Loss of H3K4 methylation destabilizes gene expression patterns and physiological functions in adult murine cardiomyocytes

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Histone H3 lysine 4 (H3K4me) methyltransferases and their cofactors are essential for embryonic development and the establishment of gene expression patterns in a cell-specific and heritable manner. However, the importance of such epigenetic marks in maintaining gene expression in adults and in initiating human disease is unclear. Here, we addressed this question using a mouse model in which we could inducibly ablate PAX interacting (with transcription-activation domain) protein 1 (PTIP), a key component of the H3K4me complex, in cardiac cells. Reducing H3K4me3 marks in differentiated cardiomyocytes was sufficient to alter gene expression profiles. One gene regulated by H3K4me3 was Kv channel-interacting protein 2 (*Kcnip2*), which regulates a cardiac repolarization current that is downregulated in heart failure and functions in arrhythmogenesis. This regulation led to a decreased sodium current and action potential upstroke velocity and significantly prolonged action potential duration (APD). The prolonged APD augmented intracellular calcium and in vivo systolic heart function. Treatment with isoproterenol and caffeine in this mouse model resulted in the generation of premature ventricular beats, a harbinger of lethal ventricular arrhythmias. These results suggest that the maintenance of H3K4me3 marks is necessary for the stability of a transcriptional program in differentiated cells and point to an essential function for H3K4me3 epigenetic marks in cellular homeostasis.

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expression and attenuates both $I_{\text{Ca,L}}$ and the sodium current ($I_{\text{Na}}$). PTIP deletion also prolongs the action potential duration (APD) resulting in increased L-type calcium current ($I_{\text{Ca,L}}$) which leads to elevated intracellular calcium ($\text{Ca}^{2+}$), and augments myocyte contractility in vivo and in vitro. Furthermore, PTIP mutant cardiomyocytes show a prolonged PR interval and are more sensitive to premature ventricular complexes (PVCs) upon stimulation, suggesting both a conduction defect and greater susceptibility to calcium-based ventricular arrhythmias. This study demonstrates a critical role of H3K4me3 marks in maintaining cellular homeostasis in fully differentiated tissue and suggests that epigenetic changes may underlie clinically relevant cardiac disease phenotypes.

Results

**Generation of an inducible, cardiac-specific PTIP-null mouse.** In order to delete the PTIP protein in cardiomyocytes, mice were bred to generate the tamoxifen-inducible Pax6p1 deletion strains. Using a floxed (fl), a null (−), and a wild-type (+) Pax6p1 allele (11), the Cre-modified estrogen receptor transgene, driven by the cardiac-specific α-myosin heavy chain promoter (αMHC<sub>CMCM</sub>), was crossed into the Pax6p1<sup>fl/fl</sup> or Pax6p1<sup>fl/+</sup> genetic background to generate αMHC<sub>CMCM</sub>Pax6p1<sup>fl/fl</sup> and αMHC<sub>CMCM</sub>Pax6p1<sup>fl/+</sup> (designated as PTIP<sup>−/−</sup>) or αMHC<sub>CMCM</sub>Pax6p1<sup>fl/fl</sup> and αMHC<sub>CMCM</sub>Pax6p1<sup>fl/+</sup> (designated as PTIP<sup>−/+</sup>) control mice. Thirty days after tamoxifen injection in 8-week-old mice, there was a marked decrease in steady-state PTIP protein levels in the tamoxifen-injected PTIP<sup>−/−</sup> mice as compared with that in the vehicle-injected PTIP<sup>−/+</sup> and the tamoxifen-injected PTIP<sup>−/−</sup> mice (Figure 1A). After tamoxifen injection, PTIP mice also have a 7- to 8-fold decrease in PTIP mRNA when compared with that in PTIP<sup>−/+</sup> mice (Figure 1B).

**PTIP is part of a histone methyltransferase complex that regulates histone methylation.** To assess whether PTIP protein affects H3K4me in ventricular whole heart samples, we examined levels of H3K4me3 by quantitative Western blotting (Figure 1C). Compared with total amounts of histone H3, levels of H3K4me3 were reduced to approximately 53% of controls. We prepared chromatin from control hearts for immunoprecipitation with anti-PTIP antibodies to examine protein-protein interactions directly on DNA. After reverse-crosslinking, proteins were separated on SDS/PAGE gels and probed for RbBP5 (Figure 1D), a component of the KMT2C/D complex (11), to show that PTIP is part of the H3K4me complex in whole heart tissue.

**Histone methylation regulates gene expression.** We next determined whether PTIP deletion and reduction in H3K4me3 was sufficient to alter gene expression profiles in the adult heart. Total RNA was isolated from cardiac tissue 30 days after tamoxifen injection from PTIP<sup>−/−</sup> ($n = 3$) and PTIP<sup>−/+</sup> ($n = 3$) mice. The RNAs were analyzed by Affymetrix microarrays to identify changes in gene expression patterns. A total of 221 genes were significantly, altered with 60% of them showing a decrease after PTIP deletion (Supplemental Table 1; supplemental material available online with this article; doi:10.1172/JCI44641DS1). These results are consistent with the hypothesis that the PTIP/KMT2C/D complex imparts H3K4me3 marks that are associated with active gene expression, at least within a subset of genes. Differential gene expression was confirmed by qPCR for selected genes (Figure 2A). Gene expression analysis was also performed on hearts from mice 8 months after tamoxifen injection (Figure 2B). Many genes that showed a decrease in expression at 1 month continued to exhibit a significant decrease 8 months after tamoxifen. In order to demonstrate that the attenuation in Kcnip2 was due to PTIP deletion and not due to genetic background or the injection of tamoxifen, Kcnip2 gene expression by qPCR was measured in Pax6p1<sup>fl/fl</sup> and Pax6p1<sup>fl/+</sup> mice, with and without the αMHC<sub>CMCM</sub> transgene and with and without tamoxifen (Supplemental Figure 1).

**Genetic evidence that PTIP and H3K4me4 regulate gene expression in differentiated tissue.** We next sought to demonstrate that PTIP deletion alters H3K4me3 marks at a specific target gene promoter region in the heart. Thus, we performed ChIP assays using antibodies to H3K4me3 to assess the methylation status at the $\alpha$ regulatory region of the Kcnip2 gene (Figure 3A). The Kcnip2 gene was selected as a target partly because Kcnip2 levels were reduced approximately 5 and 10 fold at 1 and 8 months, respectively, after PTIP deletion. In heart chromatin from PTIP<sup>−/+</sup> mice, H3K4me3 marks were enriched substantially at a DNA region −100 bp relative to the translation start site. A moderate enrichment in H3K4me3 marks was seen at +100 bp relative to the first ATG, and little enrichment was observed at −500 bp. In comparison, the H3K4me3 enrichment at −100 bp and +100 bp was significantly attenuated in the heart chromatin from the PTIP<sup>−/−</sup> group. These results show that PTIP deletion does indeed
result in an alteration in H3K4me3 marks at the S′ regulatory region of the Kcnip2 gene. Next, we examined PTIP and RbBP5 localization to the S′ regulatory region of the Kcnip2 gene (Figure 3A). In chromatin from PTIP− hearts, both PTIP and RbBP5 were enriched at a DNA region −500 bp relative to the first Kcnip2 ATG. This enrichment was not observed at DNA regions −100 bp and +100 bp relative to the first ATG. However, in chromatin from PTIP+ hearts, the PTIP and RbBP5 enrichment observed at −500 bp in PTIP+ mice was significantly reduced. No differences in PTIP and RbBP5 enrichment were observed between heart chromatin from PTIP+ and PTIP− mice at −100 and +100 bp relative to the first ATG, as these levels were closer to background. Thus, we propose that the decrease in Kcnip2 transcript expression is a direct result of PTIP deletion and the inability of PTIP+ mice to maintain a KMT2 complex and H3K4me3 marks at the S′ regulatory region of the Kcnip2 gene (Figure 3B). As a negative control, we performed ChIP assays for H3K4me3 marks at the S′ regulatory region of the Nppb gene, a gene that shows no change in mRNA expression in PTIP+ mice as compared with that in PTIP− mice (Supplemental Figure 2). We observed no changes in H3K4me3 enrichment at the S′ regulatory region of the Nppb gene in PTIP+ mice when compared with that in PTIP− mice. These results provide mechanistic evidence that maintaining histone methylation marks is critical for maintaining stable gene expression profiles in a fully differentiated, nondividing cell type.

Kcnip2, Kcn2, and Kcn3 protein expression. To determine whether mRNA profiles correspond to protein levels, hearts were harvested from PTIP+ and PTIP− mice 4 weeks after tamoxifen injection, and LVs were prepared for immunoblotting with anti-Kcnip2, anti-Kcn2, and anti-Kcn3 antibodies (Figure 4A). These data reveal that in PTIP+ mice there was a marked decrease in Kcnip2 protein as compared with that in PTIP− mice. Blotting for Kcn2 and Kcn3 revealed no significant difference in protein expression in PTIP+ and PTIP− mice, despite the fact that Kcn3 mRNA levels were significantly different in PTIP− mice as compared with those in PTIP+ mice.

Figure 2
Gene expression data in PTIP− and PTIP+ mice. (A) Eight-week-old PTIP− (n = 3−5) and PTIP+ mice (n = 3−5) were injected with tamoxifen for 5 days. Thirty days later, LV tissue was harvested, and qPCR was performed using TaqMan probes for the listed genes normalized to GAPDH. (B) Eight-week-old PTIP− (n = 5) and PTIP+ mice (n = 5) were injected with tamoxifen for 5 days. Eight months later, LV tissue was harvested, and qPCR was performed using TaqMan probes for the listed genes normalized to GAPDH. *P < 0.05 versus PTIP+. All data are mean ± SD.

EKG and action potential measurements. To investigate the significance of H3K4me marks in the heart, we studied the impact that PTIP deletion and the attenuation of Kcnip2 gene expression had on the electrophysiological phenotype. Initially, EKGs were obtained from PTIP− and PTIP+ mice 4 weeks after tamoxifen injection. As shown in Figure 4B, PTIP− mice had a marked and easily identified depression of the ST segment as compared with PTIP+ mice. In addition, analysis of the EKG also revealed a significant increase in the PR interval in PTIP− mice (n = 6) as compared with that in PTIP+ mice (n = 6) (36.9 ± 2.6 ms vs. 31.6 ± 4.0 ms, respectively; P < 0.005). There were no significant differences in the heart rate of PTIP− mice when compared with that of PTIP+ mice (666 ± 20 bpm vs. 661 ± 28 bpm, respectively). Next, LV apical myocytes were isolated from PTIP− and PTIP+ mice, and action potentials were recorded. As shown in Figure 4, C and D, the action potential profile was appreciably different in the PTIP− mice as compared with that in PTIP+ mice. Objective measurements of the APD presented in Figure 4, E and F, demonstrated that PTIP− mice had a significantly longer APD, at 30% and 90% repolarization. The APD presented in Figure 4, E and F, demonstrated that PTIP− mice (n = 6) as compared with that in PTIP+ mice (n = 4, n = 15) (–69.95 ± 1.2 mV vs. –73.55 ± 0.7 mV; P < 0.05). There were no significant differences in the heart rate of PTIP− mice when compared with that of PTIP+ mice (666 ± 20 bpm vs. 661 ± 28 bpm, respectively). Next, LV apical myocytes were isolated from PTIP− and PTIP+ mice, and action potentials were recorded. As shown in Figure 4, C and D, the action potential profile was appreciably different in the PTIP− mice as compared with that in PTIP+ mice. Objective measurements of the APD presented in Figure 4, E and F, demonstrated that PTIP− mice had a significantly longer APD, at 30% and 90% repolarization. The APD presented in Figure 4, E and F, demonstrated that PTIP− mice (n = 6) as compared with that in PTIP+ mice (n = 4, n = 15) (–69.95 ± 1.2 mV vs. –73.55 ± 0.7 mV; P < 0.05).

PTIP− mice have reduced I_{Ca_L}. As shown above, EKG data revealed that there was a significant increase in the PR interval in PTIP− mice as compared with that in PTIP+ mice, which might have indicated that PTIP− hearts had a conduction defect in the His-Purkinje system. Furthermore, analysis of ventricular action potentials revealed a decrease in maximum upstroke velocity (dV/dt) (Figure 5A). Taken together, these results suggested that there may be a decrease in the sodium inward current (I_{Na}) responsible for phase 0 of the cardiac action potential. Accordingly, we measured I_{Ca_L} in LV apical myocytes in PTIP− and PTIP+ mice. As shown in Figure 5B, PTIP deletion resulted in attenuated I_{Ca_L} in PTIP− hearts as compared with that in PTIP+ hearts.

I_{Ca_L} is reduced in PTIP+ mice. To determine whether H3K4me marks regulate I_{Ca_L}, myocytes were isolated from the apex of PTIP− and PTIP+ mice, and I_{Ca_L} currents were measured in the presence of tetrodotoxin (TTX). Figure 6A shows representative I_{Ca_L} recordings from PTIP− and PTIP+ mice. Figure 6B displays peak I_{Ca_L} amplitudes as a function of test pulse; PTIP deletion resulted in a significant attenuation of I_{Ca_L} in LV apical myocytes.

Altered calcium handling and contractility in PTIP− mice. Previous work has suggested that altering I_{Ca_L} and prolonging APD can alter [Ca^{2+}]_{i} levels and cardiac contractility (17). Accordingly, we assessed [Ca^{2+}]_{i}, I_{Ca_L}, and cellular contractility in apical LV myocytes from PTIP− and PTIP+ mice. First, cells were loaded with fluo-4AM, and calcium transients were recorded at different stimulation frequencies. As shown in Figure 7A, the amplitude of [Ca^{2+}]_{i} transients was significantly larger in PTIP− myocytes than in PTIP+ myocytes at all pacing frequencies. We also mea-
The increase in $[\text{Ca}^{2+}]_{i}$ was at least partly due to an increase in the inward current. Figure 7B shows peak $I_{\text{Ca,L}}$ as a function of test pulse. Data revealed a significantly higher $I_{\text{Ca,L}}$ in PTIP− mice as compared with that in PTIP+ mice. In addition, measurements of myocyte contractility at different stimulation frequencies revealed that PTIP− mice have a higher fractional shortening than PTIP+ mice (Figure 7C). These results suggest that attenuating $I_{\text{Ca,L}}$ and prolonging the APD alters $[\text{Ca}^{2+}]_{i}$, levels and excitation contraction coupling in cardiomyocytes.

**In vivo assessment of cardiac function.** We assessed cardiac structure and function noninvasively over time by echocardiography in isoflurane-anesthetized PTIP− and PTIP+ mice, with and without tamoxifen at 3 months, 6 months, and 9 months. Surprisingly, the echoes revealed significant and sustained improvements in LV systolic functions in the PTIP− mice after tamoxifen injection, as measured by ejection fraction (Figure 8A) and velocity of circumferential fractional shortening (Figure 8B), a load-independent assessment of systolic function (18). Despite the increase in systolic function in the PTIP− hearts, no alterations in echo parameters of chamber size, wall thickness, or heart rate were observed (Supplemental Table 2).

**Pathology.** At the end of 9 months, heart tissue was harvested, and histology was performed. The PTIP− mice showed no evidence of gross cardiac hypertrophy, as measured by LV weight to tibia length (Figure 8C), or cellular hypertrophy, as measured by myocyte cross-sectional area (Figure 8E). Myocardial fibrosis was quantified by picrosirius red staining, with no significant differences observed in the 2 groups (Figure 8D).
family of genes during development and correlate with actively with isoproterenol and caffeine. PVCs were counted for the first 5 minutes after the initial PVCs, and data were analyzed as PVCs per minute. As shown in Figure 9A, PTIP + mice showed no evidence of PVCs, and the other PTIP - mice showed intermittent isolated PVCs. In contrast, all PTIP - mice developed a sustained pattern of ventricular bigeminy, in which every other beat was a PVC (Figure 9B).

Discussion
We tested the necessity for the PTIP-dependent H3K4me pathway in a fully differentiated, nondividing cell type, the cardiomyocyte. H3K4me marks are imprinted through the actions of the TrG complex and, at least in one case, is thought to link these complexes to tissue-specific DNA-binding proteins (11). Germline deletion of Paxip1, which encodes PTIP, results in global reduction but not complete ablation of H3K4me levels and in early embryonic lethality in both flies and mice (11). Furthermore, deletion of Paxip1 in either neural stem cells or embryonic stem cells also reduces H3K4me and inhibits differentiation (11). Thus, the loss of PTIP in developing systems is consistent with the hypothesis that H3K4me is a positive epigenetic mark necessary for lineage-specific differentiation. Furthermore, Paxip1 germline null mutants are more severe and arrest earlier in development than any of the individual KMT2 mutants generated to date (20, 21). This, together with protein purification data (11, 13, 14), suggests that PTIP interacts with more than one H3K4 methyltransferase complex and, at least in one case, is thought to link these complexes to tissue-specific DNA-binding proteins (11).

Epigenetic regulatory pathways have been linked to a variety of diseases. Translocations of the KMT2A gene, which generate fusion proteins that have lost the SET domain, are gain-of-function mutations that promote leukemias (22). Overexpression of the H3K27 methylase EZH2 is linked to prostate cancer progression (23). However, loss-of-function mutations in genes encoding components of the H3K4me complexes have not been described in humans. In mice, multiple germline mutant alleles of KMT2A are embryonic lethal (20, 21, 24), thus making it difficult to assess the specific roles of KMT2A in adult cell populations. However, a neural stem cell–specific deletion of KMT2A results in increased gliogenesis at the expense of the neuronal lineage (4). A germline SET domain

Susceptibility to ventricular premature beats. To determine whether PTIP deletion and the associated changes in I Na and [Ca 2+], predispose to a susceptibility to ventricular arrhythmias, lightly anesthetized 12-week-old PTIP + and PTIP - mice were injected with isoproterenol and caffeine. PVCs were counted for the first 5 minutes after the initial PVCs, and data were analyzed as PVCs per minute. As shown in Figure 9A, PTIP - mice (n = 3) demonstrated a significantly higher number of PVCs per minute than PTIP + mice (n = 4). One PTIP + mouse showed no evidence of PVCs, and the other PTIP - mice demonstrated intermittent isolated PVCs. In contrast, all PTIP - mice developed a sustained pattern of ventricular bigeminy, in which every other beat was a PVC (Figure 9B).
the 2 types of histone methylation marks that are altered, presumably as a result of the disease state, are activating H3K4me3 marks and repressive H3K9me3 marks. Currently, it is unclear whether the changes in these marks are merely associated with disease states or whether they contribute to the initiation and progression of the disease states. The data presented here strongly argue that epigenetic changes can be sufficient to cause disease by altering the transcriptional profile of fully differentiated cardiomyocytes. Such alterations can induce abnormal electrophysiological properties, which, in our studies, impact calcium handling and actually improve contractility, but ultimately sensitizes the heart to arrhythmias.

Among the most significant gene affected by the reduction in H3K4me3 marks is Kcnip2, which controls multiple facets of the electrical cardiac phenotype. Although Kcnip2 is downregulated in human heart failure (5, 8) and confers susceptibility to ventricular tachycardia in murine models (5, 8, 9), little is known about its regulation. Previous work has demonstrated that Kcnip2 is necessary for the functional regulation of both I\textsubscript{Na} and I\textsubscript{K} \textit{g} (27). It was shown that posttranscriptional gene silencing of Kcnip2 suppressed both I\textsubscript{Na} and I\textsubscript{K} \textit{g} and reduced Na channel \alpha (Scn5a) and \beta1 (Scn1b) subunit mRNA levels in neonatal rat ventricular myocytes. On the other hand, silencing of Na\textsubscript{1.5} also reduced Kcnip2 mRNA and protein as well as K-4.x proteins, resulting in significantly decreased I\textsubscript{Na} and I\textsubscript{K} \textit{g} (27). Our data strongly support the above interactions. They demonstrate that, in addition to reducing I\textsubscript{Na}, and prolonging APD, PTIP deletion-induced attenuation of Kcnip2 results in PR prolongation on EKG and reduced I\textsubscript{Na} and slowed action potential upstroke velocity in the myocytes. These results are consistent with the notion that Kcnip2 interacts closely with K\textsubscript{4.x}, Na\textsubscript{1.5}, and that suppressing Kcnip2 attenuates I\textsubscript{Na}. Thus, the maintenance of H3K4me marks by the PTIP complex is critical to the regulation of Kcnip2 and the electrophysiological phenotype. I\textsubscript{Na} plays a significant role in regulating phase I of the cardiac action potential in rodents and in humans. In contrast to large mammals, mice and rats have small triangular action potentials that are regulated by I\textsubscript{Na} to a large extent (17). Previous work has suggested that an increased APD invokes increased [Ca\textsuperscript{2+}]., due, at least partly, to increased I\textsubscript{Ca,L}. Our data support the notion that the I\textsubscript{Na} attenuation-induced increased APD results in an increase in I\textsubscript{Ca,L} and [Ca\textsuperscript{2+}]. These changes in [Ca\textsuperscript{2+}], ultimately increase cellular cardiac contractility in vitro and systolic function in vivo. Our results are consistent with previous work that revealed that decreasing I\textsubscript{Na} by inhibitors, such as 4-AP, or by genetic manipulation prolongs the action potential, increases calcium influx, elevates [Ca\textsuperscript{2+}], and increases contractility (28, 29). Taken together with previous reports, our data reiterate the importance of the transient outward current in regulating excitation-contraction coupling in cardiac cells.

Mice expressing dominant-negative Kcnd2 have decreased I\textsubscript{Na} and improved contractility; however, those mice exhibit the early onset of cardiac hypertrophy, chamber dilatation, and interstitial fibrosis (29). One might predict that the PTIP mice would also develop hypertrophy because of increased [Ca\textsuperscript{2+}]. However, despite a significant and sustained improvement in systolic cardiac function, PTIP mice do not demonstrate a hypertrophic phenotype as measured by myocyte cross-sectional area or gravimetric analysis. Our qPCR gene expression analysis demonstrated that PTIP deletion was associated with an elevated Nppa level, both at 1 and 9 months after PTIP deletion. Although Nppa elevations are consistent with hypertrophy, PTIP mice have decreased mRNA expression of \alpha 1 skeletal muscle actin (Acta1), a protein that is upregulated in the development of hypertrophy, as compared with that of PTIP mice at 9 months of age. Furthermore, PTIP and PTIP mice have similar amounts of \beta MHC (Myb7) and Nppb, fetal genes that are upregulated in hypertrophy and failure. Thus, although PTIP mice have increased [Ca\textsuperscript{2+}], the hypertrophic phenotype may be blunted in the absence of PTIP, because of the inability to upregulate genes necessary for the development of hypertrophy. Therefore, these results suggest the possibility that at least some hypertrophy-associated genes may require new H3K4me3 marks in response to stress or injury and that such maladaptive responses may be controlled by epigenetic mechanisms.

Cardiac repolarization occurs in a heterogeneous manner such that subepicardial cells have higher repolarization gradients than subendocardial cells (7). Disruption of this gradient is a hallmark of diseased myocardium and predisposes to ventricular arrhythmias (7, 9).
Prior work has shown that genetic deletion of Kcnip2 alters the repolarization gradient and that Kcnip2 is heterogeneously expressed across the ventricular wall in humans and in mice (30, 31). How the repolarization gradient is formed and maintained is incompletely understood. Our data suggest that H3K4me3 epigenetic marks may be partly responsible for maintaining the cardiac repolarization gradient. In order to insure that the heterogeneous expression of Kcnip2 does not bias our results, we performed ChIP assays from ventricular tissue in both PTIP\(^+\) and PTIP\(^-\) mice and used apical myocytes in both groups for our patch clamp experiments.

If changes in histone methylation can alter the transcriptome of a terminally differentiated cell, how might such changes arise in the absence of mutations in PTIP or other components of the H3K4 methyltransferase machinery? Epigenetic changes are likely to correlate with altered gene expression due to stressful physiological conditions. However, these changes may not be reset once the stress is alleviated, because the epigenetic changes confer indelible changes in the transcriptome. For example, complications can continue to develop in diabetic patients even after glycemic control is achieved (32). In cell and animals models of hyperglycemia, gene expression patterns may be permanently altered even after normal glucose levels are reinstated. Such permanent alterations are thought to be due to epigenetic changes at gene-specific promoter regions. Histone deacetylase inhibitors are now used as anticancer or anticonvulsive drugs but can lead to unintended alterations in the methylation of histones in a variety of tissues (33–35).

Figure 6
PTIP deletion reduces \(I_{\text{to}}\). (A) Superimposed whole cell outward \(K^+\) current traces recorded from PTIP\(^+\) and PTIP\(^-\) mice. (B) \(I_{\text{to}}\) current-voltage relationships for PTIP\(^+\) myocytes \((N = 2, n = 6)\) and PTIP\(^-\) myocytes \((N = 2, n = 4)\) **\(P < 0.01\), ***\(P < 0.001\). Data are mean ± SEM.

Figure 7
PTIP deletion increases \(Ca^{2+}\) transients, \(I_{\text{Ca,L}}\), and contractility. (A) \(Ca^{2+}\) transients expressed as change in fluorescence (\(F\)) over control fluorescence (\(F_0\)) in PTIP\(^+\) myocytes \((N = 2, n = 7\) cells) and PTIP\(^-\) myocytes \((N = 2, n = 6\) cells). Cells were loaded with fluo-4AM and field stimulated at 1 to 6 Hz. (B) \(I_{\text{Ca,L}}\) current-voltage relationships for PTIP\(^+\) myocytes \((N = 2, n = 7)\) and PTIP\(^-\) myocytes \((N = 2, n = 6)\). (C) PTIP\(^-\) myocytes show significantly higher fractional shortening that PTIP\(^+\) myocytes at 1- to 6-Hz pacing. *\(P < 0.05\), ***\(P < 0.001\). All data are mean ± SEM.
in epigenetic machinery, such as that which may occur as a result of a pathophysiological insult, are mechanistically relevant. Our model suggests that such epigenetic changes must be considered, as they can be a cause and not just an effect of disease.

In conclusion, we believe this study is the first to demonstrate that maintenance of H3K4me marks is necessary for maintaining the expression of some genes that are critical to regulating homeostasis in fully differentiated tissue. Furthermore, our phenotypic analysis demonstrates that Kcnip2, a gene at least partly regulated by H3K4me marks, is important in regulating many facets of the electrophysiological phenotype, including APD, I_{Na}, I_{to}, \[Ca^{2+}\]), cardiac contractility, and susceptibility to ventricular premature beats. Since it is known that Kcnip2 is downregulated in human models of heart failure and attenuation of the transient outward current is a hallmark of diseased myocardium, our results provide the first evidence to our knowledge that genes that are important in pathophysiology (e.g., arrhythmogenesis and alterations in excitation contraction coupling) may, indeed, be regulated by the same epigenetic H3K4me marks that are important in establishing body patterns in Drosophila.

Methods

Animals. Mice were kept according to NIH guidelines. All animal studies were reviewed and approved by the University Committee on Use and Care of Animals at the University of Michigan.

Tamoxifen. Unless otherwise specified, mice were injected i.p. with tamoxifen (20 mg/kg) dissolved in peanut oil (vehicle) at 8 weeks of age over a 5-day period.

qPCR and array. LV samples were harvested, and RNA was isolated and sent to the University of Michigan Comprehensive Cancer Center Affymetrix and Microarray Core Facility or used for qPCR with TaqMan probes. Array data are available at the GEO database (accession no. GSE20570; http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi).

Immunoblotting. LV tissue samples were used for immunoblotting and performed using PTIP antibodies as previously described. Other antibodies used include anti-H3 (Abcam, ab1791), anti-H3K4me3 (Abcam, ab8580), anti-RbBP5 (Bethyl, A300-109A), anti-Kcnip2 (NeuroMab, 75-004), anti-Kcnd2 (Millipore), and anti-Kcnd3 (NeuroMab, 75-017).

ChIP. Ventricles were harvested and minced into 1- to 2-mm pieces. For PTIP and RbBP5 studies, minced tissue was crosslinked with 2 mM disuccinimidyl glutarate for 45 minutes and then crosslinked with 1% formaldehyde in culture media for 15 minutes. For H3K4me3 marks, minced tissue was cross-linked with 1% formaldehyde in culture media for 15 minutes. The protocol was then followed as previously described (11). Primers used for qPCR are available in Supplemental Table 3. Antibodies for ChIP include rabbit IgG (4 μg), rabbit anti-PTIP (4 μg), rabbit anti-RbBP5 (Bethyl, A300-109A), and rabbit anti-H3K4me3 (Abcam, ab8580) (4 μg).

Immunoprecipitation. Immunoprecipitation was performed on formaldehyde crosslinked chromatin, which consists of DNA and its associated
proteins. We used 4 μg rabbit anti-PTIP antibody and 4 μg rabbit IgG antibody as a control.

Echocardiography. Echo was performed as previously described using isoflurane anesthesia (36).

Myocyte isolation. For myocyte isolations, mice were injected i.p. with heparin (150 units/mouse). After 20 minutes, the mice were euthanized by CO2, followed by cervical dislocation. The heart was removed from the chest, auxiliary tissue was removed, and the aorta was cannulated. The heart was then perfused with myocyte and digestion buffer. An apical piece of LV tissue was then removed, mechanically disaggregated, and separated by centrifugation.

Fluo-4AM and calcium transients and sarcomere shortening assays. Myocytes were isolated from PTIP+ and PTIP− mice, and shortening assays and Fluo-4AM calcium transients were performed as previously described (37).

EKG. EKG was performed in conscious mice. Recordings were taken from a lead I and a lead II configuration.

Whole cell current clamp experiments. Action potentials were recorded at 37°C in current-clamp mode, using the Multiclamp 700B amplifier (Molecular Devices). Cells were paced, from 0.5 to 5 Hz, with brief depolarizing pulses at 1.5- to 2-times threshold value, generated using a programmable digital stimulator (World Precision Instruments, DS8000). Action potential parameters (RMP; dV/dt; and APD 30, 50, 70, 90) were estimated using the pClamp 10 suite of programs. The bath solution contained 148 mmol/l NaCl, 2 mmol/l CaCl2, 5 mmol/l MgATP, and 5 mmol/l HEPES (pH 7.2, with CsOH). I_{Ca,L} was assessed using the following protocol: from a holding potential of ~90 mV, cells were stepped to ~50 mV for 150 ms and then to various test potentials (~40 to 60 mV, 10 mV increments, 300 ms duration, 400 ms interpulse intervals).

I_{Isk} was measured at room temperature (21°C–22°C) with the following pipette filling solution: 5 mmol/l NaCl, 135 mmol/l CsF, 10 mmol/l EGTA, 5 mmol/l MgATP, and 5 mmol/l HEPES (pH 7.2). The extracellular solution contained 5 mmol/l NaCl, 1 mmol/l MgCl2, 1 mmol/l CaCl2, 0.1 mmol/l CdCl2, 11 mmol/l glucose, 132.5 mmol/l CsCl, and 20 mmol/l HEPES (pH 7.35). To assess the I_{Isk} density, cells were held at ~120 mV and stepped to various test potentials (~80 to 30 mV, 5 mV increments, 200 ms duration, 2,800 ms interpulse intervals).

Isoproterenol and caffeine studies. Mice were lightly anesthetized with Avertin (0.025 mg/kg) i.p., and body temperature was maintained at 37°C. Mice were hooked up to a continuous EKG recording and injected i.p. with isoproterenol (2 mg/kg) and caffeine (180 mg/kg). Analysis was performed post hoc. PVCs were counted for 5 minutes after the onset of the first PVC.

Statistics. Statistical significance was calculated using 2-tailed Student's t test to compare 2 groups and analysis of variance with Bonferroni correction to compare multiple groups. P values of less than 0.05 were considered statistically significant. All data shown are mean ± SEM unless otherwise specified.

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Figure 9

PTIP+ mice show increased susceptibility to PVCs. Twelve-week-old PTIP+ mice (n = 4) and PTIP− mice (n = 3) were anesthetized with Avertin and injected i.p. with isoproterenol (2 mg/kg) and caffeine (180 mg/kg). (A) Continuous EKG recordings were performed. PVCs were counted, and analysis revealed that PTIP+ mice had significantly more PVCs than PTIP− mice. Data are mean ± SD. *P < 0.05 vs. PTIP−. (B) Representative example of the pattern of ventricular bigeminy that was observed in all PTIP+ mice.
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