



# Ero1 $\alpha$ -Dependent ERp44 Dissociation From RyR2 Contributes to Cardiac Arrhythmia

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**BACKGROUND:** Oxidative stress in cardiac disease promotes proarrhythmic disturbances in Ca<sup>2+</sup> homeostasis, impairing luminal Ca<sup>2+</sup> regulation of the sarcoplasmic reticulum (SR) Ca<sup>2+</sup> release channel, the RyR2 (ryanodine receptor), and increasing channel activity. However, exact mechanisms underlying redox-mediated increase of RyR2 function in cardiac disease remain elusive. We tested whether the oxidoreductase family of proteins that dynamically regulate the oxidative environment within the SR are involved in this process.

**METHODS:** A rat model of hypertrophy induced by thoracic aortic banding (TAB) was used for ex vivo whole heart optical mapping and for Ca<sup>2+</sup> and reactive oxygen species imaging in isolated ventricular myocytes (VMs).

**RESULTS:** The SR-targeted reactive oxygen species biosensor ERroGFP showed increased intra-SR oxidation in TAB VMs that was associated with increased expression of Ero1 $\alpha$  (endoplasmic reticulum oxidoreductase 1 alpha). Pharmacological (EN460) or genetic Ero1 $\alpha$  inhibition normalized SR redox state, increased Ca<sup>2+</sup> transient amplitude and SR Ca<sup>2+</sup> content, and reduced proarrhythmic spontaneous Ca<sup>2+</sup> waves in TAB VMs under  $\beta$ -adrenergic stimulation (isoproterenol). Ero1 $\alpha$  overexpression in Sham VMs had opposite effects. Ero1 $\alpha$  inhibition attenuated Ca<sup>2+</sup>-dependent ventricular tachyarrhythmias in TAB hearts challenged with isoproterenol. Experiments in TAB VMs and human embryonic kidney 293 cells expressing human RyR2 revealed that an Ero1 $\alpha$ -mediated increase in SR Ca<sup>2+</sup>-channel activity involves dissociation of intraluminal protein ERp44 (endoplasmic reticulum protein 44) from the RyR2 complex. Site-directed mutagenesis and molecular dynamics simulations demonstrated a novel redox-sensitive association of ERp44 with RyR2 mediated by intraluminal cysteine 4806. ERp44-RyR2 association in TAB VMs was restored by Ero1 $\alpha$  inhibition, but not by reducing agent dithiothreitol, as hypo-oxidation precludes formation of covalent bond between RyR2 and ERp44.

**CONCLUSIONS:** A novel axis of intraluminal interaction between RyR2, ERp44, and Ero1 $\alpha$  has been identified. Ero1 $\alpha$  inhibition exhibits promising therapeutic potential by stabilizing RyR2-ERp44 complex, thereby reducing spontaneous Ca<sup>2+</sup> release and Ca<sup>2+</sup>-dependent tachyarrhythmias in hypertrophic hearts, without causing hypo-oxidative stress in the SR.

**GRAPHIC ABSTRACT:** A graphic abstract is available for this article.

**Key Words:** cardiovascular diseases ■ constriction ■ heart failure ■ homeostasis ■ oxidoreductases

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A mismatch between increased oxidative stress and defective antioxidant defenses in cardiovascular disease (CVD) contributes to aberrant Ca<sup>2+</sup> homeostasis.<sup>1,2</sup> As the main Ca<sup>2+</sup> release channel of the sarcoplasmic reticulum (SR), the cardiac RyR2 (ryanodine receptor) provides a major pathway for untimely Ca<sup>2+</sup>

release that can precipitate Ca<sup>2+</sup>-dependent ventricular tachyarrhythmias causative of sudden cardiac death.<sup>3,4</sup> It is well established that increased oxidative stress in CVD such as heart failure (HF), hypertrophy, diabetic cardiomyopathy, or aging enhances RyR2 activity via reversible posttranslational modifications including CaMKII (Ca<sup>2+</sup>/

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Supplemental Material is available at <https://www.ahajournals.org/doi/suppl/10.1161/CIRCRESAHA.121.320531>.

For Sources of Funding and Disclosures, see page 723.

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## Novelty and Significance

### What Is Known?

- Oxidative stress is a major contributor to cardiac dysfunction in the diseased heart.
- Cardiac disease-associated redox modifications of the RyR2 (ryanodine receptor type 2) sarcoplasmic reticulum (SR) Ca<sup>2+</sup> release channel are implicated in arrhythmogenesis.
- Clinical trials with antioxidants to improve cardiac function have showed limited success.

### What New Information Does This Article Contribute?

- We have identified a novel RyR2 interacting partner—ERp44 (endoplasmic reticulum protein 44)—that covalently associates with the last intraluminal loop of RyR2 Cysteine 4806 in a redox-sensitive manner, stabilizing RyR2 complex activity.
- Cardiac hypertrophy-mediated upregulation of ER stress-inducible Ero1 $\alpha$  (endoplasmic reticulum oxidoreductase 1 alpha) removes ERp44 from the complex, contributing to RyR2 dysfunction and thereby increasing propensity to Ca<sup>2+</sup>-dependent ventricular tachyarrhythmias.
- Ero1 $\alpha$  inhibition restores ERp44-RyR2 association in myocytes from hypertrophic hearts, reducing proarrhythmic spontaneous SR Ca<sup>2+</sup> release.

Reduction of sudden cardiac death incidence due to ventricular tachyarrhythmias remains a major challenge in the postindustrial world. Hyperactivity of the RyR2 SR Ca<sup>2+</sup> release channel complex due to posttranslational oxidative modifications plays a key role in Ca<sup>2+</sup>-dependent arrhythmogenesis in cardiac hypertrophy and failure. However, treatment with antioxidants to reduce RyR2 reactive cysteines, primarily located at the cytosolic face of the channel, demonstrates only partial functional recovery, which might explain limited success of clinical trials. We have identified a novel RyR2 interacting partner, intra-SR protein ERp44, which covalently associates with the RyR2 at its luminal face and stabilizes RyR2 complex activity. Importantly, RyR2 association via cysteine 4806 with ERp44 can be achieved only at oxidizing conditions and antioxidant treatment precludes disulfide bond formation. We found that in cardiac hypertrophy, RyR2-ERp44 association is disrupted by ER stress-inducible oxidoreductase Ero1 $\alpha$ , contributing to proarrhythmic spontaneous SR Ca<sup>2+</sup> release. Our data suggest that Ero1 $\alpha$  may be a promising therapeutic target to reduce arrhythmogenesis and improve cardiac function during hypertrophy and heart failure, without disturbing the finely balanced intra-SR redox environment.

## Nonstandard Abbreviations and Acronyms

<b>CVD</b>	cardiovascular disease
<b>Ero1<math>\alpha</math></b>	endoplasmic reticulum oxidoreductase 1 alpha
<b>ERp44</b>	endoplasmic reticulum protein 44
<b>HEK293</b>	human embryonic kidney 293
<b>HF</b>	heart failure
<b>hRyR2</b>	human ryanodine receptor type 2
<b>IP3R1</b>	inositol triphosphate receptor type 1
<b>IP3R2</b>	inositol triphosphate receptor type 2
<b>PDI</b>	protein disulfide isomerase
<b>PVC</b>	premature ventricular contraction
<b>ROS</b>	reactive oxygen species
<b>RyR2</b>	ryanodine receptor type 2
<b>SR</b>	sarcoplasmic reticulum
<b>TAB</b>	thoracic aortic banding
<b>VF</b>	ventricular fibrillation
<b>VM</b>	ventricular myocytes
<b>VT</b>	ventricular tachycardia

calmodulin-dependent protein kinase II)-dependent phosphorylation and oxidative modifications of reactive Cysteines in the channel.<sup>5–12</sup> Studies using ventricular myocytes

(VMs) isolated from diseased or aging hearts routinely demonstrate at least partial improvements in Ca<sup>2+</sup> handling when treated with antioxidants or scavengers of reactive oxygen species (ROS).<sup>11,13–15</sup> However, these findings have not translated into effective treatment strategies, and clinical trials aimed to improve intracellular redox balance have failed to attenuate Ca<sup>2+</sup>-dependent arrhythmogenesis.<sup>15,16</sup>

Increased RyR2 activity under oxidizing conditions has been attributed to defective regulation of the channel complex by SR luminal Ca<sup>2+</sup>, a mechanism involved in termination of systolic SR Ca<sup>2+</sup> release and RyR2 refractoriness during diastole, preventing proarrhythmic spontaneous activity of RyR2 clusters.<sup>11,13</sup> The structural region of RyR2 proposed to mediate luminal [Ca<sup>2+</sup>] sensing, that is, amino acids 4789–4844 constituting an intraluminal loop, resides within the SR,<sup>18</sup> an environment far more oxidizing than the cytosol due to a network of PDIs (protein disulfide isomerases) and oxidoreductase enzymes that facilitate protein folding.<sup>19</sup> Given that the overwhelming majority of 21 RyR2 reactive cysteines are located in the larger cytosolic assembly of the channel<sup>20,21</sup> and there exists a very different redox state of the SR when compared with the cytosol,<sup>22</sup> information on how VMs SR redox balance changes in CVD and subsequent implications of this change for RyR2 function remains limited.

Recent evidence suggests that SR oxidoreductase enzymes can modulate Ca<sup>2+</sup> homeostasis in the heart,<sup>23–25</sup>

yet the molecular mechanisms remain largely unexplored. Of particular interest is the luminal oxidoreductase Ero1 $\alpha$  (endoplasmic reticulum oxidoreductase 1 alpha), known to be induced during ER stress as a component of the PERK (protein kinase RNA-like ER kinase) branch of the unfolded protein response.<sup>26</sup> This protein can dynamically regulate the redox environment of the SR, transferring electrons from PDIs to reduce molecular oxygen to H<sub>2</sub>O<sub>2</sub>.<sup>19,27</sup> Of note, Ero1 $\alpha$  directly interacts with PDI protein ERp44 (endoplasmic reticulum protein 44), and this interaction increases with oxidative stress.<sup>19,28–30</sup> Furthermore, ERp44 was reported to inhibit Ca<sup>2+</sup> release channel IP3R1 (inositol triphosphate receptor type 1) by a direct protein-protein interaction that was redox-dependent.<sup>31,32</sup> Importantly, ERp44 interacts with an IP3R1 region<sup>31</sup> that has sequence homology to the last intraluminal loop of RyR2. However, whether ERp44 interaction with RyR2 exerts a stabilizing influence on the SR Ca<sup>2+</sup> release channel complex remains unknown.

In the present study, we investigated the role of dysregulated SR oxidoreductase system in aberrant RyR2 function and Ca<sup>2+</sup>-dependent arrhythmia using a rat model of pressure-overload cardiac hypertrophy. Our results implicate CVD-mediated upregulation of Ero1 $\alpha$  in SR oxidative stress and dissociation of ERp44 from the RyR2 complex promoting spontaneous SR Ca<sup>2+</sup> release in VMs from hypertrophic hearts. Genetic or pharmacological inhibition of Ero1 $\alpha$  restored the intra-SR redox environment and, unlike the reducing agent dithiothreitol, restored the ERp44-RyR2 interaction, markedly improving Ca<sup>2+</sup> homeostasis. Accordingly, Ero1 $\alpha$  inhibition significantly reduced malignant ventricular tachyarrhythmias induced by  $\beta$ -adrenergic stimulation in ex vivo hypertrophic rat hearts.

## METHODS

Detailed materials and methods are described in the [Supplemental Material](#). Key research materials are listed in the Major Resources Table in the [Supplemental Material](#). All procedures involving animals were performed following the National Institutes of Health Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 2011). Procedures were approved by the Institutional Animal Care and Use Committee of The Ohio State University or Rhode Island Hospital. Procedures including patient consent involving human samples were approved by the Human Study Committees of Rush University Medical Center and The Ohio State University.

## Data Availability

Data supporting findings of this study are available from corresponding authors upon reasonable request.

## RESULTS

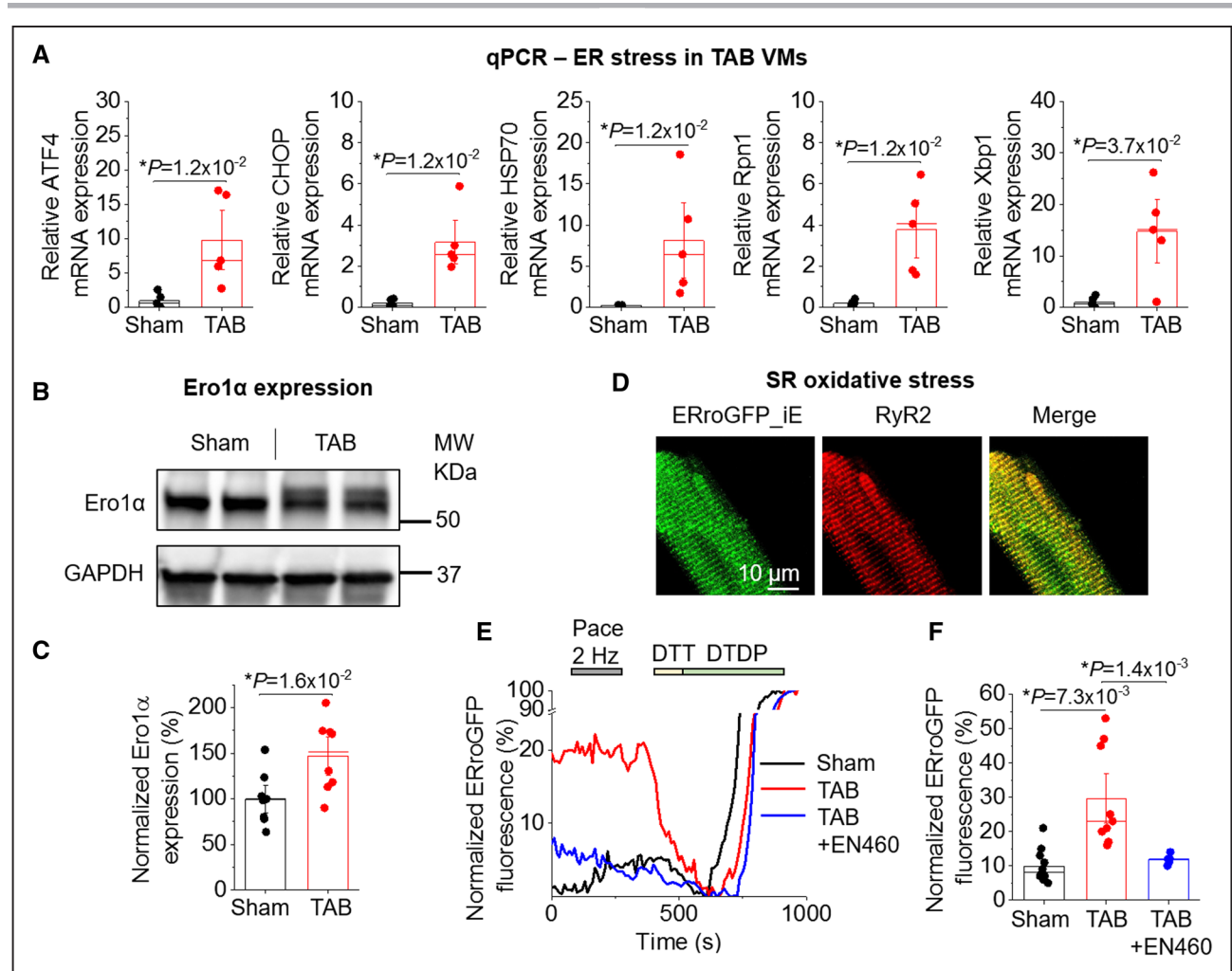
### Upregulation of Oxidoreductase Ero1 $\alpha$ Enhances Intra-SR ROS in Hypertrophic Myocytes

ER stress is critically involved in the development of cardiac hypertrophy and failure.<sup>33</sup> We first tested ER stress

induction using VMs isolated from rats with hypertrophy induced by thoracic aortic banding (TAB), an established model of arrhythmogenesis (Figure 1A).<sup>14,34</sup> Quantitative polymerase chain reaction studies confirmed ER stress this model, which closely followed the pattern observed in human congestive HF (Figure S1). Next we assessed expression levels of SR oxidoreductase, Ero1 $\alpha$ . Western blot analysis demonstrated that in TAB VMs, expression of Ero1 $\alpha$  is increased in comparison to Sham (Figure 1B and 1C). Furthermore, appearance of the slower migrating band in TABs represents the active, reduced form of Ero1 $\alpha$ .<sup>24,35</sup> Likewise, induction of Ero1 $\alpha$  was confirmed in human non-ischemic HF left ventricular tissue samples and samples from canine hearts with HF induced by rapid tachypacing (Figure S2). Next, Sham and TAB VMs were infected with adenovirus coding for intra-SR redox sensor, ERroGFP (Figure 1D).<sup>36,37</sup> Oxidation within the SR was increased in TAB versus Sham VMs, and this was reduced by preincubation with specific Ero1 $\alpha$  inhibitor EN460<sup>38</sup> (20  $\mu$ mol/L, 5 min; Figure 1E and 1F). Control experiments testing the specificity of the chosen high EN460 concentration, used to reduce exposure time, are presented in Figure S3.

### Inhibition of Ero1 $\alpha$ Attenuates Arrhythmic Potential

To directly test whether SR oxidoreductase inhibition could reduce arrhythmic potential, we performed ex vivo optical mapping experiments with Sham and TAB rat hearts. Representative ECG recordings from Sham and TAB hearts are shown in Figure 2A. Ventricular tachycardia or fibrillation (VT/VF) rarely occurred in Sham hearts exposed to  $\beta$ -adrenergic receptor agonist isoproterenol (50 nmol/L), while VF occurred in every TAB heart.<sup>14,34,36</sup> VF in isoproterenol-treated TAB hearts was initiated by repetitive, persistent focal activity from multiple locations, which degenerated into VF with wave breaks and complex waveforms (see Figure S4 and Videos S1–S3), indicating importance of focal activity in triggering VF. However, pretreatment with EN460 (10  $\mu$ mol/L, 30 min) markedly reduced VF induction in TAB hearts (N=1/8 hearts with VF). Importantly, premature ventricular contractions (PVCs) and transient VTs were observed only during the early phase of isoproterenol perfusion but PVCs and transient VTs disappeared after 22 $\pm$ 8 minutes of 50 nmol/L isoproterenol perfusion (N=7/8, Figure 2D, Figure S5). Subsequent addition of 100 nmol/L isoproterenol did not cause PVC and VT (N=7/8), demonstrating the benefit of inhibiting Ero1 $\alpha$  in TAB hearts. Transient VTs were initially observed in TAB hearts pretreated with EN460, which were generated from a single reentry that spontaneously terminated after several rotations. Maps of PVC propagation from TAB hearts with EN460 showed a typical epicardial breakout with a large area synchronously firing (Figure S6), suggesting initial PVCs may originate from the Purkinje system rather than VMs. Inhibition of Ero1 $\alpha$  with EN460 increased Ca<sup>2+</sup> transient amplitude,



**Figure 1. Upregulation of Ero1α (endoplasmic reticulum oxidoreductase 1 alpha) increases intrasarcoplasmic reticulum (SR) reactive oxygen species (ROS) in cardiac disease.**

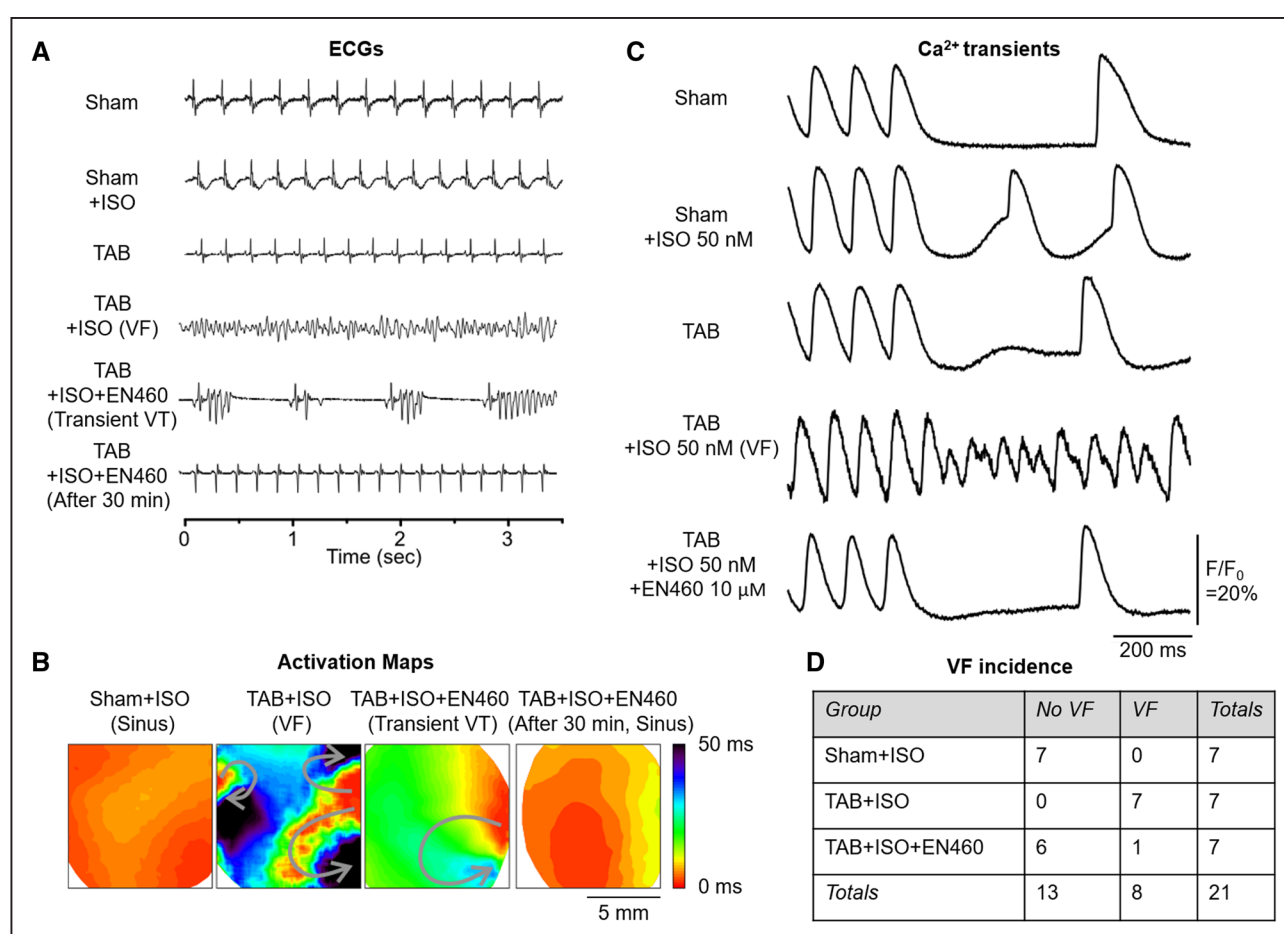
**A**, Relative mRNA expression data from Sham and thoracic aortic banding (TAB) ventricular myocytes (VMs). Data were normalized to housekeeping gene GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and are presented as the ratio of the expression values obtained with TAB RNA vs Sham RNA, which was normalized to 1.0. Relative expression levels were obtained after  $\Delta\Delta Ct$  calculation. *P* values were calculated Mann-Whitney test. **B**, Representative Western blot of Sham and TAB rat VMs and **(C)** mean  $\pm$  SEM Ero1 $\alpha$  signal, normalized to GAPDH. *N*=8 Sham, *N*=8 TAB animals. *P* value obtained using 2-sample Student *t* test. **D**, Cultured VM expressing intra-SR redox probe ERroGFP. **E**, Representative ERroGFP fluorescence traces. VMs were paced (2 Hz, 5 min) and treated with isoproterenol (ISO; 50 nmol/L) or ISO and Ero1 $\alpha$  (endoplasmic reticulum oxidoreductase 1 alpha) inhibitor EN460 (20  $\mu$ mol/L). Signal normalized to minimum (dithiothreitol [DTT], 5 mmol/L) and maximum (2,2'-dithiodipyridine [DTDP], 200  $\mu$ mol/L) fluorescence. **F**, Mean  $\pm$  SEM fluorescence (%). *N*=6 Sham, *N*=6 TAB animals, *n*=9 Sham, *n*=11 TAB, *n*=5 TAB+EN460 VMs. *P* values obtained using 2-level random intercept model with Tukey posthoc.<sup>60</sup>

suggesting EN460 antiarrhythmic effect stems from suppressing proarrhythmic spontaneous  $Ca^{2+}$  release (Figure S7). Accordingly, hearts pretreated with EN460 did not show DAD-like spontaneous  $Ca^{2+}$  release during burst-pause protocol (Figure 2C, Figure S7) nor during PVC or initiation of transient VT, which is typical of TAB hearts even before isoproterenol treatment.

We next tested effects of modulating Ero1 $\alpha$  activity at the cellular level. Adenoviral-mediated overexpression or knockdown of Ero1 $\alpha$  in Sham and TAB VMs was confirmed by Western blot analysis (Figure 3A and 3B). Measured with SR-targeted redox probe ERroGFP, oxidation levels within the SR were increased in Sham VMs with overexpression of Ero1 $\alpha$  (Figure 3 and 3D). Importantly,

Ero1 $\alpha$  knockdown in TAB VMs reduced the redox status to Sham levels. To test whether modulation of Ero1 $\alpha$  activity can modulate RyR2-mediated  $Ca^{2+}$  release, we measured intracellular  $Ca^{2+}$  handling in Fluo-3 loaded Sham and TAB VMs. Assessment of  $Ca^{2+}$  transients and spontaneous  $Ca^{2+}$  waves induced by pace-pause protocol revealed Ero1 $\alpha$  overexpression in Sham VMs recapitulates the TAB phenotype, reducing  $Ca^{2+}$  transient amplitude, spontaneous  $Ca^{2+}$  wave latency (Figure 3E and 3F) and caffeine-sensitive  $Ca^{2+}$  store load (Figure 3G and 3H). All of these changes are indicative of increased RyR2 activity. Conversely, knockdown of Ero1 $\alpha$  in TAB VMs had opposite effects, reducing proarrhythmic RyR2-mediated spontaneous SR  $Ca^{2+}$  release. Treatment





**Figure 2. Inhibition of Ero1 $\alpha$  (endoplasmic reticulum oxidoreductase 1 alpha) oxidoreductase reduces arrhythmogenic potential in hypertrophic rat hearts.**

**A**, ECG recordings of ventricular fibrillation (VF) induction in thoracic aortic banding (TAB) hearts under isoproterenol (ISO; 50 nmol/L). After EN460 (10  $\mu$ mol/L, 30 min), transient ventricular tachycardia (VTs) were observed rather than long-lasting VFs. **B**, Propagation maps of Sham heart (**left**), VF in TAB heart (**center**), and transient VT in the presence of EN460 (**right**). Arrows represent reentry. **C**, Rapid pacing followed by pause-induced spontaneous Ca<sup>2+</sup> release that triggered delayed after depolarizations. EN460 suppressed spontaneous Ca<sup>2+</sup> release. **D**, Number of ex vivo Sham, TAB, and TAB+EN460 hearts exhibiting VF. N=7 Sham+ISO, N=7 TAB+ISO, N=8 TAB+ISO+EN460 hearts. \* $P=2.1 \times 10^{-4}$ , obtained using Freeman-Halton extension of the Fisher exact test.

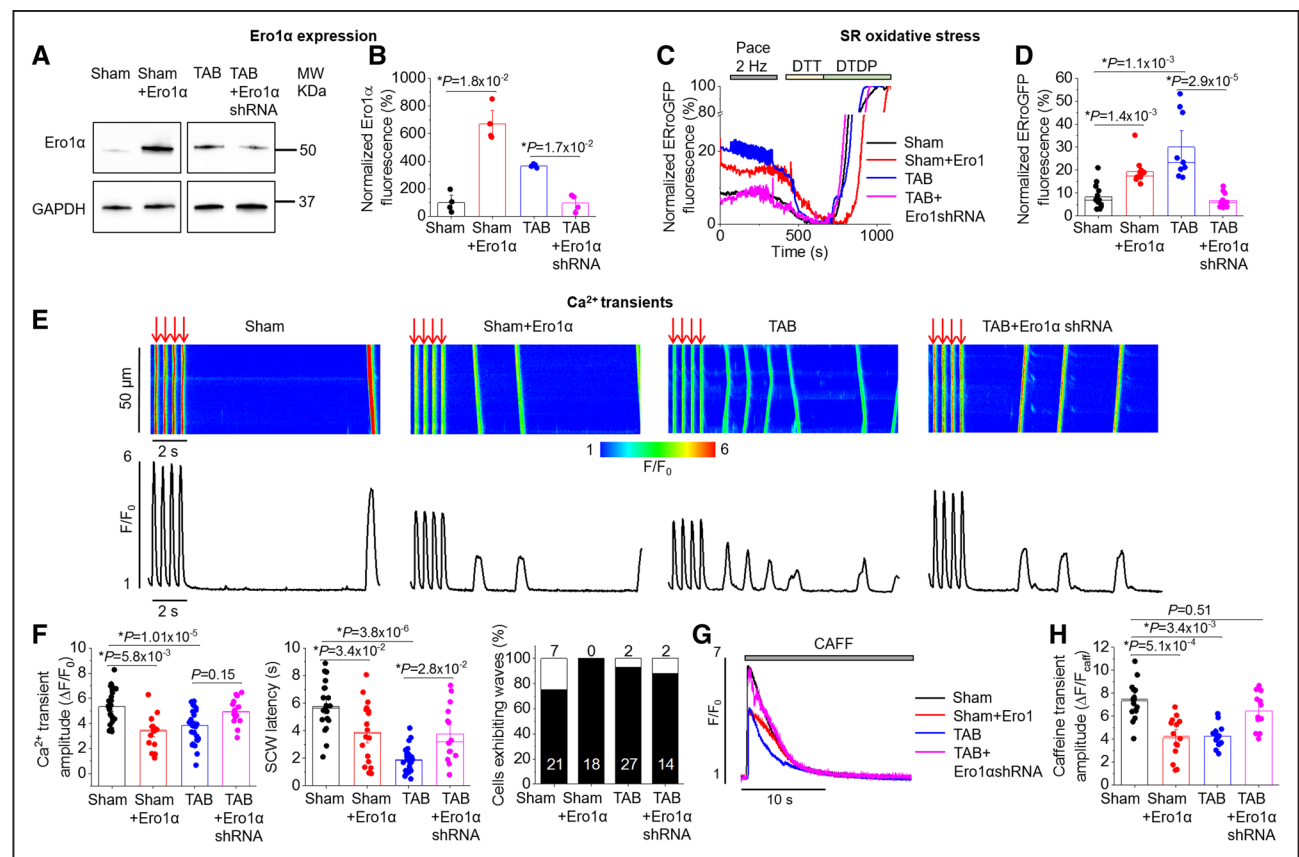
of TAB VMs with 4PBA (500 nmol/L<sup>39</sup>) for 24 hours to inhibit ER stress recapitulated findings obtained from loss-of-Ero1 $\alpha$  experiments (Figure S8). While Brefeldin A treatment of Sham VMs (1  $\mu$ mol/L, 24 hours<sup>40</sup>) followed a similar trend to findings obtained with gain-of-Ero1 $\alpha$  experiments (Figure S8), differences were not significant, perhaps indicating 24 hours was not sufficient to elicit an effect. These data strongly suggest that ER stress-linked SR oxidoreductase activity modulates SR Ca<sup>2+</sup> release in addition to SR redox homeostasis.

### ERp44 Interacts With RyR2 and the Complex Is Reduced in Hypertrophic Myocytes

We performed immunolocalization experiments with Sham and TAB VMs, probing for RyR2 and ERp44 expression (Figure 4A, negative controls Figure S9), and the extent of direct signal overlap was quantified using Manders split coefficients. RyR2/ERp44 signal overlap in TAB VMs

was reduced compared with TAB VMs (Figure 4B). Western blot analysis demonstrated this is not attributable to altered expression of ERp44, since the RyR2/ERp44 ratio remained similar (Figure 4C and 4D). Of note, co-IP experiments using HF samples from canine hearts show a trend decreasing ERp44 complexed with RyR2, consistent with results in rats (Figure S10).

Oxidoreductase Ero1 $\alpha$  is a binding partner of ERp44, and loss of Ero1 $\alpha$  function in transgenic mice has been shown to be protective in pressure-overloaded hearts.<sup>24</sup> Application of EN460 (20  $\mu$ mol/L, 30 minutes) increased RyR2-ERp44 signal coincidence. Notably, treatment of TAB VMs with general ER stress inhibitor 4PBA also increased RyR2-ERp44 coincidence (Figure S11). In contrast, thiol-reducing and disulfide bridge-breaking agent dithiothreitol (5 mmol/L, 10 minutes) did not restore protein colocalization. These findings were mirrored in assessment of native RyR2-ERp44 protein complexes of Sham, TAB and EN460/dithiothreitol



**Figure 3. Altered expression levels of Ero1 $\alpha$  (endoplasmic reticulum oxidoreductase 1 alpha) modulate intrasarcoplasmic reticulum (SR) redox state and RyR2 (ryanodine receptor type 2)-mediated Ca<sup>2+</sup> release.**

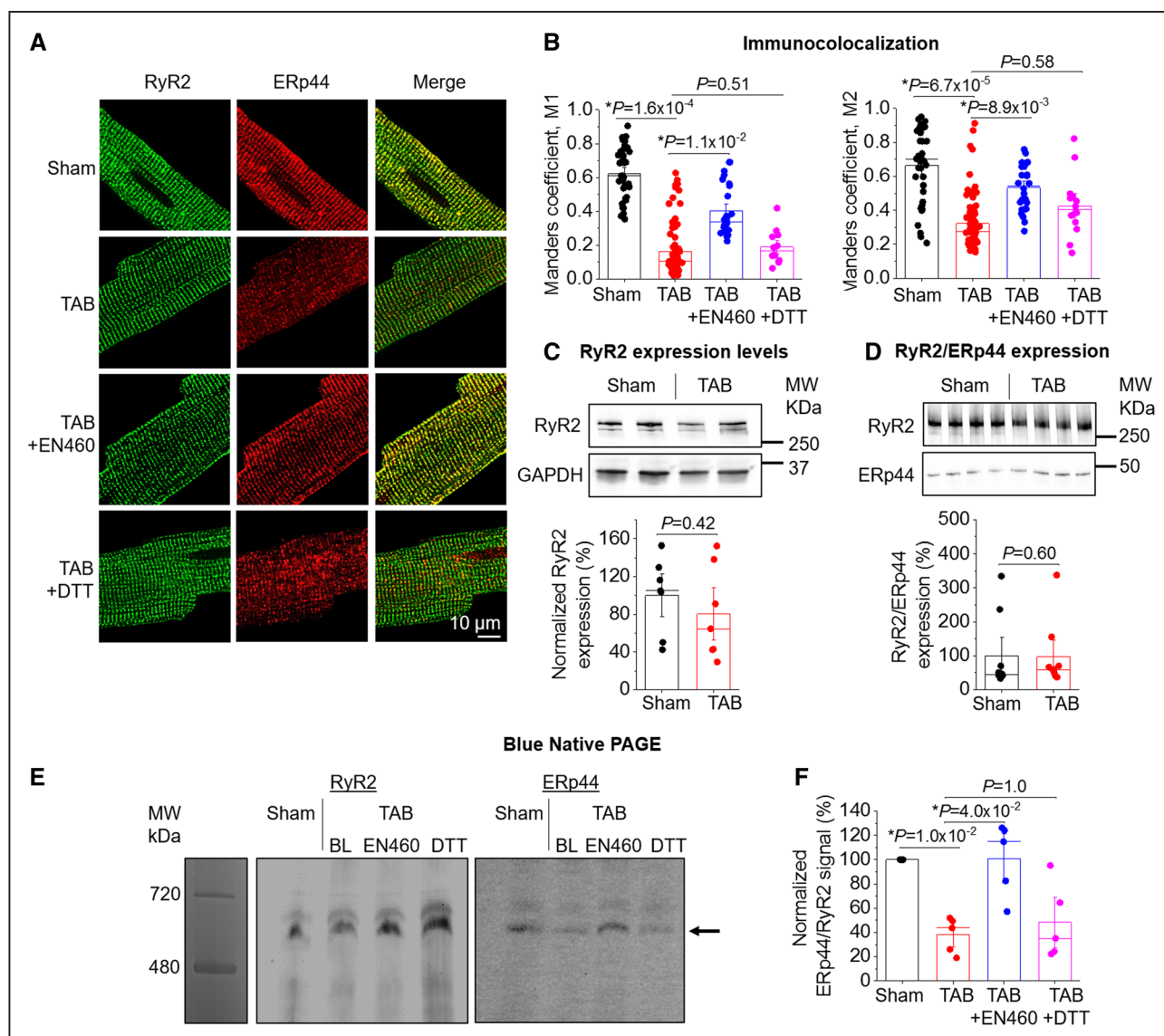
**A**, Representative Western blot demonstrating adenoviral overexpression of Ero1 $\alpha$ , and shRNA-mediated Ero1 $\alpha$  knockdown in cultured Sham and thoracic aortic banding (TAB) ventricular myocytes (VMs), respectively. **B**, Mean $\pm$ SEM Ero1 $\alpha$  signal, normalized to GAPDH (glyceraldehyde-3-phosphate dehydrogenase). N=4 Sham, N=4 TAB animals. *P* values calculated using Kruskal-Wallis with Dunn posthoc. **C**, Representative SR redox probe ERroGFP fluorescence traces. VMs were treated with isoproterenol (ISO; 50 nmol/L) and paced at 2 Hz (5 min). Dithiothreitol (DTT; 5 mmol/L) and 2,2'-dithiodipyridine (DTDP; 200  $\mu$ mol/L) were used to obtain minimum and maximum fluorescence. **D**, Mean $\pm$ SEM ERroGFP fluorescence (%). N=6 Sham, N=5 TAB animals, n=11 Sham, n=12 Sham+Ero1 $\alpha$ , n=9 TAB, n=13 TAB+Ero1 $\alpha$ -shRNA VMs. *P* values obtained using 2-level random intercept model with Tukey posthoc. **E**, Fluo-3 fluorescence ( $F/F_0$ ) profiles of ISO-treated VMs undergoing 2 Hz pace-pause protocol. **F**, Mean $\pm$ SEM Ca<sup>2+</sup> transient amplitude at 2 Hz ( $\Delta F/F_0$ ) and spontaneous Ca<sup>2+</sup> wave (SCW) latency (s). N=9 Sham, N=9 TAB animals, n=25 Sham, n=18 Sham+Ero1 $\alpha$ , n=29 TAB VMs, n=14 TAB+Ero1 $\alpha$ -shRNA VMs. *P* values obtained using 2-level random intercept model with Tukey posthoc. **G**, Representative traces of caffeine-induced Ca<sup>2+</sup> transients (10 mmol/L). **H**, Mean $\pm$ SEM caffeine-sensitive Ca<sup>2+</sup> store load. n=15 Sham, n=14 Sham+Ero1 $\alpha$ , n=15 TAB, n=12 TAB+Ero1 $\alpha$ -shRNA VMs; from the same animals used in **E** and **F**. *P* values obtained using 2-level random intercept model with Tukey posthoc.

treated TAB VMs in blue native gel electrophoresis (BN-PAGE, Figure 4E and 4F). A reduced amount of ERp44 was observed along with RyR2 in TAB versus Sham group, indicative of decreased protein-protein interaction. The amount of ERp44 that migrated with RyR2 in TAB VMs was restored by treatment with EN460 but not with dithiothreitol. These data suggest that association of ERp44 with RyR2 is redox- and Ero1 $\alpha$ -sensitive.

### Disruption of the RyR2-ERp44 Interaction Enhances RyR2 Channel Activity in a Heterologous Cell System

Although IP3R2 (inositol triphosphate receptor type 2) is established as the major isoform in VMs,<sup>41</sup> the expression of IP3R1 has been reported as increased in end-stage

human HF.<sup>42</sup> However, IP3R inhibition with xestospongin C<sup>43</sup> in Ero1 $\alpha$ -overexpressing Sham VMs did not fully restore Ca<sup>2+</sup> handling (Figure S12). Furthermore, IP3R inhibition was not as effective as Ero1 $\alpha$  knockdown in TAB VMs, suggesting aberrant RyR2 complex assembly plays a significant role in Ero1 $\alpha$ -mediated disruption in Ca<sup>2+</sup> homeostasis. To test this further, and avoid confounding effects of IP3R1-ERp44 interaction,<sup>31</sup> we investigated Ca<sup>2+</sup> release dynamics in human embryonic kidney 293 (HEK293) cells lacking all 3 isoforms of IP3R (HEK293 IP3R-3KO<sup>44</sup>). Spontaneous luminal Ca<sup>2+</sup> release events monitored with R-CEPIAer<sup>45</sup> were used as an assessment of RyR2 channel function in cells expressing recombinant hRyR2 (human RyR2) constructs<sup>46,47</sup> (Figure 5A). Co-expression of short hairpin RNA to knockdown endogenous ERp44 (Figure 5B) increased

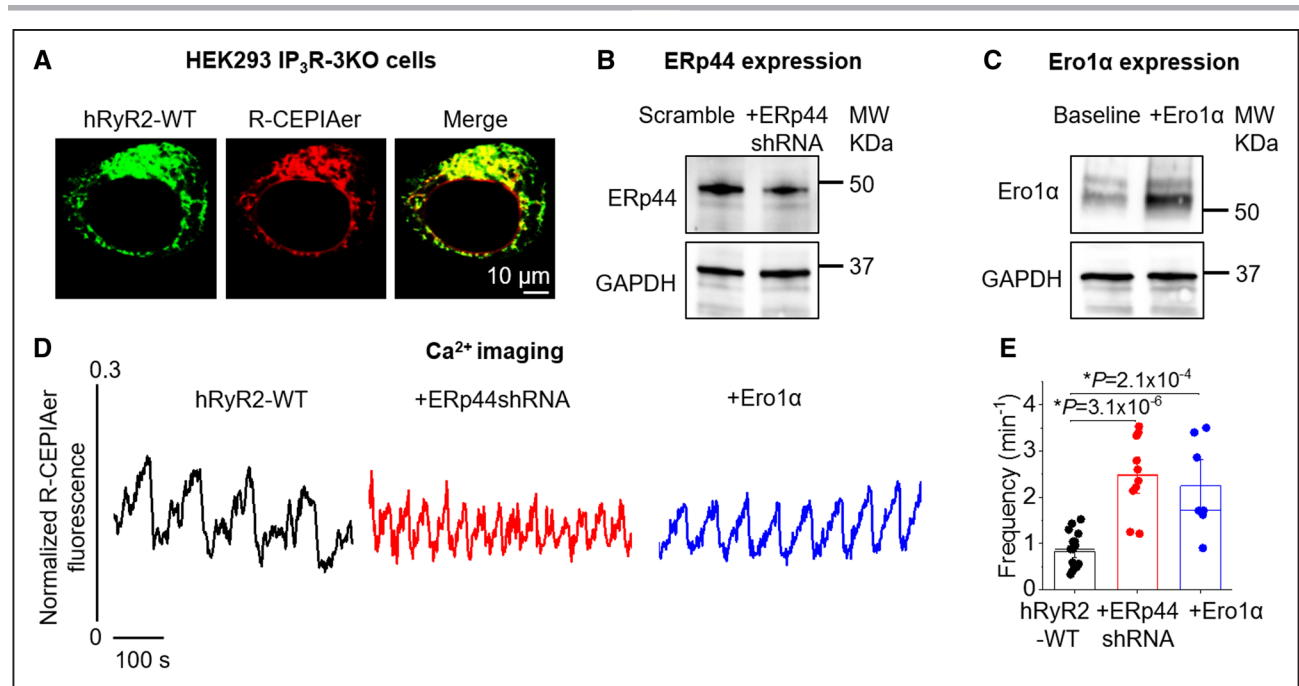


**Figure 4. ERp44 (endoplasmic reticulum protein 44) is a binding partner of RyR2 (ryanodine receptor type 2) and this interaction is reduced in cardiac disease.**

**A**, Representative images of Sham and thoracic aortic banding (TAB) ventricular myocytes (VMs) probed with anti-RyR2 and anti-ERp44 antibodies. VMs were treated with EN460 (20  $\mu$ mol/L, 30 min) or dithiothreitol (DTT; 5 mmol/L, 10 min). **B**, Mean  $\pm$  SEM for Manders coefficients M1 and M2. N=4 Sham, N=6 TAB animals, n=36 Sham, n=81 TAB, n=26 TAB+DTT, N=14 TAB+EN460 VMs. *P* values obtained using 2-level random intercept model with Tukey posthoc. **C**, Representative Western blot demonstrating expression of RyR2 and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) in Sham and TAB VMs, and mean  $\pm$  SEM RyR2 signal, normalized to GAPDH. N=7 Sham animals, N=7 TAB animals. *P* values obtained using 1-way ANOVA with Bonferroni posthoc. **D**, Representative Western blot of Sham and TAB VMs probed for RyR2 and ERp44 expression, and mean  $\pm$  SEM normalized ERp44/RyR2 signal (%). N=6 Sham, N=6 TAB animals. *P* value obtained by Mann-Whitney test. **E**, Representative BN-PAGE images of RyR2 from fresh Sham and TAB VMs, immunoblotted for RyR2 and ERp44. VMs were treated with EN460 (20  $\mu$ mol/L, 30 min) or DTT (5 mmol/L, 10 min). Arrow indicates native RyR2 protein complexes. **F**, Mean  $\pm$  SEM normalized ERp44/RyR2 signal (%). N=5 Sham, N=5 TAB animals. *P* values obtained using Kruskal-Wallis with Dunn posthoc.

hRyR2-WT (wild type)-mediated  $\text{Ca}^{2+}$  release events (Figure 5D and 5E). To corroborate the hypothesis that increased expression/activity of oxidoreductase Ero1 $\alpha$  promoted dissociation of ERp44 from RyR2, Ero1 $\alpha$  was overexpressed (Figure 5C). hRyR2-WT-mediated  $\text{Ca}^{2+}$  release events increased with Ero1 $\alpha$  overexpression (Figure 5D and 5E), indicative that modulation of PDI proteins within the SR effectively modulate RyR2 activity.

We reasoned that interaction of specific Cysteine residues of RyR2 with ERp44 underlies luminal  $\text{Ca}^{2+}$  regulation of the channel, and this interaction is disrupted with Ero1 $\alpha$  overexpression. Figure 6A shows Cysteine 4806 (Cys4806, human nomenclature) within the last luminal loop of RyR2 that we posit is the primary Cysteine residue mediating RyR2-ERp44 interaction. Cys4806Ser substitution (hRyR2-MUT, Figure 6B) eliminated any putative thiol group-mediated covalent bond



formation with ERp44. Confocal microscopy revealed that the frequency of Ca<sup>2+</sup>-release events in cells expressing hRyR2-MUT was increased when compared with hRyR2-WT (Figure 6C–6E). These data highlight a putative role for luminal Cys4806 in regulating RyR2-mediated Ca<sup>2+</sup> release. Critically, increased Ero1 $\alpha$  or reduced ERp44 expression levels significantly modulated Ca<sup>2+</sup> release via hRyR2-WT (Figure 5D and 5E) but were unable to change Ca<sup>2+</sup> release via hRyR2-MUT (Figure 6F and 6G).

### RyR2-ERp44 Interaction Is Redox-Dependent

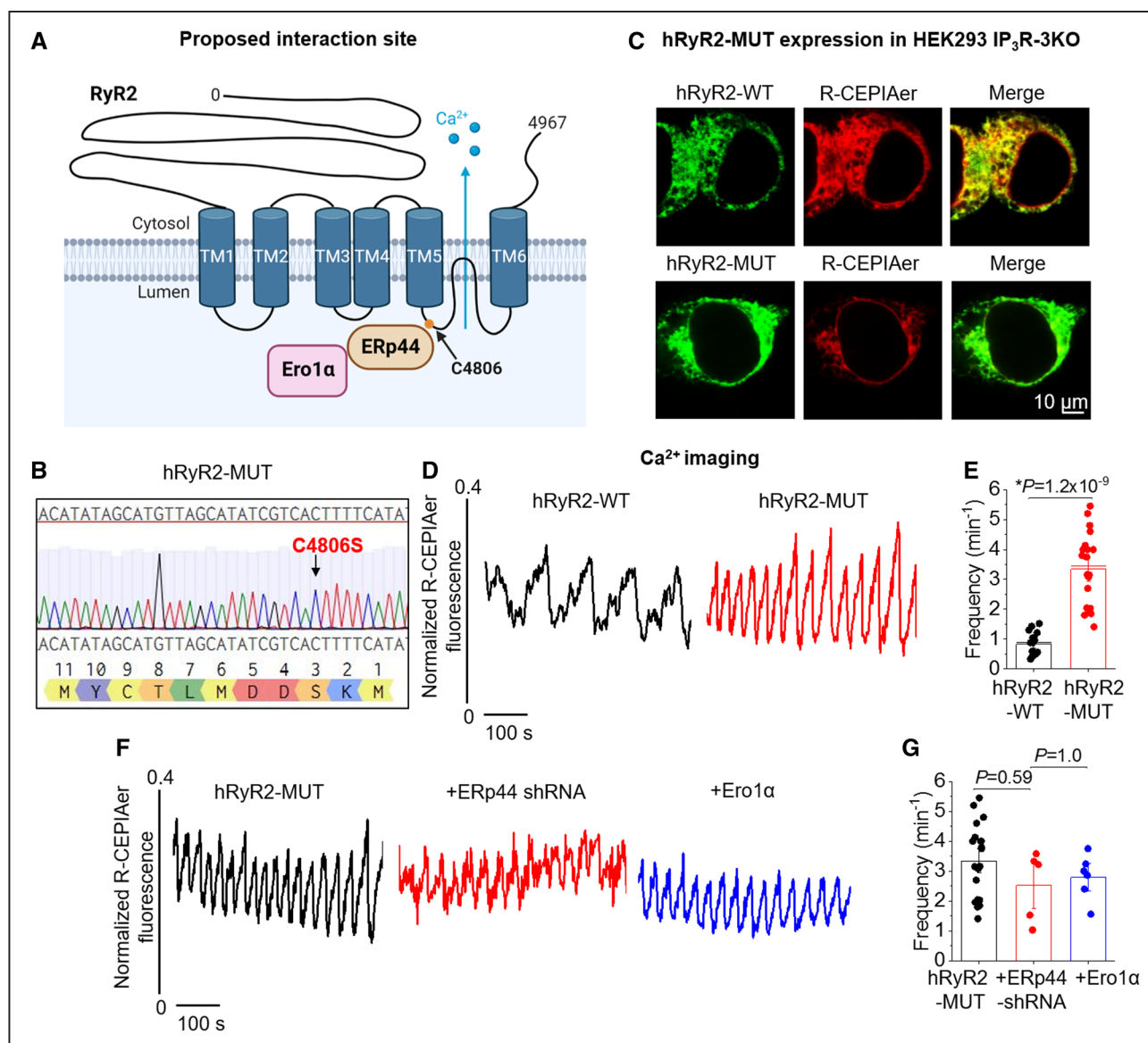
We next performed *in silico* calculations to model redox-dependent RyR2-ERp44 interaction (Figure 7). ERp44 forms mixed disulfides with other proteins via cysteine 29. Yang et al<sup>48</sup> resolved the crystal structure of ERp44 in complex with SR-resident protein PRXD4 (peroxiredoxin 4), and showed that ERp44 binds the oxidized, but not reduced, form. The reduced segment of PRXD4 (PDB ID: 3tkp, 3tjb) involved in thiol-disulfide interchange reactions with ERp44 structurally aligned with reduced structures of RyR2 (PDB ID: 6jh6, amino acids 4751–4882) and IP3R1 (PDB ID: 3jav; Figure 7A). High degree of structural homology with the ERp44-PRXD4 crystal structure allowed us to estimate how the ERp44-RyR2 complex might form using molecular docking

software (Figure 7B). We repeated multiple iterations of docking and short molecular dynamics simulations (up to 100 ns) of both the reduced and oxidized RyR2 forms in complex with the opened state of ERp44. Docking studies revealed that RyR2-Cys4806 and ERp44-cysteine 29 could proximally associate to form a disulfide bridge (Figure 7C), and predicted oxidized RyR2 retained RyR2-ERp44 interaction, whereas the reduction of RyR2 caused the dissociation of proteins within picoseconds (Figure 7D, Videos S4 and S5). Cys4806Ser substitution resulted in more pronounced RyR2-ERp44 distance fluctuations but not complete dissociation. These data suggest RyR2-Cys4806 is an important mediator of interaction with ERp44, possibly forming a disulfide bridge with cysteine 29 of ERp44 during normal luminal redox states.

### DISCUSSION

In the present study, we aimed to unravel molecular determinants of the RyR2 luminal redox sensor. The basal SR redox potential is higher than that in the cytosol, and we have shown that this promotes association of the SR-resident protein ERp44 with the last intraluminal loop of RyR2 to modulate SR Ca<sup>2+</sup> release. Activation of oxidoreductase Ero1 $\alpha$  in cardiac hypertrophy, in addition to increasing SR oxidation, dissociates ERp44 from the





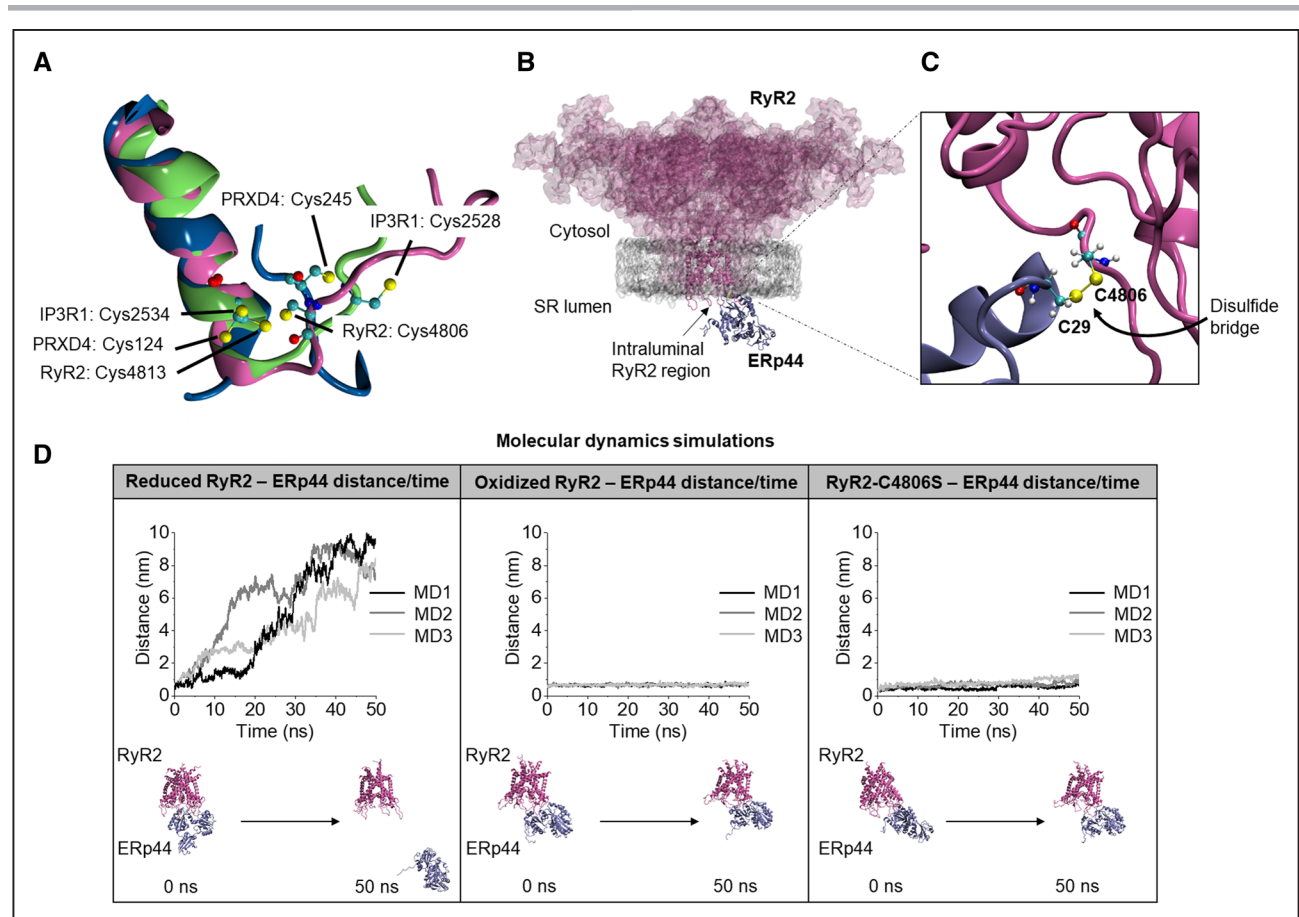
**Figure 6. Mutation of intraluminal cysteine Cys4806 increases human RyR2 (ryanodine receptor type 2) activity.**

**A**, Schematic of RyR2 monomer with proposed ERp44 (endoplasmic reticulum protein 44)-interacting Cys4806. **B**, DNA chromatogram of recombinant hRyR2 (human RyR2) plasmid indicating Cys4806Ser substitution, to prevent interaction with ERp44. Image created with Biorender.com. **C**, HEK293 IP<sub>3</sub>R-3KO cell transfected with hRyR2-WT (wildtype) or hRyR2-MUT (hRyR2-Cys4806Ser) and R-CEPIAer. **D**, Representative R-CEPIAer fluorescence traces in cells co-transfected with hRyR2-WT or hRyR2-MUT. Caffeine (20 mmol/L) was applied to deplete the store. Signal normalized to minimum (2 mmol/L EGTA and 20 μmol/L ionomycin) and maximum (20 mmol/L Ca<sup>2+</sup> and 20 μmol/L ionomycin) fluorescence. **E**, Mean±SEM frequency of Ca<sup>2+</sup> waves. Cells were assessed from 6 transfections. n=17 hRyR2-WT, n=22 hRyR2-MUT cells. *P* value obtained using 2-sample Student *t* test. **F**, Representative R-CEPIAer fluorescence traces in cells co-expressing hRyR2-MUT±ERp44-shRNA or Ero1α (endoplasmic reticulum oxidoreductase 1 alpha). **G**, Mean±SEM for Ca<sup>2+</sup> wave frequency (min<sup>-1</sup>). Cells were assessed from 6 transfections. n=20 hRyR2-MUT, n=5 hRyR2-MUT+ERp44-shRNA, n=6 hRyR2-MUT+Ero1α cells. *P* values obtained using Kruskal-Wallis with Dunn posthoc.

complex and augments RyR2-mediated spontaneous SR Ca<sup>2+</sup> release. We also showed that inhibition of Ero1α in VMs from hypertrophic hearts restored SR ROS levels and RyR2 association with ERp44, improving intracellular Ca<sup>2+</sup> handling and most importantly, reducing the propensity for spontaneous Ca<sup>2+</sup> release following β-adrenergic stimulation. In ex vivo hypertrophic hearts challenged with β-adrenergic agonist, inhibition of Ero1α produced striking attenuation of Ca<sup>2+</sup>-dependent ventricular arrhythmia.

### Ero1α Upregulation Promotes Proarrhythmic Spontaneous SR Ca<sup>2+</sup> Release in Cardiac Hypertrophy

Abnormally high RyR2 channel activity has a central role in contractile deficiency and arrhythmogenesis underlying sudden cardiac death<sup>3,4,49</sup> and posttranslational modifications of the channel including PKA/CaMKII-mediated phosphorylation or oxidation are involved, as well as their interplay (ie, ROS-CaMKII-RyR2) at the cytosolic face of



**Figure 7. Molecular dynamics simulations suggest RyR2 (ryanodine receptor type 2)-ERp44 (endoplasmic reticulum protein 44) interaction is redox dependent.**

**A**, Structural alignment of RyR2 and IP3R1 (inositol triphosphate receptor type 1) intraluminal regions to peroxiredoxin-4 (PRDX4) region involved in formation of a disulfide bridge with ERp44. RyR2 is in purple (PDB ID: 6JH6), IP3R1 is in green (PDB ID: 3JAV), reduced PRDX4 is in blue (PDB ID: 3TJF). **B**, General view of docked RyR2-ERp44 complex. The intraluminal region of RyR2 with Cys4806 is exposed to the sarcoplasmic reticulum (SR) lumen, making it possible for ERp44 to bind. **C**, Zoomed disulfide bridge between Cys4806 of RyR2 and Cys29 of ERp44. RyR2 is highlighted in purple (PDB ID: 6JH6), ERp44 is highlighted in blue (PDB ID: 5XWM), and the POPC membrane is translucent. **D**, Graphs showing distance between  $\beta$ -carbon atom of RyR2's Cys4806 and sulfur atom of ERp44's Cys29 for reduced, oxidized, and mutated RyR2-ERp44 complexes as a function of time for 3 full-length simulations (MD1, MD2, and MD3). The reduced form shows increasing distance and completely dissociates during the early period of simulations. Both oxidized and mutated forms show complex retention.

RyR2.<sup>5,50</sup> Here, we show a novel regulatory axis for RyR2 ROS modulation involving the luminal ROS sensor Ero1 $\alpha$  and the luminal RyR2-binding protein ERp44. Our previous studies showed that  $\beta$ -adrenergic stimulation induces Ca<sup>2+</sup>-dependent VT/VF in 100% of ex vivo hearts from rats with pressure-overload induced hypertrophy.<sup>14,34,36</sup> Complementary cellular studies confirmed that arrhythmogenic RyR2 dysfunction was, to a large extent, triggered by the effects of oxidative stress on the channel.<sup>14,34</sup> Here, we aimed to delineate the mechanisms by which disease-associated dysregulation of PDIs, responsible for oxidation and reduction within the SR, modulates RyR2 activity. Oxidoreductase Ero1 $\alpha$ , constitutively expressed in many tissues including the heart, is activated under conditions of stress.<sup>19,25,27</sup> Our results show increased Ero1 $\alpha$  expression as part of the ER stress response in VMs from TAB rat hearts (Figure 1) and human and canine HF samples (Figures S1 and S2). These data are consistent with previous

findings from a mouse model of HF induced by thoracic aortic constriction.<sup>24</sup> Ero1 $\alpha$  upregulation is expected to increase H<sub>2</sub>O<sub>2</sub> production within the ER, and we confirmed this in TAB rat VMs using the SR-targeted redox state biosensor ERroGFP (Figures 1 and 3). Both acute Ero1 $\alpha$  inhibition by small molecule inhibitor EN460 and more chronic short hairpin RNA-mediated knockdown normalized SR oxidation levels in TAB VMs. Conversely, Ero1 $\alpha$  overexpression in Sham VMs increased SR oxidation levels. These data implicate Ero1 $\alpha$  as an important mediator of dysregulated SR redox potential in cardiac pathology. Furthermore, as H<sub>2</sub>O<sub>2</sub> is capable of crossing intracellular membranes and traveling substantial distances within the cell,<sup>51</sup> our results support the notion that the SR is a significant source of ROS, in addition to well established sources such as NOX2 (NADPH oxidase 2) or mitochondria.<sup>8,52–54</sup> The possibility also exists that H<sub>2</sub>O<sub>2</sub> may directly modulate reactive cysteines located in the cytosolic domains

of RyR2. Indeed, immunoprecipitation experiments demonstrated a significant reduction of RyR2 oxidation level in TAB VMs treated with EN460 (Figure S13). Moreover, Ero1 $\alpha$  knockdown in TABs reduced mitochondrial matrix H<sub>2</sub>O<sub>2</sub> levels measured using mitochondria-targeted biosensor MLS-HyPer7 (Figure S14). While the latter might be a direct result of reduced H<sub>2</sub>O<sub>2</sub> emission from the SR, our recent demonstration of RyR2 Ca<sup>2+</sup>-leak-dependent disruption of mitochondrial Ca<sup>2+</sup> and ROS homeostasis<sup>52</sup> would favor a mechanism that involved RyR2 channel stabilization.

Ero1 $\alpha$  overexpression in Sham VMs treated with isoproterenol decreased Ca<sup>2+</sup> transient amplitude and SR Ca<sup>2+</sup> content and shortened spontaneous Ca<sup>2+</sup> wave latency, consistent with increased RyR2 activity (Figure 3). In TAB VMs, Ero1 $\alpha$  knockdown normalized Ca<sup>2+</sup> homeostasis, increasing Ca<sup>2+</sup> transient amplitude, loading of the SR, and reducing the propensity for proarrhythmic spontaneous Ca<sup>2+</sup> waves. Further investigations of WT- and Cys4806Ser hRyR2 in HEK293 cells devoid of IP3R isoforms revealed that Ero1 $\alpha$  overexpression potently increases RyR2 activity (Figure 5). Accordingly, treatment of ex vivo TAB hearts with an Ero1 $\alpha$  inhibitor attenuated Ca<sup>2+</sup>-dependent tachyarrhythmia evoked by  $\beta$ -adrenergic challenge (Figure 2, Figure S5). Previous work using VMs from transgenic mouse model of Ero1 $\alpha$  loss-of-function showed a marked decrease in Ca<sup>2+</sup> transient amplitudes and SR Ca<sup>2+</sup> content, both in baseline conditions and under isoproterenol.<sup>24</sup> However, pressure-overloaded Ero1 $\alpha$  mutant hearts exhibited significant preservation of cardiac function in comparison to WTs, pointing to Ero1 $\alpha$ 's key role in aberrant excitation-contraction coupling during HF development. Our data extend these findings and indicate that strategies to reduce Ero1 $\alpha$  activity, which is increased in CVD, can be of benefit in therapeutically improving contractility and limiting proarrhythmic spontaneous RyR2-mediated SR Ca<sup>2+</sup> release.

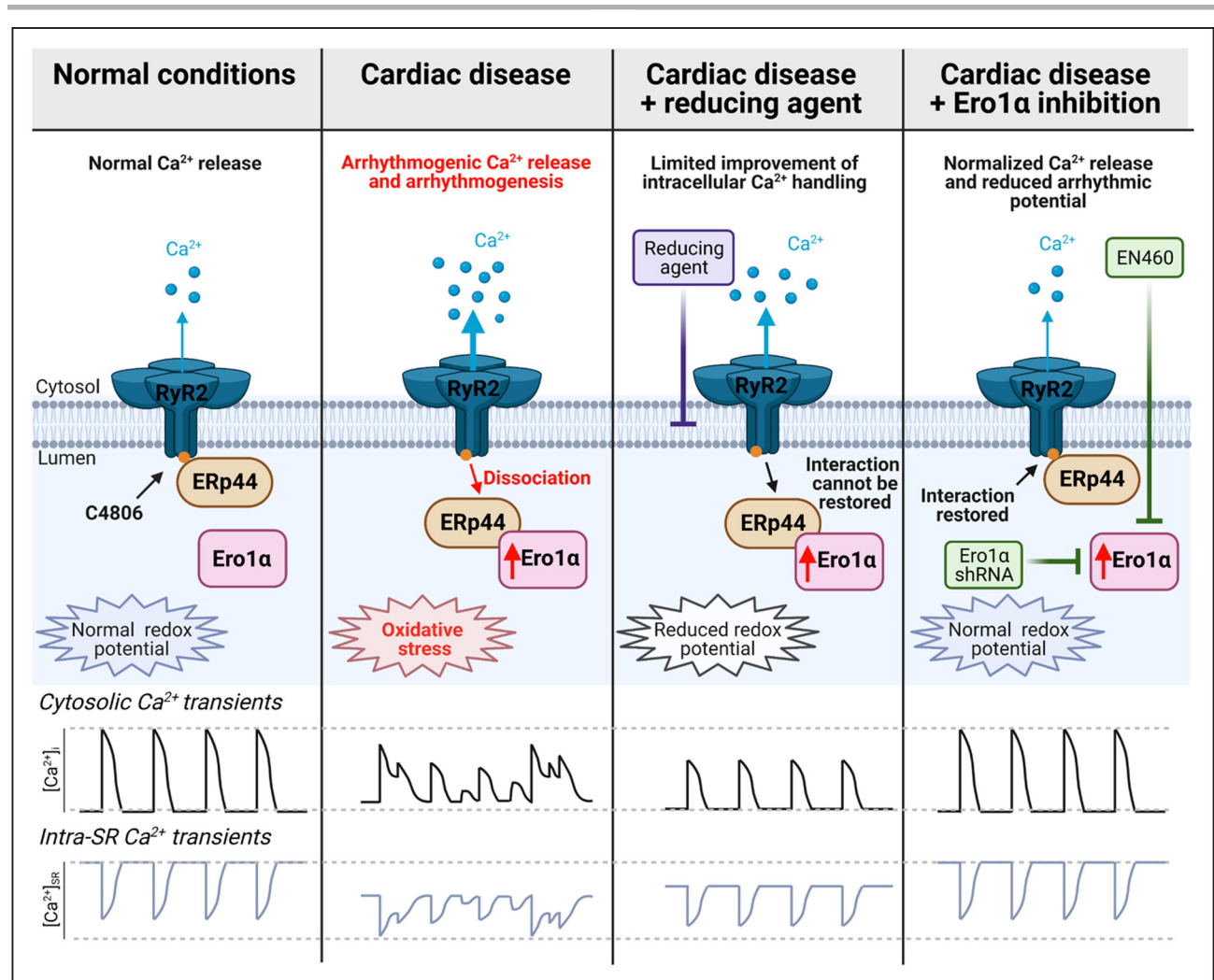
A recent study established antiarrhythmic effects induced by PERK knockout in myocardial infarct mice.<sup>55</sup> Of note, Ero1 $\alpha$  is upregulated as part of the ER stress-induced unfolded protein response, downstream of PERK.<sup>56</sup> Indeed, modulation of ER stress produced similar effects to altering Ero1 $\alpha$  levels on Ca<sup>2+</sup> handling (Figure 3, Figures S8 and S10). These data support that Ero1 $\alpha$  upregulation is a key factor contributing to Ca<sup>2+</sup>-dependent arrhythmogenesis in conditions associated with ER stress and the unfolded protein response including hypertrophy, myocardial infarct and HF.

### RyR2 Interacts With ERp44 in a Redox-Sensitive Manner

Besides regulating the redox environment, Ero1 $\alpha$  was shown to modulate function of SR Ca<sup>2+</sup> channel multimolecular complexes, such as IP3R1, by disrupting tethering of luminal protein ERp44 to the complex.<sup>31</sup> While

our results do not exclude the importance of Ero1 $\alpha$ -mediated IP3R1-ERp44 disassociation in hypertrophic VMs (Figure S12), we demonstrate that Ero1 $\alpha$  inhibition in TAB VMs restores the association of ERp44 with RyR2 (Figure 4), stabilizing channel activity.

RyR2 is established as a major redox sensor in cardiac myocytes.<sup>211</sup> Reversible modifications of reactive cysteines modulate channel activity.<sup>21</sup> Increased disulfide bond formation has been linked to pathological increase in RyR2 activity in cardiac disease, and reducing agents that attenuated this increase were associated with improvements in Ca<sup>2+</sup> handling.<sup>11,13,57</sup> However, clinical studies did not substantiate a therapeutic effect of antioxidants in improving outcomes for patients with HF.<sup>16,17</sup> In line with this, reducing agents such as dithiothreitol or mercaptopropionyl glycine produced only partial recovery in Ca<sup>2+</sup> handling in VMs from HF or infarcted hearts.<sup>11,13</sup> Furthermore, sequential application of oxidizing agent 2,2'-dithiodipyridine, which promotes disulfide bond formation, followed by dithiothreitol in VMs from healthy hearts did not result in full restoration of SR Ca<sup>2+</sup> content,<sup>11</sup> implying only partial stabilization of RyR2 activity when reactive cysteines at both cytosolic and luminal RyR2 sides are reduced. Our new data demonstrates that oxidative stress promotes the dissociation of important regulatory molecular component(s) from the RyR2 complex, which cannot be reversed by application of a reducing agent. Given the major difference in redox potential between the cytosol and the SR,<sup>58</sup> we surmised this regulatory component was most likely a resident SR protein that associates with a small luminal portion of RyR2 in a redox-sensitive manner. ERp44 expression is restricted to endoplasmic reticulum and its major role is thought to be in forming disulfide bonds with the target proteins in the Golgi and for transport to its final location in the ER.<sup>28,59</sup> Higo et al<sup>31</sup> showed that ERp44 associates with an IP3R1 intraluminal loop, a region homologous to the last intraluminal loop of RyR2, to affect IP3R1 regulation. This mechanism was subsequently shown to operate in VMs from ERp44 knockdown mice.<sup>32</sup> Our immunolocalization studies showed spatial colocalization between RyR2 and ERp44 in VMs from Sham hearts was lost in VMs from TAB hearts (Figure 4). Furthermore, treatment of TAB VMs with dithiothreitol did not reverse this pattern. Further evidence of RyR2-ERp44 interaction was obtained using BN-PAGE, which confirmed the presence of ERp44 in the RyR2 macromolecular complex of healthy VMs, as well as its dissociation in hypertrophic VMs that was not reversible by dithiothreitol (Figure 4). Using HEK293 cells, we showed that ERp44 knockdown with short hairpin RNAs increases RyR2 activity (Figure 5). Moreover, substitution of RyR2 Cys4806Ser disrupted putative disulfide bond formation with ERp44 and led to an increase in RyR2 activity. ERp44 knockdown failed to produce additional effects (Figure 6). Molecular docking computer simulations confirmed that RyR2-ERp44 association is highly unstable in reducing conditions requiring high redox potential (Figure 7).



**Figure 8.** Scheme depicting the putative multimolecular RyR2 (ryanodine receptor type 2) intraluminal redox sensor and sarcoplasmic reticulum (SR) oxidoreductase-dependent mechanisms of cardiac Ca<sup>2+</sup>-dependent arrhythmia.

Figure created with Biorender.com.

Importantly, given the very high redox potential in the SR of TAB VMs, which must favor RyR2-ERp44 association, the fact that ERp44 is lost from the complex implies involvement of active enzymatic reaction to break the bond between RyR2 and ERp44. It has been shown that activated oxidoreductase Ero1 $\alpha$  associates with ERp44, removing it from its binding partners.<sup>28,29</sup> Indeed Ero1 $\alpha$  inhibition in TAB VMs, unlike dithiothreitol treatment, restored RyR2-ERp44 colocalization and the abundance of ERp44 in the RyR2 complex (Figure 4). Moreover, the importance of the Ero1 $\alpha$  effect on ERp44-RyR2 association versus its effect on ROS was underscored by the lack of additional effects of Ero1 $\alpha$  overexpression in HEK293 cells expressing hRyR2-MUT (Figure 6).

## Conclusions

To summarize, we have identified a novel RyR2 interacting partner—ERp44—that covalently associates with the last intraluminal loop of RyR2 in a redox-sensitive manner,

stabilizing RyR2 complex activity. In addition to increasing SR redox potential, Ero1 $\alpha$  upregulation removes ERp44 from the complex, contributing to RyR2 dysfunction and thereby increased propensity to Ca<sup>2+</sup> dependent ventricular tachyarrhythmias in hypertrophic hearts. Our data suggest that ER stress-induced Ero1 $\alpha$  may be a promising therapeutic target to reduce arrhythmogenesis and improve cardiac function during the development of HF, without compromising the finely balanced intra-SR redox environment (Figure 8).

## ARTICLE INFORMATION

Received November 19, 2021; revision received January 5, 2022; accepted January 11, 2022.

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### Acknowledgments

We thank the Genomics Shared Resource at The Ohio State University, supported by the NCI CCSG P30CA016058, for Sanger sequencing and The Ohio Supercomputer Center.

### Sources of Funding

This work was supported by The Ohio State University President's Postdoctoral Scholars Award (S. Hamilton), National Institutes of Health (NIH) National Heart, Lung, and Blood Institute (NHLBI) K99HL155492 (S. Hamilton), NIH NIAAA R01AA024769 and NIH NHLBI R01HL146744 (X. Ai), British Heart Foundation RG/15/6/31436 (C.H. George), NIH NHLBI R01HL132213 (J.P. Davis), NIH NHLBI R01HL063043, NIH NHLBI R01HL074045 (S. Gyorke), NIH NHLBI R01HL138579 (S. Gyorke and J.P. Davis), NIH NHLBI R01HL142588, and NIH NHLBI HL121796 (D. Terentyev).

### Disclosures

None.

### Supplemental Materials

Expanded Materials and Methods

Figures S1–S14

Videos S1–S5

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