Notch-Mediated Epigenetic Regulation of Voltage-Gated Potassium Currents

Aditi Khandekar, Steven Springer, Wei Wang, Stephanie Hicks, Carla Weinheimer, Ramon Diaz-Trelles, Jeanne M. Nerbonne, Stacey Rentschler

**Rationale:** Ventricular arrhythmias often arise from the Purkinje–myocyte junction and are a leading cause of sudden cardiac death. Notch activation reprograms cardiac myocytes to an induced Purkinje-like state characterized by prolonged action potential duration and expression of Purkinje-enriched genes.

**Objective:** To understand the mechanism by which canonical Notch signaling causes action potential prolongation.

**Methods and Results:** We find that endogenous Purkinje cells have reduced peak K+ current, I_{to} and I_{k,slow} when compared with ventricular myocytes. Consistent with partial reprogramming toward a Purkinje-like phenotype, Notch activation decreases peak outward K+ current density, as well as the outward K+ current components I_{to} and I_{k,slow}. Gene expression studies in Notch-activated ventricles demonstrate upregulation of Purkinje-enriched genes Contactin-2 and Scn5a and downregulation of K+ channel subunit genes that contribute to I_{to} and I_{k,slow}. In contrast, inactivation of Notch signaling results in increased cell size commensurate with increased K+ current amplitudes and mimics physiological hypertrophy. Notch-induced changes in K+ current density are regulated at least in part via transcriptional changes. Chromatin immunoprecipitation demonstrates dynamic RBP-J (recombination signal binding protein for immunoglobulin kappa J region) binding and loss of active histone marks on K+ channel subunit promoters with Notch activation, and similar transcriptional and epigenetic changes occur in a heart failure model. Interestingly, there is a differential response in Notch target gene expression and cellular electrophysiology in left versus right ventricular cardiac myocytes.

**Conclusions:** In summary, these findings demonstrate a novel mechanism for regulation of voltage-gated potassium currents in the setting of cardiac pathology and may provide a novel target for arrhythmia drug design. (Circ Res. 2016;119:1324-1338. DOI: 10.1161/CIRCRESAHA.116.309877.)

**Key Words:** action potential ■ Brugada syndrome ■ cardiomyopathies ■ cellular reprogramming ■ electrophysiology ■ Notch receptors ■ Purkinje cells

Components of the Notch signaling pathway are expressed at higher levels within chamber myocardium when compared with nodal-like tissue, whereas canonical Wnt signaling is active within nodal regions.1 The interplay between Notch and Wnt pathways may affect global electric programming with relevance to disease phenotypes. Genome-wide association studies implicate variants in the direct Notch target HEY2 (HRT2) with Brugada syndrome, characterized by ST-segment elevation in the right precordial leads and a propensity for sudden death because of ventricular arrhythmias.2 Our recent work has shown that ectopic Notch activation within the developing atrioventricular junction myocardium results in accessory atrioventricular pathway formation and ventricular pre-excitation, similar to Wolff–Parkinson–White syndrome.3 Ectopic Notch activation within atrioventricular myocardium leads to blurring of the boundary between the atrioventricular junction and ventricle and reprograms atrioventricular cardiac myocytes to a chamber-like phenotype as assessed by upregulation of genes normally excluded from the atrioventricular junction, including Scn5a.1,3 The phenotype is at least partially mediated by Notch-induced repression of canonical Wnt signaling and can be rescued by preventing Wnt signaling downregulation.1

**Editorial, see p 1265
In This Issue, see p 1255

Impulse propagation through the intricate Purkinje fiber network is complex, and efforts to understand Purkinje–myocyte junctions are compounded by the fact that only a subset propagate.
electric impulses. Stressors that lead to gap junction remodeling are speculated to increase the proportion of Purkinje–ventricular junctions capable of impulse propagation, which may initiate re-entry and lead to ventricular arrhythmias. We have recently demonstrated that Notch activation in the myocardium reprograms ventricular cardiac myocytes to a specialized Purkinje-like phenotype. Although activation of Notch was sufficient to induce electrophysiological changes, the efficiency is relatively low, and the majority of cells exhibit partial reprogramming as assessed by conversion to a Purkinje-like action potential morphology. In addition, ectopic Notch activation within developing ventricular myocardium blurs the boundary between ventricular and Purkinje cells, leading to abnormal Purkinje–myocyte junctions. Given the propensity of arrhythmias to arise from the Purkinje–myocyte junction, we hypothesize that aberrant Notch signaling may increase the risk of arrhythmias.

Voltage-gated K⁺ (Kv) currents regulating cardiac repolarization play an important role in synchronizing cardiac excitation and contraction. Changes in K⁺ current expression promote cardiac arrhythmias, which are a leading cause of death in patients with heart failure and cardiomyopathies. The rapidly activating and inactivating transient outward K⁺ current, I_{to,fast} (I_{to}), is generated by the pore-forming α (Kv) channel subunits Kv4.2 (encoded by Kcnq2) and Kv4.3 (encoded by Kcnq3) in association with Kv accessory subunits, including Kv channel interacting protein 2 (KChIP2), encoded by Kcnd2. There are two components of the slowly inactivating repolarizing K⁺ current, I_{k,slow}*, in mouse, generated by Kv2.1 (encoded by Kcnb1) and Kv1.5 (encoded by Kcnj5). Despite the critical role of repolarizing K⁺ currents in regulating cellular electrophysiological properties and arrhythmogenesis, little is known about the transcriptional regulation of ion channel components that encode these channels. In this article, we demonstrate that Notch signaling regulates I_{to,fast} and I_{k,slow}* at least in part through transcriptional and epigenetic regulation of the subunits that underlie these currents.

Binding of a Delta/Serrate/LAG-2 (DSL) ligand on a signal-sending cell with the Notch receptor on a signal-receiving cell initiates a cascade of cleavages ultimately releasing the Notch intracellular domain (NICD) from the cell membrane. NICD translocates to the nucleus where it forms a complex with its main effector molecule, the DNA-binding transcription factor RBP-J (recombination signal binding protein for immunoglobulin kappa J region, also known as CSL). In the non-activated state, RBP-J bound to target gene promoters can be associated with repressor proteins, such as histone deacetylases and SHARP (SMRT and HDAC associated repressor protein), whereas it acts in a Notch-independent manner to inhibit target gene activation. Binding of NICD to RBP-J can lead to displacement of corepressors and transform the complex into an activating complex. To further add to the complexity whereby Notch signaling regulates gene expression, RBP-J is constitutively bound to a subset of Notch-regulated genes, whereas it is dynamically recruited to additional Notch-responsive genes in the presence of NICD. The precise mechanism(s) for how the RBP-J/NICD complex functions in a cell type–specific manner to mediate target gene regulation are actively being dissected. Here, we demonstrate dynamic regulation of genes encoding subunits of voltage-gated K⁺ currents by Notch. Notch signaling alters the epigenetic landscape of these ion channel target genes, leading to a loss of the H3K4 activating histone mark. Taken together, the data provide rationale for inhibition of Notch signaling as a potential target for development of novel antiarrhythmic drugs in the setting of cardiac pathology.

**Methods**

Expanded methods are presented in the Online Data Supplement. Western immunoblotting was performed using anti-KChIP2 (UC Davis/NIH NeuroMab Facility), anti-Kv2.1 (obtained from UC Davis), and anti-GAPDH (Cell Signaling Technology 14C10) antibodies. Whole-cell current- and voltage-clamp recordings were obtained within 12 hours of cardiac myocyte isolation at room temperature (22–23 °C). Cardiac myocyte isolation was performed according to standard techniques, with the exception of Purkinje cell isolation, which required an additional hour of incubation in collagenase-containing solution with elastase and protease type XIV. Pressure overload with progression to heart failure was induced by moderate transaortic constriction with distal left anterior descending ligation according to previously described methodology.

**Results**

**Developmental Notch Activation Programs a Purkinje-Like Phenotype**

Lineage tracing studies in both avian and murine models demonstrated that His-Purkinje cells share a common origin with ventricular cardiac myocytes, and these lineages diverge during midgestation. It has been well established in multiple organ systems that activation of Notch signaling has distinct effects depending on its timing of activation. Within the heart, Notch regulates diverse processes including cardiac morphogenesis and programming of cardiac myocyte electric properties. Our previous work demonstrated that expression of a constitutively active form of the Notch1 receptor (NICD) within ventricular myocytes under control of the Myosin Light Chain 2v promoter programs ventricular myocytes toward a Purkinje-like phenotype (Mlc2v^{Cox} / NICD, hereafter referred...
Given this finding, we next asked whether Notch signaling is involved in Purkinje lineage restriction and whether Notch-activated cells within the ventricle are preferentially located within the conduction system. To demarcate cells where Notch1 signaling has been active during development, we used mice where the Notch1 activity-trap line is combined with the 

\[ \text{Notch1}^\text{activity-trap} \times \text{TdTomato} \]

reporter allele.\(^{25}\) We observed that Notch signaling has been active within both Contactin-2\(^+\) (Cntn2\(^+\)) Purkinje cells and ventricular myocytes (Figure 1A and 1B). Together with our previous work demonstrating that Notch activation in ventricular myocardium expands Purkinje cell markers most robustly in regions neighboring the native conduction system,\(^7\) these data suggest that Notch signaling may not restrict cardiac myocytes to the Purkinje lineage per se but rather that Notch may prime ventricular cardiac myocytes to respond to additional local signaling cues, which in combination result in robust conversion to a Purkinje-like phenotype.

### Notch Activation Prolongs Action Potentials in Left Ventricular Myocytes

Representative action potential waveforms recorded from left ventricular (LV) myocytes isolated from control and Notch GOF mice in response to brief depolarizing current injections are shown (Figure 1D; Online Figure I). As is evident, the action potential is substantially broader in the Notch GOF, compared with the control, LV myocyte. Analyses of current-clamp recordings from many control and Notch GOF LV cells revealed considerably more heterogeneity in action potential durations measured at 50% (action potential duration [APD\(_{50}\]) and 70% (APD\(_{70}\)) repolarization (data not shown), in the Notch GOF LV myocytes (Figure 1E). Mean±SEM APD\(_{50}\) (P=0.015), APD\(_{70}\) (P=0.014), and APD\(_{90}\) (P=0.013), however, were all significantly longer in Notch GOF (n=32), than that in control (n=27), LV myocytes (Table 1). In contrast to action potential durations, resting membrane potentials, input resistances, and action potential amplitudes measured in Notch GOF and control LV myocytes were not significantly different (Table 1). Additional current-clamp recordings were obtained from visually identified Purkinje cells (Figure 1C) isolated from Contactin2-eGFP mice (Cntn2-eGFP).\(^{26}\) Representative action potential waveforms recorded from isolated Cntn2-eGFP\(^+\) Purkinje cells are shown (Figure 1D; Online Figure I). Mean±SEM APD\(_{50}\), APD\(_{70}\), and APD\(_{90}\) values in Cntn2-eGFP\(^+\) Purkinje cells (n=18) were significantly (P<0.0001) longer than that in control LV myocytes (Figure 1E; Table 1). In addition, as previously reported,\(^{26}\) action potential amplitudes were significantly (P=0.0032) larger in Cntn2-eGFP\(^+\) Purkinje cells,

\[ \text{Notch activation prolonged left ventricular (LV) action potential duration.} \]

**Figure 1.** Notch activation prolonged left ventricular (LV) action potential duration. A and B, Immunofluorescence images of Notch1 activation revealed by N1IP::Cre\(^{10}\); TdTTomato reporter line is combined with the 

\[ \text{TdTTomato} \]

reporter allele.\(^{25}\) We observed that Notch signaling has been active within both Contactin-2\(^+\) (Cntn2\(^+\)) Purkinje cells (arrowheads) and ventricular cardiac myocytes (arrows). C, Fluorescence images of eGFP\(^+\) Purkinje cells in the intact ventricular free wall of a Cntn2-eGFP mouse (left) and an individual Cntn2-eGFP\(^+\) Purkinje cell following isolation for whole-cell patch clamp recordings (right). D, Representative recordings of action potentials elicited by brief (2–5 ms) depolarizing current injections delivered at 1 Hz from a littermate R26R\(^{c dose}\) control left ventricular (LV) myocyte, a Notch GOF LV myocyte, and a Cntn2-eGFP\(^+\) Purkinje cell, are shown. E, Action potential durations measured at 50% and 70% repolarization in individual control LV myocytes (●, n=27 cells from 5 mice), Notch gain-of-function (GOF) LV myocytes (▲, n=33 cells from 7 mice), and Cntn2-eGFP\(^+\) Purkinje cells (■, n=18 cells from 8 mice). Mean±SEM values are also indicated. Mean values are significantly (*P<0.05 and §P<0.0001) different from those measured in LV control myocytes, ns, not significant. Bar (A)=20 \(\mu\)m, (B)=20 \(\mu\)m, and (C)=50 \(\mu\)m.
compared with control LV myocytes. The resting membrane properties and input resistances of Cntn2-eGFP+ Purkinje cells, however, were similar to those measured in control LV myocytes (Table 1). On the basis of the morphology and duration of the action potential, as well as expression of the conduction system marker Cntn2,26 these results are consistent with the suggestion that Notch activation programs a subset of ventricular myocytes to closely resemble Purkinje-like cells, whereas the remainder appears partially programmed. To begin to address which cells are most responsive to Notch signaling, we compared APD of LV and right ventricle (RV) separately. Interestingly, Notch activation does not result in action potential prolongation in adult mouse RV myocytes (Online Figure II; Table 1).

**Notch Activation Selectively Attenuates Voltage-Gated K+ (Kv) Currents in LV Myocytes**

To explore the hypothesis that alterations in repolarizing K+ currents underlie the action potential prolongation observed in Notch GOF LV myocytes, whole-cell voltage-clamp recordings were obtained under conditions optimized to record K+ currents uncontaminated by inward Na+ and Ca2+ currents. Briefly, whole-cell, depolarization-activated outward K+ (Kv) currents were evoked during voltage steps to test potentials between −60 and +40 mV (in 10 mV increments) from a holding potential of −80 mV from the same holding potential, was also recorded in each cell. Representative recordings from control and Notch GOF LV myocytes are shown in Figure 2A and 2B; the voltage-clamp paradigm is illustrated below the current records. No differences in LV myocyte cell size, determined from measurements of whole-cell membrane capacitances (C_m), were observed (Table 2). The currents in individual cells, therefore, were normalized to the C_m (in the same cell), and current densities are plotted. The density of the peak outward Kv current (I_{K,peak}) is substantially lower in the Notch GOF LV myocyte (Figure 2B), compared with the control LV myocyte (Figure 2A).

To determine the amplitudes of the individual Kv components, I_{K,slow} and I_{K,fast}, of the peak outward Kv current, I_{K,peak}, in control and Notch GOF LV myocytes, the decay phases of the currents were fitted to the sum of 2 exponentials.27,28 These analyses revealed that mean±SEM I_{K,peak}, I_{K,slow} and I_{K,fast} amplitudes/densities were significantly lower in Notch GOF, than in control, LV myocytes (Figure 2D; Table 2), whereas I_{K,slow} and I_{K,fast} amplitudes/densities in control and Notch GOF LV myocytes were similar (Figure 2D; Table 2). Further whole-cell recordings revealed significantly lower I_{K,peak} amplitudes/densities in Notch GOF, than in control, LV myocytes (Figure 2D; Table 2), whereas I_{K,slow} and I_{K,fast} amplitudes/densities in control and Notch GOF LV myocytes were similar (Figure 2D; Table 2). Further whole-cell recordings revealed significantly lower I_{K,peak} amplitudes/densities, and the waveforms of the currents are distinct in Cntn2-eGFP+ Purkinje cells (Figure 2C), compared with control LV myocytes (Figure 2A). Interestingly, and as evident in Figure 2C, I_{K,slow} was not detected (Table 2) in Cntn2-eGFP–expressing Purkinje cells. Given that ventricular myocytes from the same preparation exhibit I_{K,slow}, the absence of I_{K,slow} in Purkinje cells is not an artifact of the enzymatic digestion protocol (Online Figure III). Analyses of the voltage-clamp records revealed that I_{K,slow} densities were significantly lower in Cntn2-eGFP–expressing Purkinje cells than in control LV myocytes, whereas I_{K,fast} and I_{K,slow} densities in Purkinje cells and LV myocytes were similar (Figure 2D; Table 2). In conclusion, Notch-induced changes in I_{K,slow} and I_{K,fast} current densities trended toward Purkinje cells, with some cells experiencing a reduction to levels akin to the Purkinje cells.

**Notch Signaling Regulates Expression of K+ Channel Subunits in LV Myocytes**

We next sought to delineate the mechanism by which Notch signaling regulates K+ current densities by analyzing the expression of transcripts encoding K+ channel subunits. As expected, we observe significant upregulation of the known direct Notch targets Hrt2 by 8-fold (P=2.3E-0.4) and Hes1 by 3-fold (P=1.1E-0.3), as well as of Purkinje-enriched genes Cntn2 by 8-fold (P=3.6E-0.5) and Scn5a by 9-fold (P=4.7E-0.5), in Notch GOF, compared with control, LV myocytes (n=6 each; Figure 3A). Moreover, we see 3-fold reduction in the expression of transcripts encoding the K+ channel pore-forming subunit of I_{K,slow} Kcnf1 by 4-fold (P=4.1E-0.5). Transcripts of genes encoding the pore-forming subunits of I_{K,slow}, namely, Kcnf1 and Kcnf2, are both downregulated ≈2.5-fold (P=1.7E-0.3 and 3.4E-0.3, respectively; Figure 3B). To determine whether there is a corresponding decrease in protein levels, we performed Western

---

**Table 1. Summary of Myocyte Resting and Active Membrane Properties**

<table>
<thead>
<tr>
<th>Condition</th>
<th>V_m (mV)</th>
<th>R_m (MΩ)</th>
<th>APA (mV)</th>
<th>APD_{50} (ms)</th>
<th>APD_{70} (ms)</th>
<th>APD_{90} (ms)</th>
<th>dv/dt (mV/ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control LV n=27</td>
<td>−82±1</td>
<td>273±117</td>
<td>118±3</td>
<td>4±1</td>
<td>9±1</td>
<td>36±8</td>
<td>178±13</td>
</tr>
<tr>
<td>Notch GOF LV n=32</td>
<td>−81±1</td>
<td>291±57</td>
<td>124±2</td>
<td>9±2†</td>
<td>16±3†</td>
<td>54±7†</td>
<td>226±12†</td>
</tr>
<tr>
<td>Cntn2-eGFP n=18</td>
<td>−81±2</td>
<td>403±104</td>
<td>130±3†</td>
<td>37±4‡</td>
<td>68±7‡</td>
<td>115±8‡</td>
<td>191±17</td>
</tr>
<tr>
<td>Control RV n=33</td>
<td>−81±1</td>
<td>301±106</td>
<td>115±2</td>
<td>3±1</td>
<td>6±1</td>
<td>20±5</td>
<td>157±10</td>
</tr>
<tr>
<td>Notch GOF RV n=22</td>
<td>−80±1</td>
<td>115±15</td>
<td>111±2</td>
<td>3±1</td>
<td>5±1</td>
<td>14±2</td>
<td>137±13</td>
</tr>
</tbody>
</table>

All values are means±SEM. n=numbers of cells. APA indicates action potential amplitude; APD, action potential duration; GOF, gain of function; LV, left ventricular; RV, right ventricular; and V_m, resting membrane potential.

*Current-clamp recordings were obtained as described in Materials and Methods.

†P<0.05, significantly different from those measured in control LV cells.

‡P<0.0001, significantly different from those measured in control LV cells.
blotting with antibodies directed against KChIP2, encoded by Kcnip2, and Kv2.1, encoded by Kcnb1. Relative band densities were used for protein quantification, which reveals a 2.5-fold reduction of KChIP2 in Notch GOF LV free wall (P=0.004), as well as a 2-fold (P=0.007) reduction of Kv2.1 when compared with littermate controls (n=4; Figure 3C and 3D). Taken together, these data suggest that the decrease in K+ current densities seen in Notch GOF mice is at least partially mediated via transcriptional downregulation of the subunits encoding the repolarizing K+ channels Ito,f and IK,slow.

**Haploinsufficiency of KChIP2 Selectively Attenuates Ito,f in LV Myocytes**

Previous studies have shown that complete loss of KChIP2 in mouse ventricular myocardium results in elimination of Ito,f.29 In Notch GOF mice, KChIP2 protein levels are reduced by ≈50% (Figure 3D). To explore the hypothesis that the reduction of KChIP2 in Notch GOF is sufficient to attenuate Ito,f, whole-cell Kv current recordings were obtained from LV myocytes isolated from KChIP2+/− mice lacking one copy of Kcnip2.29 Analyses of the voltage-clamp data obtained in these experiments revealed that mean±SEM Ipeak (P=0.018) and Ito,f (P=0.0026) densities were significantly lower in KChIP2+/− LV myocytes, than in control LV myocytes (Online Figure IV; Table 2), whereas IK,slow, I1,s, and IK1 densities in KChIP2+/− and control LV myocytes were not significantly different (Online Figure IV; Table 2). This suggests that the reduction in KChIP2 levels seen in Notch GOF is likely responsible for diminished Ito,f but not the decrease observed in IK,slow.

**Notch Inactivation Increases Kv Current Amplitudes and LV Myocyte Size**

To further demonstrate that K+ currents are regulated by canonical Notch signaling, we tested whether Notch signaling is required for regulation of ventricular K+ currents. Given the redundancy of the 4 mammalian Notch receptors, we used a dominant-negative approach to inhibit Notch-mediated transcription. The dominant-negative mastermind-like protein (DNMAML) specifically inhibits Notch-mediated transcription downstream of all 4 Notch receptors.30 Voltage-clamp recordings from LV myocytes isolated from Notch loss of function Mlc2vCre/+; DNMAML mice, hereafter referred to as
Notch loss of function (LOF), reveals that \( I_{\text{K,peak}} \) amplitudes are significantly \((P<0.001)\) higher than in control LV myocytes (Figure 4A). Analyses of the waveforms of the currents further revealed that mean±SEM \( I_{\text{K,slow}} \) \((P<0.0001)\), \( I_{\text{ss}} \) \((P<0.01)\), and \( I_{\text{K1}} \) \((P<0.001)\) amplitudes were all significantly larger than that in control LV myocytes. Summary of Myocyte Repolarizing \( K^+ \) Current Densities*

<table>
<thead>
<tr>
<th>Cells</th>
<th>( C_m ), pF</th>
<th>( I_{\text{K,peak}} ) Density, pA/pF</th>
<th>( I_{\text{K,slow}} ) Density, pA/pF</th>
<th>( I_{\text{ss}} ) Density, pA/pF</th>
<th>( I_{\text{K1}} ) Density, pA/pF</th>
<th>( \tau_{\text{decay}} ), ms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control LV n=18</td>
<td>120±5</td>
<td>55.7±3.0</td>
<td>26.4±2.5</td>
<td>21.9±1.4</td>
<td>9.1±0.5</td>
<td>12.5±0.8</td>
</tr>
<tr>
<td></td>
<td>( \tau_{\text{decay}} ), ms</td>
<td>65±4</td>
<td>805±62</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Notch GOF LV n=19</td>
<td>114±11</td>
<td>31.7±4.9†</td>
<td>14.0±3.0‡</td>
<td>12.3±2.3‡</td>
<td>8.0±0.5</td>
<td>11.6±1.0</td>
</tr>
<tr>
<td></td>
<td>( \tau_{\text{decay}} ), ms</td>
<td>65±3</td>
<td>1386±175</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cntn2-eGFP n=25</td>
<td>75±7‡</td>
<td>13.8±1.0†</td>
<td>5.5±0.4‡</td>
<td>8.6±0.7</td>
<td>13.8±1.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>( \tau_{\text{decay}} ), ms</td>
<td>1946±251</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KChIP2−/− LV n=53</td>
<td>132±7‡</td>
<td>42.1±2.7§</td>
<td>16.7±1.3‡</td>
<td>16.7±1.4</td>
<td>8.0±0.4</td>
<td>15.1±1.0</td>
</tr>
<tr>
<td></td>
<td>( \tau_{\text{decay}} ), ms</td>
<td>128±10</td>
<td>1475±69</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Notch LOF LV n=20</td>
<td>180±10†</td>
<td>56.5±3.3</td>
<td>22.8±2.0</td>
<td>25.1±1.3</td>
<td>8.7±0.7</td>
<td>12.0±1.1</td>
</tr>
<tr>
<td></td>
<td>( \tau_{\text{decay}} ), ms</td>
<td>103±7</td>
<td>1041±47</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

All values are means±SEM; Peak outward \( K^+ \) currents \((I_{\text{K,peak}})\) were measured as the maximal outward current evoked at +40 mV, and \( I_{\text{ss}} \) was measured as the maximal inward current evoked at −120 mV. The amplitudes of the individual \( K^+ \) current components, \( I_{\text{K,slow}} \), \( I_{\text{ss}} \), and \( I_{\text{K1}} \), were determined from exponential fits to the decay phases of the currents and normalized to the whole-cell membrane capacitance \((C_m)\) to provide current densities. LOF indicates loss of function; and \( n \), numbers of cells.

*Voltage-clamp recordings were obtained as described in Materials and Methods.

†\( P<0.0001 \), significantly different from those measured in control LV cells.
‡\( P<0.001 \), significantly different from those measured in control LV cells.
§\( P<0.05 \), significantly different from those measured in control LV cells.
‖\( P<0.01 \), significantly different from those measured in control LV cells.

Figure 3. Notch activation dysregulates expression of \( K^+ \) channel subunits. A, Notch gain of function (GOF) upregulates expression of the Purkinje cell marker \( Cntn2 \) 8-fold, \( Scn5a \) 9-fold as well as ventricle-enriched Notch targets \( Hrt2 \) (9-fold) and \( Hes1 \) (3-fold), whereas the atrial-enriched Notch target \( Hrt1 \) was unchanged \((n=6)\) compared with littermate controls. B, Notch GOF represses expression of transcripts encoding potassium channel subunits comprising \( I_{\text{ss}} \) \((Kcnb1 \text{ and } Kcnb2 \text{ 2-fold}); \) \( I_{\text{K,slow}} \) \((Kcnb1 \text{ and } Kcnb5 \text{ 2-fold}), \) whereas subunits encoding \( I_{\text{K1}} \) \((Kcnj2)\) are not changed \((n=6)\) compared with littermate controls. C, Western blot showing decreased protein levels of \( KChIP2 \) (35 kDa doublet) and \( Kv2.1 \) (114 kDa) in Notch GOF when compared with \( GAPDH \) protein levels. D, Quantification of protein levels based on band density shows significantly reduced levels of \( KChIP2 \) (3-fold) and of \( Kv2.1 \) (2-fold) in Notch GOF compared with littermate controls \((n=4)\). Equal variance, 2-tailed Student t test was performed to determine statistical significance. *\( P<0.05 \), †\( P<0.01 \), ‡\( P<0.001 \), ns, not significant. Controls are littermate \( R26\text{-NICD/+} \).
When current amplitudes were normalized to whole-cell capacitance, all current densities in Notch LOF LV myocytes are similar to those measured in control LV myocytes (Figure 4E; Table 2), suggesting that Notch inactivation leads to an increase in LV myocyte capacitance with appropriate scaling of voltage-dependent K+ currents to increased amplitudes of Ipeak, Ito,f, IK,slow, and Iss for all K+ currents recorded (C), current density in Notch LOF myocytes was not significantly different than control, which suggests physiological hypertrophy (E). Mean values are significantly (†P<0.01, ‡P<0.001, §P<0.0001) different from those measured in control LV myocytes; ns, not significantly different. (F, In consistence with the physiological hypertrophy observed in Notch LOF currents, cytoplasmic genes β-actin, Kcnd3, Kcnip2, and Kcnb1 are significantly upregulated in Notch LOF by ≈2-fold, relative to nuclear housekeeping gene Tbp. n=6. Equal variance, 2-tailed Student t test was performed to determine statistical significance. *P<0.05, †P<0.01, ‡P<0.001, §P<0.0001, ns, not significant.)
accommodate the increase in cell size. These types of changes are reminiscent of findings in physiological hypertrophy,\textsuperscript{31} where transcriptional upregulation of the channel subunits occurs in parallel with an increase in myocyte size, thus preserving action potential waveforms. Consistent with this, we observe significant upregulation of \( K^{+} \) channel subunit genes \( Kcnb1(P=0.001) \) and \( Kcnb1(P=0.03) \) by \( \approx2\)-fold, relative to expression of nuclear housekeeping gene TATA-box–binding protein (\( \text{Tbp} \); n=5; Figure 4F). We also see 2.5-fold upregulation of the cytoplasmic housekeeping gene \( \beta \)-actin (P=0.004) relative to \( \text{Tbp} \).

**Notch Loss of an Activating Histone Methylation Mark in the Proximal Promoters of \( Kcnip2 \), \( Kcnb1 \), and \( Kcna5 \)**

Epigenetic factors are essential for the establishment of gene expression patterns in a cell–specific and heritable manner during development and enable maintenance of adult gene expression. Importantly, epigenetic modifications can also be acquired in response to stress.\textsuperscript{32,33} Histone methylation is associated with both active and repressed genes, depending on the specific lysine or arginine residues methylated.\textsuperscript{34} Histone H3 lysine 4 methylation (H3K4me) marks are imprinted through the Trithorax group proteins (TrxG), and high levels of H3 lysine 4 methylation (H3K4me) marks are imprinted at the specific lysine or arginine residues methylated.\textsuperscript{35} Histone methylation is associated with both active and repressed genes, depending on the specific lysine or arginine residues methylated. Histone H3 lysine 4 methylation (H3K4me) marks are imprinted through the Trithorax group proteins (TrxG), and high levels of Histone H3 lysine 4 trimethylation (H3K4me3) are associated with the 5′ regions of nearly all actively expressed genes.\textsuperscript{35,36}

Interestingly, maintenance of the H3K4me3 mark is required to sustain ion channel gene expression, and loss of H3K4 methylation in adult murine cardiac myocytes results in a robust reduction in expression of \( Kcnip2 \) and reduction of \( I_{\text{Kav}} \).\textsuperscript{37}

To date, the minimal promoters for \( Kcnip2 \), \( Kcnb1 \), and \( Kcna5 \) have not been fully delineated; however, the genomic region from \( +1524 \) bp upstream to \( +312 \) bp downstream of the rat \( Kcnip2 \) transcriptional start site is responsive to stimuli such as isoproterenol and NF (nuclear factor)-\( \kappa B \) in reporter assays.\textsuperscript{38} Therefore, we analyzed the comparable highly conserved proximal promoter region in mouse \( Kcnip2 \) for regulatory elements. Similarly, genomic regions from 2000 bp upstream to \( +500 \) bp downstream of the transcriptional start site were used to scan for histone modifications in \( Kcnb1 \) and \( Kcna5 \). On the basis of the adult mouse heart H3K4me3 ChIP-seq data set obtained from the UCSC Genome Browser, we identified several putative regions of H3K4me3 within the proximal promoters of \( Kcnip2 \), \( Kcnb1 \), and \( Kcna5 \) (Figure 5A). To begin to define the molecular mechanisms whereby Notch signaling regulates expression of voltage-gated \( K^{+} \) subunits, we performed chromatin immunoprecipitation (ChIP) for H3K4me3. Activation of Notch signaling significantly decreases H3K4me3 in the region located \( +100 \) bp from the transcription start site in the \( Kcnip2 \) promoter by \( >5\)-fold (P=0.00015), whereas the H3K4me3 region located more upstream is not affected (Figure 5B). Interestingly, enhanced histone methylation at the \( +100 \) bp

---

**Figure 5. Notch induces removal of activating histone methylation in \( Kcnip2 \) and \( Kcna5 \) promoters.** A, Schematic showing H3K4me3 marks in proximal promoters of \( Kcnip2 \), \( Kcnb1 \), and \( Kcna5 \). Numbers below the marks indicate location with respect to transcription start site. B, Chromatin immunoprecipitation (ChIP) quantitative polymerase chain reaction assay shows loss of H3K4me3 marks by over 4-fold in \( Kcnip2 \), \( Kcnb1 \), and \( Kcna5 \) promoters in Notch gain of function (GOF), compared with littermate controls. H3K4me3 region in the proximal promoter of \( Kcnip2 \), a gene unresponsive to Notch GOF, was used as negative control (n=6). C, Compared with littermate controls, doxycycline-induced Notch activation (INICD) at 1 d after birth shows significant upregulation of \( \text{Cntn2}, \text{Hrt2}, \) and \( \text{Hes1} \) to about 2-fold and downregulation of \( Kcnip2 \) by 5-fold and \( \text{Kcnb1} \) by 4-fold (n=6). One-way ANOVA with post hoc Tukey test was performed to determine statistical significance. D, Compared with littermate controls, INICD at 8 wk results in over 4-fold loss of H3K4me3 in the proximal promoter of \( Kcnip2 \) in the same location as in Notch GOF; H3K4me3 region in the proximal promoter of \( Kcnj2 \), a gene unresponsive to Notch, was used as negative control. Equal variance, 2-tailed Student \( t \) test was performed to determine statistical significance. *P<0.05, †P<0.01, ‡P<0.001, ns, not significant. Controls are littermate \( \text{R26R}^{\text{ NICD}} \) (B) or littermate \( \alpha\text{MHC}-\text{rtTA} \) on doxycycline (C and D).
region has previously been correlated with maintenance of expression of Kcnip2 within adult cardiac myocytes.37 Similarly, we identified loss of H3K4me3 in the vicinity of the transcription start sites of Kcnb1 of >4-fold (P=0.008) and Kcna5 by >5-fold (P=0.016; Figure 5B) in Notch GOF LV myocytes. In contrast, histone methylation at a region within the Kcnb2 promoter near the transcription start site remains unchanged (Figure 5B), which correlates well with the absence of changes in Kcnb2 expression (Figure 3B) and Ik1 current observed in Notch-activated mice (Table 2).

Notch signaling is normally not active in adult myocardium, however, it is reactivated in response to injuries such as myocardial infarction.39 To test whether Notch can regulate K+ channel subunit gene expression after developmental programming has occurred, we generated a doxycycline-inducible model to allow us to activate Notch in perinatal and adult myocytes (αMHCrtTA; tetO-NICD; subsequently referred to as iNICD).1 Notch induction at birth, a time point after which Purkinje and ventricular myocyte lineages have diverged,32 leads to significant upregulation of the Purkinje-enriched gene Cntn2 by 4-fold (P=0.044), as well as significant downregulation of Kcnip2 by 2-fold (P=0.03), Kcnb1 by 2-fold (P=0.02) and Kcna5 1.5-fold (P=0.012; Figure 5C). Similarly, when Notch is induced at 8 weeks of age, we see significant downregulation of Kcnip2 (P=7.7E-05) and Kcnb1 (P=0.013) by 5-fold (Figure 5C); however, other Purkinje-enriched genes remain unchanged. Terminal differentiation and binucleation of cardiac myocytes coincident with decreased cellular plasticity occurs shortly after birth in rodents,40 the timing of which also coincides with the partial effects we observe in response to Notch activation in adult versus neonatal mice. Interestingly, induction of Notch at 8 weeks of age also results in loss of the H3K4me3 mark in the Kcnip2 +100 bp location by 4-fold (P=4.5E-04), similar to that seen during developmental Notch GOF (Figure 5D). Previous literature has shown that maintenance of the H3K4me3 mark in the Kcnip2 promoter of adult cardiac myocytes is necessary for electric homeostasis.37 Therefore, we hypothesize that Notch-mediated chromatin remodeling in response to pathological stress may predispose to altered repolarization.41

**Dynamic RBP-J–Binding Sites Within Kv Channel Subunit Promoters**

When the RBP-J regulatory complex is bound to DNA in the absence of Notch, it has been shown to be capable of recruiting the Junonji, AT Rich Interactive Domain 1A (JARID1A, also known as KDM5A) demethylase and removing the H3K4me3 mark from target genes.42 In addition, Notch-regulated genes often contain dynamic RBP-J–binding sites. Therefore, using TRANSFAC motif finding software, we next screened Kcnip2, Kcnb1, and Kcna5 proximal promoters (~2000 bp to +500 bp, as described above) for consensus RBP-J motifs. Interestingly, putative RBP-J binding sites were found within the proximal promoters of Kcnip2, Kcnb1, and Kcna5 in the vicinity of the Notch-regulated histone methylation sites (Figure 6A). To determine whether Notch activation dynamically increases RBP-J complex binding to these promoters, we performed ChIP assays using anti–RBP-J antibody. Interestingly, we observed basal RBP-J binding at the Kcnip2, but not the Kcnb1 or Kcna5 promoters, based on fold enrichment over IgG (data not shown). Notch activation resulted in >3.5-fold enrichment of RBP-J binding at both the Kcnip2 (P=0.003) and Kcnb1 (P=0.001) promoters (Figure 6B), consistent with a Notch-regulated dynamic binding profile. In contrast, RBP-J could not be detected at the putative site within the proximal promoter of Kcna5, suggesting that Notch signaling may indirectly regulate expression of Kcna5.

**Downregulation of K+ Channel Subunits Requires Canonical Notch Signaling**

The NICD/RBP-J/MAML complex is a canonical Notch regulatory complex. To provide further evidence for regulation of voltage-gated K+ channel subunit expression by canonical Notch signaling, we performed a genetic rescue experiment. Activation of Notch signaling through ectopic expression of NICD results in 3-fold upregulation of NICD (P=0.044), and the direct Notch target Hrt2 (P=0.026), whereas Kcnip2 is downregulated 3-fold (P=0.003), Kcnb1 is downregulated 5-fold (P=0.002), and Kcna5 is downregulated 2-fold (P=0.002; Figure 6C). We concurrently expressed NICD and DNAMAML to inhibit binding between NICD and MAML, thus interfering with formation of the Mastermind-containing transcriptional complex.43 Whereas concurrent expression of NICD/DNAMAML does not affect overall NICD levels, the expression levels of Hrt2, Kcnip2, Kcnb1, and Kcna5 are all normalized in NICD/DNAMAML ventricles, suggesting that Notch-mediated downregulation of potassium channel genes occurs through the canonical NICD/ RBP-J/MAML complex (Figure 6C).

To explore the kinetics of Notch-mediated transcriptional events, we examined the time course of dysregulation of K+ channel subunit expression after acute induction of Notch signaling. Cardiac myocytes were isolated from adult iNICD mice, followed by induction of NICD through addition of doxycycline to the media. NICD transcript was upregulated 4-fold within 30 minutes of doxycycline induction (P=0.028), and further increased as high as 26-fold at 5 hours (P=0.003; Figure 6D). The direct Notch target Hes1 was significantly upregulated 6-fold at 5 hours (P=0.03). Interestingly, Kcnip2 was significantly downregulated within 30 minutes, and both Kcnip2 and Kcnb1 are significantly downregulated 4-fold at 5 hours (P=0.008 and P=0.012, respectively), whereas levels of Kcna5 remain unchanged. Taken together, Kcnip2 and Kcnb1 contain dynamic RBP-J–binding sites, and transcript down-regulation occurs within the timeframe of the direct Notch target gene Hes1. In contrast, we did not detect dynamic RBP-J binding at the Kcna5 promoter, and regulation of Kcna5 transcript levels does not occur within the timeframe of known direct Notch target genes.

**Mechanism for Differential Electrophysiological Effects in LV Versus RV**

Because Notch activation does not result in action potential prolongation in adult mouse RV myocytes (Online Figure II; Table 1), we asked whether Notch downregulates K+ currents within the RV. In contrast to the effects in the LV, Notch activation in the RV did not reduce Ito,f nor IK,slow (Online Figure VA), whereas reduction of KChIP2 in the KChIP2+/- RV results in a similar reduction of Ito,f to that seen in the LV (Online Figure VB). In contrast to Notch GOF LV, we found that expression
of KChIP2 and Kv2.1 protein levels remain unchanged in Notch GOF RV, which explains the lack of Notch effects on $I_{to}$ and $I_{K_{slow}}$ in the RV (Online Figure VC).

Interestingly, although at baseline, the expression of Hrt2 is similar in the LV and RV, Notch activation in the LV upregulates Hrt2, whereas, in contrast, Notch activation in the RV downregulates Hrt2 expression (Online Figure VIA).

Given the importance of Hrt2 in regulating cellular electrophysiology,2,43 we assessed for H3K4me3 and RBP-J at predicted binding sites in the Hrt2 promoter 44 and enhancer 21 Figure 6.

Dysregulation of Kcnip2 and Kcnb1 is responsive to canonical Notch regulatory complex with dynamic RBP-J binding. A, Schematic showing H3K4me3 marks and putative RBP-J–binding sites in the proximal promoters of Kcnip2, Kcnb1, and Kcna5. Numbers indicate location with respect to transcription start site. B, Chromatin immunoprecipitation (ChIP) quantitative polymerase chain reaction assay using anti–RBP-J antibody shows ≈3.5-fold higher binding of RBP-J on Kcnip2 and Kcnb1 but not on Kcna5 promoter in Notch GOF, compared with littermate controls (n=5). Kcnb1 −500 bp site has no putative RBP-J–binding site and was used as negative control to verify sensitivity of the assay. The proximal promoter of Kcn2, with no putative RBP-J site, was also used as negative control. Equal variance, 2-tailed Student t test was used to determine statistical significance.

C, Schematic illustrating the Notch loss-of-function (LOF) model through expression of a small MAML peptide that functions as a dominant-negative (DNMAML). Notch GOF (n=4) through ectopic expression of NICD results in ≈3-fold upregulation of NICD, and the direct Notch target Hrt2, and 3-fold downregulation of Kcnip2, 5-fold downregulation of Kcnb1, and 2-fold downregulation of Kcna5 compared with controls (n=5). Concurrent expression of DNMAML in the setting of Notch GOF (n=6) shows NICD expression increase by 2-fold, with normalization of expression levels of Hrt2, Kcnip2, Kcnb1, and Kcna5. One-way ANOVA was performed to determine statistical significance.

D, Doxycycline-induced activation of Notch signaling in isolated adult iNICD left ventricular (LV) free wall myocytes (n=3) results in NICD upregulated by 4-fold in 30 min and increases to ≈26-fold by 5 h post doxycycline treatment, compared with cells from the same animal not treated with doxycycline. Direct target Hes1 is significantly upregulated by 6-fold within 5 h after doxycycline induction of NICD. Similarly, Kcnip2 and Kcnb1 are significantly downregulated by 5-fold within 5 h of doxycycline induction of NICD. Kcna5 expression does not change within 5 h. β-actin was used as negative control and shows stable expression throughout the time course. One-way ANOVA with post hoc Tukey was performed to determine statistical significance. *P<0.05, †P<0.01, ns, not significant. Controls in (B and C) are littermate R26RNICD/DNMAML, controls in (D) are from the same αMHC-rtTA; tetO-NICD animals without doxycycline.
through ChIP assays. Coincident with gene expression changes, Notch activation results in increased H3K4me3 at the Hrt2 promoter in the LV, whereas, in contrast, there is loss of H3K4me3 at the promoter in the RV (Online Figure VIIB). To provide further mechanistic insight for the differential effects, we assessed for the presence of RBP-J. At baseline, RBP-J is bound to the proximal Hrt2 promoter in the LV, whereas, in contrast, RBP-J is not bound to the proximal Hrt2 promoter in the RV (Online Figure VIC). RBP-J binding to this proximal promoter site has previously been shown to be both required and sufficient for mediating Hrt2 expression.44 In contrast to the dynamic RBP-J binding to Kcnip2 and Kcnb1 promoters in the presence of NICD (Figure VIIB), we did not detect an increase in RBP-J binding to the Hrt2 promoter and enhancer sites in LV nor RV in the presence of NICD (Online Figure VIC). These results are consistent with a classical model of Notch regulation of Hrt2 expression in the LV, whereas Hrt2 expression in the RV seems to be regulated through an alternative mechanism (model Online Figure VID).

### Notch Activation in Cardiac Myocytes in Response to Pressure-Induced Heart Failure

Notch is normally quiescent in adult cardiac myocytes; however, several recent studies have suggested that Notch signaling is transiently activated in the setting of cardiac injuries, including myocardial infarction, in mouse and zebrafish models.19,39,45,46 In response to myocardial infarction, Notch induction occurs primarily in border zone cardiac myocytes.39 In the setting of relatively large myocardial infarctions with subsequent activation of β-adrenergic signaling, I_{s} and I_{slow} are reduced through transcriptional mechanisms.27 To determine whether Notch1 is activated in response to a stress that results in downregulation of the promoters (Figure 7F; Online Figure VIC). These results are consistent with a classical model of Notch regulation of Hrt2 expression in the LV, whereas Hrt2 expression in the RV seems to be regulated through an alternative mechanism (model Online Figure VID).

#### Discussion

### Purkinje Cell Currents

Previous studies in mice have shown that action potential durations in Purkinje cells are prolonged in relation to ventricular myocytes that is a gradual and predictable progression of adverse left ventricular remodeling leading to heart failure, as previously described.49 Mice were euthanized 4 weeks after surgery for analysis, revealing pathological hypertrophy, as observed in left ventricular remodeling,48 assessed by increased heart weight/tibia length and upregulation of Nppa and Nppb in the heart failure animals when compared with sham (Figure 7A and 7B). Transcripts encoding the Jagged-1 ligand, Notch1 receptor, and direct Notch target Hes1 are upregulated in heart failure (Figure 7C). In addition, although not upregulated globally at the transcript level, we observed increased expression of the Notch ligand Dll4 within vascular endothelium throughout the heart after transverse aortic constriction (Online Figure VII).

To determine which cells express Notch1 in heart failure, we made use of a tamoxifen-inducible Notch1 activation–dependent reporter knock-in mouse line, NIP1::CreER<sup>22</sup>.49 In this line, the intracellular domain of Notch1 was replaced with a complementary DNA encoding a 6× myc-tagged CreERT2 (6mtCreER<sup>22</sup>). Binding of Notch ligands to the NIP1::CreERT2 will trigger release of the 6mtCreER<sup>22</sup> from the membrane, but only in the presence of tamoxifen can CreERT2 enter the nucleus. Within the nucleus, CreERT2 can mediate the excision of a floxed stop cassette in Rosa26R<sup>T-Albinoe</sup> reporter mice, thereby permanently labeling Notch1-expressing cells and their offspring red (Figure 7D). Although in the absence of tamoxifen no cells are labeled (data not shown), sham surgical animals treated with tamoxifen display active Notch1 signaling only within PECAM-1<sup>+</sup> (platelet and endothelial cell adhesion molecule-1) endothelial cells, as expected, and labeling of α-actinin<sup>+</sup> cardiac myocytes was not detected (Figure 7D; Online Figure VIII). In contrast, induction of left ventricular remodeling with administration of tamoxifen results in Notch1 activation (red) in many α-actinin<sup>+</sup> cardiac myocytes (green) throughout the left ventricle, as well as an increase in vascularity (Figure 7D; Online Figure VIII). As expected, Kcnip2 and Kcnb1 transcripts are downregulated (Figure 7E), coincident with loss of H3K4me3 at the promoters (Figure 7F). Interestingly, we observe dynamic binding of RBP-J to the Kcnip2 promoter in heart failure similar to what we observe in adult Notch-activated mice, whereas dynamic RBP-J binding to the Kcnb1 promoter was not detected in heart failure (Figure 7G).

### Mechanisms of Gene Regulation by Canonical Notch Signaling

Left and right ventricular cardiac myocytes may respond differently to instructive signals because of their distinct embryological origins, namely, the first and second heart fields, respectively. Intrinsic differences in LV versus RV developmental programming and gene regulatory networks may govern distinct myocardial electrophysiology and could have profound implications for understanding disease pathogenesis.
In this context, it is interesting to note that Brugada syndrome, characterized by electric remodeling primarily of the RV predisposing to ventricular arrhythmias, is associated with variants in a direct target of the Notch signaling pathway (HEY2 or HRT2). Interestingly, we note distinct electric differences between LV and RV in response to Notch activation, as well as in expression of Hrt2 (Figure 2; Table 1; Online Figures II, V, and VI). Activation of Notch signaling downregulates Kv currents only within the LV, where RBP-J is bound to the Hrt2 promoter, and Hrt2 gene expression is upregulated. This chamber-specific difference in RBP-J binding and response to Notch activation is potentially of interest for future investigation and may be mediated by differential chromatin landscape present at the Hrt2 promoter or differences in expression of co-operating...
factors between the chambers. Indeed, it has previously been suggested that Htr2 expression within ventricular myocardium may not be dependent on Notch signaling status, and our data are generally consistent with the idea that Htr2 expression in the RV may be regulated by additional factors.

Here, we demonstrate that voltage-gated K+ currents are regulated by Notch signaling via transcriptional mechanisms. Although the majority of inducible RBP-J-binding sites are located within gene enhancers, the direct Notch target Hes1 contains an inducible binding site within its proximal promoter. The Kcnip2 and Kcnb1 proximal promoters also demonstrate Notch-induced RBP-J binding similar to Hes1, as well as transcriptional kinetics similar to Hes1. In contrast, we did not detect RBP-J binding at the putative consensus site within the Kcnas promoter either in the presence or absence of Notch and the kinetics of Kcnas transcriptional regulation are consistent with indirect regulation. Notch-mediated transcriptional regulation of Kv currents is inhibited by DNMAML and, therefore, occurs through the canonical NICD/RBP-J/MAML complex. The NICD/RBP-J/MAML complex is considered an activating complex; however, βHLH proteins such as Hrt have been suggested to bind to the NICD/RBP-J complex with subsequent recruitment of transcriptional corepressors. Therefore, Notch signaling may regulate expression of voltage-gated potassium channel subunits through multiple transcriptional mechanisms, potentially also involving binding of βHLH transcription factors to consensus E box–binding sites within the promoters.

Notch Activation in Cardiac Pathology
Developmental inactivation of Notch signaling results in transcriptional upregulation of ion channel subunits in parallel with increases in myocyte size, consistent with findings seen in physiological hypertrophy. In contrast to physiological hypertrophy, pathological hypertrophy can occur in response to biomechanical stresses. In the setting of pathological hypertrophy, in part because of a failure to upregulate expression of the underlying K+ channel subunits proportionally with the increase in myocyte size, repolarizing K+ current amplitudes are not increased, and K+ current densities are decreased. Interestingly, many of these cardiac stresses can reactivate Notch signaling in the adult. Therefore, although Notch GOF mice do not exhibit cellular hypertrophy (Table 2, Cm), it is possible that activation of Notch signaling in the setting of pathological hypertrophy might contribute to decreased K+ current densities.

Many recent studies have shown that Notch signaling is also transiently activated in the setting of injury, including myocardial infarction, in mouse and zebrafish models. Notch induction occurs primarily in border zone cardiac myocytes and, although it may exert cardioprotective functions such as decreased cardiac myocyte apoptosis and may promote some regenerative effects, Notch activation could also potentially increase the risk of adverse outcomes. By analogy, transient Notch activation in response to adult liver injuries that provoke a biliary response initiates a cascade that reprograms hepatocytes into biliary epithelial cells. We postulate that perhaps a similar injury paradigm exists in the heart resulting in transcriptional and epigenetic changes akin to induced Purkinje-like cells, which could potentially contribute to postinfarction arrhythmias.

In support of this model, genome-wide histone profiling in human heart failure demonstrates that the H3K4me3 and H3K9me3 marks are among those most dynamically regulated. In addition, previous literature shows that loss of H3K4me in terminally differentiated cardiac myocytes results in significant changes in cellular physiology predisposing to ventricular arrhythmias, suggesting that epigenetic regulators may contribute to the arrhythmic phenotype. In this study, we demonstrate loss of H3K4me3 at the Kcnip2 promoter in response to transient Notch activation in adult cardiac myocytes (Figure 6), as well as in heart failure (Figure 7). Future global transcriptional and epigenetic profiling may further elucidate additional Notch-regulated targets, which may contribute to pathology.

The 4 members of the JARID1 family of demethylases specifically remove methyl marks from the trimethylated lysine 4 of histone H3 (H3K4me3). Given that Notch is activated in adult cardiac pathology, specific inhibitors targeting the Notch pathway itself, or perhaps inhibitors targeting the epigenetic machinery required downstream of Notch, could provide novel therapeutic targets for arrhythmias. It may prove to be important to specifically target cardiac myocytes because Notch activity plays a homeostatic role in other organs and cell types. Overexpression of lysine demethylases and activation of the Notch signaling pathway is known to be pathogenic in many types of cancers, and inhibitors of these pathways are currently being pursued as potential cancer therapeutics. Given that drug discovery pipelines are hampered by cardiac toxicity, and specifically proarrhythmic effects are worrisome, a better understanding of the effects of these Notch inhibitors and epigenetic modifiers on cardiac electrophysiology is relevant to patient care.

Acknowledgments
We would like to acknowledge Rick Wilson for invaluable technical assistance, Ralf Kopan for providing the NIP1::CreERT2 and NIP1::CreER27 mice, and the Developmental Biology Histology and Microscopy Core at Washington University.

Sources of Funding
This work was supported by NHLBI R01 HL130212 (Dr Rentschler), NHLBI K08 HL107449 (Dr Rentschler), NHLBI R01 HL034161 (J.M. Nerbonne), AHA grant in aid 14GRNT19510011 (Dr Rentschler), Center for the Investigation of Membrane Excitability Diseases (Dr Rentschler), and Department of Medicine funds from Washington University (Dr Rentschler). Dr Rentschler holds a Career Award for Medical Scientists from the Burroughs Wellcome Fund. S. Springer was supported by the NHLBI-sponsored Institutional Training grant T32HL007275. R. Diaz-Trelles was supported by AHA Scientist Development Grant 11SDG7610174.

Disclosures
None.

References


Novelty and Significance

What Is Known?

- Genome-wide association studies have linked the direct Notch target HEY2 (Hairy and Enhancer of Split-Related 2/HRT2) with Brugada syndrome, a syndrome that primarily affects the right ventricle and is associated with sudden cardiac death.

- Ventricular arrhythmias often arise from the Purkinje–myocyte junction, and Notch induces ventricular myocytes near the conduction system to become Purkinje-like cells.

What New Information Does This Article Contribute?

- Notch activation decreases voltage-gated potassium currents in left ventricular cardiac myocytes through transcriptional and epigenetic changes.

- There is a differential response in voltage-gated potassium currents and HRT2 expression in left versus right ventricular cardiac myocytes in response to Notch activation.

- Induction of pathological hypertrophy with left ventricular remodeling is associated with Notch1 activation in cardiac myocytes, dynamic RBP-J binding, and epigenetic changes to the promoters encoding voltage-gated K+ channels associated with downregulation of the transcripts.

K+ current dysregulation occurs in many hereditary and acquired diseases, where it can contribute to dispersion of refractoriness and result in a proarrhythmic substrate. Many studies have focused on the molecular and functional components of the ion channels themselves, and comparatively little is known with regard to the transcriptional and epigenetic mechanisms regulating expression of the genes that comprise these channels. Notch signaling is normally quiescent in adult cardiac myocytes and is activated in response to several pathological stimuli with global effects on cellular electrophysiology. Here, we demonstrate the mechanisms underlying Notch-mediated regulation of voltage-gated K+ currents in left ventricular remodeling using a model of pressure overload associated with myocardial injury. Interestingly, we find significant differences in transcription and cellular electrophysiology in left versus right ventricular cardiac myocytes in response to Notch activation, which could be related to their distinct embryological origins and developmental programming. Therefore, targeted modulation of the Notch pathway in the setting of cardiac pathology may present a novel opportunity for antiarrhythmic drug design.
Notch-Mediated Epigenetic Regulation of Voltage-Gated Potassium Currents
Aditi Khandekar, Steven Springer, Wei Wang, Stephanie Hicks, Carla Weinheimer, Ramon Diaz-Trelles, Jeanne M. Nerbonne and Stacey Rentschler

Circ Res. 2016;119:1324-1338; originally published online October 3, 2016;
doi: 10.1161/CIRCRESAHA.116.309877

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2016 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://circres.ahajournals.org/content/119/12/1324

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2016/10/03/CIRCRESAHA.116.309877.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published
in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the
Editorial Office. Once the online version of the published article for which permission is being requested is
located, click Request Permissions in the middle column of the Web page under Services. Further information
about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/
Supplemental Material

Detailed Methods

Mice

Notch GOF (Mic2v^{Cre+/+}; R26R^{NICD+/}) \(^1\), Notch LOF (Mic2v^{Cre+/+}; R26R^{DNMAML+/}) \(^1\), R26R^{StTomato} \(^2\), iN1ICD (aMHCrtTA; TetO-NICD) \(^3\), NT1P::Cre \(^4\) and NIP1::CreET2 \(^5\) mice have been described previously, and were maintained on a mixed genetic background. The KChIP2\(^{+-}\) \(^6\) and Cntn2-eGFP \(^7\) mouse lines were maintained on a C57BL6/J background. The Cntn2-eGFP line was obtained from \(^7\) (Bar Harbor, ME). Wild type CD-1 outbred mice were obtained from Charles River Laboratories. All animal protocols were approved by the Animal Studies Committee at Washington University. Animals were handled in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Control data for molecular biology and electrophysiology was obtained from littermate animals with genotypes: R26R^{NICD+} for Notch GOF, R26R^{DNMAML+} for Notch LOF, aMHCrtTA for iN1ICD and R26R^{NICD/DNMAML} for Notch GOF/ DNMAML unless otherwise noted in the Figure legends. Statistical analysis revealed no differences in EP properties among the various control groups, and the R26R^{NICD+} control group was included in figures 1, 2, and 4.

Electrophysiological Recordings

Adult (8-15 weeks old) control, Notch GOF (Mic2v^{Cre+/+}: NICD), Notch LOF (Mic2v^{Cre+/+}: DNMAML), KChIP2\(^{+-}\), and Cntn2-eGFP mice were anaesthetized (5 % isoflurane; Butler Shein, Dublin, Ohio), and ventricular myocytes were isolated using described procedures \(^8\), \(^9\). In brief, hearts were rapidly removed from anaesthetized animals, mounted on a Langendorf apparatus and perfused retrogradely with 25 ml of 0.2 mg/ml collagenase-containing (type II, Worthington Biochemical Corp., Lakewood, NJ, USA) solution. Following the perfusion, the left ventricular free wall, right ventricular free wall and/or the interventricular septum was separated using iridectomy scissors. LV and RV myocytes were mechanically dissociated, collected, resuspended in Medium-199 (M-199) (Irvine Scientific, Santa, Ana, CA), plated on laminin-coated coverslips and maintained in M-199; 95% air/ 5% CO\(_2\) incubator at 37\(^\circ\)C. For the isolation of Purkinje cells, the micro-dissected interventricular septum samples from the Cntn2-eGFP mice were minced and incubated for an additional hour in collagenase-containing solution with (10 \(\mu\)M) elastase and (30 \(\mu\)M) protease Type XIV (Sigma, St Louis, MO, USA) added. After the secondary enzyme incubation, Purkinje cells were mechanically dispersed, collected, resuspended in M-199, plated on laminin-coated coverslips and maintained in a 95% air/ 5% CO\(_2\) incubator at 37\(^\circ\)C until used. Control experiments in which LV myocytes were dissociated using the same protocol revealed that the properties of LV cells isolated with and without the secondary enzyme incubation were indistinguishable.

Whole-cell current- and voltage-clamp recordings were obtained from left ventricular (LV), right ventricular (RV) and interventricular septum (IVS) (Cntn2-eGFP mice only) myocytes within 12 h of isolation at room temperature (22–23\(^\circ\)C). Voltage- and current-clamp experiments were performed and data were collected using an Axopatch 1D (Molecular Devices, Sunnyvale, CA, USA) or a Dagan 3900A (Dagan Corp., Minneapolis, MN, USA) patch clamp amplifier interfaced to a microcomputer with a Digidata 1332 analog/digital interface and the pCLAMP10 software package (Molecular Devices). Data were filtered at 5 kHz before digitization and storage.

For voltage-clamp recordings of K\(^+\) currents, pipettes contained (in mM): KCl 135, EGTA 10, HEPES 10 and glucose 5 (pH 7.2; 310 mOsm). For current-clamp recordings, pipettes
contained (in mM): K aspartate 76, KCl 20, MgCl\(_2\) 2.5, HEPES 10, NaCl 4, CaCl\(_2\) 6, K\(_2\)EGTA 10, K\(_4\)ATP 5 and Na-GTP 0.1 (pH 7.2; 310 mOsm). For voltage-clamp recordings, the bath solution contained (in mM): NaCl 136, KCl 4, MgCl\(_2\) 2, CaCl\(_2\) 1, CoCl\(_2\) 0.5, HEPES 10, glucose 10 and Tetrodotoxin (TTX) 0.02, (pH 7.4; 300 mOsm). The TTX and the CoCl\(_2\) were omitted from the bath solution for current-clamp recordings. Whole-cell voltage-gated K\(^+\) (Kv) currents were recorded in response to 4.5 s depolarizing voltage steps to test potentials between -60 and +40 mV from a holding potential (HP) of -70 mV. Currents (I\(_{K1}\)) through inwardly rectifying K\(^+\) (Kir) channels, evoked in response to (4.5 s) hyperpolarizations to -120 mV from the same HP, were also recorded in each cell. Action potentials were elicited in response to brief (2–5 ms) depolarizing current injections of varying amplitudes, delivered at 1 Hz; recordings were obtained after the waveforms reached a steady state, typically after 8-10 action potentials.

Voltage-clamp and current-clamp data were compiled and analyzed using Clampfit Version 10.3 (Molecular Devices) and MATLAB (MathWorks) software. Integration of the capacitance transients, recorded during brief ± 5 mV voltage steps from the holding potential (-70 mV) provided whole-cell membrane capacitances (C\(_{m}\)). Input resistances were calculated from the same records. Leak currents were always ≤200 pA and were not corrected. Series resistances (<10 MΩ) were routinely compensated by > 80%. Voltage errors resulting from the uncompensated series resistance were always ≤ 8 mV and were not corrected.

Peak Kv currents (I\(_{K,peak}\)) were measured as the maximal amplitudes of the outward currents evoked at each test potential; I\(_{K1}\) amplitudes were measured as the maximal currents evoked at -120 mV. Using previously described methods (Xu et al. 1999; Brunet et al. 2004), the decay phases of the outward currents recorded from control, Notch GOF, Notch LOF, and KChIP2\(^-\) LV myocytes were analyzed to provide the amplitudes of the individual Kv current components, I\(_{to,f}\), I\(_{K,slow}\) and I\(_{ss}\) and the time constants of decay (\(t_{decay}\)) of the inactivating components, I\(_{to,f}\) and I\(_{K,slow}\). The decay phases of the Kv currents in Cntn2-eGFP-expressing cells, in contrast, were well described by one exponential component, reflecting I\(_{K,slow}\), and the steady-state outward current, I\(_{ss}\). Resting membrane potentials, action potential amplitudes and action potential durations at 50, 70 and 90% repolarization were also measured in control LV, Notch GOF and Cntn2-eGFP-expressing Purkinje cells. Voltage-clamp data from the various littermate controls were not significantly different from the littermate controls of the Notch GOF mice, which were then used as the control group for statistical comparisons.

Surgical Instrumentation to Induce Left Ventricular Remodeling and Heart Failure

We utilized a previously validated surgical approach that combines transverse aortic constriction (TAC) and distal left anterior coronary ligation (MI) to produce a gradual and predictable progression of adverse left ventricular (LV) remodeling that leads to heart failure (HF) \(^{10}\). Briefly, either wild-type C57BL/6 mice (Jackson Laboratories) or NIP1::CreER\(^{T2}\); R26\(^{RdTomato}\) mice were subjected to a combined small apical infarct plus moderate constriction of the transverse aorta using a 26 gauge constrictor (instead of the commonly used 27 gauge needle for this mouse weight) with the goal of achieving a moderate band. Adult mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) administered i.p. To perform the HF surgery, mice were restrained supine, intubated, and ventilated using a Harvard respirator. Following dissection through the intercostal muscles, the aorta was identified and freed by blunt dissection. 7.0 silk suture was placed around the great vessel, tied around a blunt 26 gauge needle and then rapidly removed. Immediately following this procedure, a left thoracotomy was performed, the LV and the left main coronary artery system exposed, and the apical portion of the LAD ligated with a 9-0 silk suture. The surgical incision was closed and the mice were recovered on a warmer until being returned to their cage. Total time to perform this dual surgical
procedure was less than 15 minutes. 5 doses of 4-OH tamoxifen (Sigma) prepared as 5 mg/ml in corn oil were administered by gavage at 0.1 mg per gram of body weight within 120 hours (5 days) before the surgery. Post-surgery, tamoxifen chow (Envigo, TD.130860) was administered starting 2 weeks post surgery following 2 cycles of 5 days ON, 2 days OFF. Mice were sacrificed 4 weeks post-surgery for immunohistochemistry and molecular experiments.

**Histology and Immunohistochemistry**

Immunohistochemistry was performed on frozen sections with antibodies recognizing Contactin-2 (AF4439, R&D Systems) with secondary anti-goat Alexa 488 (Invitrogen), Platelet and endothelial cell adhesion molecule-1 (PECAM-1) (Dianova, DIA-310) with secondary anti-rat Alexa 488 (Life Technologies, A11006), α-actinin (Abcam, ab9465, and anti-Delta-Like 4 (Dll-4) (Santa Cruz Biotechnology).

**Reverse Transcription-Quantitative Polymerase Chain Reaction**

Total RNA was isolated from ventricles using Trizol (Invitrogen) and DNase treated using TURBO DNA-free DNase Treatment Kit (Ambion). First-strand cDNA was synthesized using a high Capacity cDNA Reverse Transcription kit (Applied Biosystems). Gene expression was assayed using the Power SYBR Green PCR Master Mix (Applied Biosystems) with primers listed in the attached Table S1 and quantified using the ViiA™ 7 qRT-PCR system (Applied Biosystems). Relative fold changes were calculated using the comparative threshold cycle methods (2-ΔΔCt).

**Chromatin Immunoprecipitation**

Tissue samples were processed for ChIP as described previously with a few modifications. Briefly, for each biological replicate, 4 X 10^7 cells from single adult LV free wall were cross-linked with 1% formaldehyde for 30 mins at room temperature and flash frozen until further use. Chromatin was sheared to approximately 500 bp using a Bioruptor sonicator (Diagenode, Sparta, NJ). Chromatin was precleared using protein G, precipitated with anti–RBP-J (Abcam ab25949), anti-H3K4me3 (Milipore 07-473), or negative control anti-IgG antibody (Abcam ab46540). After reverse cross-linking, immunoprecipitated DNA was purified and analyzed using quantitative PCR (qPCR) and SYBR Green chemistry. Primer sequences for qPCR are indicated in Table S2. Fold enrichment was calculated as 2^(ΔΔCt) (Ct_input - Ct_test) and expressed relative to the IgG negative control.

**Western Blotting**

Protein lysates were prepped from LV free wall and used for immunoblotting as previously described. Briefly, ventricles were harvested and homogenized (separately) in ice-cold 1X RIPA (Cell Signaling Technology #9806) lysis buffer and protease inhibitor cocktail tablet (Roche, Mannheim, Germany). Samples were centrifuged at 15000 g for 30 min at 4°C. The supernatants were used for protein analysis. Immunoblotting was performed using anti-KChIP2 (UC Davis/NIH NeuroMab Facility), anti-Kv2.1 (obtained from UCDavis) and anti-GAPDH (Cell Signaling Technology 14C10) antibodies.

**Doxycycline treatment assay**

Cardiomyocytes isolated from MHC-rtTA; tetO-NICD left ventricular free wall were plated, followed by addition of doxycycline (1μg/ml) diluted in molecular grade water. No doxycycline
was added to the control well. Cells were harvested for gene expression analysis before doxycycline addition (time = 0), at 30 minutes, one hour and five hours after doxycycline treatment. qRT-PCR was used to measure gene expression, and fold change relative to the no doxycycline control was calculated and plotted for each gene.

**Bioinformatics analysis**

Chromosomal regions spanning from 2000bp upstream to 500bp downstream of the transcription start site for respective K⁺ channel subunits were scanned for transcription factor binding sites and H3K4me3 peaks. Putative RBP-J, Hrt2 and Hes1 binding sites in the proximal promoters of *Kcnip2*, *Kcnb1* and *Kcna5* were identified using TRANSFAC professional V10.2 library. H3K4me3 peaks were identified using UCSC Genome Browser/ ENCODE from a ChIP-seq dataset in 8 week old adult mouse heart performed at Ludwig Institute of Cancer Research (LICR).

**Statistical Analysis**

All data are expressed as means ± standard error of the mean (SEM). For comparison of more than two experimental groups, the statistical significance of observed differences in mean was evaluated using a one-way analysis of variance (ANOVA), followed by post hoc Tukey's multiple comparison test. For comparison of two experimental groups, an equal variance Student's t test was used; a two-tailed P value of <0.05 was considered statistically significant. Significant differences are indicated by * p<0.05, † p < 0.01, ‡ p < 0.001, § p< 0.0001, ns: not significant.
Online Figures

Online Figure I: Heterogeneity of action potential waveforms recorded from Notch GOF myocytes and Purkinje cells. Action potentials were recorded as described in Figure 1. As shown in Figure 1E, action potentials recorded from Notch GOF LV myocytes and Cntn2-eGFP+ Purkinje cells displayed a range of repolarization durations; here, individual recordings are shown which illustrate variability of repolarization and action potential morphology. The calibration bar shown applies to all panels (A, B and C), x axis is 100 ms and y axis is 50 mV. Representative traces are from cells isolated from Control n=5, Notch GOF LV n=7, Cntn2-eGFP n=8 individual mice.
Online Figure II: Notch activation does not prolong action potential durations in right ventricular myocytes. Action potentials were elicited as described in the legend to Figure 1, and representative recordings from a right ventricular (RV) myocyte isolated from a control mouse (n=5 mice) or from a Notch GOF mouse (n=6 mice), are shown in (A). Action potential durations measured at 50 and 70 percent repolarization in individual control RV myocytes (Δ, n = 33) and Notch GOF (▽, n = 22) RV myocytes are shown in (B); mean ± SEM values are also indicated. Equal variance Students' t-test was performed to compare means. ns = not significant
Online Figure III. eGFP⁻ myocytes isolated from Cntn2-eGFP mice exhibit robust Kv currents. Whole-cell K⁺ currents were recorded, as described in the legend to Figure 1, from eGFP⁻ and eGFP⁺ cells isolated from Cntn2-eGFP mice. In response to outward voltage steps, eGFP⁻ cells exhibited a high peak current density with a level of I_{to,f} current density comparable to that seen in recordings from control mice. 3 recordings were performed to ensure that the added enzymatic digestion steps required to successfully isolate eGFP⁺ cells did not cause a reduction in Kv currents.
Online Figure IV: KChIP2 haplo-insufficiency selectively attenuates $I_{\text{to,f}}$ in adult LV myocytes. Whole-cell K$^+$ currents in control, KChIP2$^{+/-}$, and Notch GOF LV myocytes were recorded as described in the legend to Figure 2. Representative recordings are shown in (A-C). Analyses of the decay phases of the outward Kv currents revealed the selective attenuation of $I_{\text{to,f}}$ in KChIP2$^{+/-}$ LV myocytes, compared with control $R26R^{NICD/+}$ LV myocytes; $I_{K,\text{slow}}$ and $I_{ss}$ densities in KChIP2$^{+/-}$ and control LV myocytes were not significantly different. (D) Current densities measured in individual control (●, n = 18 cells from 3 mice), KChIP2$^{+/-}$ (♦, n = 33 cells from 6 mice), and Notch GOF (▲, n = 19 cells from 2 mice) LV myocytes are plotted and mean ± SEM values are indicated. Equal variance Students’ t-test was performed to compare means. * p<0.05, † p < 0.01, ‡ p < 0.001, § p< 0.0001, ns = not significant.
Online Figure V: KChIP2 haploinsufficiency, but not Notch activation, causes a reduction in $I_{to,f}$ density in RV myocytes. $K_v$ currents were evoked as previously described in age-matched control RV myocytes and in RV myocytes isolated from Notch GOF mice (n=2) (A) and KChIP2+/− mice (n=2) (B). When the inactivation was fit with the sum of two exponentials, $I_{to,f}$ density was revealed to be reduced relative to control in RV KChIP2+/− myocytes but not in Notch GOF myocytes. Although $I_{to,f}$ was significantly reduced, $I_{K,Peak}$ was not significantly reduced relative to control in RV KChIP2+/− myocytes, as expected. Equal variance Students’ t-test was performed to compare means. * p<0.05. (C) Western blots demonstrate similar amounts of KChIP2 and Kv2.1 protein in Notch GOF and littermate control R26R<sup>NICD/+</sup> RV.
Online Figure VI: Differential regulation of Hrt2 in left versus right ventricle in response to Notch activation. (A) Baseline expression of the direct Notch target Hrt2 is similar between the LV and RV. Notch GOF up-regulates expression of Hrt2 in the LV while, in contrast, Hrt2 is significantly down-regulated in Notch GOF RV compared to littermate R26R NICD/+ controls (n = 6). One-way ANOVA was performed to determine statistical significance. (B) ChIP-qPCR shows increased H3K4me3 at the Hrt2 transcription start site in the LV, and loss of H3K4me3 in the RV (n = 4). (C) ChIP-qPCR shows that RBP-J, an effector of Notch signaling, constitutively binds at the known Hrt2 promoter and enhancer in the LV, but not in RV. In contrast to the dynamic RBP-J seen at the Kcnip2 and Kcnb1 promoters, there was no increased RBP-J binding at the Hrt2 promoter in LV or RV. Equal variance, two-tailed Students’ t-test was performed to determine statistical significance. (D) Schematic showing that in LV Notch GOF upregulates Hrt2 through canonical Notch regulatory complex comprising of RBP-J, MAML and other co-activators. However, in Notch GOF RV RBP-J does not bind Hrt2 proximal promoter. * \( p < 0.05 \), † \( p < 0.01 \), ‡ \( p < 0.001 \).
Online Figure VII: Histological immunostaining for Delta-like-4 (Dll-4) in left ventricular myocardium in sham operated (control) versus 14 days after TAC banding demonstrates up-regulation of Dll-4 in vascular endothelium in response to TAC. Scale bars represent 50 μM.
Online Figure VIII: NIP1::CreER\textsuperscript{T2}; R26R\textsuperscript{tdTomato} mice were subjected to sham versus TAC +MI surgery to induce gradual and progressive heart failure, and tamoxifen was administered similar to the procedure described in Figure 7. Vascular endothelial cells are identified with PECAM-1 immunostaining (green), demonstrating increased vascularity in the heart failure model. NIP1::CreER\textsuperscript{T2}; R26R\textsuperscript{tdTomato} cells that have experienced active Notch1 signaling during the 4 week period following surgery are labeled red. In the Sham LV, all of the active Notch1 signaling is within vascular endothelial cells (arrows), while in the heart failure model Notch is active within many ventricular cardiomyocytes throughout the LV (arrowheads), as well as within PECAM-1\textsuperscript{+} vascular endothelial cells (arrows).
<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer 1</th>
<th>Reverse Primer 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cntn2</td>
<td>5' GGCCAGGACTCGGTAGAT 3'</td>
<td>5' TCCCTCCCTCCCTCTACT 3'</td>
</tr>
<tr>
<td>Scn5a</td>
<td>5' GAAGAAGCTGGGCTCCAGA 3'</td>
<td>5' CATCGAGGCTCTGTTGGTC 3'</td>
</tr>
<tr>
<td>Hrt1</td>
<td>5' GAAGCGGCCAGAGACCGAATCAA 3'</td>
<td>5' CAGGGCGTGCGTCAAAAAATAACC3'</td>
</tr>
<tr>
<td>Hrt2</td>
<td>5' CGACGTGGGGAGCGAGAAACTA 3'</td>
<td>5' GGCAGAGCATGGGCAATCAAAGTA 3'</td>
</tr>
<tr>
<td>Hes1</td>
<td>5' AAAGCCTATCATGGAGAAGACG 3'</td>
<td>5' GGAATGCCGGAGCTATTTCTT 3'</td>
</tr>
<tr>
<td>Kcnd2</td>
<td>5' GCCGCGCACCTAGCTCGTT 3'</td>
<td>5' CACCCACGCTGATGATCTTA 3'</td>
</tr>
<tr>
<td>Kcnd3</td>
<td>5' CCTAGCTCCAGCGACAAGA 3'</td>
<td>5' CCACCTACGTTGAGGACG 3'</td>
</tr>
<tr>
<td>Kcnip2</td>
<td>5' GGCTGTATCATGGAGAAGAGA 3'</td>
<td>5' CCGTCTTTTTCTGTC 3'</td>
</tr>
<tr>
<td>Kcnb1</td>
<td>5' CGACGTGGGGAGCGAGAACAATG 3'</td>
<td>5' GGCAGAGCATGGGCAATCAAAGTA 3'</td>
</tr>
<tr>
<td>Kcnf2</td>
<td>5' AAGAGCCTCTTGTGGAAAGA 3'</td>
<td>5' CACGGAGATGCTGCTCAGGTC 3'</td>
</tr>
<tr>
<td>Notch1</td>
<td>5' GATGGACGACAATCGAGAAGA 3'</td>
<td>5' ATCAGTAGGAGTCGGAAGA 3'</td>
</tr>
<tr>
<td>Nppa</td>
<td>5' TCGATAGATCGCTCCCTT 3'</td>
<td>5' CTGATATCTGTCATCCTACC 3'</td>
</tr>
<tr>
<td>Nppb</td>
<td>5' ACCACCTTTGAGATCCCTATT 3'</td>
<td>5' GCAAGTTTTGTGTCAAGATAA 3'</td>
</tr>
<tr>
<td>Jag1</td>
<td>5' CTACTGTGAAGCCTTCCCTTG 3'</td>
<td>5' GTGACAGATAAGAAGATACC 3'</td>
</tr>
<tr>
<td>Tbp</td>
<td>5' GGGATTCAAGAAGACCACATG 3'</td>
<td>5' CTGACCAACTGTCACATGCAG 3'</td>
</tr>
<tr>
<td>β-actin</td>
<td>5' CTGCCTGACGGGCACAGTCAT 3'</td>
<td>5' GTCACAGCTACACTTCATGATGG 3'</td>
</tr>
<tr>
<td>Actc1</td>
<td>5' GACCTCAGCTACCTCGT 3'</td>
<td>5' TCTCGTTCTCAGGAGG 3'</td>
</tr>
</tbody>
</table>
## Online Table II: ChIP-qPCR oligonucleotide sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kcnip2</td>
<td>5' GGTCTAGGGGGATGGAGTCT 3'</td>
<td>5' AGGCCGAAAGGAGAGTTGT 3'</td>
</tr>
<tr>
<td>Kcnip2</td>
<td>5' GTGAAGGGGTAAGTCTGTA 3'</td>
<td>5' AGAGGTGCTGAGAATTG 3'</td>
</tr>
<tr>
<td>Kcnb1</td>
<td>5' GGAGGAACAGCGAGAGATTT 3'</td>
<td>5' CTGACCTCCCAGGCTTA 3'</td>
</tr>
<tr>
<td>Kcnb1</td>
<td>5' CTGGAGAGCAGAGAGATT 3'</td>
<td>5' TCCTCCTCGCTCCTCCT 3'</td>
</tr>
<tr>
<td>Kcna5</td>
<td>5' CAGCGGGTCTCCTCTAAACAT 3'</td>
<td>5' CATTTCCTCAAAGGGATCGAAGTAG 3'</td>
</tr>
<tr>
<td>Kcna5</td>
<td>5' GAACGCGACTGCCATGA 3'</td>
<td>5' CTCCTCAGGTGATTTCTTTT 3'</td>
</tr>
<tr>
<td>Kcnj2</td>
<td>5' GTTGTACAGGTCTGTTGTAATTG 3'</td>
<td>5' TCCAGCGTGCTCAGTATTTT 3'</td>
</tr>
<tr>
<td>Kcnip2</td>
<td>5' GTCTAGGGGGATGGAGTCT 3'</td>
<td>5' AGGCCGAAAGGAGAGTTGT 3'</td>
</tr>
<tr>
<td>Kcnb1</td>
<td>5' GGAGGAACAGCGAGAGATTT 3'</td>
<td>5' CTGACCTCCCAGGCTTA 3'</td>
</tr>
<tr>
<td>Kcna5</td>
<td>5' GAACGCGACTGCCATGA 3'</td>
<td>5' CTCCTCAGGTGATTTCTTTT 3'</td>
</tr>
<tr>
<td>Kcnj2</td>
<td>5' GTTGTACAGGTCTGTTGTAATTG 3'</td>
<td>5' TCCAGCGTGCTCAGTATTTT 3'</td>
</tr>
<tr>
<td>Kcnb1</td>
<td>5' CTGGAGAGCAGAGAGATT 3'</td>
<td>5' TCCTCCTCGCTCCTCCT 3'</td>
</tr>
<tr>
<td>Kcnb1</td>
<td>5' CAGCGGGTCTCCTCTAAACAT 3'</td>
<td>5' CATTTCCTCAAAGGGATCGAAGTAG 3'</td>
</tr>
<tr>
<td>Kcna5</td>
<td>5' GAACGCGACTGCCATGA 3'</td>
<td>5' CTCCTCAGGTGATTTCTTTT 3'</td>
</tr>
<tr>
<td>Hrt2</td>
<td>5' CAGGCGAAGAACCTCCAGA 3'</td>
<td>5' AAACTCCTCCAGCAGATG 3'</td>
</tr>
<tr>
<td>Hrt2</td>
<td>5' CAGGCGAAGAACCTCCAGA 3'</td>
<td>5' AAACTCCTCCAGCAGATG 3'</td>
</tr>
<tr>
<td>Hrt2</td>
<td>5' CAGGCGAAGAACCTCCAGA 3'</td>
<td>5' AAACTCCTCCAGCAGATG 3'</td>
</tr>
<tr>
<td>Negative</td>
<td>5' GTGTCCTGGCGATAACCTAT 3'</td>
<td>5' CACTGCTGGGTTGAGAATA 3'</td>
</tr>
<tr>
<td>Control</td>
<td>5' GTGTCCTGGCGATAACCTAT 3'</td>
<td>5' CACTGCTGGGTTGAGAATA 3'</td>
</tr>
</tbody>
</table>
References:


