synergized with AAV6-N1ICD when this was administered immediately after cell plating (25.9%; P<0.01 versus AzaC alone). Most relevant, the drug was able to rescue the relatively poorer efficacy of AAV6-N1ICD when this was administered at day 4 (26.2% versus
10.9%; BrdU+ cardiomyocytes with or without AzaC). This result is consistent with the conclusion that impeding the establishment of methylation favors the effect of N1ICD. The effectiveness of AzaC in preventing methylation was verified at the Notch1, Hes1 promoters, and glial fibrillary acidic protein promoters by bisulfite sequencing.

Figure 7. Notch1 and Hes1 promoters in nonproliferative cardiomyocytes show markedly increased levels of DNA methylation. A–G, Results of bisulfite sequencing data from DNA obtained from day-3, day-7, and adult rat cardiomyocytes using primer pairs encompassing the indicated promoter regions of the Notch1, Hes1, Hey1, Hey2, Cyclin D1, GAPDH, and glial fibrillary acidic protein (GFAP) genes (the last 2 serving as negative and positive controls for methylation respectively). Each row represents a single analyzed DNA clone; white and black circles show unmethylated and methylated CpG dinucleotides, respectively. The Table at the bottom of each figure shows the percentages of methylated CpGs. TSS indicates transcription start site.
Suppression of the Notch pathway by chromatin modification at the Notch target promoters also occurs in other developmental conditions. In Drosophila, the PcG PRC1 binds to multiple components in the Notch signaling pathway to control proliferation. In the dystrophic skeletal muscle, tumor necrosis factor-α suppresses Notch1 by promoting recruitment of Ezh2 to the Notch-responsive promoters. Also in the developing myocardium, endothelial deletion of Jarid2, a member of the Jumonji family of proteins that is not active as a demethylase but associates with the PcG complex to modulate its function, derepresses endocardial Notch1 expression. In addition, deletion of Ezh2 in cardiac progenitors is known to cause postnatal myocardial pathology.

In neonatal cardiomyocytes, chromatin-mediated suppression of the Notch target genes was effectively counteracted by the forced activation of the Notch pathway on AAV-mediated gene transfer. Transduction correlated with persistence of active chromatin marks at the investigated Notch promoters and higher levels of transcription of the corresponding genes, with the ultimate result of driving proliferation of the transduced cells. Of interest, a proliferative response was observed by both the constitutively active N1ICD and the soluble form of its ligand Jagged1. The target cell population of the 2 factors is conceptually different: although N1ICD transduction forces Notch pathway activation by directly acting on the target genes in the nucleus, the soluble form of the ligand requires the cognate receptor to be expressed on the cell surface. Therefore, the timing and strength of the response were different: although N1ICD was able to increase cardiomyocyte proliferation immediately, such proliferative response was only detectable after 1 week in the Jagged1-transduced cells. This delayed response also accounts for the need of soluble Jagged1 to reach a minimum concentration in the extracellular environment to activate downstream signaling.

The inefficacy of Notch pathway reactivation by gene transfer to drive adult cardiomyocyte proliferation ex vivo and cardiac regeneration after MI does not seem to be the consequence of an intrinsic incapacity of adult cardiomyocytes to proliferate. Other stimuli, including peristin, neuregulin, FGF1 together with a p38 inhibitor, have all been shown capable to stimulate cardiomyocyte DNA synthesis, karyokinesis, and cytokinesis. In particular, we have recently described a series of microRNAs that induce re-entry of adult cardiomyocytes into the cell cycle and promote cardiac regeneration after MI. Of interest, cell cycle reactivation by 2 of these microRNAs (miR-590 and miR-199a) does not seem to correlate with the reactivation of the Notch pathway, as concluded by the lack of transcriptional activation of the Notch target genes. Taking the lack of effect of Notch transduction and the lack of Notch gene activation after miRNA treatment together, it
can be concluded that the induction of adult cardiomyocyte proliferation can no longer be sustained by the Notch pathway because it is in neonatal cells.

Why is N1CD ineffective in adult cardiomyocytes? This can be correlated to the high level of CpG DNA methylation observed in adult cardiomyocytes at the Notch1 and Hes1 gene promoter proximal regions. Indeed, DNA methylation is a well-established modification that correlates with the permanent suppression of gene expression. A context-dependent crosstalk between PcG and DNA methylation has already been demonstrated in different systems. These include the Notch promoter in skeletal muscle satellite and B acute leukemia cells.

The lack of a regenerative effect of AAV9-mediated Notch1 and soluble Jagged1 gene transfer in vivo does not necessarily contrast with previous reports that have shown a beneficial effect of the genetic reactivation of the Notch pathway in transgenic animals. In this respect, it should be considered that AAV vectors are outstanding tools for gene transfer into postmitotic cells and that their efficiency, in the heart, increases with terminal cardiomyocyte differentiation. Thus, although our experiments rule out a direct effect of Notch pathway reactivation in driving adult cardiomyocyte proliferation in vitro and in vivo, they still remain compatible with the possibility that Notch signaling in adults hearts might exert beneficial effects in other cell types, such as in epicardial cells, mesenchymal stromal cells, bone marrow–derived cells, or cardiomyocyte precursors derived from cardiac stem cells. Reactivation of Notch1 expression after MI in α-myosin heavy chain-mER-Cre+ mice showed a positive effect, which was mainly attributed to the preservation of cardiomyocyte viability and the stimulation of angiogenesis, with no evidence of a major regenerative effect. A possible beneficial effect of Notch pathway stimulation in cardiac cells other than cardiomyocytes can also be inferred from our study, where soluble Jagged1 transduction produced a marginal improvement in cardiac function, albeit not statistically significant in the groups of animals analyzed, which can be attributed to a paracrine effect.

In conclusion, contrary to other conditions, such as brain ischemia and skeletal muscle injury, where reactivation of Notch signaling promotes tissue repair, attempts at inducing cardiac regeneration after MI by reactivation of the Notch pathway in cardiomyocytes by gene transfer are ineffective. This observation contrasts with the assumption that tissue regeneration in adult organisms has to recapitulate the events occurring during development. A parallel situation also exists for other organs. For instance, the satellite cell transcription factor Pax7 is required for skeletal muscle regeneration in neonatal mice but is dispensable for regeneration during juvenile and adult stages. In both cardiac and skeletal muscle, therefore, it seems that a distinction exists between the molecular mechanisms driving development and those responsible for further maintenance, expansion, and repair of the differentiated tissues. How to exploit these adult mechanisms for therapeutic purposes continues to remain a highly sought after target for future investigation.

Acknowledgments

We are grateful to Marina Dapas and Michela Zotti for technical support in adenoassociated virus production, to Mauro Sturmeza for help in animal experimentation and to Suzanne Kerbavcic for editorial assistance.

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Disclosures

None.

References


**Novelty and Significance**

**What Is Known?**

- After birth, cardiomyocyte proliferation stops and cardiac damage is repaired through a scarring mechanism.
- Before birth, activation of the Notch pathway during development is a key determinant of embryonic and fetal cardiomyocyte proliferation.

**What New Information Does This Article Contribute?**

- Overexpression of Notch1 or its ligand Jagged1 using adenoassociated virus vectors maintains robust neonatal cardiomyocyte proliferation immediately after birth.
- The same adenoassociated virus vectors, however, fail to induce cardiac repair in adult hearts after damage because of irreversible epigenetic modifications at Notch target genes in adult cardiomyocytes, involving deposition of repressive chromatin marks and DNA methylation.

The Notch pathway plays a key role in stimulating mammalian cardiomyocyte proliferation during development; in adult zebrafish, reactivation of this pathway is also essential to drive cardiac regeneration after injury. We thus wondered whether forced activation in this pathway might induce cardiac regeneration and repair in adult, damaged mouse hearts. We developed adenoassociated virus vectors expressing activated Notch1 and its ligand Jagged1. These vectors were capable to expand the proliferative capacity of neonatal cardiomyocytes significantly. The same adenoassociated virus vectors, however, were largely ineffective in stimulating cardiac repair after myocardial infarction in adult mice, despite optimal and long-lasting transgene expression. Analysis of Notch-responsive promoters in adult cardiomyocytes showed marks of repressed chromatin and irreversible DNA methylation at the Notch1 target genes. We conclude that Notch pathway activation, despite being crucial in regulating cardiomyocyte proliferation during the early postnatal life, fails to drive cardiac regeneration in adults, because of permanent epigenetic modification at the Notch-responsive promoters.
Epigenetic Modification at Notch Responsive Promoters Blunts Efficacy of Inducing Notch Pathway Reactivation After Myocardial Infarction
Giulia Felician, Chiara Collesi, Marina Lusic, Valentina Martinelli, Matteo Dal Ferro, Lorena Zentilin, Serena Zacchigna and Mauro Giacca

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Online Figure I. Analysis of Ezh2 expression levels along cardiomyocyte differentiation and after Notch pathway activation.

(A) Expression levels of Ezh2 in neonatal rat cardiomyocytes at day 3 and 7 after plating, and in adult cardiomyocytes.

(B) Transcription levels of Ezh2 in neonatal rat cardiomyocytes analyzed at days 3 and 7 after AAV6 vector transduction.

Data are expressed to cellular HPRT mRNA levels. Shown are the means±sem of at least three independent experiments.
Online Figure II. Transduction of neonatal and adult cardiomyocytes by AAV serotype 6 vectors

(A) Representative images of neonatal rat cardiomyocytes at days 3 and 7 of culture after transduction with AAV6-EGFP. Scale bar 100 μm.

(B) Quantification of neonatal cardiomyocyte positivity to EGFP at days 3 and 7 after transduction with AAV6-EGFP.

(C) Top panel: contrast phase representative images of adult rat cardiomyocytes cultured in absence of FBS at days 4, 5 and 6 after AAV6-EGFP transduction. Bottom panel: Representative immunofluorescence stainings of adult rat cardiomyocytes cultured in medium with 5% FBS at days 6, 9 and 12 after AAV6-EGFP transduction.

(D) Quantification of EGFP-positive adult cardiomyocytes after transduction with AAV6-EGFP vector in the absence or presence of FBS in the culture medium.
Online Figure III. Transduced N1ICD binds endogenous RBP-Jk protein in cardiomyocytes.
Detergent solubilized proteins from rat cardiomyocytes transduced with AAV6-N1ICD were immunoprecipitated with an anti-Myc tag antibody and analyzed by Western blotting using an anti-RBP-Jk antibody.
Online Figure IV. AAV6-N1ICD or AAV6-sJ1 transduction increase mitosis and cytokinesis of neonatal cardiomyocytes

(A) Representative images of rat neonatal cardiomyocytes transduced with the indicated vectors and analyzed, at day 7, by immunofluorescence against histone 3 phosphorylated on serine 10 (pH3; red). Cardiomyocytes are stained in green with an anti-α-actinin antibody; nuclei are visualized in blue by DAPI. The leftmost panels show image splitting. Arrows point at mitotic cardiomyocytes. Scale bar: 100 μm.

(B) Quantification of pH3+ cardiomyocytes. Shown are the means ± sem of at least three independent experiments. *: P<0.05.

(C) Representative images of rat neonatal cardiomyocytes transduced with the indicated vectors and analyzed, at day 7, by immunofluorescence against Aurora B (red). Cardiomyocytes are stained in green with an anti-α-actinin antibody; nuclei are visualized in blue by DAPI. Arrows point at midbodies. Scale bar: 30 μm.

(D) Quantification of midbodies in cardiomyocytes. Shown are the means ± sem of at least three independent experiments. *: P<0.05.
Online Figure V. The effects of AAV6-N1ICD are not mediated by endogenous Notch1 receptor signaling.

(A) Neonatal rat cardiomyocytes were transfected with either a Hes1-Luciferase or a 4xRBP-Jk-Luciferase reporter constructs (panels a-b and c-d respectively) and analyzed at day 3 or day 7 (panels a-c and b-d respectively). Transfection efficiency was standardized by co-transfecting a constitutively expressed Renilla luciferase reporter. The histograms show mean±sem; n = 6; *: P<0.05; **: P<0.01 vs. control.

(B) Quantification of BrdU incorporation in neonatal rat cardiomyocytes at days 3 and 7 after transduction with either AAV6-N1ICD or AAV6-sJ1, in the presence or not of DAPT. Shown are the means ± sem of at least three independent experiments. *: P<0.05; **: P<0.01 vs. control.
Online Figure VI. Effect of promoter methylation in transient reporter assays.

(A and B) Rat neonatal cardiomyocytes were transiently transfected with Sss I-methylated (grey bars) or unmethylated (white bars) plasmids containing either Notch1 full length or the Notch1 core region promoter, or Hes1, or Hey1 or Hey2 driving expression of firefly luciferase. As a control of the methylation reaction, each plasmid DNA vector was also treated with equal amounts of methylating enzyme, but without the methyl group donor SAM (black bars). Luciferase activities were normalized for transfection efficiency by cotransfection of an unmethylated Renilla construct. The empty vector (pGL3) was used as negative control. Values are the mean ± sem obtained from three independent experiments. **: \( P < 0.01 \). Reporter gene expression was analyzed at days 3 (A) and 7 (B).

(C) Control of in vitro methylation efficiency. Before transfection, each sample was separately digested with the methylation-sensitive HpaII or methylation-insensitive MspI restriction enzyme isoschizomer. For all plasmids, Hpa II was unable to digest the constructs in which the methylation reaction was carried out in the presence of SAM, proving effective methylation.
Online Figure VII. AzaC treatment decreases DNA methylation in the analyzed genes.

(A-C) Results of bisulfite sequencing data from DNA obtained from day 7 cardiomyocytes treated, between day 1 and day 3, with 5 μM AzaC. Eight different clones are shown for Notch1 (A), Hes1 (B) and GFAP (C).

(D) Summary table showing differences in DNA methylation found in day 7 cardiomyocytes comparing untreated cells (data shown in Fig. 7) and cells treated with AzaC. The same results are shown in a histogram format in Fig. 8B.
### Online Table I. Primers used to analyze gene expression levels

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<th>Gene</th>
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### Online Table II. Primers used for competitive PCR quantification of AAV transduction levels

#### Primers for competitor construction

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#### Primers for competitive PCR analysis

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### Online Table III. Primers used for chromatin immunoprecipitation

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### Online Table IV. Primers used for bisulfite sequencing

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<td><strong>Hey2</strong></td>
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<tr>
<td><strong>Cyclin D1</strong></td>
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<td>ACCAAATTTCCAAAAAAAAAAAAA</td>
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<td><strong>GAPDH</strong></td>
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<td><strong>GFAP</strong></td>
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DETAILED METHODS

Animals
Animal care and treatment were conducted in conformity with institutional guidelines in compliance with national and international laws and policies (European Economic Community Council Directive 86/609, OJL 358, December 12, 1987). Wistar rats and CD1 mice were purchased from Charles River Laboratories Italia Srl and maintained under controlled environmental conditions.

Culture of neonatal rat cardiomyocytes
Neonatal rat ventricular cardiomyocytes were extracted from day 0 or day 1 newborn Wistar rats, as described previously 1, with minor modifications. Briefly, ventricles were separated from atria and great vessels and cut in smaller pieces. The dissociation was performed in CBFHH (calcium and bicarbonate-free Hanks with Hepes) buffer containing 1.75 mg/ml of trypsin (BD Difco) and 10 μg/ml DNAse II (Sigma). Digestions were performed on a stirrer in eight- to ten-10 minutes steps; after each digestion, the supernatant was collected and trypsin was inactivated with Fetal Bovine Serum (FBS, Life Technologies). The collected supernatant was centrifuged and the cell pellet was resuspended in the medium DMEM (Dulbecco’s modified Eagle medium, Life Technologies) 4.5 g/l glucose supplemented with 5% FBS, 20 mg/ml vitamin B12 (Sigma), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma). Cells were filtered through a cell strainer (40 μm, BD Falcon) and then pre-plated on plastic 10 cm dishes for 2 hr at 37° in 5% CO₂. After the pre-plating step, medium containing cardiomyocytes was collected, cells were counted and then plated at the appropriate density on Primaria plates (BD Falcon), or on glass slides pre-coated with 0.2% gelatin in PBS. The culture medium was replaced with fresh one the next day after plating.

When indicated, the Ezh2 inhibitor GSK1262 (BioVision; 1 μM) was added to the medium for 2 or 4 days. DMSO was added as a vehicle for the same time as a control. Again when indicated, 5 μM 5-Aza-2’déoxycytidine (AzaC, Sigma) was added to the culture medium for the first 2 days of culture, between day 1 and day 3, and then removed. In both cases, DMSO was used as a control. When indicated, the gamma secretase inhibitor DAPT (N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester, Sigma, 10 μM for 8 hours) was added.

Culture of adult rat cardiomyocytes
Adult ventricular cardiomyocytes from 2-months-old adult female Wistar rats were extracted with Langendorff perfusion system. Animals were anesthetized with Zoletil/xylazine and heparinized with 2ml of heparin (Sigma) 1000U/ml. The heart was cannulated through the aorta and perfused with a perfusion buffer composed of NaCl, 120.4 mM (Sigma), KCl 14.7 mM (Sigma), KH₂PO₄ 0.6 mM (Sigma), Na₂HPO₄ 0.6 mM (Sigma), MgSO₄-7H₂O 1.2 mM (Sigma), NaHCO₃ 4.6 mM (Sigma), Na-Hepes 10 mM, taurine 30 mM (Sigma), 2,3-butanedione monoxime (BDM, Sigma), 10 mM, glucose 5.5mM (Sigma) in H₂O pH 7.2). After 5 min, 1 mg/ml Liberase TM (Roche) was added to the perfusion buffer for 10-12 min. The heart was detached from the apparatus; atria and great vessels were removed and the ventricles were cut in smaller pieces. Mechanical digestion was performed pipetting up and down the tissue fragments in wash medium (50:50 perfusion buffer: DMEM 1g/l glucose). The collected cell suspension was filtered (100 μm cell strainer, BD Falcon) and centrifuged at low speed (30 g) 3 min at room temperature. The cell pellet was resuspended in wash buffer and then added to the BSA gradient prepared with 0.645 g BSA (Sigma) in DMEM 1g/l glucose. The separation of cardiomyocytes from other cell types lasted 15 min at room temperature. Then, the cardiomyocyte pellet was resuspended in ACCT medium (2 g/l BSA, 2 mM L-carnitine (Sigma), 5 mM creatine (Sigma), 5 mM taurine (Sigma), 1 mM BDM (Sigma), 100 U/ml of penicillin and 100 μg/ml of streptomycin in DMEM 1g/l glucose). Cells were plated on Primaria plates coated with Laminin (Sigma) and kept in culture at 37° in 5% CO₂. Two hr after plating, the medium was replaced with fresh ACCT medium to remove cardiomyocytes not attached to the plate. The medium was changed 24 h later with fresh ACCT medium or with DMEM 4.5 g/l glucose supplemented with 5% FBS and Vitamin B12.
RNA isolation and quantitative real-time PCR

Total mRNA was purified either from cultured neonatal cardiomyocytes at day 3 and 7 of culture, or from adult cardiomyocytes 3 or 9 days after plating or from total heart homogenates. Extracted mRNA (1 μg) was reverse-transcribed using MLV-RT (Invitrogen) with random hexamers (10 μM) in a 20 μl reaction, following the manufacturer’s instruction. mRNA levels for Notch1, Hes1, Hey1, Hey2, Cyclin D1 genes (primer sequences listed in Primer Table I) were quantified by qRT-PCR and GoTaq qPCR Master Mix (Promega). The real-time qPCR program was performed with a melting curve dissociation protocol (from 60°C to 95°C), according to manufacturer’s instruction. The final dilution of the primers in the reaction was 900 nm; for each primer set, optimal conditions were established and efficiency of the amplification was calculated. The housekeeping gene GAPDH was used for normalization with predesigned TaqMan assay (Applied Biosystems) and iQ Supermix (Biorad) according to the manufacturer’s instruction.

Analysis of protein expression

For the transgene expression, heart samples were collected at different time points after myocardial infarction, homogenized in 1 ml of RIPA Buffer (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 5 mM EDTA, 0.1% sodium dodecyl sulfate [SDS], 1% Triton X-100, 1% deoxycholate), supplemented with 90 μg/ml PMSF, 1 mM Na3VO4 (all from Sigma) and proteases inhibitors (Roche), using Magna Lyser (Roche). After sonication and pre-clearing, protein lystate concentration was determined by Bradford Assay (Biorad). Equal amounts of proteins were resolved on 6% SDS-PAGE minigels and transferred to nitrocellulose membranes (GE Healthcare). Immunoblots were blocked in 5% skim milk in TBST (50 mM Tris-HCl, pH 7.4, 200 mM NaCl, and 0.1% Tween 20). Membranes were incubated with primary antibodies overnight and washed in Tris-buffered saline, 0.1% Tween 20. Secondary antibodies were diluted in blocking buffer and incubated with the membranes for 45 min at room temperature. Proteins were detected with the ECL detection kit (GE Health Care Bio-Sciences).

For the analysis of H3K27 methylation, the same procedure was performed, but proteins were resolved on 15% if SDS-PAGE minigels. Membrane stripping was performed in stripping buffer (100mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl pH 6.7) at 56°C for 15 min, followed by extensive washing in TBST. To detect the in vivo physical interaction between N1ICD and RBP-Jk, 1x10⁶ neonatal rat cardiomyocytes, transduced with AAV6-N1ICD, were lysed in IPLS buffer (50 mM Tris-HCL pH 7.5, 120 mM NaCl, 0.5 mM EDTA and 0.5% Nonidet P-40) supplemented with 90 μg/ml PMSF (Sigma) and proteases inhibitors (Roche). After sonication and pre-clearing, protein lystate concentration was determined by Bradford Assay (Biorad). N1ICD was immunoprecipitated from total cell lysates with 1 mg/ml of monoclonal 9B11 Myc-tag antibody (#2279, mouse monoclonal, 1:1000, Cell Signaling) for 2 hr at 4°C with gentle rotation, followed by incubation with protein A/G agarose beads (Santa Cruz) for additional 2 h. Immunoprecipitates were resolved on 10% SDS-PAGE minigels and transferred to nitrocellulose membranes (GE Healthcare). Immunoblots were blocked in 2% BSA (Roche) in TBST (50 mM Tris-HCl, pH 7.4, 200 mM NaCl, and 0.1% Tween 20). Membranes were incubated with RBP-Jk (# AB 2284, Millipore) primary antibodies (overnight and washed in Tris-buffered saline, 0.1% Tween 20. Secondary antibodies were diluted in blocking buffer and incubated with the membranes for 45 min at room temperature. Proteins were detected with the ECL detection kit (GE Health Care Bio-Sciences).

The following antibodies were used for Western Blot: anti-Myc 9B11 (#2279, mouse monoclonal, 1:1000, Cell Signaling), anti-Tubulin (T5168, mouse monoclonal, 1:10000, Sigma), anti-H3K27trimethyl (ab6002, mouse monoclonal, 1:1000, Abcam), anti-Histone-H3 (06-755, rabbit monoclonal, 1:1000, Millipore), anti RBP-Jk (AB 2284,1:1000, rabbit, Millipore). The following antibody were used as secondary: goat anti-mouse conjugated to HRP (P0447, DAKO-CYTOMATION), goat anti-rabbit conjugated to HRP (#31460, Thermo Scientific), protein A conjugated to HRP (18-160, Millipore).
Luciferase assays
To detect Notch1 activity under different experimental conditions, neonatal cardiomyocytes were seeded onto 96-well primary cells culture plates (1x10^4 cells per well) and co-transfected after either 2 or 6 days after isolation with 0.5 μg of either pHes1 (kindly provided by R. Kageyama, Kyoto University, Japan) or 4xRBP-Jk-Luc (provided by S.D. Hayward, John Hopkins University School of Medicine, Baltimore, MD) reporter plasmids and 0.05 μg pRL-Renilla (which was used as a control) by using Lipofectamine 2000 transfection reagent (Invitrogen). Twenty-four hours after transfection, cells were harvested and both firefly and Renilla Luciferase activities were assayed with the Dual-luciferase reporter assay kit (Promega). The value of Firefly Luciferase activity was corrected for the transfection efficiency by using the Renilla Luciferase activity in each sample.

Promoter methylation assays
A series of plasmids were generated, in which the promoter regions of the following genes were cloned upstream of the firefly luciferase gene: Hes1, kindly provided by R. Kageyama, Kyoto University, Japan, Hey1, kindly provided by Manfred Gessler, Theodor-Boveri-Institut fuer Biowissenschaften, Wuerzburg, Germany, Hey2, kindly provided by Stefano Zanotti, Saint Francis Hospital and Medical Center, Hartford, CT and Notch1, kindly provided by Warren S. Pear, University of Pennsylvania, Philadelphia. Plasmid pGL3 contained and empty polylinker (Promega). Each reporter construct (10 μg) was methylated in vitro using 20 U of SssI methylase (New England Biolabs, Beverly, MA) in the presence of S-adenosyl-methionine (160 μM; New England Biols, Beverly, MA) at 37°C for 4 h, with subsequent inactivation of the enzyme at 65°C for 20 min. Mock-methylation reactions were performed using the same conditions, but omitting SAM. Complete methylation was ascertained by digesting the methylated DNA with an excess (20 U/mg) of restriction enzymes HpaII or MspI. All the constructs were purified using Wizard DNA purification columns (Promega).

Neonatal cardiomyocytes were seeded onto 96-wells primary cells culture plates (1x10^4 cells per well) and co-transfected at either day 2 or 6 with 0.5 μg methylated or mock-methylated constructs and 0.05 μg pRL-Renilla (which was used for standardization) by using Lipofectamine 2000 transfection reagent (Invitrogen). Twenty-four hours after transfection, cells were harvested and both firefly and Renilla luciferase activities were assayed with the Dual-luciferase reporter assay kit (Promega). The value of Firefly Luciferase activity was corrected for the transfection efficiency by using the Renilla luciferase activity in each sample.

Viral genome quantification through competitive PCR
DNA was extracted from frozen hearts of transduced animals collected at different time points with Blood and Tissue Kit (Qiagen). Competitive PCR was performed as previously described, using a multicompetitor plasmid carrying a deleted form of the cellular gene β-globin flanked by CMV primer sequences to quantify total genomic DNA as a reference and AAV vector DNA, respectively. Fixed amounts of sample DNA were mixed with scalar amounts of the multicompetitor DNA and PCR was performed with the 2 primer sets. The PCR reaction was run on 8% polyacrylamide gel and stained with ethidium bromide; the different bands corresponding to competitor-, AAV- or the β-globin-DNA were quantified. The primers used for the competitor and for the PCR amplification are listed in Online Table II.

Chromatin immunoprecipitation (ChIP)
The protocol for immunoprecipitation of cardiomyocyte chromatin was adapted from that used in ref. 9. Day 3, day 7 and adult cardiomyocyte (approximately 10x10^6 cells for each time point), were cross-linked with 11% formaldehyde for 15 min at room temperature, followed by termination of the reaction with 125 mM glycine on ice. The cell pellet was washed twice in PBS, lysed in 2% NP-40 buffer (10 mM Tris-HCl pH7.4, 10 mM NaCl, 3mM MgCl2, 1 mM PMSF and protease inhibitors) to obtain purified nuclei, followed by dounce homogenization in the same buffer. Lysis of the nuclei was performed using the same buffer containing 4% NP-40 and left at 37°C for 15 min, after which micrococcal nuclease (120 U, Roche) was
added. The reaction was stopped after 15 min with 3 mM EGTA. DNA was additionally sheared by sonication to an average size of DNA fragments below 0.5 kb. Extracts were pre-cleared by 2 rounds of incubation with IgGs and agarose beads, followed by centrifugation at 3500 rpm for 5-10 min. The lysate (400 μl) was then incubated with 4 μg of the indicated antibody overnight at 4°C, followed by incubation for 4 hr with MagnaChIP Protein Protein A/G magnetic beads (Millipore). Beads were then washed thoroughly with RIPA150, with LiCl – containing buffer and with TE, RNAse-treated for 30 min at 37°, and Proteinase K-treated for at least 2 hr at 56°. De-crosslinking of protein–DNA complexes was performed by an overnight incubation at 65°C. DNA was then subjected to phenol–chloroform extraction, followed by ethanol precipitation and quantified by real time PCR using specific set of primers for each promoter, listed in Primer table 3, previously tested to evaluate their amplification efficiency, and GoTaq qPCR Mater Mix (Promega).

The following antibodies were used for ChIP: anti-H3K4trimethyl (ab8580, Abcam), anti-acetyl-Histone-H3 (06-599, Millipore), anti Ezh2 (17-662, Millipore), anti-H3K27trimethyl (ab6002, Abcam). For each antibody, as a negative control, ChIP was also performed with total IgGs.

**Immunofluorescence and BrdU detection**

Cells were seeded on 24-well primaria plates (~5-10x10⁴ cells per well) and, after 3, 7 or 10 day of culture, were fixed with 4% paraformaldehyde (PFA) for 10 min, washed twice in PBS, permeabilized with 1% Triton X-100 in PBS three times for 10 min, followed by 1 hr blocking in 2% BSA (Roche) in PBS. Cells were then stained overnight at 4°C with primary antibodies diluted in blocking buffer. In the case of BrdU pulse labeling (10 μM for 8 hours for neonatal, 48 hr for adult cardiomyocytes), following cells permeabilization, DNA denaturation was performed incubating 10 min in 1M HCl on ice and 20 min in 2 M HCl at 37°C. Cells were incubated with 0.1 M sodium-borate buffer pH 8.4 12 min at room temperature, then washed three times with PBS 1% Triton X-100. Blocking was performed as previously described. Cells were washed with PBS 0.2% Tween20 and incubated for 1 hr with appropriate secondary antibodies. Three more washes in PBS 0.2% Tween20 were performed and then DAPI (Vectashield) or Hoechst 33342 (Life Technologies) were used to stain DNA. The following antibodies were used as primary antibody: anti-α-sarcomeric actinin (ab9465, Abcam), anti-GFP (A6455, Invitrogen), anti-BrdU (ab6326, Abcam), anti-histone H3 phosphorylated at serine 10 (06-570, Millipore), anti- Aurora B kinase (A5102, Sigma). The following antibodies were used as secondary: goat anti-mouse conjugated to Alexa Fluor 488 (Molecular Probes), goat anti-rat conjugated to Alexa Fluor 555 (Molecular Probes), donkey anti-rabbit conjugated to Alexa Fluor 594 (Molecular Probes).

Image acquisition was performed using an ImageXpress Micro automated high-content screening fluorescence microscope or by manual acquisition of 10 fields/slide, and by counting in a double blinded way, scoring the number of BrdU+ cardiomyocytes, over the total number of α-actinin+ cells.

**Myocardial infarction and echocardiography analysis**

Myocardial infarction was produced in adult female CD1 mice (8–12 weeks old), by permanent left anterior descending (LAD) coronary artery ligation. Briefly, mice were anesthetized by intraperitoneal injection of ketamine and xylazine, endotracheally intubated and placed on a rodent ventilator. Body temperature was maintained at 37°C on a heating pad. The beating heart was accessed via a left thoracotomy. After removing the pericardium, a descending branch of the LAD coronary artery was visualized with a stereomicroscope (Leica) and occluded with a nylon suture. Ligation was confirmed by the whitening of a region of the left ventricle, immediately post-ligation. Recombinant AAV vectors, at a dose of 1x10¹¹ viral genome particles per animal, were injected immediately after LAD ligation into the myocardium bordering the infarct zone (single injection), using an insulin syringe with incorporated 30-gauge needle. The chest was closed, and the animals moved to a prone position until the occurrence of spontaneous breathing. To evaluate left ventricular function and dimensions, transthoracic two-dimensional echocardiography was performed on mice sedated with 5% isoflurane at 7,14, 30 and 60 days after myocardial infarction, using a Visual Sonics Vevo 770 Ultrasound (Visual Sonics) equipped with a 30-MHz linear array transducer. M-mode tracings in parasternal short axis view were used to measure
left ventricular anterior and posterior wall thickness and left ventricular internal diameter at end-systole and end-diastole, which were used to calculate left ventricular fractional shortening and ejection fraction.

**Heart collection and histological analysis**
Heart samples were collected at days 7 and 60 after myocardial infarction to perform further analysis. At the end of the studies, animals were anaesthetized with 5% isoflurane and then killed by injection of 10% KCl, to stop the heart at diastole. The heart was excised, briefly washed in PBS, fixed in 10% formalin at room temperature, embedded in paraffin and further processed for histology staining. Masson's trichrome stainings were performed according to standard procedures, and analyzed for regular morphology and extent of fibrosis. Infarct size was measured as the percentage of the total left ventricular area showing fibrosis.

**Production, purification and characterization of rAAV vectors**
All the AAV vectors used in this study were generated by the AAV Vector Unit (AVU) at ICGEB Trieste (http://www.icgeb.org/avu-core-facility.html) as described previously 10. In brief, infectious AAV6 or AAV9 vector particles were generated in HEK293 cells by cotransfecting each vector plasmid together with the packaging plasmid/s expressing AAV and adenovirus helper functions, pDP6 (PlasmidFactory) for AAV6; pSE18 11 plus helper plasmid (pHELP; Stratagene) for AAV9. Viral stocks were obtained by CsCl gradient centrifugation; rAAV titers, determined by measuring the copy number of viral genomes in pooled, dialyzed gradient fractions, as described previously 12 were in the range of 1x10^{12} to 1x10^{13} genome copies per milliliter.

**In vitro AAV transduction of cardiomyocytes**
Cardiomyocytes were transduced with the AAV6-EGFP, AAV6-Control, AAV6-sJ1 or AAV6-N1ICD contextually to plating of neonatal cardiomyocytes, at an m.o.i. of 1x10^{4} vg/cell. Adult cardiomyocytes were transduced the day after plating with AAV6-EGFP, AAV6-Control, AAV6-mir199a, AAV6-mir590, AAV6-N1ICD, AAV6-sJ1 at an m.o.i. of 1x10^{4} vg/cell.

**In vivo transduction of neonatal hearts**
To evaluate AAV persistence, animals were injected intraperitoneally with AAV9-Control, AAV9-N1ICD or AAV9-sJ1 at a dose of 2.25x10^{10} vg per g of body weight. An appropriate volume of viral vector solution (~30 μl in neonatal mice) was injected slowly using a U-100 insulin syringe. Heart samples were collected at 7, 14, 30 and 60 days post injection, slowly frozen using isopentane/liquid nitrogen and stored at −80°C until processed for genomic DNA or RNA extraction.

**Bisulfite sequencing**
Genomic DNA was extracted from day 3, day 7 and adult cardiomyocytes in culture using DNeasy Blood and Tissue Kit (Qiagen). Genomic DNA (1 μg) of each sample was treated for DNA conversion with sodium bisulfite using EpiTect Bisulfite Kit (Qiagen) according to the manufacturer’s instruction. Promoter sequences were analyzed with using Methyl Primer Express Software v1.0 (Applied biosystems) to predict CpG islands. Primer sequences were designed using the same software and are listed in Online Table IV. Each primer set was tested to optimize PCR conditions. Amplified fragments were separated on 2% agarose gel, visualized by ethidium bromide and then purified from the gel using Wizard SV Gel and PCR Clean-up system kit (Promega). The purified DNA was cloned into TOPO T/A cloning kit (Invitrogen). Eight randomly picked clones were sequenced and analyzed using BiQ analyzer 13.
References cited in the Online Supplements


