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Loss of Protein Phosphatase 1 Regulatory Subunit PPP1R3A Promotes Atrial Fibrillation

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BACKGROUND: Abnormal calcium (Ca^{2+}) release from the sarcoplasmic reticulum (SR) contributes to the pathogenesis of atrial fibrillation (AF). Increased phosphorylation of 2 proteins essential for normal SR-Ca^{2+} cycling, the type-2 ryanodine receptor (RyR2) and phospholamban (PLN), enhances the susceptibility to AF, but the underlying mechanisms remain unclear. Protein phosphatase 1 (PP1) limits steady-state phosphorylation of both RyR2 and PLN. Proteomic analysis uncovered a novel PP1-regulatory subunit (PPP1R3A [PP1 regulatory subunit type 3A]) in the RyR2 macromolecular channel complex that has been previously shown to mediate PP1 targeting to PLN. We tested the hypothesis that reduced PPP1R3A levels contribute to AF pathogenesis by reducing PP1 binding to both RyR2 and PLN.

METHODS: Immunoprecipitation, mass spectrometry, and complexome profiling were performed from the atrial tissue of patients with AF and from cardiac lysates of wild-type and *Pln*-knockout mice. *Ppp1r3a*-knockout mice were generated by CRISPR-mediated deletion of exons 2 to 3. *Ppp1r3a*-knockout mice and wild-type littermates were subjected to in vivo programmed electrical stimulation to determine AF susceptibility. Isolated atrial cardiomyocytes were used for Stimulated Emission Depletion superresolution microscopy and confocal Ca^{2+} imaging.

RESULTS: Proteomics identified the PP1-regulatory subunit PPP1R3A as a novel RyR2-binding partner, and coimmunoprecipitation confirmed PPP1R3A binding to RyR2 and PLN. Complexome profiling and Stimulated Emission Depletion imaging revealed that PLN is present in the PPP1R3A-RyR2 interaction, suggesting the existence of a previously unknown SR nanodomain composed of both RyR2 and PLN/sarco/endoplasmic reticulum calcium ATPase-2a macromolecular complexes. This novel RyR2/PLN/sarco/endoplasmic reticulum calcium ATPase-2a complex was also identified in human atria. Genetic ablation of *Ppp1r3a* in mice impaired binding of PP1 to both RyR2 and PLN. Reduced PP1 targeting was associated with increased phosphorylation of RyR2 and PLN, aberrant SR-Ca^{2+} release in atrial cardiomyocytes, and enhanced susceptibility to pacing-induced AF. Finally, PPP1R3A was progressively downregulated in the atria of patients with paroxysmal and persistent (chronic) AF.

CONCLUSIONS: PPP1R3A is a novel PP1-regulatory subunit within the RyR2 channel complex. Reduced PPP1R3A levels impair PP1 targeting and increase phosphorylation of both RyR2 and PLN. PPP1R3A deficiency promotes abnormal SR-Ca^{2+} release and increases AF susceptibility in mice. Given that PPP1R3A is downregulated in patients with AF, this regulatory subunit may represent a new target for AF therapeutic strategies.

Key Words: atrial fibrillation ■ calcium release activated calcium channels ■ protein phosphatase 1 ■ ryanodine receptor 2 ■ ryanodine receptor calcium release channel

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Clinical Perspective

What Is New?

- This study demonstrates for the first time that reduced levels of the protein phosphatase 1 (PP1) regulatory subunit PPP1R3A in human atria are causally linked to abnormal calcium handling and atrial fibrillation pathogenesis.
- In the absence of PPP1R3A, reduced binding of PP1 catalytic subunit increases phosphorylation levels of the ryanodine receptor calcium release channel and phospholamban.
- Complexome profiling, a technique that combines native gel electrophoresis with mass spectrometry to obtain the composition of multiprotein assemblies, revealed that PPP1R3A is part of a macromolecular protein complex containing the ryanodine receptor calcium release channel and the sarco/endoplasmic reticulum calcium ATPase-2a/phospholamban calcium reuptake transporter.

What Are the Clinical Implications?

- Reduced levels of a PP1 regulatory subunit contribute to abnormal sarcoplasmic reticulum calcium release and reuptake in atrial myocytes, thereby promoting atrial fibrillation pathogenesis.
- Normalizing levels of the PPP1R3A phosphatase subunit represents a novel therapeutic approach for atrial fibrillation.

Atrial fibrillation (AF) is the most common sustained cardiac arrhythmia and is associated with an increased risk of stroke and mortality.[1] Current AF therapy with drugs is of moderate efficacy, because AF is increasingly considered as a symptom or marker of an atrial cardiomyopathy resulting from a variety of risk factors, including genetic predisposition, sex, advanced age, and cardiovascular and noncardiovascular diseases.[2] The limitations of current AF therapies have, at least in part, been attributed to a one-size-fits-most approach that ignores the diversity of underlying cardiomyopathy and the related mechanisms that predispose to AF.[3,4] Altered calcium (Ca^{2+}) handling within atrial cardiomyocytes is widely regarded as a hallmark of AF.[5] Emerging evidence has revealed abnormal Ca^{2+} release from the sarcoplasmic reticulum (SR) as a key mechanism promoting atrial arrhythmogenesis and AF development.[6–9]

Normal excitation-contraction coupling depends on the balanced influx and efflux of cytosolic Ca^{2+}.[10] During an action potential, Ca^{2+} enters the cell through L-type Ca^{2+} channels, activating the type 2 ryanodine receptor (RyR2), which in turn releases a much larger amount of Ca^{2+} from the SR into the cytosol to trigger myocyte contraction. Relaxation occurs when cytosolic Ca^{2+} is resequestered into the SR via the SR-Ca^{2+}-ATPase (SERCA2a), or extruded from the myocyte via the Na^+/Ca^{2+}-exchanger.

In patients with early-stage (paroxysmal) or long-standing persistent (chronic) AF (pAF and cAF, respectively), enhanced activities of both RyR2 and SERCA2a are associated with increased SR-Ca^{2+} leak, increased SR-Ca^{2+} load, and delayed afterdepolarizations that promote cellular arrhythmogenic events.[9,11,12] Studies using human atrial tissue revealed that, in cAF, hyperphosphorylation of RyR2 promotes spontaneous SR-Ca^{2+} leak, whereas hyperphosphorylation of the SERCA2a regulator phospholamban (PLN) promotes increased SR-Ca^{2+} reuptake through reduced inhibition of SERCA2a.[13] This enhanced reuptake likely compensates for the reduction in SR-Ca^{2+} load during AF and may have a permissive effect on SR-Ca^{2+} leak. The mechanisms underlying enhanced phosphorylation of SR-Ca^{2+}–handling proteins in AF, especially that of RyR2, have remained subject of substantial controversy.[14–16] Until recently, a majority of work has focused on understanding the role of kinases such as protein kinase A (PKA) and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) in promoting RyR2 and PLN dysfunction.[17,18] However, several studies including ours revealed that cardiac diseases lead to extensive changes in subcellular targeting of protein phosphatases as well.[13,19,20]

Protein phosphatase type 1 (PP1)—one of the most abundant serine-threonine phosphatases in the heart—dephosphorylates both RyR2 and PLN.[21] Proper targeting of the PP1-catalytic (PP1c) subunit depends on a variety of regulatory subunits (R-subunits) that confer substrate specificity, and we have recently shown that altered binding between PP1c and its R-subunits may contribute to impaired phosphatase targeting in AF.[19,22] For example, PP1c is targeted to RyR2 by the R-subunit PPP1R9B (spinophilin). Genetic ablation of spinophilin in mice reduced the amount of RyR2-bound PP1c by ≈60%, leading to increased phosphorylation of RyR2 at S2814 and enhanced AF susceptibility.[22] It is interesting to note that the amount of residual PP1c bound to RyR2 in spinophilin-deficient mice suggests that additional R-subunits may also mediate the interaction between PP1c and RyR2. In contrast, targeting of PP1c to PLN is mediated by the R-subunit PPP1R3A (also known as GM/R_{GL}); however, little is known about the role of this R-subunit in cardiac function.[23]

Here, we report that PPP1R3A is a novel RyR2-binding partner, and demonstrate that loss of PPP1R3A impairs targeting of PP1c to both PLN and RyR2. This leads to increased SR-Ca^{2+} leak and enhanced AF susceptibility in *Ppp1r3A*-KO mice. Complexome profiling[24,25] suggests that PPP1R3A itself plays an integral part of a never-before-identified extended protein complex within the SR that comprises, among others, RyR2, PLN, and SERCA2a, revealing that Ca^{2+} release/reuptake complexes physically interact. Furthermore, PPP1R3A is

progressively downregulated and its extended SR protein complex disrupted in patients with pAF and cAF, implicating this R-subunit as a potential contributor to AF progression to more persistent forms, and, hence, a novel therapeutic target for AF treatment.

METHODS

Please see the online-only Data Supplement for detailed materials and methods. The data that support the findings of this study are available from the corresponding authors upon reasonable request.

Human Samples

Right atrial appendages of patients undergoing open-heart surgery were collected with patients' written informed consent. All experimental protocols were approved by the ethics committee of the Medical Faculty at the University of Duisburg-Essen (AZ:12-5268-BO). Patient characteristics are listed in Tables I and II in the online-only Data Supplement.

Animal Studies

Studies involving animals were performed according to protocols approved by the Institutional Animal Care and Use Committee at Baylor College of Medicine, and conform to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). *Ppp1r3a*-KO mice were generated by CRISPR-Cas9–targeted deletion of exons 2 and 3 and were maintained on a C57Bl6/J background. Male and female mice were used for all experiments, with wild-type (WT) littermates as controls. Mice were anesthetized with 2% isoflurane in 0.5 L/min 100% O_2 before surgery or tissue collection. Mice that appeared unhealthy or runted (body weight <2 SD below average) were excluded. Analyses were performed in blinded manner.

Coimmunoprecipitation and Mass Spectrometry

Coimmunoprecipitation and mass spectrometry were performed as described.[19]

Quantitative Real-Time Polymerase Chain Reaction

Quantitative real-time polymerase chain reaction was performed as described.[26] Primers are listed in Table III in the online-only Data Supplement.

Intracardiac Electrophysiology Studies

In vivo electrophysiology studies were performed in 4- to 5-month-old *Ppp1r3a*-KO mice and WT littermates.[27]

Stimulated Emission Depletion and Confocal Microscopy

Atrial cardiomyocytes were isolated via retrograde Langendorff perfusion using a modified collagenase protocol.[6,12] Cells were either fixed and stained with PPP1R3A, RyR2, and PLN antibodies for Stimulated Emission Depletion (STED) super-resolution imaging, or loaded with Fluo-4 AM for live-cell confocal Ca^{2+} imaging.[8,28]

Complexome Profiling

Total membrane pellets (100 000×*g*) from isolated mouse ventricular cardiomyocytes or human right-atrial tissue were solubilized with digitonin. Higher-molecular-weight complexes including mitochondrial oxidative phosphorylation supercomplexes were separated by Blue Native gel electrophoresis, and protein composition and migration profiling were analyzed by mass spectrometry as described.[29,30]

Statistical Analysis

Results are expressed as mean± SEM. The Fisher exact test, χ^2 test, or unpaired 2-tailed Student *t* test was used for comparisons between 2 groups, as specified in the figure legends. Generalized estimating equation was used for analysis of hierarchical/nonindependent data (see the online-only Data Supplement). A *P* value of <0.05 was considered statistically significant.

RESULTS

Identification of PPP1R3A as a Novel Regulator of RyR2

To identify novel regulators of RyR2 phosphorylation, RyR2 was coimmunoprecipitated from WT mouse hearts followed by mass spectrometry analysis. As a negative control, each sample was also immunoprecipitated with murine nonspecific immunoglobulin G. Of the proteins identified, 6 were either serine/threonine kinases, phosphatases, or phosphatase regulatory subunits (Table IV in the online-only Data Supplement). In comparison with the mass spectrometry signal from the control immunoprecipitation (IP) using nonspecific immunoglobulin G, 2 of these proteins were found exclusively in the RyR2 IP (top 2 rows with ratio of Infinity). The protein with the highest abundance in the IP as estimated by the label-free quantification signal normalized to molecular weight was the PP1 catalytic subunit PP1c (right-most column). The protein with the second highest abundance was PPP1R3A, a known regulatory subunit of PP1c.[31] Because PP1c is known to regulate RyR2 function, but not through PPP1R3A, we conducted in-depth studies to investigate potentially important roles of PPP1R3A in regulating RyR2.

PPP1R3A Mediates Targeting of PP1c to Both RyR2 and PLN in Mouse Heart

PPP1R3A is a 126-kDa protein reported to target PP1c to glycogen and PLN.[23,32,33] However, there were no previous reports suggesting that PPP1R3A mediates the in-

teraction between PP1c and RyR2. We therefore sought to validate the mass spectrometry results implicating PPP1R3A as a novel RyR2 interactor. Reciprocal coimmunoprecipitation from mouse heart lysates using either RyR2 or PPP1R3A antibodies confirmed that PPP1R3A binds to both RyR2 and PP1c in mouse heart (Figure 1A and 1B). Superresolution microscopy using STED revealed that the interaction of PPP1R3A with RyR2 in ventricular or atrial cardiomyocytes occurs in a highly localized fashion in SR nanodomains, often near Z-line striations (Figure 1C and 1D). Magnified views of atrial myocytes depict larger, axially extended RyR2 clusters, apparently containing distinct PPP1R3A colocalization domains (Figure 1E). In addition, triple-color STED imaging further confirmed PPP1R3A nanodomains typically aligned along the main cell axis and colocalized with PLN right next to RyR2 clusters (Figure I in the online-only Data Supplement). Validation of the signal specificity of the PPP1R3A antibody used for STED experiments can be found in Figure II in the online-only Data Supplement. Taken together, these findings confirm PPP1R3A as a novel RyR2-binding partner and suggest this PP1 regulatory (R) subunit may play a dual or integrative role in regulating both PLN and RyR2.

To explore the functional importance of PPP1R3A in the heart, we generated *Ppp1r3a* knockout (KO) mice by CRISPR-Cas9–mediated deletion of exons 2 and 3 (Figure III in the online-only Data Supplement). Coimmunoprecipitation experiments revealed a 47% reduction in the amount of PP1c bound to RyR2 in *Ppp1r3a*-KO mouse hearts ($P<0.05$ versus WT; Figure 2A and 2B) confirming that PPP1R3A is necessary for mediating (at least in part) the interaction between PP1c and RyR2. It is important to note that the loss of PPP1R3A did not alter protein phosphatase 2A catalytic subunit binding to RyR2. Similarly, we found a 58% reduction in the amount of PP1c bound to PLN ($P<0.05$ versus WT; Figure 2C and 2D), in agreement with previous reports that PPP1R3A mediates targeting of PP1c to PLN.[23,32] These findings suggest that some PP1c might bind to PLN through another yet-to-be-identified PP1 R-subunit.

Impaired Local PP1 Regulation Enhances RyR2 and PLN Phosphorylation

Enhanced phosphorylation of RyR2 at both S2808 and S2814 promotes SR-Ca^{2+} release, whereas phosphorylation of PLN at S16 and T17 promotes SR-Ca^{2+}

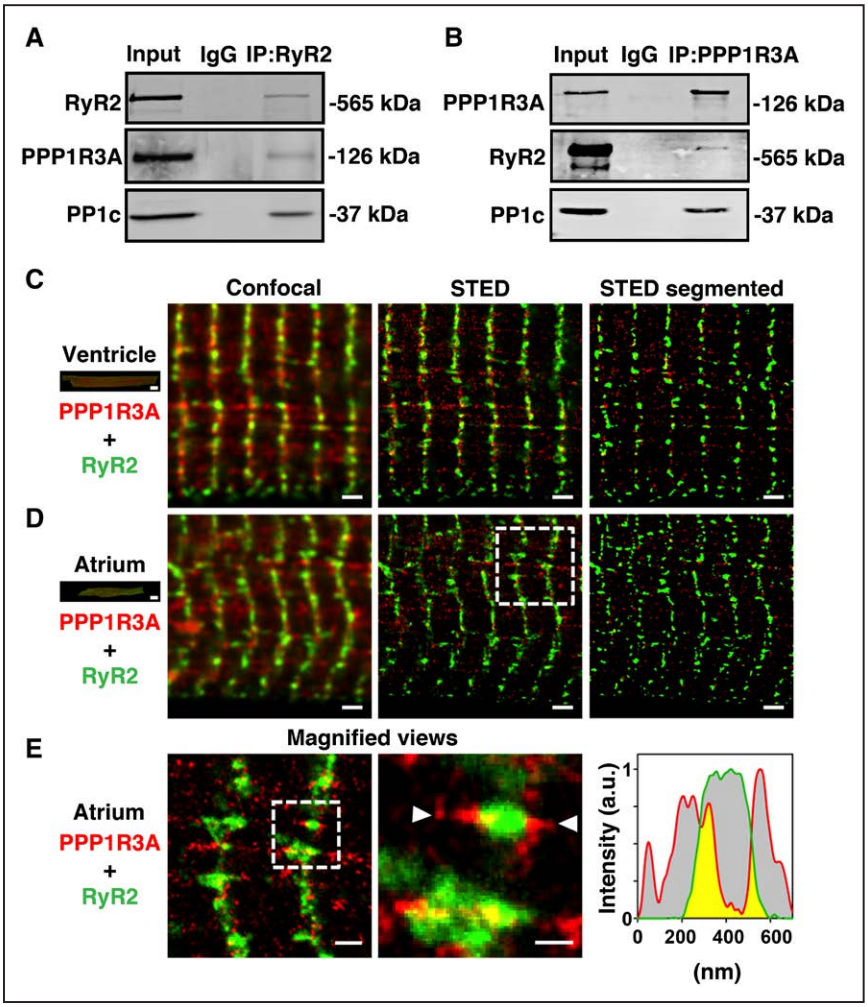

Figure 1. Validation of binding between RyR2, PPP1R3A, and PP1c in mouse heart. Representative Western blots confirming the interaction between RyR2, PPP1R3A, and PP1c in WT mouse heart lysates immunoprecipitated with RyR2 antibody (**A**) or PPP1R3A antibody (**B**). Confocal and STED imaging of coimmunostained ventricular (**C**) and atrial (**D**) mouse cardiomyocytes. STED, but not confocal imaging, resolves the PPP1R3A (red) and RyR2 (green) signals, which enables image segmentation for regional nanodomain visualization (right). Of note, RyR2 and PPP1R3A clusters show considerable differences in their subcellular distribution between ventricular and atrial myocytes. Scale bars, 10 μm (left, cell overview) or 1 μm (image panels). **E**, **Left**, Magnified view (as indicated in **D**). **Center**, Zoom-in showing the local association of PPP1R3A with RyR2 clusters at nanometric scale. The white triangles indicate the nanodomain orientation used for signal intensity profiling. **Right**, The PPP1R3A (red) and RyR2 (green) signal distribution confirms subcluster areas exhibiting colocalized signals (yellow). Scale bars, 500 nm (**Left**) or 200 nm (**Center**). Dashed boxes indicate magnified views in **D** and **E**. IgG indicates immunoglobulin G; IP, immunoprecipitation; PP1c, protein phosphatase 1 catalytic subunit; PPP1R3A, protein phosphatase 1 regulatory subunit type 3A; RyR2, ryanodine receptor type 2; STED, Stimulated Emission Depletion; and WT, wild type.

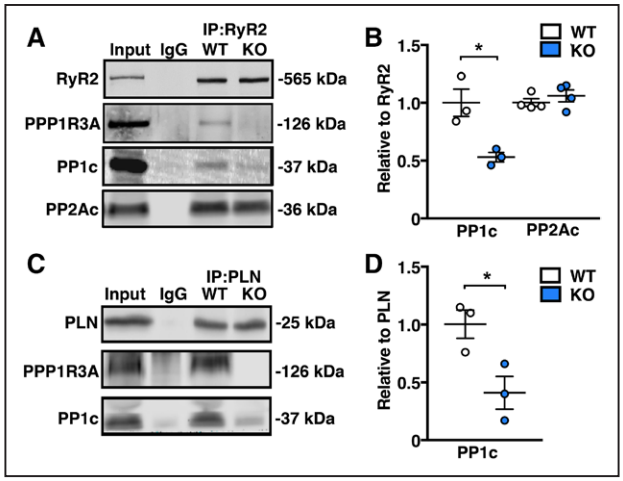

Figure 2. Genetic ablation of PPP1R3A impairs binding of PP1c to both RyR2 and PLN.
Representative Western blots and corresponding dot plots showing that with PPP1R3A ablation, the association between RyR2 and PP1c is significantly reduced with no change in the association between RyR2 and PP2A (**A** and **B**), and the association between PLN and PP1c is significantly reduced (**C** and **D**). Data represent mean±SEM and were analyzed using unpaired 2-tailed Student *t* test (**P*<0.05 vs WT). IgG indicates immunoglobulin G; IP, immunoprecipitation; KO, knockout; PLN, phospholamban; PP1c, protein phosphatase 1 catalytic subunit; PP2A, protein phosphatase 2A; PP2Ac, protein phosphatase 2A catalytic subunit; PPP1R3A, protein phosphatase 1 regulatory subunit type 3A; RyR2, ryanodine receptor type 2; and WT, wild type.

reuptake through SERCA2a by reducing PLN's inhibition of the channel. Because we observed impaired targeting of PP1c to both RyR2 and PLN in *Ppp1r3a*-KO hearts, we expected an increase in the phosphorylation levels of both proteins. Relative phosphorylation of RyR2 was consistently increased at the primary PKA site S2808 by 50% (*P*<0.01 versus WT), with no change at the CaMKII site S2814 (Figure 3A and 3B). Moreover, phosphorylation of PLN was increased at both the PKA site S16 by 62% and the CaMKII site T17 by 100% in *Ppp1r3a*-KO atria (*P*<0.05 versus WT for both; Figure 3C and 3D).

To demonstrate that the observed changes in RyR2 and PLN phosphorylation were attributable exclusively to decreased targeting of PP1c, we investigated global changes in protein expression of the phosphatases and kinases known to regulate the 2 proteins. We did not observe compensatory changes in expression levels of PP1 and protein phosphatase 2A catalytic subunits, or of the PP1c R-subunit spinophilin in *Ppp1r3a*-KO atria (Figure IVA and IVB in the online-only Data Supplement). Atrial protein levels of kinases PKA and CaMKII were also unchanged between *Ppp1r3a*-KO and WT (Figure IVC and IVD in the online-only Data Supplement). Priming phosphorylation sites of PKA (T197) and CaMKII (T286), which are essential for their catalytic activity, were also unchanged, indicating that the changes in RyR2 and PLN phosphorylation are specifically attributable to reduced phosphatase targeting, and not enhanced kinase activities (Figure IVE and IVF in the online-only Data Supplement).

Impaired Local PP1c Regulation by *Ppp1r3a*-KO Increases AF Susceptibility

To understand how locally reduced PP1c targeting to RyR2 and PLN affects cardiac function, we performed in vivo programmed electrical stimulation studies in 4-month-old *Ppp1r3a*-KO mice and WT littermates. Heart rate and baseline ECG parameters were similar between groups (Figure 4A; Table V in the online-only Data Supplement). Furthermore, we found no indication of baseline ventricular dysfunction in *Ppp1r3a*-KO mice (Table VI in the online-only Data Supplement). However, after atrial burst pacing, 40% of the *Ppp1r3a*-KO mice had reproducible episodes of AF, in comparison with none of the WT mice (*P*<0.05; Figure 4B and 4C). Ventricular programmed electrical stimulation did not induce ventricular tachycardia in any *Ppp1r3a*-KO mice (data not shown). Of note, PPP1R3A protein levels were 2.9-fold higher in mouse atria than in ventricles (Figure V in the online-only Data Supplement). These data indicate that genetic ablation of PPP1R3A results in an atrial-specific phenotype, and position PPP1R3A as a novel atrial-selective therapeutic target.

To determine the underlying cause of AF in *Ppp1r3a*-KO mice, we looked for signs of structural and electrical remodeling. We observed no changes in heart morphology, atrial weight, or baseline ECG parameters (Figure VIA in the online-only Data Supplement; Table V in the online-only Data Supplement). Quantitative real-time

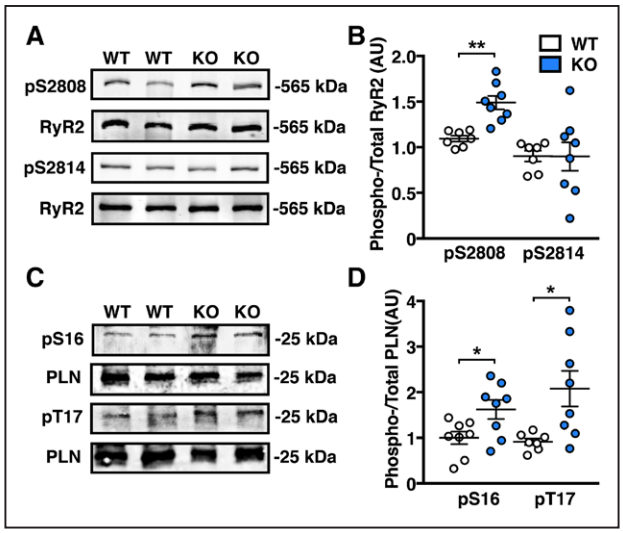

Figure 3. Loss of local PP1c regulation in the absence of PPP1R3A increases RyR2 and PLN phosphorylation.
A and **B,** Representative Western blots and corresponding dot plots showing increased phosphorylation of RyR2 at S2808 but not at S2814 in *Ppp1r3a*-KO (KO) mouse atria in comparison with wild-type (WT) atria. **C** and **D**, Representative Western blots and corresponding dot plots showing increased phosphorylation of PLN at both S16 and T17 in *Ppp1r3a*-KO mouse atria. Data represent mean±SEM and were analyzed using unpaired 2-tailed Student *t* test (**P*<0.05, ***P*<0.01 vs WT). KO indicates knockout; PLN, phospholamban; PP1c, protein phosphatase 1 catalytic subunit; PPP1R3A, protein phosphatase 1 regulatory subunit type 3A; RyR2, ryanodine receptor type 2; and WT, wild type.

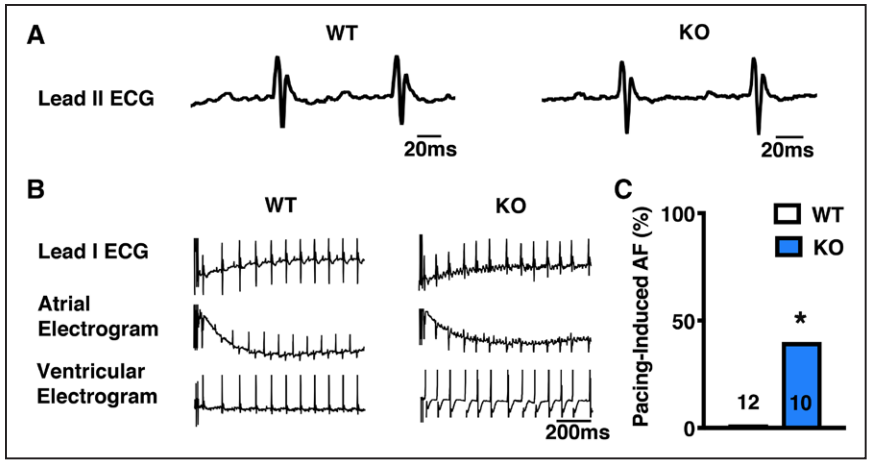

Figure 4. *Ppp1r3a*-KO mice exhibit increased susceptibility to pacing-induced AF. **A**, Representative recordings of surface ECG lead II showing no change in baseline ECG parameters comparing 4-month-old *Ppp1r3a*-KO mice and WT littermates. **B**, Simultaneous recordings from surface and intracardiac (atrial and ventricular) leads demonstrating AF in *Ppp1r3a*-KO mice (**Right**) and normal sinus rhythm in WT littermate (**Left**) after atrial burst pacing. **C**, Bar graph summarizing the incidence of inducible AF in *Ppp1r3a*-KO mice. Data were analyzed using Fisher exact test (**P*<0.05 vs WT). AF indicates atrial fibrillation; KO, knockout; and WT, wild type.

polymerase chain reaction showed unchanged expression of fibrotic gene markers (collagen-I, collagen-III, and transforming growth factor β) in *Ppp1r3a*-KO atria (Figure VIB in the online-only Data Supplement), and Masson's trichrome staining confirmed the absence of fibrosis in *Ppp1r3a*-KO atria (Figure VIC and VID in the online-only Data Supplement). Similarly, we found no changes in atrial expression of the ion-channel genes *Cacna1c*, *Kcna5*, *Kcnj3*, or *Kcnj5* (Figure VII in the online-only Data Supplement). Although *Cacna1c* mRNA levels showed a nonsignificant increase in KO mice, no change was found at the protein level. Overall, these results indicate that the AF phenotype observed in *Ppp1r3a*-KO mice is unlikely to be attributable to structural or electrical remodeling.

Ca^{2+} Handling Is Altered in *Ppp1r3a*-KO Atrial Cardiomyocytes Because of Increased Activities of RyR2 and SERCA2a

Because RyR2 and PLN are both critical mediators of SR-Ca^{2+} release and reuptake, we expected their dysregulation to lead to altered SR-Ca^{2+} handling in *Ppp1r3a*-KO atrial cardiomyocytes. Although total protein expression of SR-Ca^{2+}–handling proteins was unaltered (Figure VIII in the online-only Data Supplement), line scan confocal Ca^{2+} imaging revealed an increase in the frequency of RyR2-mediated Ca^{2+} sparks in *Ppp1r3a*-KO atrial cardiomyocytes (3.3±0.5 a.u.) in comparison with WT (1.1±0.2 a.u.; *P*<0.01; Figure 5A and 5B). Total SR-Ca^{2+} content (SR load) was not significantly different between groups (4.8±0.5 a.u. for KO versus 4.2±0.4 a.u. for WT; Figure 5C), and the increase in SR-Ca^{2+} leak was maintained after normalizing the frequency of RyR2-mediated Ca^{2+} sparks to SR load (1.1±0.2 a.u. for KO versus 0.34±0.1 a.u. for WT; *P*<0.01; Figure 5D). Sparks characteristics are listed in Table VII in the online-only Data Supplement. The amplitude of the Ca^{2+} transient was also increased in cardiomyocytes from *Ppp1r3a*-KO mice (4.2±0.6 a.u. for KO versus 1.9±0.2 a.u. for WT; *P*<0.01; Figure 5E and 5F). In addition to enhanced RyR2-mediated SR-Ca^{2+} leak, *Ppp1r3a*-KO atrial cardiomyocytes exhibited faster reuptake of cytosolic Ca^{2+} into the SR proportional to a 30% increase in SERCA2a activity (*P*<0.05 versus WT; Figure 5E and 5G). These results reveal aberrant SR-Ca^{2+} release and reuptake in atrial cardiomyocytes from *Ppp1r3a*-KO mice consistent with enhanced activities of both RyR2 and SERCA2a. In addition, challenging atrial cardiomyocytes with 100 nmol/L isoprenaline further exacerbated RyR2-mediated SR-Ca^{2+} leak in *Ppp1r3a*-KO cells in comparison with WT, with no difference in SERCA2a activity between groups (Figure IX in the online-only Data Supplement).

Although RyR2 phosphorylation at S2808 was increased in *Ppp1r3a*-KO ventricle (Figure XA and XB in the online-only Data Supplement), RyR2-mediated Ca^{2+} leak was comparable between WT and *Ppp1r3a*-KO ventricular cardiomyocytes (Figure XIA through XIC in the online-only Data Supplement). Nevertheless, as in atria, Ca^{2+} transient amplitude and SERCA2a activity were significantly higher in *Ppp1r3a*-KO ventricular cardiomyocytes (Figure XID and XIE in the online-only Data Supplement), and PLN phosphorylation was increased at S16, but not T17 (Figure XC and XD in the online-only Data Supplement). Similar to atria, no change in ventricular Na^{+}/Ca^{2+}-exchanger activity was observed (Figure XIF in the online-only Data Supplement). Thus, unlike in atrial cardiomyocytes, PPP1R3A appears to be less important in regulating ventricular excitation-contraction coupling.

Identification of PPP1R3A Within an Extended RyR2/PLN/SERCA2a Complex

Our findings reveal PPP1R3A as a novel integrator of SR-Ca^{2+} cycling in cardiomyocytes. This is of great interest because little is known about the interplay between RyR2 and PLN/SERCA2a and how they work together to precisely coordinate SR-Ca^{2+} release and reuptake locally. To better understand how PPP1R3A interacts with both RyR2 and the PLN/SERCA2a complex, we performed Blue Native polyacrylamide gel electrophoresis/

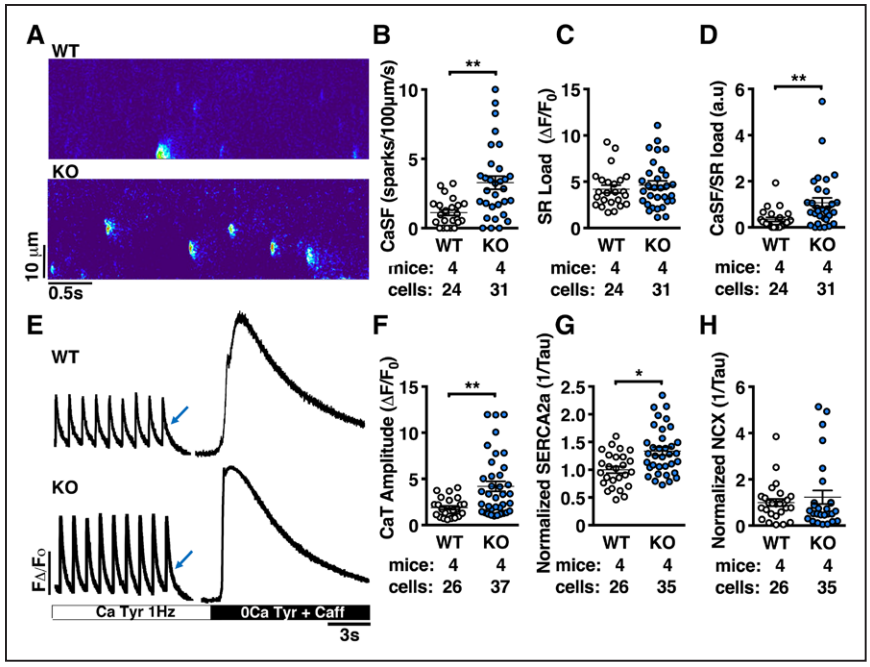

Figure 5. Ca^{2+} handling is altered in atrial cardiomyocytes from *Ppp1r3a*-KO mice because of increased activities of RyR2 and SERCA2a.
A, Representative confocal line-scan images of atrial myocytes from *Ppp1r3a*-KO mice and WT littermates. **B**, Dot plots summarizing spontaneous Ca^{2+} spark frequency (CaSF). Summary of SR-Ca^{2+} load (**C**) and CaSF normalized to caffeine-induced SR-Ca^{2+} load (**D**). **E**, Representative tracings of Ca^{2+} transient recordings during 1-Hz pacing and after exposure to 10 mmol/L caffeine. **F**, Dot plot summarizing Ca^{2+} transient amplitude. **G**, SERCA2a activity calculated as the difference between the decay of the pacing-induced transient and the caffeine-induced transient. **H**, NCX activity calculated from the decay of the caffeine-induced transient. Data represent mean±SEM and were analyzed using the Generalized Estimating Equation function in SPSS (*P<0.05; **P<0.01 vs WT). Caff indicates caffeine; CaT, Ca^{2+} transient; KO, knockout; NCX, Na^{+}/Ca^{2+}-exchanger; RyR2, ryanodine receptor type 2; SERCA2a, sarco/endoplasmic reticulum calcium ATPase-2a; SR, sarcoplasmic reticulum; Tyr, Tyrode buffer; and WT, wild type.

mass spectrometry–based complexome profiling from total membrane fractions of mouse ventricular cardiomyocytes. To identify large (10 kDa to 5 MDa) multiprotein complexes, samples were fractionated by Blue Native gel electrophoresis, and 60 gel slices subjected to in-gel tryptic digestion for analysis by mass spectrometry. Apparent molecular weight calibration for soluble and integral membrane proteins including larger complexes from the gel slices can be found in Figure XII in the online-only Data Supplement.[34] It is notable that mitochondrial oxidative phosphorylation complexes of known molecular weight were preserved in PLN KO membranes, confirming proteomic quality and quantitative profiling as a viable approach for ventricular cardiomyocyte membranes (Figure XIII in the online-only Data Supplement).[29]

Complexome profiling identified PPP1R3A as part of previously unknown higher-molecular-weight complexes that include PLN, SERCA2a, and RyR2, as indicated by their overlapping migration pattern after Blue Native gel electrophoresis (Figure 6A through 6C). It is interesting to note that the abundance of both the SERCA2a and RyR2 complexes was disrupted in PLN-deficient (PLN-KO) cardiomyocytes (Figure 6D through 6F). This unexpected finding positions PLN as a necessary mediator of PPP1R3A binding to RyR2, strongly supporting the existence of a novel extended complex within SR nanodomains that includes PPP1R3A, RyR2, PLN, and SERCA2a. In contrast to prevailing models of functionally discrete core complexes, these results together with our STED imaging data indicate that RyR2 and PLN/SERCA2a may in fact physically interact and function as joint Ca^{2+} release/reuptake regulatome complexes.

PPP1R3A Expression Is Downregulated With Progression of AF in Patients

Because PPP1R3A deficiency enhances the susceptibility to inducible AF in mice, we assessed PPP1R3A protein levels in atrial tissues from patients with pAF and cAF. Patient characteristics are listed in Table I in the online-only Data Supplement, and human PPP1R3A antibody validation can be found in Figure XIV in the online-only Data Supplement. PPP1R3A expression was not significantly decreased in patients with pAF (Figure 7A and 7B), but was 60% lower in patients with cAF (P<0.01 versus control patients in sinus rhythm; Figure 7C and 7D). These findings suggest that PPP1R3A is a clinically relevant PP1 R-subunit whose downregulation may contribute to proarrhythmic alterations in atrial cardiomyocyte Ca^{2+} homeostasis in patients with AF.

PPP1R3A deficiency in mice prevented PP1c from targeting to the SR, resulting in increased phosphorylation of RyR2 and PLN. Because previous work showed that phosphorylation of RyR2 and PLN is increased in patients with cAF, we correlated PPP1R3A protein levels with those of P-RyR2, P-PLN, and PP1c in a second cAF cohort. Consistent with other groups, we found significant increases in phosphorylation of RyR2 (at S2814) and of PLN (at S16) in patients with cAF, and confirmed a significant reduction in PPP1R3A protein expression (Figure XV in the online-only Data Supplement). Our correlation analyses revealed that PPP1R3A expression negatively correlates with phosphorylation levels of RyR2 and PLN. Similarly, PP1c protein expression also showed a negative correlation with the degree of RyR2 and PLN phosphorylation (Figure XVI in the online-only Data Supplement). These patient data are consistent with our mouse studies, where we found

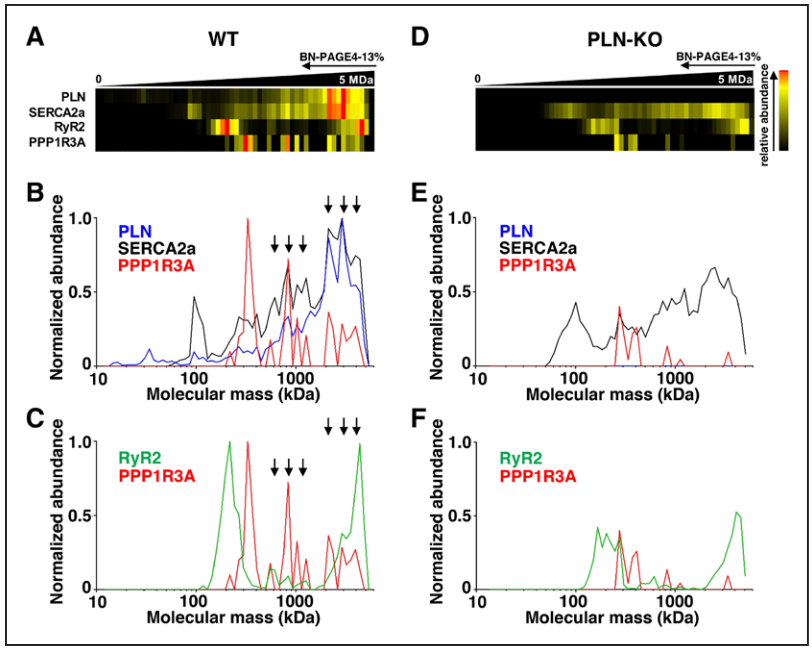

Figure 6. Complexome profiling reveals PPP1R3A as a protein within a novel high-molecular-weight RyR2/PLN/SERCA2a complex.
Heat map (**A**) and migration profiles (**B** and **C**) of PLN, SERCA2a, RyR2, and PPP1R3A in cardiomyocytes from PLN wild-type (WT) mice. Arrows indicate high-molecular-weight complexes. Heat map (**D**) and migration profiles (**E** and **F**) of SERCA2a, RyR2, and PPP1R3A in cardiomyocytes from PLN-KO mice. BN-PAGE indicates Blue Native polyacrylamide gel electrophoresis; KO, knockout; PLN, phospholamban; PPP1R3A, protein phosphatase 1 regulatory subunit type 3A; RyR2, ryanodine receptor type 2; SERCA2a, sarco/endoplasmic reticulum calcium ATPase-2a; and WT, wild type.

that a reduction in local PP1c targeting to the SR can cause hyperphosphorylation of key SR-Ca^{2+}–handling proteins, thereby increasing diastolic SR-Ca^{2+} leak and susceptibility to AF.

Disruption of the Extended RyR2/PLN/SERCA2a Complex in Human AF

Our complexome study identified a novel, extended SR-Ca^{2+} cycling complex in the mouse heart that included RyR2 and PLN/SERCA2a. To determine the relevance of this complex in human atria, we performed complexome profiling on right-atrial samples from 1 patient each in sinus rhythm (pseudo-control, because of coronary artery disease), pAF and cAF (Table II in the online-only Data Supplement). Samples were size-fractionated by Blue Native gel electrophoresis and 60 gel slices were subjected to digestion for mass spectrometry profiling (Figure XVIIA in the online-only Data Supplement). Soluble and integral membrane proteins/complexes showed a robust linear calibration throughout apparent molecular weights (Figure XVIIB in the online-only Data Supplement).[34] Moreover, human mitochondrial oxidative phosphorylation complexes appeared intact in pAF and cAF samples (Figure XVIII in the online-only Data Supplement), confirming a similar quality of complexome profiling as in mouse cardiomyocytes (Figure XIII in the online-only Data Supplement).

In the patient in sinus rhythm, complexome profiling confirmed higher-molecular-weight complexes composed of PLN, SERCA2a, RyR2, and Junctophilin-2 (JPH2) by their overlapping migration profiles (Figure 8A and 8B, sinus rhythm). In contrast, in pAF, RyR2 channels interacting with JPH2 proteins were nearly abolished in higher complexes, with only monomeric JPH2 detected (Figure 6A and 6B, pAF). Similarly, in cAF, the abundance of higher-molecular-weight complexes was clearly reduced and again JPH2 was not detected (Figure 8A and 8B, cAF). Although PPP1R3A was not detected for technical reasons in these samples, coimmunoprecipitation confirmed the presence of PPP1R3A in the complex in human atria (Figure XIX in the online-only Data Supplement). These data identify a previously unknown qualitative and quantitative disruption of higher-molecular-weight RyR2/SERCA2a/PLN

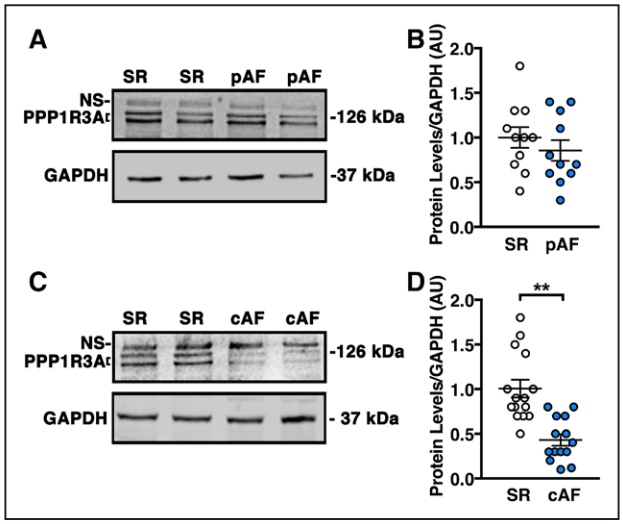

Figure 7. PPP1R3A protein expression is reduced in atria of patients with cAF.
Representative Western blots and corresponding dot plots from human atrial biopsy samples showing slight but nonsignificant decrease in PPP1R3A expression levels (bottom 2 bands) in patients with early-stage (paroxysmal) AF (**A** and **B**) and significant decrease in PPP1R3A expression levels in patients with late-stage (chronic) AF (**C** and **D**). Data represent mean±SEM and were analyzed using unpaired 2-tailed Student *t* test (**P<0.01 vs SR control). cAF indicates chronic atrial fibrillation; NS, nonsignificant; pAF, paroxysmal atrial fibrillation; PPP1R3A, protein phosphatase 1 regulatory subunit type 3A; and SR, sinus rhythm.

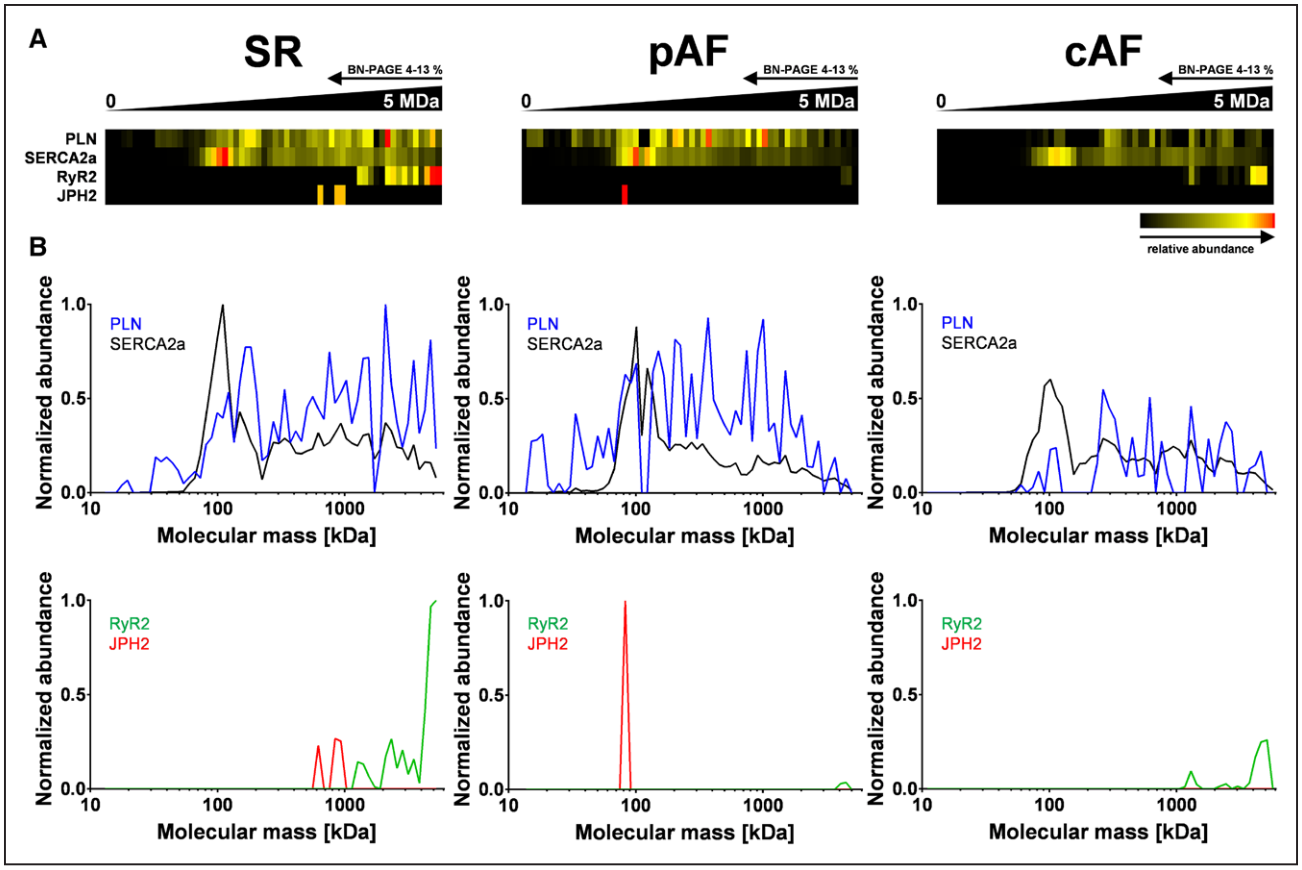

Figure 8. Higher-molecular-weight complexes in human atrial tissue are disrupted in atrial fibrillation.
A, Heat map representation of the migration profile of PLN, SERCA2a, RyR2, and JPH2 in human atrial tissue (total membrane fraction). Atrial samples were obtained from patients with SR, pAF, or chronic AF. **B**, In SR (pseudo-control; see Table II in the online-only Data Supplement), profiling shows comigration of PLN/SERCA2a and RyR2 in higher-molecular-weight complexes (>1 MDa) and, in addition, JPH2 (≤1 MDa). In contrast, in pAF the RyR2 channel was nearly abolished in higher-molecular-weight complexes, and only monomeric JPH2 exists. In contrast, in cAF, the abundance of higher-molecular-weight complexes was decreased and JPH2 was not detected any longer. BN-PAGE indicates Blue Native polyacrylamide gel electrophoresis; cAF, chronic atrial fibrillation; JPH2, Junctophilin-2; pAF, paroxysmal atrial fibrillation; PLN, phospholamban; RyR2, ryanodine receptor type 2; SERCA2a, sarco/endoplasmic reticulum calcium ATPase-2a; and SR, sinus rhythm.

complexes, which confirms and extends our data from mouse ventricles to human atria, and shows a potentially relevant loss of JPH2 in AF.

DISCUSSION

Dysregulation of steady-state protein phosphorylation plays an important role in atrial arrhythmogenesis, but the mechanisms remain poorly understood because of a lack of understanding of global versus local phosphatase regulation. In the heart, PP1c associates with nearly 200 known R-subunits that govern its local regulation by modulating subcellular localization, substrate specificity, and catalytic activity. We demonstrate for the first time that the PP1c R-subunit PPP1R3A mediates PP1c regulation of both RyR2 and PLN, and is an essential integrator of cardiac SR-Ca^{2+} cycling. Our IP/mass spectrometry data identified PPP1R3A as a novel RyR2-binding partner, and further studies confirmed that PPP1R3A mediates binding of the PP1-catalytic subunit PP1c to RyR2. Similarly, we confirmed that PPP1R3A also mediates binding of PP1c to PLN. Using complexome profiling, we identified PPP1R3A within an extended SR macromolecular complex that included RyR2, PLN, and SERCA2a. We discovered that cardiac PPP1R3A deficiency in mice is associated with increased atrial RyR2 and PLN phosphorylation, arrhythmogenic atrial SR-Ca^{2+} leak, and susceptibility to AF. Finally, we found that PPP1R3A is reduced in patients with cAF, revealing PPP1R3A downregulation as a potentially novel mechanism underlying aberrant phosphatase targeting in AF pathogenesis.

RyR2 Regulation by PP1 in AF

The impact of altered RyR2 phosphorylation in cardiac diseases remains controversial.[14] Nevertheless, the majority of studies exploring dysregulation of RyR2 in AF have shown that hyperphosphorylation of RyR2 at residues S2814 and S2808 promotes SR-Ca^{2+} leak, spontaneous Ca^{2+}-release events, and delayed afterdepolarizations that may underlie arrhythmogenic triggered activity in atrial cardiomyocytes.[21,35,36] Phosphorylation of RyR2 is mediated by PKA and CaMKII, whereas

dephosphorylation is mediated by PP1 and protein phosphatase 2A. However, it is still incompletely understood which RyR2 phosphorylation sites are dephosphorylated by PP1 and protein phosphatase 2A.[21] Chiang et al[22] demonstrated that PP1 R-subunit PPP1R9B (spinophilin) targets PP1c to RyR2. However, genetic ablation of spinophilin in mice reduced PP1c binding to RyR2 by only ≈60%, suggesting that PP1c may bind to the channel through alternative targeting mechanism(s). Consistent with this observation, our findings from *Ppp1r3a*-KO mice revealed that PPP1R3A also mediates PP1c targeting to RyR2, accounting for ≈47% of total PP1c binding. It is interesting to note that, whereas ablation of spinophilin increased RyR2 phosphorylation exclusively at S2814, ablation of PPP1R3A increased RyR2 phosphorylation exclusively at S2808. These findings suggest differential roles for spinophilin versus PPP1R3A in site-specific regulation of RyR2, the significance of which remains to be elucidated.

One unexpected finding was that *Ppp1r3a* ablation resulted in an atrial-specific phenotype in 4- to 5-month-old mice. Although RyR2-S2808 phosphorylation was increased in both atria and ventricles of *Ppp1r3a*-KO mice, only atrial cardiomyocytes exhibited excessive RyR2-mediated SR-Ca^{2+} leak, and only atrial arrhythmias were detected with programmed electrical stimulation in *Ppp1r3a*-KO mice. This may be attributable to 3-fold higher PPP1R3A protein levels in atria, but also suggests a differential role for RyR2-S2808 phosphorylation in atrial versus ventricular excitation-contraction coupling. Indeed, recently we identified a conserved, differential subcellular phosphorylation mechanism specific for atrial cardiomyocytes, where only junctional RyR2 clusters are constitutively phosphorylated by PKA and CaMKII, in contrast to nonjunctional clusters.[6,37] The Ca^{2+}-handling alterations observed in ventricular cardiomyocytes of *Ppp1r3a*$^{-/-}$ mice (≈30% increase in SERCA2a activity without diastolic SR-Ca^{2+} leak) may even be beneficial in the setting of chronic cytosolic Ca^{2+} overload; however, further studies are needed to fully understand the importance of PPP1R3A in the ventricle.

PLN Regulation by PP1

PLN is a critical mediator of SR-Ca^{2+} reuptake through regulation of SERCA2a. In its dephosphorylated form, PLN inhibits SERCA2a activity. Phosphorylation by PKA at PLN-S16 and by CaMKII at PLN-T17 relieves this inhibition, increasing the rate of SR-Ca^{2+} transport and SERCA2a activity. Early work established that dephosphorylation of PLN is mediated primarily by PP1.[38] However, recently, Akaike et al[39] reported that the newly identified PP2Ce is also able to dephosphorylate cardiac PLN, specifically at T17, but not at S16. Our findings support the notion that PP1 dephosphorylates both S16 and T17 of PLN, because both sites were hyperphosphorylated in the absence of PPP1R3A. Nevertheless, it is possible that PPP1R3A also mediates targeting of PP2Ce to PLN, which may explain why we observed a larger increase in T17 than in S16 phosphorylation in *Ppp1r3a*-KO mice. This possibility may be the subject of future investigation, because currently no antibodies sensitive enough to detect cardiac PP2Ce are available.

PLN is apparently less important in regulating atrial (versus ventricular) SERCA2a function because of a lower PLN/SERCA2a ratio in murine atrial tissue.[6] In our studies, *Ppp1r3a* ablation resulted in increased phosphorylation of atrial PLN at S16 and T17, which was associated with a 30% increase in SERCA2a activity in *Ppp1r3a*-KO atrial cardiomyocytes. Although RyR2-mediated SR-Ca^{2+} leak was exacerbated with 100 nmol/L isoprenaline in KO cardiomyocytes, the difference in SERCA2a activity disappeared, indicating that maximal SERCA2a activity was restored in *Ppp1r3a*-KO atrial cardiomyocytes, consistent with 2-fold higher expression of SERCA2a in atria versus ventricles.[6,37]

New Insights Into the SR-Ca^{2+}–Handling Regulatome

Complexome profiling is a powerful, unbiased method for analysis of higher-molecular-weight complexes, previously established to identify protein constituents of mitochondrial oxidative phosphorylation supercomplexes (S_0-S_n).[25,30] Here, we used complexome profiling in combination with superresolution STED microscopy to study the dual role of PPP1R3A in regulating both RyR2 and SERCA2a/PLN complexes.[24,40] Our data suggest that PPP1R3A forms an integral part of a previously unappreciated SR membrane complex comprising both the RyR2 Ca^{2+} release channel and the SERCA2a/PLN Ca^{2+} reuptake unit. In addition, we found that this higher-molecular-weight Ca^{2+} cycling complex is disrupted in patients with AF. Recent work has revealed that membrane proteins including RyR2 form supramolecular clusters with inbuilt functional heterogeneity to enhance and coordinate the critical threshold to external stimuli.[41] Our findings provide fundamental and potential clinical insight into the spatial and molecular architecture of these important SR-Ca^{2+}–handling machines, and suggest that the RyR2 and SERCA2a complexes may physically interact to precisely coordinate SR-Ca^{2+} release and reuptake in local SR nanodomains.

Global Versus Local PP1 Dysfunction in AF

We found that PPP1R3A targets PP1c to both RyR2 and PLN in mouse heart, and that PPP1R3A protein levels are reduced in patients with AF. It is interesting to note that, although global PP1c levels were decreased in our cAF tissue samples, earlier studies from tissues of

patients with AF have reported paradoxically increased PP1c expression and activity.[42,43] Nevertheless, this was associated with heterogeneous steady-state phosphorylation of contractile and Ca^{2+}-handling proteins.[13,44] For example, PLN and RyR2 were found to be hyperphosphorylated in cAF (consistent with our findings), whereas cMyBP-C (cardiac myosin binding protein C) was hypophosphorylated.[21] The surprising finding that PP1c targeting to RyR2 seems to be locally reduced despite globally increased expression and activity may be explained by altered binding to one (or more) of the PP1 regulatory subunits. Recent IP/mass spectrometry findings by Chiang and colleagues[19] have revealed that the PP1 R-subunit interactome is altered in patients with AF, underscoring the importance of investigating changes in local phosphatase regulation rather than changes in global phosphatase levels and activity. Atrial cardiomyocytes from *Ppp1r3a*-KO mice revealed increased diastolic SR-Ca^{2+} leak, which may promote arrhythmogenic delayed afterdepolarizations. This, together with our data from patients with AF, suggests that reduced PPP1R3A levels contribute to aberrant atrial Ca^{2+} handling by altering local targeting of PP1c to both RyR2 and PLN.

Potential Limitations

For this work, we generated and validated a *Ppp1r3a*-KO mouse model. One limitation of this model is that ablation of PPP1R3A was not atrial-specific. Nevertheless, we did not observe ventricular abnormalities that may have confounded the AF phenotype, nor did we find gross morphological changes in *Ppp1r3a*-KO mice, in contrast to others who reported increased weight gain in *Ppp1r3a*-KO attributable to impaired glycogen metabolism in skeletal muscle.[45] Another potential limitation is that *Ppp1r3a*-KO mice did not exhibit spontaneous AF at the age studied (4–5 months); however, studies in older mice may reveal an aging phenotype with subsequent development of spontaneous AF. PPP1R3A mediates phosphatase regulation of 2 key Ca^{2+}-handling proteins. Future studies crossing *Ppp1r3a*-KO mice to either PLN or RyR2 mutants should be performed to decipher the precise functional effects of PPP1R3A on each protein. We cannot exclude the possibility that PPP1R3A mediates PP1 targeting to proteins other than RyR2 and PLN; however, we believe this is unlikely because PPP1R3A binds to the SR membrane and is therefore not easily accessible to non–SR-bound proteins.[30] Finally, in the present cohort of patients with AF, we did not observe an increase in RyR2 phosphorylation at Ser2808 as reported in some,[8,11,36,46] but not all[47–49] previous studies. Differences in type and evolution of the underlying atrial cardiomyopathy may potentially contribute to and explain these inconsistent results.

Conclusions

We have shown for the first time that the PP1-regulatory subunit PPP1R3A is an RyR2-binding protein responsible for targeting PP1c to the PKA site (S2808) of RyR2. We also discovered that PPP1R3A targets PP1c to both the PKA site (S16) and CaMKII site (T17) of PLN, ultimately forming higher-molecular-weight protein complexes that include RyR2, PLN, and SERCA2a. To our knowledge, our study is the first to detect this native, extended SR-Ca^{2+} cycling complex in the heart. Furthermore, our finding that PPP1R3A modulates PP1 regulation of both RyR2 and PLN to promote SR-Ca^{2+} leak sheds light on the complex mechanisms by which altered phosphatase targeting may contribute to aberrant SR-Ca^{2+} cycling in AF pathophysiology.

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Disclosures

Dr Wehrens is a founding partner of Elex Biotech, a start-up company that developed drug molecules to target ryanodine receptors for the treatment of cardiac arrhythmias. The other authors report no conflicts.

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