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REGULAR ARTICLE

Ero1α-Dependent ERp44 Dissociation From RyR2 Contributes to Cardiac Arrhythmia

Shanna Hamilton, Radmila Terentyeva, Vladimir Bogdanov, Tae Yun Kim, Fruzsina Perger, Jiajie Yan, Xun Ai, Cynthia A. Carnes, Andriy E. Belevych, Christopher H. George, Jonathan P. Davis, Sandor Gyorke, Bum-Rak Choi, Dmitry Terentyev

BACKGROUND: Oxidative stress in cardiac disease promotes proarrhythmic disturbances in Ca^{2+} homeostasis, impairing luminal Ca^{2+} regulation of the sarcoplasmic reticulum (SR) Ca^{2+} 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^{2+} 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 oxidoreductase Ero1α. Pharmacological (EN460) or genetic Ero1α inhibition normalized SR redox state, increased Ca^{2+} transient amplitude and SR Ca^{2+} content, and reduced proarrhythmic spontaneous Ca^{2+} waves in TAB VMs under β-adrenergic stimulation (isoproterenol). Ero1α overexpression in Sham VMs had opposite effects. Ero1α inhibition attenuated Ca^{2+}-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 Ero1α-mediated increase in SR Ca^{2+}-channel activity involves dissociation of intraluminal protein ERp44 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 Ero1α 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 Ero1α has been identified. Ero1α inhibition exhibits promising therapeutic potential by stabilizing RyR2-ERp44 complex, thereby reducing spontaneous Ca^{2+} release and Ca^{2+}-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 disease ■ constriction ■ heart failure ■ homeostasis ■ oxidoreductase

A mismatch between increased oxidative stress and defective antioxidant defenses in cardiovascular disease (CVD) contributes to aberrant Ca^{2+} homeostasis.[1,2] As the main Ca^{2+} release channel of the sarcoplasmic reticulum (SR), the cardiac RyR2 (ryanodine receptor) provides a major pathway for untimely Ca^{2+} release that can precipitate Ca^{2+}-dependent ventricular tachyarrhythmias causative of sudden cardiac death.[3,4] It is well established that increased oxidative stress in CVD such as heart failure (HF), hypertrophy, diabetic cardiomyopathy, or aging

Correspondence to: Shanna Hamilton, Physiology and Cell Biology, The Ohio State University, 460 Medical Center Dr, Columbus, OH 43210, Email shanna.hamilton@osumc.edu or Dmitry Terentyev, Physiology and Cell Biology, The Ohio State University, 460 Medical Center Dr, Columbus, OH 43210, Email dmitry.terentyev@osumc.edu
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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^{2+} 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—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 oxidoreductase Ero1α removes ERp44 from the complex, contributing to RyR2 dysfunction and thereby increasing propensity to Ca^{2+}-dependent ventricular tachyarrhythmias.
- Ero1α inhibition restores ERp44-RyR2 association in myocytes from hypertrophic hearts, reducing proarrhythmic spontaneous SR Ca^{2+} 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^{2+} release channel complex due to posttranslational oxidative modifications plays a key role in Ca^{2+}-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 Ero1α, contributing to proarrhythmic spontaneous SR Ca^{2+} release. Our data suggest that Ero1α 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

CVD	cardiovascular disease
HEK293	human embryonic kidney 293
HF	heart failure
HF	heart failure
hRyR2	human ryanodine receptor type 2
IP3R1	inositol triphosphate receptor type 1
IP3R2	inositol triphosphate receptor type 2
PDI	protein disulfide isomerase
PRXD4	peroxiredoxin 4
PVC	premature ventricular contraction
ROS	reactive oxygen species
RyR2	ryanodine receptor type 2
SR	sarcoplasmic reticulum
TAB	thoracic aortic banding
VF	ventricular fibrillation
VM	ventricular myocytes
VT	ventricular tachycardia

enhances RyR2 activity via reversible posttranslational modifications including CaMKII-dependent phosphorylation and oxidative modifications of reactive Cysteines in the channel.[5–12] Studies using ventricular myocytes (VMs) isolated from diseased or aging hearts routinely demonstrate at least partial improvements in Ca^{2+} handling when treated with antioxidants or scavengers of reactive oxygen species (ROS).[11,13–15] However, these findings have not translated into effective treatment strategies, and clinical trials aimed to improve intracellular redox balance have failed to attenuate Ca^{2+}-dependent arrhythmogenesis.[15,16]

Increased RyR2 activity under oxidizing conditions has been attributed to defective regulation of the channel complex by SR luminal Ca^{2+}, a mechanism involved in termination of systolic SR Ca^{2+} release and RyR2 refractoriness during diastole, preventing proarrhythmic spontaneous activity of RyR2 clusters.[11,13] The structural region of RyR2 proposed to mediate luminal [Ca^{2+}] sensing, that is, amino acids 4789-4844 constituting an intraluminal loop, resides within the SR,[18] an environment far more oxidizing than the cytosol due to a network of PDIs (protein disulfide isomerases) and oxidoreductase enzymes that facilitate protein folding.[19] Given that the overwhelming majority of 21 RyR2 reactive cysteines are located in the larger cytosolic assembly of the channel[20,21] and there exists a very different redox state of the SR when compared with the cytosol,[22] 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^{2+} homeostasis in the heart,[23–25] yet the molecular mechanisms remain largely unexplored. Of particular interest is the luminal oxidoreductase Ero1α, known to be induced during ER stress as a component of the PERK branch of the unfolded protein response.[26] This protein can dynamically regulate the redox environment of the SR, transferring electrons from PDIs to reduce molecular oxygen to H_2O_2.[19,27] Of note, Ero1α directly interacts with PDI protein ERp44, and this interaction increases with oxidative stress.[19,28–30] Furthermore, ERp44 was reported to inhibit Ca^{2+} release channel IP3R1 (inositol triphosphate receptor type 1) by a direct protein-protein interaction that was redox-dependent.[31,32] Importantly, ERp44 interacts with an IP3R1 region[31] 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^{2+} release channel complex remains unknown.

In the present study, we investigated the role of dysregulated SR oxidoreductase system in aberrant RyR2 function and Ca^{2+}-dependent arrhythmia using a rat model of pressure-overload cardiac hypertrophy. Our results implicate CVD-mediated upregulation of Ero1α in SR oxidative stress and dissociation of ERp44 from the RyR2 complex promoting spontaneous SR Ca^{2+} release in VMs from hypertrophic hearts. Genetic of pharmacological inhibition of Ero1α restored the intra-SR redox environment and, unlike the reducing agent dithiothreitol, restored the ERp44-RyR2 interaction, markedly improving Ca^{2+} homeostasis. Accordingly, Ero1α inhibition significantly reduced malignant ventricular tachyarrhythmias induced by β-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 supporting findings of this study are available from corresponding authors upon reasonable request.

RESULTS

Upregulation of Oxidoreductase Ero1α Enhances Intra-SR ROS in Hypertrophic Myocytes

ER stress is critically involved in the development of cardiac hypertrophy and failure.[33] 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).[14,34] 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, Ero1α. Western blot analysis demonstrated that in TAB VMs, expression of Ero1α 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 Ero1α.[24,35] Likewise, induction of Ero1α was confirmed in human nonischemic 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).[36,37] Oxidation within the SR was increased in TAB versus Sham VMs, and this was reduced by preincubation with specific Ero1α inhibitor EN460[38] (20 μmol/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 Ero1α 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 β-adrenergic receptor agonist isoproterenol (50 nmol/L), while VF occurred in every TAB heart.[14,34,36] 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 μmol/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±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 Ero1α 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

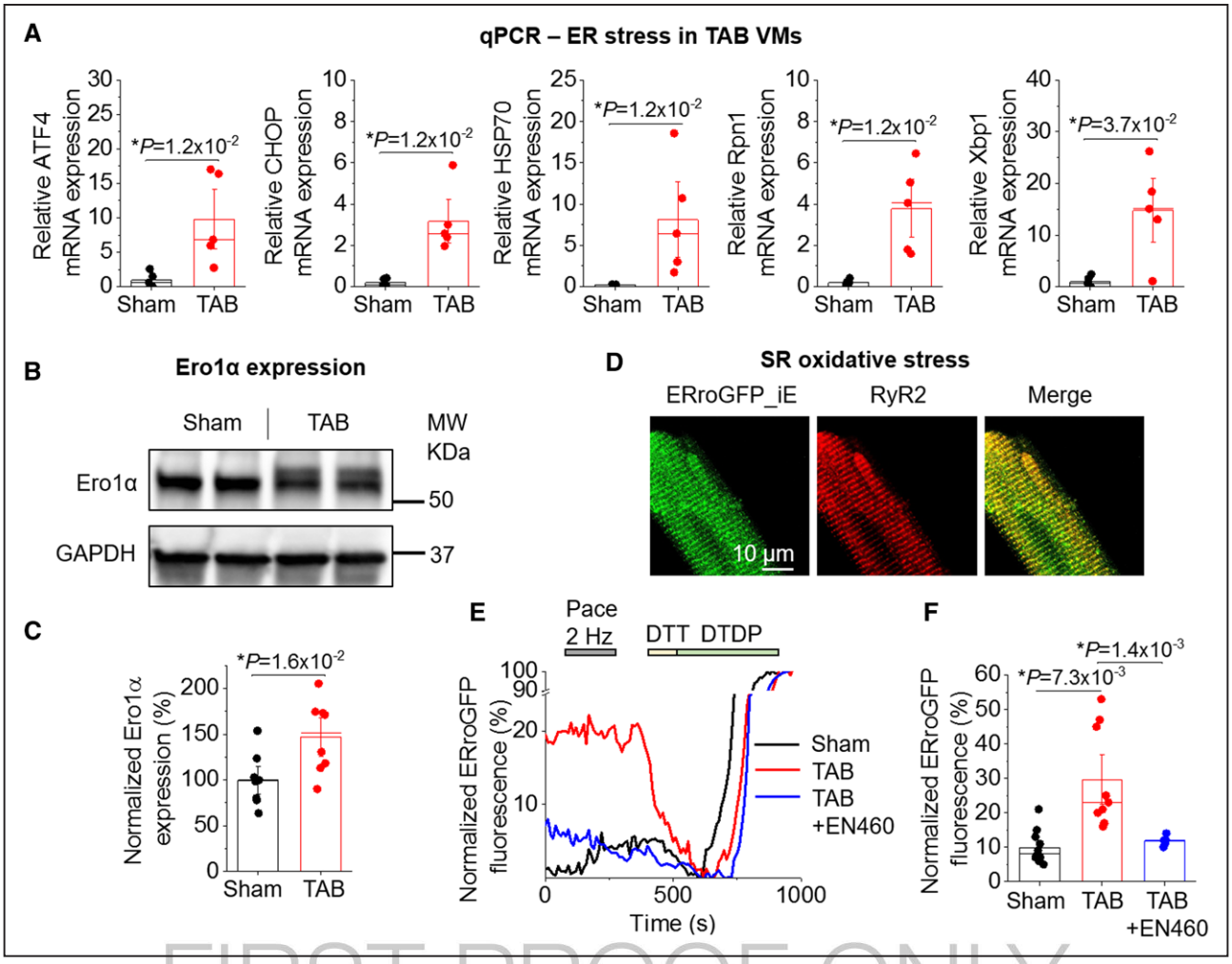
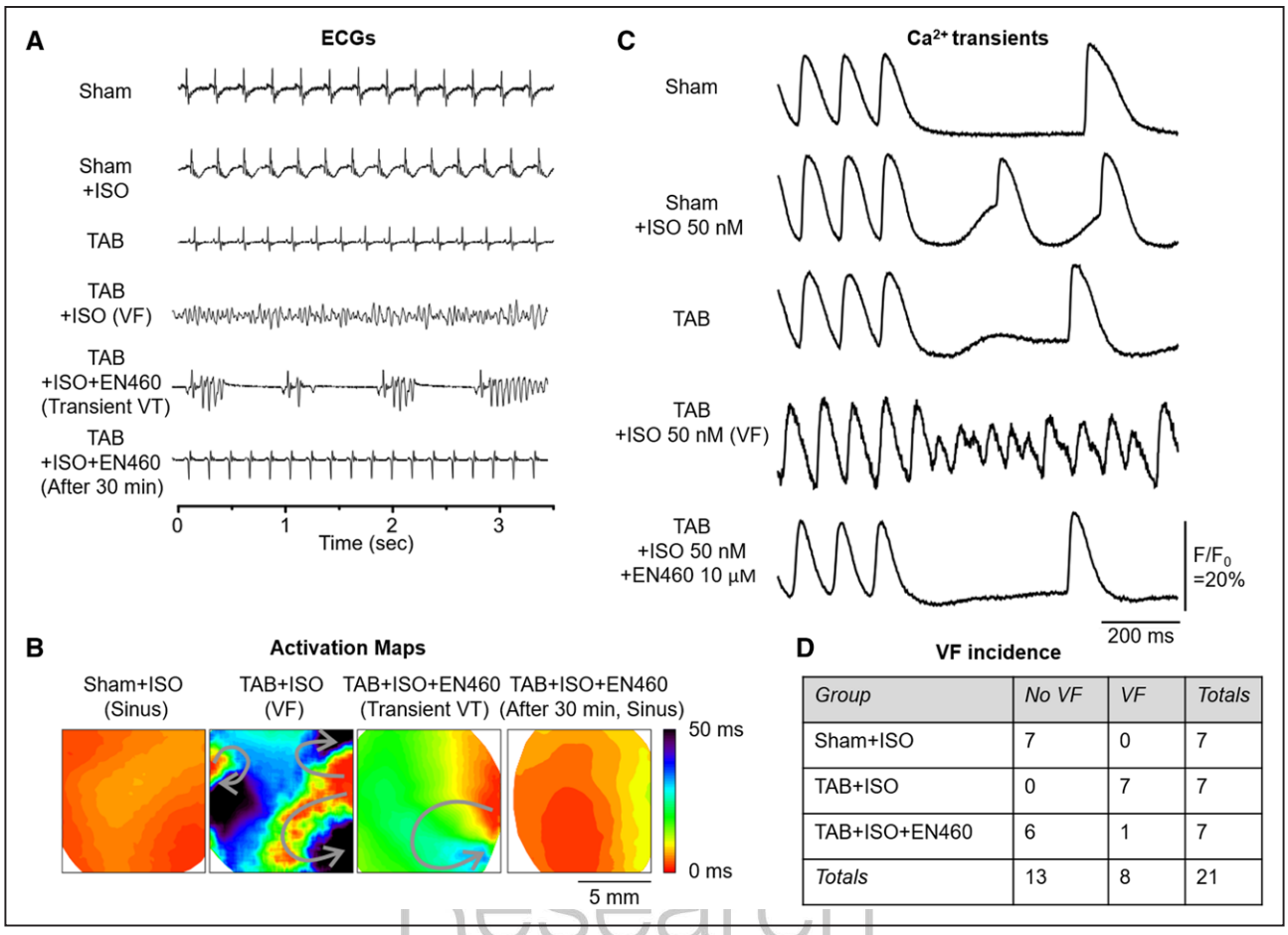


Figure 1. Upregulation of Ero1α increases intrasarcoplasmic reticulum (SR) reactive oxygen species (ROS) in cardiac disease. **A**, Relative mRNA expression data from Sham and thoracic aortic banding (TAB) ventricular myocytes (VMs). Data were normalized to housekeeping gene GAPDH and are presented as 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 ΔΔCt calculation. *P* values were calculated Mann-Whitney test. **B**, Representative Western blot of Sham and TAB rat VMs and (**C**) mean±SEM Ero1α 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 Ero1α inhibitor EN460 (20 μmol/L). Signal normalized to minimum (dithiothreitol [DTT], 5 mmol/L) and maximum (2,2′-dithiodipyridine [DTDP], 200 μmol/L) fluorescence. **F**, Mean±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.[60]

S6), suggesting initial PVCs may originate from the Purkinje system rather than VMs. Inhibition of Ero1α with EN460 increased Ca^{2+} transient amplitude, 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 Ero1α activity at the cellular level. Adenoviral-mediated overexpression or knockdown of Ero1α 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 Ero1α (Figure 3 and 3D). Importantly, Ero1α knockdown in TAB VMs reduced the redox status to Sham levels. To test whether modulation of Ero1α 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 Ero1α 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 Ero1α in TAB VMs had opposite effects,



Group	No VF	VF	Totals
Sham+ISO	7	0	7
TAB+ISO	0	7	7
TAB+ISO+EN460	6	1	7
Totals	13	8	21

Figure 2. Inhibition of Ero1α 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 µmol/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^{2+} release that triggered DADs. EN460 suppressed spontaneous Ca^{2+} 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=2.1×10^{-4}, obtained using Freeman-Halton extension of the Fisher exact test.

reducing proarrhythmic RyR2-mediated spontaneous SR Ca^{2+} release. Treatment of TAB VMs with 4PBA (500 nmol/L[39]) for 24 hours to inhibit ER stress recapitulated findings obtained from loss-of-Ero1α experiments (Figure S8). While Brefeldin A treatment of Sham VMs (1 µmol/L, 24 hours[40]) followed a similar trend to findings obtained with gain-of-Ero1α 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^{2+} 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 Ero1α is a binding partner of ERp44, and loss of Ero1α function in transgenic mice has been shown to be protective in pressure-overloaded hearts.[24] Application of EN460 (20 µmol/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

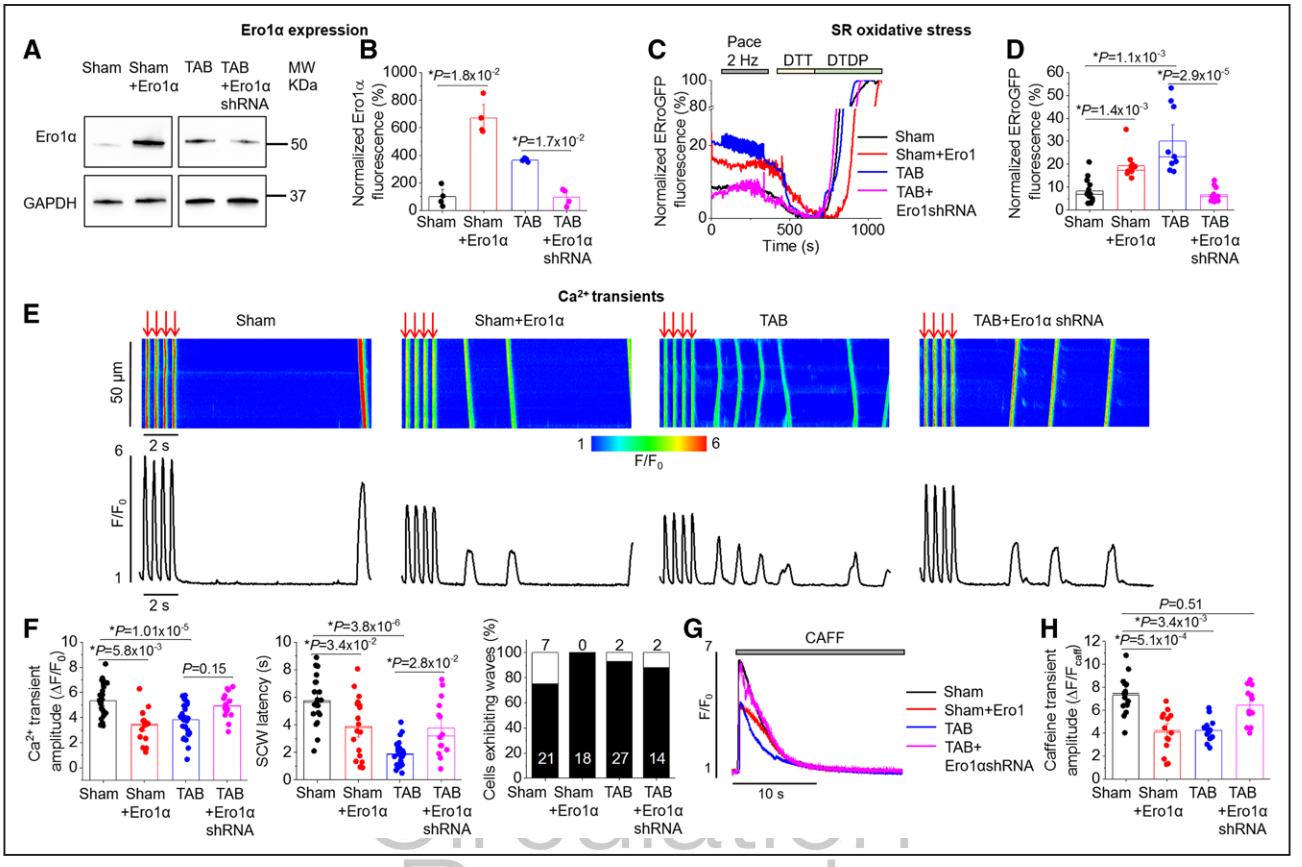


Figure 3. Altered expression levels of Ero1α modulate intrasarcoplasmic reticulum (SR) redox state and RyR2 (ryanodine receptor type 2)-mediated Ca^{2+} release.
A, Representative Western blot demonstrating adenoviral overexpression of Ero1α, and shRNA-mediated Ero1α knockdown in cultured Sham and thoracic aortic banding (TAB) ventricular myocytes (VMs), respectively. **B**, Mean±SEM Ero1α signal, normalized to GAPDH. 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 µmol/L) were used to obtain minimum and maximum fluorescence. **D**, Mean±SEM ERroGFP fluorescence (%). N=6 Sham, N=5 TAB animals, n=11 Sham, n=12 Sham+Ero1α, n=9 TAB, n=13 TAB+Ero1α-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±SEM Ca^{2+} transient amplitude at 2 Hz ($\Delta F/F_0$) and spontaneous Ca^{2+} wave (SCW) latency (s). N=9 Sham, N=9 TAB animals, n=25 Sham, n=18 Sham+Ero1α, n=29 TAB VMs, n=14 TAB+Ero1α-shRNA VMs. *P* values obtained using 2-level random intercept model with Tukey posthoc. **G**, Representative traces of caffeine-induced Ca^{2+} transients (10 mmol/L). **H**, Mean±SEM caffeine-sensitive Ca^{2+} store load. n=15 Sham, n=14 Sham+Ero1α, n=15 TAB, n=12 TAB+Ero1α-shRNA VMs; from the same animals used in **E** and **F**. *P* values obtained using 2-level random intercept model with Tukey posthoc.

complexes of Sham, TAB and EN460/dithiothreitol 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 Ero1α-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,[41] the expression of IP3R1 has been reported as increased in end-stage human HF.[42] However, IP3R inhibition with xestospongin C[43] in Ero1α–overexpressing Sham VMs did not fully restore Ca^{2+} handling (Figure S12). Furthermore, IP3R inhibition was not as effective as Ero1α knockdown in TAB VMs, suggesting aberrant RyR2 complex assembly plays a significant role in Ero1α-mediated disruption in Ca^{2+} homeostasis. To test this further, and avoid confounding effects of IP3R1-ERp44 interaction,[31] we investigated Ca^{2+} release dynamics in human embryonic kidney 293 (HEK293) cells lacking all 3 isoforms of IP3R (HEK293 IP3R-3KO[44]). Spontaneous luminal Ca^{2+} release events monitored with R-CEPIAer[45] were used as an assessment of RyR2 channel function in cells expressing recombinant hRyR2 (human RyR2) constructs[46,47] (Figure 5A).

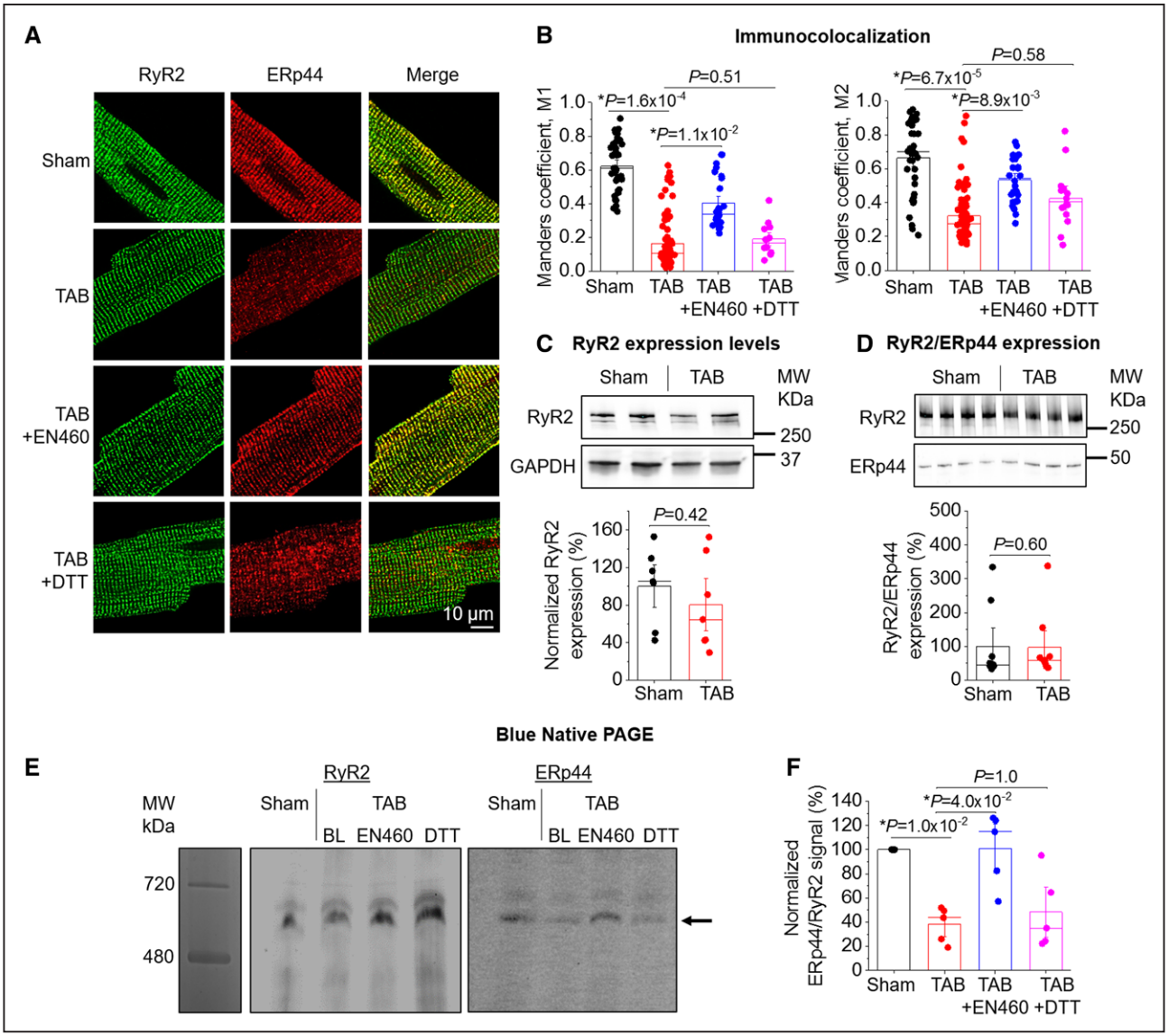


Figure 4. ERSp44 is a binding partner of RyR2 (ryanodine receptor type 2) and this interaction is reduced in cardiac disease. **A**, Representative images of Sham and thoracic aortic banding (TAB) ventricular myocytes (VMs) probed with anti-RyR2 and anti-ERp44 antibodies. VMs were treated with EN460 (20 µmol/L, 30 min) or dithiothreitol (DTT; 5 mmol/L, 10 min). **B**, Mean±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 in Sham and TAB VMs, and mean±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±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 µmol/L, 30 min) or DTT (5 mmol/L, 10 min). Arrow indicates native RyR2 protein complexes. **F**, Mean±SEM normalized ERp44/RyR2 signal (%). N=5 Sham, N=5 TAB animals. *P* values obtained using Kruskal-Wallis with Dunn posthoc.

Co-expression of short hairpin RNA to knockdown endogenous ERp44 (Figure 5B) increased hRyR2-WT-mediated Ca^{2+} release events (Figure 5D and 5E). To corroborate the hypothesis that increased expression/activity of oxidoreductase Ero1α promoted dissociation of ERp44 from RyR2, Ero1α was overexpressed (Figure 5C). hRyR2-WT-mediated Ca^{2+} release events increased with Ero1α 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 Ca^{2+} regulation of the channel, and this interaction is disrupted with Ero1α 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

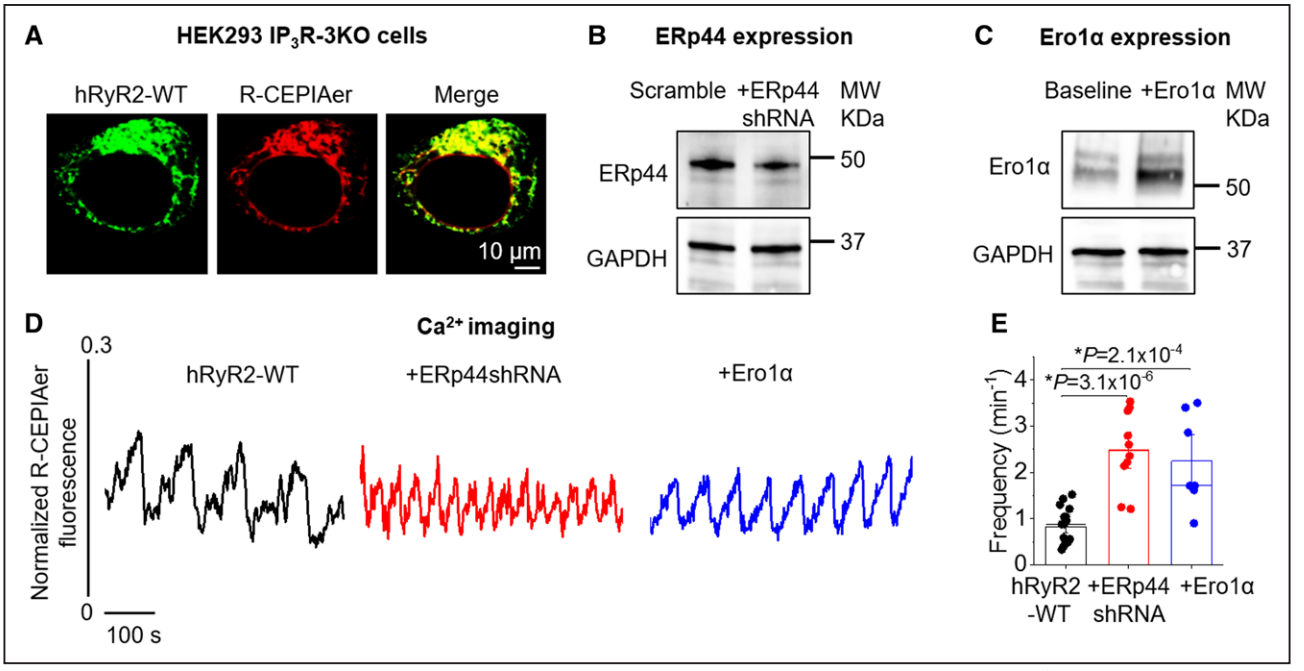


Figure 5. Knockdown of ERp44 and overexpression of Ero1α increases human RyR2 (ryanodine receptor type 2) activity in a heterologous cell system.

A, Images of HEK293 IP3R (inositol triphosphate receptor)-3KO cell co-expressing EGFP-hRyR2-WT and R-CEPIAer. **B**, Western blot demonstrating ERp44 knockdown. **C**, Western blot demonstrating Ero1α overexpression. **D**, Representative R-CEPIAer fluorescence traces in cells co-expressing hRyR2±ERp44-shRNA or Ero1α. Signal was normalized to minimum (2 mmol/L EGTA and 20 µmol/L ionomycin) and maximum (20 mmol/L Ca^{2+} and 20 µmol/L ionomycin) fluorescence. **E**, Mean±SEM Ca^{2+} wave frequency (min^{-1}). Cells were assessed from four transfections. n=17 hRyR2-WT, n=10 hRyR2-WT+ERp44-shRNA, n=7 hRyR2-WT+Ero1α cells. *P* values obtained using 1-way ANOVA with Bonferroni posthoc.

revealed that the frequency of Ca^{2+}-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^{2+} release. Critically, increased Ero1α or reduced ERp44 expression levels significantly modulated Ca^{2+} release via hRyR2-WT (Figure 5D and 5E) but were unable to change Ca^{2+} 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[48] 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^{2+} release. Activation of oxidoreductase Ero1α in cardiac hypertrophy, in addition to increasing SR oxidation, dissociates ERp44 from the

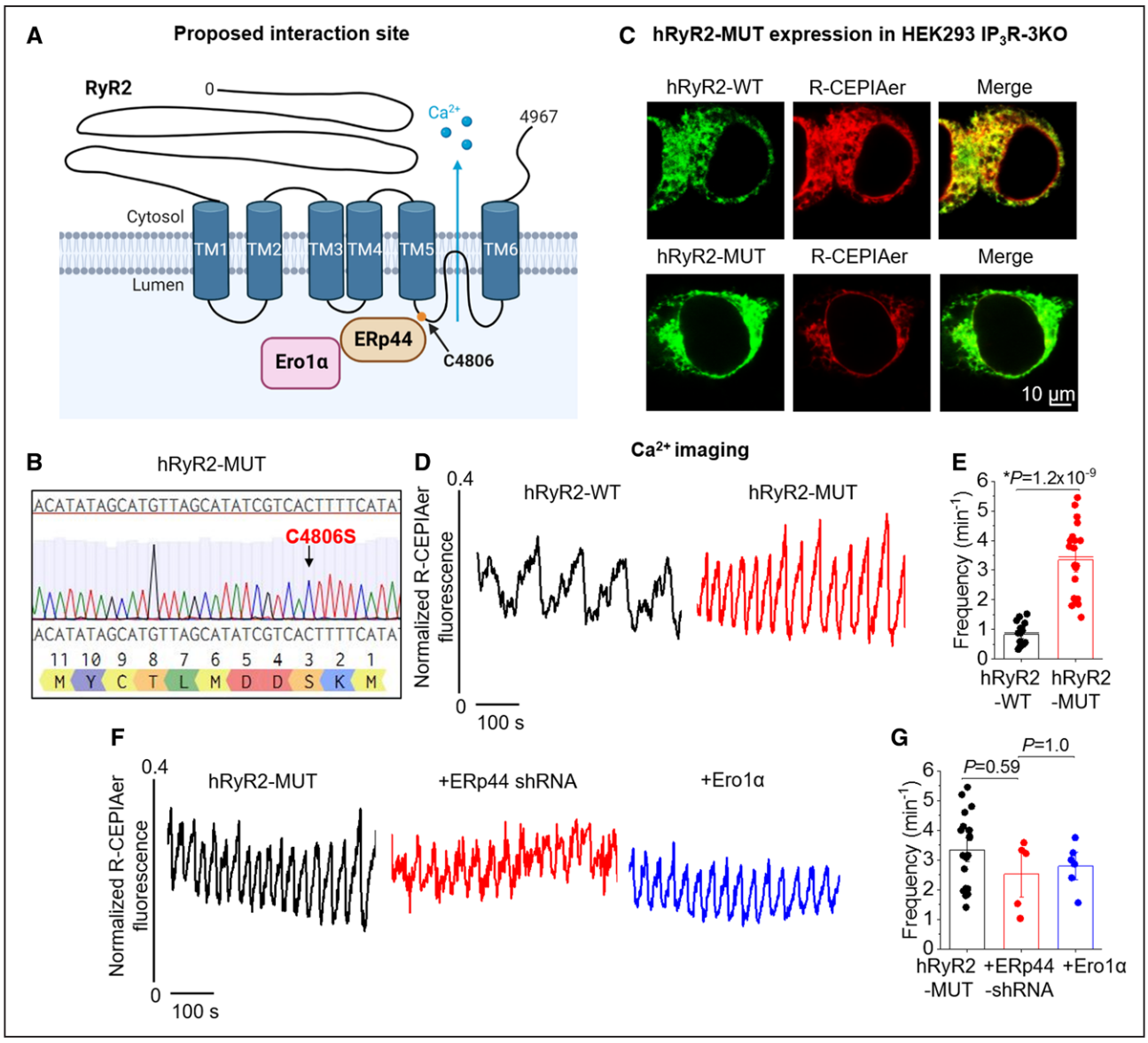


Figure 6. Mutation of intraluminal cysteine Cys4806 increases human RyR2 (ryanodine receptor type 2) activity.
A, Schematic of RyR2 monomer with proposed ERp44-interacting Cys4806. **B**, DNA chromatogram of recombinant hRyR2 (human RyR2) plasmid indicating Cys4806Ser substitution, to prevent interaction with ERp44. Image created with Biorender.com. **C**, HEK293 IP3R-3KO cell transfected with hRyR2-WT 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^{2+} and 20 μmol/L ionomycin) fluorescence. **E**, Mean±SEM frequency of Ca^{2+} 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. **G**, Mean±SEM for Ca^{2+} wave frequency (min^{-1}). 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^{2+} 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^{2+} handling and most importantly, reducing the propensity for spontaneous Ca^{2+} release following β-adrenergic stimulation. In ex vivo hypertrophic hearts challenged with β-adrenergic agonist, inhibition of Ero1α produced striking attenuation of Ca^{2+}-dependent ventricular arrhythmia.

Ero1α Upregulation Promotes Proarrhythmic Spontaneous SR Ca^{2+} Release in Cardiac Hypertrophy

Abnormally high RyR2 channel activity has a central role in contractile deficiency and arrhythmogenesis underlying sudden cardiac death[3,4,49] 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

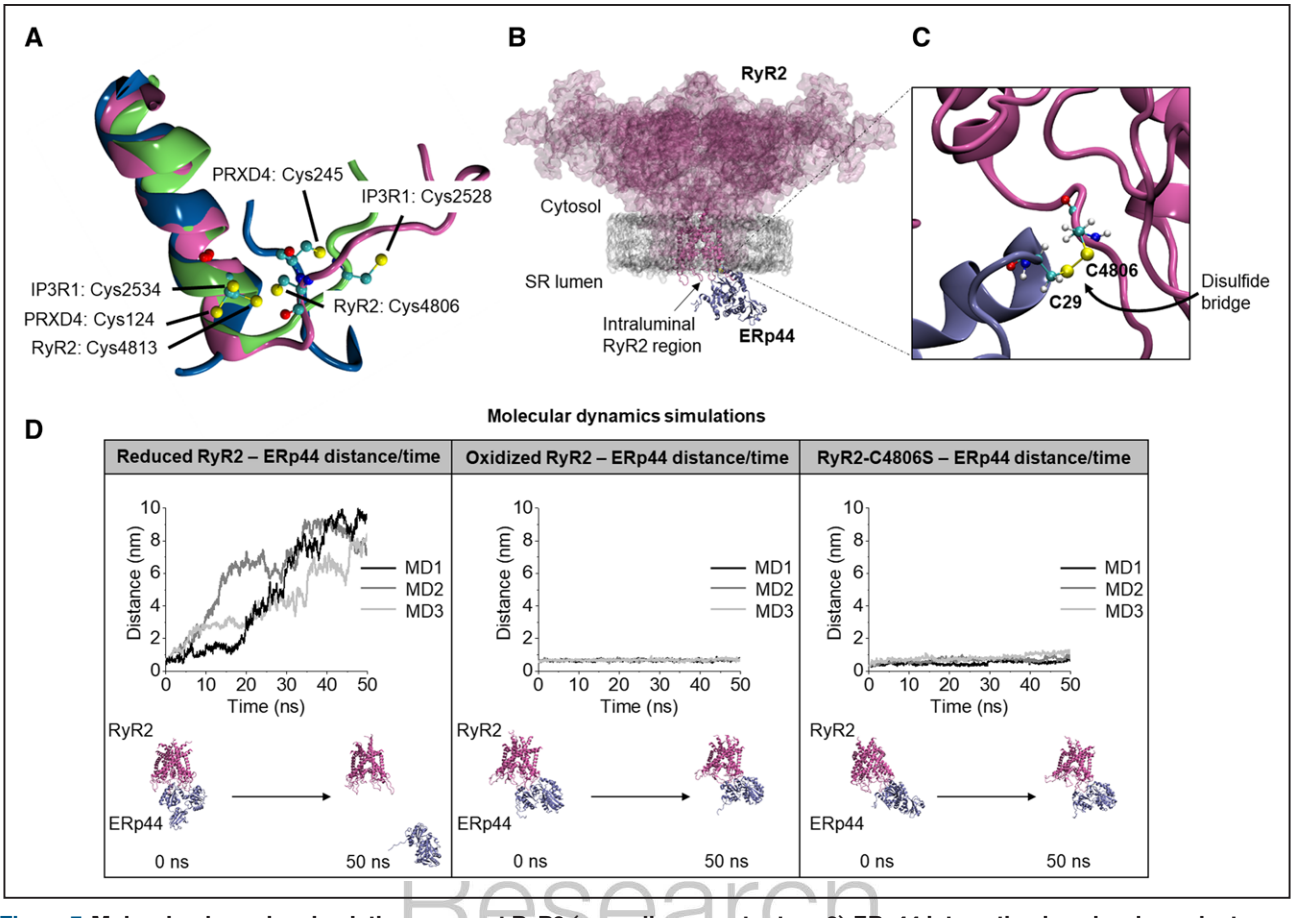


Figure 7. Molecular dynamics simulations suggest RyR2 (ryanodine receptor type 2)-ERp44 interaction is redox dependent. **A**, Structural alignment of RyR2 and IP3R1 (inositol triphosphate receptor type 1) intraluminal regions to peroxiredoxin-4 (PRDX4) region involved in formation of a disulfide bridge with ERp44. RyR2 is in purple (PDB ID: 6JH6), IP3R1 is in green (PDB ID: 3JAV), reduced PRXD4 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 β-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.[5,50] Here, we show a novel regulatory axis for RyR2 ROS modulation involving the luminal ROS sensor Ero1α and the luminal RyR2-binding protein ERp44. Our previous studies showed that β-adrenergic stimulation induces Ca^{2+}-dependent VT/VF in 100% of ex vivo hearts from rats with pressure-overload induced hypertrophy.[14,34,36] Complementary cellular studies confirmed that arrhythmogenic RyR2 dysfunction was, to a large extent, triggered by the effects of oxidative stress on the channel.[14,34] 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 Ero1α, constitutively expressed in many tissues including the heart, is activated under conditions of stress.[19,25,27] Our results show increased Ero1α 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.[24] Ero1α upregulation is expected to increase H_2O_2 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 Ero1α inhibition by small molecule inhibitor EN460 and more chronic short hairpin RNA-mediated knockdown normalized SR oxidation levels in TAB VMs. Conversely, Ero1α overexpression in Sham VMs increased SR oxidation levels. These data implicate Ero1α as an important mediator of dysregulated SR redox potential in cardiac pathology. Furthermore, as H_2O_2 is capable of crossing intracellular membranes and traveling substantial distances within the cell,[51] our results support the notion that the SR is a significant source of ROS, in addition to well established sources such as NOX2 or mitochondria.[8,52–54] The possibility also exists that H_2O_2 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, Ero1α knockdown in TABs reduced mitochondrial matrix H_2O_2 levels measured using mitochondria-targeted biosensor MLS-HyPer7 (Figure S14). While the latter might be a direct result of reduced H_2O_2 emission from the SR, our recent demonstration of RyR2 Ca^{2+}-leak-dependent disruption of mitochondrial Ca^{2+} and ROS homeostasis[52] would favor a mechanism that involved RyR2 channel stabilization.

Ero1α overexpression in Sham VMs treated with isoproterenol decreased Ca^{2+} transient amplitude and SR Ca^{2+} content and shortened spontaneous Ca^{2+} wave latency, consistent with increased RyR2 activity (Figure 3). In TAB VMs, Ero1α knockdown normalized Ca^{2+} homeostasis, increasing Ca^{2+} transient amplitude, loading of the SR, and reducing the propensity for proarrhythmic spontaneous Ca^{2+} waves. Further investigations of WT- and Cys4806Ser hRyR2 in HEK293 cells devoid of IP3R isoforms revealed that Ero1α overexpression potently increases RyR2 activity (Figure 5). Accordingly, treatment of ex vivo TAB hearts with an Ero1α inhibitor attenuated Ca^{2+}-dependent tachyarrhythmia evoked by β-adrenergic challenge (Figure 2, Figure S5). Previous work using VMs from transgenic mouse model of Ero1α loss-of-function showed a marked decrease in Ca^{2+} transient amplitudes and SR Ca^{2+} content, both in baseline conditions and under isoproterenol.[24] However, pressure-overloaded Ero1α mutant hearts exhibited significant preservation of cardiac function in comparison to WTs, pointing to Ero1α's key role in aberrant excitation-contraction coupling during HF development. Our data extend these findings and indicate that strategies to reduce Ero1α activity, which is increased in CVD, can be of benefit in therapeutically improving contractility and limiting proarrhythmic spontaneous RyR2-mediated SR Ca^{2+} release.

A recent study established antiarrhythmic effects induced by PERK knockout in myocardial infarct mice.[55] Of note, Ero1α is upregulated as part of the ER stress-induced unfolded protein response, downstream of PERK.[56] Indeed, modulation of ER stress produced similar effects to altering Ero1α levels on Ca^{2+} handling (Figure 3, Figures S8 and S10). These data support that Ero1α upregulation is a key factor contributing to Ca^{2+}-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, Ero1α was shown to modulate function of SR Ca^{2+} channel multimolecular complexes, such as IP3R1, by disrupting tethering of luminal protein ERp44 to the complex.[31] While our results do not exclude the importance of Ero1α-mediated IP3R1-ERp44 disassociation in hypertrophic VMs (Figure S12), we demonstrate that Ero1α 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.[2,11] Reversible modifications of reactive cysteines modulate channel activity.[21] 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^{2+} handling.[11,13,57] However, clinical studies did not substantiate a therapeutic effect of antioxidants in improving outcomes for patients with HF.[16,17] In line with this, reducing agents such as dithiothreitol or mercaptopropionyl glycine produced only partial recovery in Ca^{2+} handling in VMs from HF or infarcted hearts.[11,13] 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^{2+} content,[11] 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,[58] 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.[28,59] Higo et al[31] 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.[32] 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).

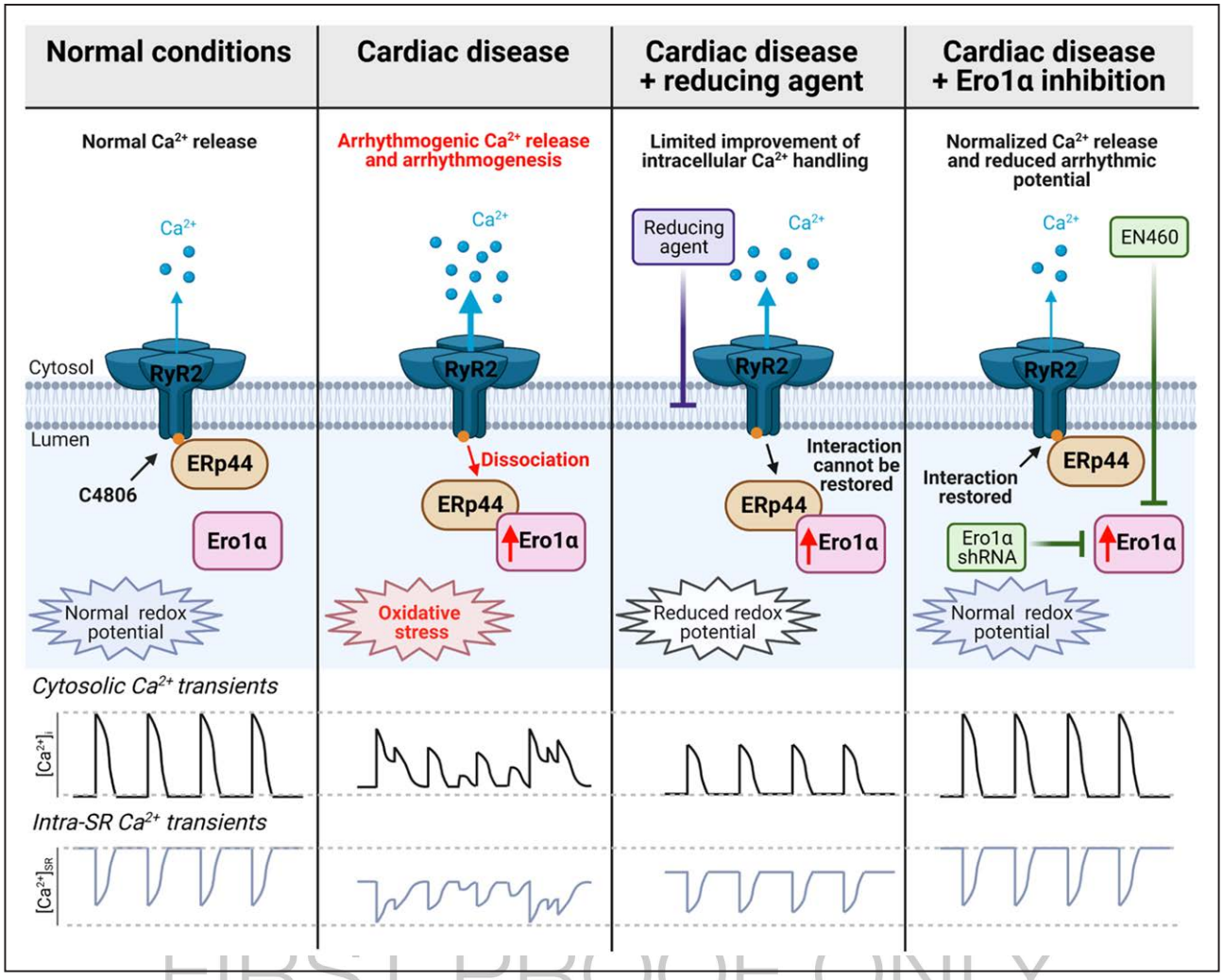


Figure 8. Scheme depicting the putative multimolecular RyR2 (ryanodine receptor type 2) intraluminal redox sensor and sarcoplasmic reticulum (SR) oxidoreductase-dependent mechanisms of cardiac Ca^{2+}-dependent arrhythmia.
Figure created with Biorender.com.

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 Ero1α associates with ERp44, removing it from its binding partners.[28,29] Indeed Ero1α 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 Ero1α effect on ERp44-RyR2 association versus its effect on ROS was underscored by the lack of additional effects of Ero1α 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, Ero1α upregulation removes ERp44 from the complex, contributing to RyR2 dysfunction and thereby increased propensity to Ca^{2+} dependent ventricular tachyarrhythmias in hypertrophic hearts. Our data suggest that ER stress-induced Ero1α 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

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Affiliations

Department of Physiology and Cell Biology (S.H., R.T., V.B., F.P., J.Y., X.A., A.E.B., J.P.D., S.G., D.T.) and College of Pharmacy (C.A.C.), The Ohio State University. Dorothy M. Davis Heart and Lung Research Institute, College of Medicine, The Ohio State University Wexner Medical Center, Columbus (S.H., R.T., V.B., F.P., J.Y., X.A., C.A.C., A.E.B., J.P.D., S.G., D.T.). Cardiovascular Research Center, Rhode Is-

land Hospital, Department of Medicine, Warren Alpert Medical School of Brown University, Providence, RI (T.Y.K., B.-R.C.). Medical School, Swansea University, United Kingdom (C.H.G.).

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Disclosures

None.

Supplemental Materials

Expanded Materials and Methods
Figures S1–S14
Videos S1–S5
References[61–75]

REFERENCES

1. Hamilton S, Terentyev D. Proarrhythmic remodeling of calcium homeostasis in cardiac disease; implications for diabetes and obesity. *Front Physiol.* 2018;9:1517. doi: 10.3389/fphys.2018.01517
2. Zima AV, Blatter LA. Redox regulation of cardiac calcium channels and transporters. *Cardiovasc Res.* 2006;71:310–321. doi: 10.1016/j.cardiores.2006.02.019
3. Bers DM. Cardiac excitation-contraction coupling. *Nature.* 2002;415:198–205. doi: 10.1038/415198a
4. Landstrom AP, Dobrev D, Wehrens XHT. Calcium signaling and cardiac arrhythmias. *Circ Res.* 2017;120:1969–1993. doi: 10.1161/CIRCRESAHA.117.310083
5. Mattiazzi A, Bassani RA, Escobar AL, Palomeque J, Valverde CA, Vila Petroff M, Bers DM. Chasing cardiac physiology and pathology down the CaMKII cascade. *Am J Physiol Heart Circ Physiol.* 2015;308:H1177–H1191. doi: 10.1152/ajpheart.00007.2015
6. Boraso A, Williams AJ. Modification of the gating of the cardiac sarcoplasmic reticulum Ca(2+)-release channel by H2O2 and dithiothreitol. *Am J Physiol.* 1994;267:H1010–H1016. doi: 10.1152/ajpheart.1994.267.3.H1010
7. Bovo E, Lipsius SL, Zima AV. Reactive oxygen species contribute to the development of arrhythmogenic Ca^{2+} waves during β-adrenergic receptor stimulation in rabbit cardiomyocytes. *J Physiol.* 2012;590:3291–3304. doi: 10.1113/jphysiol.2012.230748
8. Cooper LL, Li W, Lu Y, Centracchio J, Terentyeva R, Koren G, Terentyev D. Redox modification of ryanodine receptors by mitochondria-derived reactive oxygen species contributes to aberrant Ca2+ handling in ageing rabbit hearts. *J Physiol.* 2013;591:5895–5911. doi: 10.1113/jphysiol.2013.260521
9. Eager KR, Dulhunty AF. Activation of the cardiac ryanodine receptor by sulfhydryl oxidation is modified by Mg2+ and ATP. *J Membr Biol.* 1998;163:9–18. doi: 10.1007/s002329900365
10. Liu T, Yang N, Sidor A, O'Rourke B. MCU overexpression rescues inotropy and reverses heart failure by reducing SR Ca2+ Leak. *Circ Res.* 2021;128:1191–1204. doi: 10.1161/CIRCRESAHA.120.318562
11. Terentyev D, Györke I, Belevych AE, Terentyeva R, Sridhar A, Nishijima Y, de Blanco EC, Khanna S, Sen CK, Cardounel AJ, et al. Redox modification of ryanodine receptors contributes to sarcoplasmic reticulum Ca2+ leak in chronic heart failure. *Circ Res.* 2008;103:1466–1472. doi: 10.1161/CIRCRESAHA.108.184457
12. Salama G, Menshikova EV, Abramson JJ. Molecular interaction between nitric oxide and ryanodine receptors of skeletal and cardiac sarcoplasmic reticulum. *Antioxid Redox Signal.* 2000;2:5–16. doi: 10.1089/ars.2000.2.1-5
13. Belevych AE, Terentyev D, Terentyeva R, Ho HT, Gyorke I, Bonilla IM, Carnes CA, Billman GE, Györke S. Shortened Ca2+ signaling refractoriness underlies cellular arrhythmogenesis in a postinfarction model of sudden cardiac death. *Circ Res.* 2012;110:569–577. doi: 10.1161/CIRCRESAHA.111.260455
14. Kim TY, Terentyeva R, Roder KH, Li W, Liu M, Greener I, Hamilton S, Polina I, Murphy KR, Clements RT, et al. SK channel enhancers attenuate Ca2+-dependent arrhythmia in hypertrophic hearts by regulating mito-ROS-dependent oxidation and activity of RyR. *Cardiovasc Res.* 2017;113:343–353. doi: 10.1093/cvr/cvx005
15. Mochizuki M, Yano M, Oda T, Tateishi H, Kobayashi S, Yamamoto T, Ikeda Y, Ohkusa T, Ikemoto N, Matsuzaki M. Scavenging free radicals by low-dose carvedilol prevents redox-dependent Ca2+ leak via stabilization of ryanodine receptor in heart failure. *J Am Coll Cardiol.* 2007;49:1722–1732. doi: 10.1016/j.jacc.2007.01.064
16. Sawyer DB. Oxidative stress in heart failure: what are we missing? *Am J Med Sci.* 2011;342:120–124. doi: 10.1097/MAJ.0b013e3182249fcd
17. van der Pol A, van Gilst WH, Voors AA, van der Meer P. Treating oxidative stress in heart failure: past, present and future. *Eur J Heart Fail.* 2019;21:425–435. doi: 10.1002/ejhf.1320
18. Györke I, Hester N, Jones LR, Györke S. The role of calsequestrin, triadin, and junctin in conferring cardiac ryanodine receptor responsiveness to luminal calcium. *Biophys J.* 2004;86:2121–2128. doi: 10.1016/S0006-3495(04)74271-X
19. Zito E. ERO1: a protein disulfide oxidase and H2O2 producer. *Free Radic Biol Med.* 2015;83:299–304. doi: 10.1016/j.freeradbiomed.2015.01.011
20. Aracena-Parks P, Goonasekera SA, Gilman CP, Dirksen RT, Hidalgo C, Hamilton SL. Identification of cysteines involved in S-nitrosylation, S-glutathionylation, and oxidation to disulfides in ryanodine receptor type 1. *J Biol Chem.* 2006;281:40354–40368. doi: 10.1074/jbc.M600876200
21. Nikolaienko R, Bovo E, Zima AV. Redox dependent modifications of ryanodine receptor: basic mechanisms and implications in heart diseases. *Front Physiol.* 2018;9:1775. doi: 10.3389/fphys.2018.01775
22. Ushioda R, Nagata K. Redox-Mediated regulatory mechanisms of endoplasmic reticulum homeostasis. *Cold Spring Harb Perspect Biol.* 2019;11:a033910. doi: 10.1101/cshperspect.a033910
23. Caillard A, Sadoune M, Cescau A, Meddour M, Gandon M, Polidano E, Delcayre C, Da Silva K, Manivet P, Gomez AM, et al. QSOX1, a novel actor of cardiac protection upon acute stress in mice. *J Mol Cell Cardiol.* 2018;119:75–86. doi: 10.1016/j.yjmcc.2018.04.014
24. Chin KT, Kang G, Qu J, Gardner LB, Coetzee WA, Zito E, Fishman GI, Ron D. The sarcoplasmic reticulum luminal thiol oxidase ERO1 regulates cardiomyocyte excitation-coupled calcium release and response to hemodynamic load. *FASEB J.* 2011;25:2583–2591. doi: 10.1096/fj.11-184622
25. Okatan EN, Tuncay E, Turan B. Cardioprotective effect of selenium via modulation of cardiac ryanodine receptor calcium release channels in diabetic rat cardiomyocytes through thioredoxin system. *J Nutr Biochem.* 2013;24:2110–2118. doi: 10.1016/j.jnutbio.2013.08.002
26. Higa A, Chevet E. Redox signaling loops in the unfolded protein response. *Cell Signal.* 2012;24:1548–1555. doi: 10.1016/j.cellsig.2012.03.011
27. Ramming T, Appenzeller-Herzog C. The physiological functions of mammalian endoplasmic oxidoreductin 1: on disulfides and more. *Antioxid Redox Signal.* 2012;16:1109–1118. doi: 10.1089/ars.2011.4475
28. Anelli T, Alessio M, Mezghrani A, Simmen T, Talamo F, Bachi A, Sitia R. ERp44, a novel endoplasmic reticulum folding assistant of the thioredoxin family. *EMBO J.* 2002;21:835–844. doi: 10.1093/emboj/21.4.835
29. Otsu M, Bertoli G, Fagioli C, Guerini-Rocco E, Nerini-Molteni S, Ruffato E, Sitia R. Dynamic retention of Ero1alpha and Ero1beta in the endoplasmic reticulum by interactions with PDI and ERp44. *Antioxid Redox Signal.* 2006;8:274–282. doi: 10.1089/ars.2006.8.274
30. Ramming T, Okumura M, Kanemura S, Baday S, Birk J, Moes S, Spiess M, Jenö P, Bernèche S, Inaba K, et al. A PDI-catalyzed thiol-disulfide switch regulates the production of hydrogen peroxide by human Ero1. *Free Radic Biol Med.* 2015;83:361–372. doi: 10.1016/j.freeradbiomed.2015.02.011
31. Higo T, Hattori M, Nakamura T, Natsume T, Michikawa T, Mikoshiba K. Subtype-specific and ER lumenal environment-dependent regulation of inositol 1,4,5-trisphosphate receptor type 1 by ERp44. *Cell.* 2005;120:85–98. doi: 10.1016/j.cell.2004.11.048
32. Wang DY, Abbasi C, El-Rass S, Li JY, Dawood F, Naito K, Sharma P, Bousette N, Singh S, Backx PH, et al. Endoplasmic reticulum resident protein 44 (ERp44) deficiency in mice and zebrafish leads to cardiac developmental and functional defects. *J Am Heart Assoc.* 2014;3:e001018. doi: 10.1161/JAHA.114.001018
33. Wang S, Binder P, Fang Q, Wang Z, Xiao W, Liu W, Wang X. Endoplasmic reticulum stress in the heart: insights into mechanisms and drug targets. *Br J Pharmacol.* 2018;175:1293–1304. doi: 10.1111/bph.13888
34. Hamilton S, Polina I, Terentyeva R, Bronk P, Kim TY, Roder K, Clements RT, Koren G, Choi BR, Terentyev D. PKA phosphorylation underlies functional recruitment of sarcolemmal SK2 channels in ventricular

myocytes from hypertrophic hearts. *J Physiol*. 2020;598:2847–2873. doi: 10.1113/JP277618
35. Baker KM, Chakravarthi S, Langton KP, Sheppard AM, Lu H, Bulleid NJ. Low reduction potential of Ero1alpha regulatory disulphides ensures tight control of substrate oxidation. *EMBO J*. 2008;27:2988–2997. doi: 10.1038/emboj.2008.230
36. Hamilton S, Terentyeva R, Kim TY, Bronk P, Clements RT, O-Uchi J, Csordás G, Choi BR, Terentyev D. Pharmacological modulation of mitochondrial Ca2+ content regulates sarcoplasmic reticulum Ca2+ release via oxidation of the ryanodine receptor by mitochondria-derived reactive oxygen species. *Front Physiol*. 2018;9:1831. doi: 10.3389/fphys.2018.01831
37. Avezov E, Cross BC, Kaminski Schierle GS, Winters M, Harding HP, Melo EP, Kaminski CF, Ron D. Lifetime imaging of a fluorescent protein sensor reveals surprising stability of ER thiol redox. *J Cell Biol*. 2013;201:337–349. doi: 10.1083/jcb.201211155
38. Blais JD, Chin KT, Zito E, Zhang Y, Heldman N, Harding HP, Fass D, Thorpe C, Ron D. A small molecule inhibitor of endoplasmic reticulum oxidation 1 (ERO1) with selectively reversible thiol reactivity. *J Biol Chem*. 2010;285:20993–21003. doi: 10.1074/jbc.M110.126599
39. Wiersma M, Meijering RAM, Qi XY, Zhang D, Liu T, Hoogstra-Berends F, Sibon OCM, Henning RH, Nattel S, Brundel BJJM. Endoplasmic reticulum stress is associated with autophagy and cardiomyocyte remodeling in experimental and human atrial fibrillation. *J Am Heart Assoc*. 2017;6:e006458. doi: 10.1161/JAHA.117.006458
40. Dalal S, Foster CR, Das BC, Singh M, Singh K. B-adrenergic receptor stimulation induces endoplasmic reticulum stress in adult cardiac myocytes: role in apoptosis. *Mol Cell Biochem*. 2012;364:59–70. doi: 10.1007/s11010-011-1205-7
41. Perez PJ, Ramos-Franco J, Fill M, Mignery GA. Identification and functional reconstitution of the type 2 inositol 1,4,5-trisphosphate receptor from ventricular cardiac myocytes. *J Biol Chem*. 1997;272:23961–23969. doi: 10.1074/jbc.272.38.23961
42. Gutstein DE, Marks AR. Role of inositol 1,4,5-trisphosphate receptors in regulating apoptotic signaling and heart failure. *Heart Vessels*. 1997;Suppl 12:53–57.
43. Gafni J, Munsch JA, Lam TH, Catlin MC, Costa LG, Molinski TF, Pessah IN. Xestospongins: potent membrane permeable blockers of the inositol 1,4,5-trisphosphate receptor. *Neuron*. 1997;19:723–733. doi: 10.1016/s0896-6273(00)80384-0
44. Alzayady KJ, Wang L, Chandrasekhar R, Wagner LE 2nd, Van Petegem F, Yule DI. Defining the stoichiometry of inositol 1,4,5-trisphosphate binding required to initiate Ca2+ release. *Sci Signal*. 2016;9:ra35. doi: 10.1126/scisignal.aad6281
45. Suzuki J, Kanemaru K, Ishii K, Ohkura M, Okubo Y, Iino M. Imaging intraorganellar Ca2+ at subcellular resolution using CEPIA. *Nat Commun*. 2014;5:4153. doi: 10.1038/ncomms5153
46. Thomas NL, George CH, Lai FA. Functional heterogeneity of ryanodine receptor mutations associated with sudden cardiac death. *Cardiovasc Res*. 2004;64:52–60. doi: 10.1016/j.cardiores.2004.06.009
47. George CH, Sorathia R, Bertrand BM, Lai FA. In situ modulation of the human cardiac ryanodine receptor (hRyR2) by FKBP12.6. *Biochem J*. 2003;370:579–589. doi: 10.1042/BJ20021433
48. Yang K, Li DF, Wang X, Liang J, Sitia R, Wang CC, Wang X. Crystal structure of the ERp44-Peroxiredoxin 4 complex reveals the molecular mechanisms of thiol-mediated protein retention. *Structure*. 2016;24:1755–1765. doi: 10.1016/j.str.2016.08.002
49. Hamilton S, Veress R, Belevych A, Terentyev D. The role of calcium homeostasis remodeling in inherited cardiac arrhythmia syndromes. *Pflugers Arch*. 2021;473:377–387. doi: 10.1007/s00424-020-02505-y
50. Hegyi B, Pölönen RP, Hellgren KT, Ko CY, Ginsburg KS, Bossuyt J, Mercola M, Bers DM. Cardiomyocyte Na+ and Ca2+ mishandling drives vicious cycle involving CaMKII, ROS, and ryanodine receptors. *Basic Res Cardiol*. 2021;116:58. doi: 10.1007/s00395-021-00900-9
51. Mishina NM, Tyurin-Kuzmin PA, Markvicheva KN, Vorotnikov AV, Tkachuk VA, Laketa V, Schultz C, Lukyanov S, Belousov VV. Does cellular hydrogen peroxide diffuse or act locally? *Antioxid Redox Signal*. 2011;14:1–7. doi: 10.1089/ars.2010.3539
52. Hamilton S, Terentyeva R, Martin B, Perger F, Li J, Stepanov A, Bonilla IM, Knollmann BC, Radwański PB, Györke S, et al. Increased RyR2 activity is exacerbated by calcium leak-induced mitochondrial ROS. *Basic Res Cardiol*. 2020;115:38. doi: 10.1007/s00395-020-0797-z
53. Bertero E, Maack C. Calcium signaling and reactive oxygen species in mitochondria. *Circ Res*. 2018;122:1460–1478. doi: 10.1161/CIRCRESAHA.118.310082
54. Prosser BL, Ward CW, Lederer WJ. X-ROS signaling: rapid mechano-chemo transduction in heart. *Science*. 2011;333:1440–1445. doi: 10.1126/science.1202768
55. Liu M, Liu H, Parthiban P, Kang GJ, Shi G, Feng F, Zhou A, Gu L, Karnopp C, Tolkacheva EG, et al. Inhibition of the unfolded protein response reduces arrhythmia risk after myocardial infarction. *J Clin Invest*. 2021;131:147836. doi: 10.1172/JCI147836
56. Marciniak SJ, Yun CY, Oyadomari S, Novoa I, Zhang Y, Jungreis R, Nagata K, Harding HP, Ron D. CHOP induces death by promoting protein synthesis and oxidation in the stressed endoplasmic reticulum. *Genes Dev*. 2004;18:3066–3077. doi: 10.1101/gad.1250704
57. Bovo E, Mazurek SR, Zima AV. Oxidation of ryanodine receptor after ischemia-reperfusion increases propensity of Ca2+ waves during β-adrenergic receptor stimulation. *Am J Physiol Heart Circ Physiol*. 2018;315:H1032–H1040. doi: 10.1152/ajpheart.00334.2018
58. Pessah IN, Feng W. Functional role of hyperreactive sulfhydryl moieties within the ryanodine receptor complex. *Antioxid Redox Signal*. 2000;2:17–25. doi: 10.1089/ars.2000.2.1-17
59. Tempio T, Anelli T. The pivotal role of ERp44 in patrolling protein secretion. *J Cell Sci*. 2020;133:jcs240366. doi: 10.1242/jcs.240366
60. Sikkel MB, Francis DP, Howard J, Gordon F, Rowlands C, Peters NS, Lyon AR, Harding SE, MacLeod KT. Hierarchical statistical techniques are necessary to draw reliable conclusions from analysis of isolated cardiomyocyte studies. *Cardiovasc Res*. 2017;113:1743–1752. doi: 10.1093/cvr/cvx151
61. Long VP 3rd, Bonilla IM, Baine S, Glynn P, Kumar S, Schober K, Mowrey K, Weiss R, Lee NY, Mohler PJ, et al. Chronic heart failure increases negative chronotropic effects of adenosine in canine sinoatrial cells via A1R stimulation and GIRK-mediated IKado. *Life Sci*. 2020;240:117068. doi: 10.1016/j.lfs.2019.117068
62. Sridhar A, Nishijima Y, Terentyev D, Khan M, Terentyeva R, Hamlin RL, Nakayama T, Gyorke S, Cardounel AJ, Carnes CA. Chronic heart failure and the substrate for atrial fibrillation. *Cardiovasc Res*. 2009;84:227–236. doi: 10.1093/cvr/cvp216
63. Yan J, Zhao W, Thomson JK, Gao X, DeMarco DM, Carrillo E, Chen B, Wu X, Ginsburg KS, Bakhos M, et al. Stress signaling JNK2 crosstalk with CaMKII underlies enhanced atrial arrhythmogenesis. *Circ Res*. 2018;122:821–835. doi: 10.1161/CIRCRESAHA.117.312536
64. Yan J, Bare DJ, DeSantiago J, Zhao W, Mei Y, Chen Z, Ginsburg K, Solaro RJ, Wolska BM, Bers DM, et al. JNK2, a newly-identified SERCA2 enhancer, augments an arrhythmic [Ca2+]SR leak-load relationship. *Circ Res*. 2021;128:455–470. doi: 10.1161/CIRCRESAHA.120.318409
65. Ai X, Pogwizd SM. Connexin 43 downregulation and dephosphorylation in nonischemic heart failure is associated with enhanced colocalized protein phosphatase type 2A. *Circ Res*. 2005;96:54–63. doi: 10.1161/01.RES.0000152325.07495.5a
66. Choi BR, Salama G. Simultaneous maps of optical action potentials and calcium transients in guinea-pig hearts: mechanisms underlying concordant alternans. *J Physiol*. 2000;529(pt 1):171–188. doi: 10.1111/j.1469-7793.2000.00171.x
67. Terentyev D, Rees CM, Li W, Cooper LL, Jindal HK, Peng X, Lu Y, Terentyeva R, Odening KE, Daley J, et al. Hyperphosphorylation of RyRs underlies triggered activity in transgenic rabbit model of LQT2 syndrome. *Circ Res*. 2014;115:919–928. doi: 10.1161/CIRCRESAHA.115.305146
68. Suzuki J, Kanemaru K, Ishii K, Ohkura M, Okubo Y, Iino M. Imaging intraorganellar Ca2+ at subcellular resolution using CEPIA. *Nat Commun*. 2014;5:4153. doi: 10.1038/ncomms5153
69. Abraham MJ, Murtola T, Schulz R, Páll S, Smith JC, Hess B, Lindahl E. GROMACS: High performance molecular simulations through multi-level parallelism from laptops to supercomputers. *SoftwareX*. 2015;1–2,19–25. doi: 10.1016/j.softx.2015.06.001
70. Jo S, Kim T, Iyer VG, Im W. CHARMM-GUI: a web-based graphical user interface for CHARMM. *J Comput Chem*. 2008;29:1859–1865. doi: 10.1002/jcc.20945
71. Essmann U, Perera L, Berkowitz ML, Darden T, Lee H, Pedersen LG. A smooth particle mesh ewald potential. *J Chem Phys*. 1995;103:8577–8592.
72. Huang J, MacKerell AD Jr. CHARMM36 all-atom additive protein force field: validation based on comparison to NMR data. *J Comput Chem*. 2013;34:2135–2145. doi: 10.1002/jcc.23354
73. Hess B, Bekker H, Berendsen HJC, Fraaije JGEM. LINCS: a linear constraint solver for molecular simulations. *J Comp Chem*. 1997;18:1463–1472.
74. Ohio Supercomputer Center. 1987. http://osc.edu/ark:/19495/f5s1ph73.
75. Gao X, Dong X, Li X, Liu Z, Liu H. Prediction of disulfide bond engineering sites using a machine learning method. *Sci Rep*. 2020;10:10330. doi: 10.1038/s41598-020-67230-z