

Overexpression of ryanodine receptor type 1 enhances mitochondrial fragmentation and Ca^{2+} -induced ATP production in cardiac H9c2 myoblasts

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¹Center for Translational Medicine, Department of Medicine, Jefferson Medical College, Thomas Jefferson University, Philadelphia, Pennsylvania; ²Institute of Cardiovascular and Medical Science, University of Glasgow, Glasgow, United Kingdom; ³Cell Signaling Laboratory, Cancer Research Institute, Department of Pharmacology, College of Medicine, Chungnam National University, Daejeon, Republic of Korea; ⁴Department of Pharmacology, School of Medicine, Showa University, Shinagawa-ku, Tokyo, Japan; and ⁵Department of Pharmacology, Juntendo University School of Medicine, Bunkyo-ku, Tokyo, Japan

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O-Uchi J, Jhun BS, Hurst S, Bisetto S, Gross P, Chen M, Kettlewell S, Park J, Oyamada H, Smith GL, Murayama T, Sheu S. Overexpression of ryanodine receptor type 1 enhances mitochondrial fragmentation and Ca^{2+} -induced ATP production in cardiac H9c2 myoblasts. *Am J Physiol Heart Circ Physiol* 305: H1736–H1751, 2013. First published October 11, 2013; doi:10.1152/ajpheart.00094.2013.— Ca^{2+} influx to mitochondria is an important trigger for both mitochondrial dynamics and ATP generation in various cell types, including cardiac cells. Mitochondrial Ca^{2+} influx is mainly mediated by the mitochondrial Ca^{2+} uniporter (MCU). Growing evidence also indicates that mitochondrial Ca^{2+} influx mechanisms are regulated not solely by MCU but also by multiple channels/transporters. We have previously reported that skeletal muscle-type ryanodine receptor (RyR) type 1 (RyR1), which expressed at the mitochondrial inner membrane, serves as an additional Ca^{2+} uptake pathway in cardiomyocytes. However, it is still unclear which mitochondrial Ca^{2+} influx mechanism is the dominant regulator of mitochondrial morphology/dynamics and energetics in cardiomyocytes. To investigate the role of mitochondrial RyR1 in the regulation of mitochondrial morphology/function in cardiac cells, RyR1 was transiently or stably overexpressed in cardiac H9c2 myoblasts. We found that overexpressed RyR1 was partially localized in mitochondria as observed using both immunoblots of mitochondrial fractionation and confocal microscopy, whereas RyR2, the main RyR isoform in the cardiac sarcoplasmic reticulum, did not show any expression at mitochondria. Interestingly, overexpression of RyR1 but not MCU or RyR2 resulted in mitochondrial fragmentation. These fragmented mitochondria showed bigger and sustained mitochondrial Ca^{2+} transients compared with basal tubular mitochondria. In addition, RyR1-overexpressing cells had a higher mitochondrial ATP concentration under basal conditions and showed more ATP production in response to cytosolic Ca^{2+} elevation compared with nontransfected cells as observed by a matrix-targeted ATP biosensor. These results indicate that RyR1 possesses a mitochondrial targeting/retention signal and modulates mitochondrial morphology and Ca^{2+} -induced ATP production in cardiac H9c2 myoblasts.

fluorescence resonance energy transfer; mitochondrial Ca^{2+} uniporter; mitochondria; mitochondrial morphology; ryanodine receptor type 1

MITOCHONDRIAL Ca^{2+} is critical for the regulation of various cellular functions, including energy metabolism, ROS generation, spatiotemporal dynamics of Ca^{2+} signaling, and cell growth/development and death (11, 18, 23). Historically, Ca^{2+}

was found to be accumulated by mitochondria over 5 decades ago (for reviews, see Refs. 24, 64, and 69), and, shortly thereafter, it was also recognized that Ca^{2+} stimulates the oxidative phosphorylation [tricarboxylic acid (TCA) cycle] and electron transport chain activity, which results in the stimulation of ATP synthesis (for a review, see Ref. 23). Additionally, the coexistence of mitochondrial dysfunction and loss of cellular Ca^{2+} homeostasis are frequently observed in various cardiovascular diseases, but it is still not clear how altered mitochondrial Ca^{2+} handling and/or mitochondrial dysfunction are involved in the pathogenesis of each different disease setting (23, 33, 56).

Although the basic functional and pharmacological properties of mitochondrial Ca^{2+} uptake mechanisms have been discovered over 50 yr ago, the molecular identities responsible for these mechanisms have remained a mystery until very recently. Mitochondrial Ca^{2+} influx was principally considered to result from a single transport mechanism mediated by the mitochondrial Ca^{2+} uniporter (MCU) initially due to near complete inhibition by ruthenium red and lanthanides. Finally, through RNA interference studies, several groups (4, 17, 52, 67) discovered the molecular identity of MCU and its regulatory proteins in nonexcitable cells. Although in these studies MCU was confirmed as the most dominant Ca^{2+} influx mechanism (see also Ref. 42), interestingly, mitochondrial morphology, respiration, and cell viability remain normal in MCU knockout or MCU-overexpressing cells (4, 17, 52, 67) even though Ca^{2+} influx is critical in the regulation of these main mitochondrial functions. In addition, recent reports using mathematical analysis (81) and electrophysiological experiments (21) have suggested that cardiac mitochondria Ca^{2+} influx through MCU might be slow and small under the physiological range of cytosolic Ca^{2+} concentrations ($[\text{Ca}^{2+}]_c$; i.e., 0.1–20 μM) compared with other cytosolic Ca^{2+} fluxes such as sarco(endo)plasmic reticulum Ca^{2+} -ATPase (SERCA). Therefore, these results have raised another hypothesis: that additional Ca^{2+} uptake pathways must exist in the regulation of these critical mitochondrial functions. Indeed, subsequent studies have identified additional Ca^{2+} uptake pathways, such as leucine zipper-EF hand-containing transmembrane protein 1 (Letm1) (36), rapid mode of uptake (RaM) (75), and coenzyme Q₁₀ (10), which exhibit different Ca^{2+} affinity, uptake kinetics, and pharmacological characteristics from the original MCU

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theory (for a review, see Ref. 64). Moreover, it has been recently shown that cardiac mitochondria contain two modes of binuclear ruthenium complex-sensitive Ca^{2+} uptake, a high Ca^{2+} affinity rapid uptake mode and a low Ca^{2+} affinity slow uptake mode, that are responsible for modulating oxidative phosphorylation and Ca^{2+} buffering, respectively (80). However, the detailed molecular compositions of the ion channels/transporters responsible for the Ca^{2+} uptake mechanism, especially in excitable cells, including cardiomyocytes, remain elusive.

Among these studies, a skeletal muscle-type ryanodine receptor (RyR), RyR type 1 (RyR1), was also found as one of the mitochondrial Ca^{2+} influx mechanisms in cardiomyocytes with a known molecular identity reported from our group, termed “mitochondrial RyR1” (mRyR1) (for reviews, see Refs. 64, 71, and 72). We first found that a low level of RyR1 is expressed at the mitochondrial inner membrane in cardiomyocytes, as shown by a combination of biochemical and cell biological experiments, and finally confirmed its role in cardiomyocytes as a fast Ca^{2+} uptake pathway by electrophysiological experiments (1, 7, 8, 72). Since mRyR1 shows a bell-shaped Ca^{2+} -dependent activation (bimodal activation) in a physiological range of $[\text{Ca}^{2+}]_c$, this unique property places mRyR1 as an ideal candidate for sequestering Ca^{2+} quickly and transiently under physiological $[\text{Ca}^{2+}]_c$ oscillation, such as during heart beats. The molecular identity of RyR1 in cardiac mitochondria was carefully analyzed and confirmed using not only native cardiomyocytes but also those obtained from RyR1 knockout mouse hearts (8). Interestingly, in RyR1 knockout mouse heart mitochondria, the respiratory control index is very low (≈ 1) and $[\text{Ca}^{2+}]_c$ does not stimulate O_2 consumption, suggesting that mRyR1 is required for both the basal metabolic state and Ca^{2+} -dependent acceleration of the TCA cycle in cardiomyocytes (8) even though its expression level is much lower than RyR2, which is the main RyR isoform expressed in the cardiac sarcoplasmic reticulum (SR)/endoplasmic reticulum (ER) (44).

In addition, recent reports have indicated that changes in mitochondrial dynamics/morphology are closely related to mitochondrial function, including ATP production and cellular function, suggesting that mitochondrial form and function are tightly linked (for a review, see Ref. 25). We (26) have also reported that mitochondrial Ca^{2+} influx is a key regulator for mitochondrial morphology in cardiac cells. Therefore, we hypothesize that Ca^{2+} sequestration via mRyR1 into cardiac mitochondria regulates mitochondrial morphology, concomitantly efficiently stimulating oxidative phosphorylation for ATP production during excitation-contraction coupling to meet metabolic demands. To deal with the potential issue of low expression of RyR1 in native cardiomyocytes (8), we overexpressed RyR1 in cardiac H9c2 myoblasts and tested whether RyR1 overexpression can increase both basal metabolism and Ca^{2+} -dependent ATP production. We also observed the effect of RyR1 overexpression on mitochondrial morphology. Here, we showed that 1) overexpression of RyR1 (but not RyR2 or MCU) leads to mitochondrial fragmentation and 2) cells that overexpress RyR1 had a higher mitochondrial ATP concentration ($[\text{ATP}]_{\text{mt}}$) under basal conditions and accelerated $[\text{Ca}^{2+}]_c$ -dependent ATP production. We also found that fragmented mitochondria possess bigger and sustained mitochondrial Ca^{2+} transients compared with basal tubular mitochondria in H9c2

cells, suggesting the existence of a critical link between the mitochondrial form (morphology) and function (Ca^{2+} -dependent ATP production). These results indicate that mRyR1 and MCU have distinct roles in the regulation of mitochondrial form and function in cardiac H9c2 myoblasts.

MATERIALS AND METHODS

Plasmids, antibodies, and reagents. The following plasmids were used in the experiments: rabbit RyR1 in pCI-neo (provided by Dr. Paul Allen, Brigham and Women's Hospital, Boston, MA) (60); green fluorescent protein (GFP)-tagged rabbit RyR1 in pGFP37 and [kindly provided by Dr. Robert T. Dirksen, University of Rochester, Rochester NY; modified GFP is fused at the NH_2 -term of rabbit RyR1 provided by Dr. Paul D. Allen (46)]; multifunctional (mf)GFP-tagged RyR1 (RyR1-mfGFP) in pcDNA5/FRT/TO (mfGFP is inserted in the middle of the coding sequence after Ala¹³⁹⁷) (43); mouse RyR2 in pcDNA3 and GFP-tagged RyR2 in pcDNA3 (kindly provided by Dr. S. R. Wayne Chen, University of Calgary, AB, Canada; GFP is inserted in the middle of the coding sequence after Asp⁴³⁶⁵) (49); mouse wild-type enhanced GFP (EGFP)-tagged MCU in pEGFP-N1 and EGFP-tagged mitochondrial Ca^{2+} uptake 1 (MICU1) in pEGFP-N1 (kindly provided by Dr. Rosario Rizzuto at University of Padua, Padua, Italy; tags are fused at COOH-term of MCU and MICU1) (17); rat dynamin-like protein 1 (DLP1)-K38A in pcDNA3, mitochondrial matrix-targeted DsRed (mt-RFP) in pDsRed1-N1 [the mitochondrial transit sequence of human isovaleryl coenzyme A dehydrogenase was fused to the NH_2 -terminus of red fluorescent protein (RFP)], and mitochondrial matrix-targeted (mt)-GFP in pEGFP-N1 (kindly provided by Dr. Yisang Yoon, Georgia Health Sciences University, Augusta GA) (83); mitochondria-targeted Ca^{2+} biosensor (Mitycam) in pcDNA3.1(+) (40) and mitochondria-targeted ATP biosensor Ateam (mt-Ateam) in pcDNA3 (kindly provided by Dr. Hiromi Imamura, Kyoto University, Kyoto, Japan) (29); SR-targeted GFP (GFP-KDEL or SR-GFP) in pcDNA3.1/Zeo(+) (an ER targeting sequence that corresponds to the NH_2 -terminal 17 amino acids of mouse calreticulin was fused to the NH_2 -terminus of EGFP and an ER retention signal peptide, KDEL, was inserted into its COOH terminus; kindly provided by Drs. Katsuhiko Mikoshiba and Hiroko Bannai, RIKEN, Wako, Japan) (2); small interfering (si)RNA-GFP in pLKO.1 puro (63) (Addgene, Cambridge MA); GFP-tagged Letm1 in pEGFP-N1 (68); pcDNA3.1(+) (Invitrogen, Grand Island, NY); and pEGFP-C1 (Clontech, Mountain View, CA). Full-length Mitycam was dissected from pcDNA3.1(+) and was inserted into pCI-neo (Clontech) at *Eco*R1 and *Not*I sites. Full-length RyR1-mfGFP was dissected from pcDNA5/FRT/TO and was inserted into pcDNA3.1(+) at *Hind*III and *Eco*RV sites.

The following antibodies were used in the experiments: anti-RyR1 antibody (ARR-001) from Alomone Labs (Jerusalem, Israel); anti-voltage-dependent anion channel (VDAC; No. 4866S), anti-SERCA2a (No. 9580), and anti-GFP (No. 2956S) from Cell Signaling Technology (Danvers, MA); anti-SERCA2 (ab2861) from Abcam (Cambridge, MA); anti-tubulin (T5168) and anti-RyR2 (clone C3-33, R-128) from Sigma-Aldrich (St. Louis, MO); and anti-DRP1 (611738) from BD Bioscience (San Jose, CA).

All reagents were purchased from Sigma-Aldrich unless otherwise indicated. Coenzyme Q₁₀ (TCI America, Portland, OR) was dissolved in ethanol. Fulo-3-AM, fura red-AM, MitoSox red, and tetramethyl rhodamine ester (TMRE) (Invitrogen) were dissolved in DMSO.

Cell culture, transfection, and infection. A rat cardiac myoblast cell line (H9c2 cells; American Type Culture Collection, Manassas, VA) was maintained in high-glucose (4.5 g/l) DMEM (Mediatech/Corning, Corning NY) supplemented with 10% FBS (GIBCO, Grand Island, NY), 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin (Mediatech/Corning), and 1% L-glutamax (Invitrogen) at 37°C with 5% CO₂ in a humidified incubator (84). This cell line possesses cardiac characteristics in the undifferentiated myoblast condition, expressing both

cardiac muscle and skeletal muscle-featured proteins. After differentiation, cells form skeletal-type myotubes and lose their cardiac characteristics (39, 65, 66). Therefore, experiments were performed under the myoblast condition. A mouse skeletal myoblast cell line (C2C12 cells; kindly provided by Dr. Robert T. Dirksen) and an immortalized RyR1-dyspedic mouse myoblast cell line (1B5 cells; kindly provided by Dr. Paul D. Allen) were maintained in low-glucose (1.0 g/l) DMEM (Sigma-Aldrich), 20% FBS (GIBCO), 100 U/ml penicillin, 100 μ g/ml streptomycin, 0.25 μ g/ml fungizone (Lonza, Allendale, NJ), and 2% L-glutamax (Invitrogen) in gelatin-covered dishes at 37°C with 5% CO₂ in a humidified incubator (54). Cells were transfected with plasmids using FUGENE HD transfection reagents (Promega, Madison, WI) as subconfluent cultures (80–90% cell confluence), replated 24 h after transfection using Acutase (Innovative Cell Technologies, San Diego, CA) for single cell analysis, and used for experiments 48 h after transfection. An equimolar ratio of GFP-tagged proteins and mt-RFP was transfected.

Generation of the RyR1 stably expressing H9c2 cell line. Stable cells carrying RyR1-mfGFP were generated by transfection with mfGFP-RyR1 in pcDNA3.1(+) and selected with 1,600 μ g/ml G-418 (Mediatech/Corning). One month after selection was started under 1,200 μ g/ml G-418, cell colonies carrying EGFP expression were isolated under fluorescence microscope and were maintained in 800 μ g/ml G-418. The expression level of RyR1 was also confirmed by Western blot analysis.

Mitochondrial protein isolation and Western blot analysis. The expression level of RyR1 in mitochondria was determined by isolating mitochondria-enriched protein fractions as previously described (26). Briefly, H9c2 cells were cultured in 10-cm dishes, washed with isolation buffer (320 mM sucrose, 1 mM EDTA, and 10 mM Tris-HCl; pH 7.4), removed from dishes, and collected by centrifuge at 700 g. Cells were resuspended with isolation buffer containing protease inhibitor cocktail (Sigma-Aldrich) and phosphatase inhibitor cocktail (Roche, Mannheim, Germany), homogenized with a Dounce homogenizer, and centrifuged again at 700 g. The supernatant was then collected, and mitochondrial proteins and cytosolic proteins containing SR proteins were separated by centrifugation at 17,000 g. The pellet (mitochondrial protein fraction) was resuspended in lysis buffer (Cell Signaling Technology) containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.2% SDS, 2.5 mM sodium pyrophosphate, 1 μ M β -glycerophosphate, 1 mM NaVO₄, 50 mM NaF, 1 mM PMSF, and 1% protease inhibitor cocktail (Sigma-Aldrich).

Whole cell lysates were also prepared from H9c2 cells (35). Cells were washed with cold PBS and then incubated in lysis buffer on ice for 30 min. Cell lysates were centrifuged at 20,000 g for 15 min at 4°C, and supernatants were collected.

The cytosolic fraction containing the SR was isolated from the whole heart or skeletal muscle of adult male c57BL/6 mice using procedures we have previously reported (7, 8). The protein concentration was determined using the BCA method (Thermo Scientific, Rockford, IL). Cell lysates were separated by SDS-PAGE and transferred to nitrocellulose membranes (Santa Cruz Biotechnology, Santa Cruz, CA) and incubated with primary antibodies followed by an incubation with fluorescence-conjugated secondary antibodies (LI-COR Biotechnology, Lincoln, NE). Immunoreactive bands were visualized using the Odyssey Infrared Imaging System (LI-COR Biotechnology).

All animal experiments were performed in accordance with the guidelines on animal experimentation of Thomas Jefferson University. The study protocol was approved by the Animal Care Committee of Thomas Jefferson University. The investigation conformed with the National Institutes of Health (NIH) *Guide for the Care and Use of Laboratory Animals*.

Confocal microscopy. Localization of GFP-tagged proteins (3, 35), measurements of [Ca²⁺]_c by fluo-3 and fura red (30, 31, 38), measurements of mitochondrial membrane potential (Ψ_m) by the Ψ_m -

sensitive probe TMRE (Invitrogen) (28), mitochondrial ROS levels by the mitochondrial superoxide indicator MitoSox red (26), and measurements of mitochondrial matrix Ca²⁺ concentrations ([Ca²⁺]_{mt}) by the mitochondria-targeted Ca²⁺ biosensor Mitycam (29, 40) and [ATP]_{mt} by the ATP biosensor Ateam (29) were observed in live H9c2 cells using laser scanning confocal microscopes (FV-500 or FV-1000) with images obtained using Fluoview software (Olympus, Tokyo, Japan) at room temperature. Briefly, cells were plated on glass bottom 35-mm dishes (Matek, Ashland, MA) for observation. The cell culture medium was replaced with Tyrode solution (136.9 mM NaCl, 5.4 mM KCl, 1 mM CaCl₂, 0.5 mM MgCl₂, 0.33 mM NaH₂PO₄, 5 mM HEPES, and 5 mM glucose; pH 7.40, adjusted with NaOH) during observation.

Quantitative colocalization coefficient analysis. The mitochondrial localization of GFP-tagged proteins was observed by the coexpression of the mitochondrial marker mt-RFP (83). The expression of mt-RFP itself did not exhibit any significant changes in mitochondrial morphology compared with classical mt-tracker red staining (data not shown). Two-dimensional catterplots of pixel intensities in red (mt-RFP) and green (GFP-tagged proteins) channels were generated with ImageJ software (NIH; see Fig. 2C). *Region 1* and *2* pixels represent signals in *channels 1* or *2* only, respectively, and *region 3* represents colocalized pixels (see Fig. 2C). Quantitative colocalization analysis was performed using ImageJ software (NIH) with an Intensity Correlation Analysis plug-in (The Bob and Joan Wright Cell Imaging Facility, Toronto Western Hospital). Colocalization was estimated using Pearson's correlation coefficient and overlap coefficient according to Manders (86). The values for Pearson's correlation are ranged from 1 to -1. A value of 1 represents perfect correlation, -1 represents perfect exclusion, and 0 represents random localization. Cells overexpressing both mt-GFP and mt-RFP were used as positive controls for perfect correlation and colocalization, and cells overexpressing both SR-GFP and mt-RFP were used as negative controls for perfect exclusion (see Fig. 2).

Quantitative analyses of mitochondrial morphology. Quantitative analyses of mitochondrial morphology were performed using methods we have previously described (26, 27, 84). Digital images obtained by confocal microscopy were processed through a convolve filter of ImageJ software (NIH) to obtain isolated and equalized fluorescent pixels. After a conversion to masks, individual mitochondria (particles) were subjected to particle analyses to acquire values for the form factor (FF; the reciprocal of circularity value) and aspect ratio (AR; major axis/minor axis). Both parameters have a minimal value of 1 when it is a perfect circle. High values for FF represent elongated tubular mitochondria, and increased AR values indicate an increase of mitochondrial complexity (length and branching; see also Fig. 3B) (84).

Measurements of [Ca²⁺]_c. Resting [Ca²⁺]_c was measured with a double-indicator ratiometric procedure by loading cells with fluo-3 and fura red (30, 31, 38). Cells were incubated with fluo-3-AM (5 μ M) and fura red-AM (10 μ M, Invitrogen) in culture medium for 10 min at 37°C. Cells were washed with Tyrode solution and observed using the FV-1000 confocal system (Olympus). The dyes were excited by a 488-nm laser line, and fluorescence was detected in two channels collected through 505- to 605-nm (for fluo-3) and 655- to 755-nm (for fura red) filters. For line scans, a single pixel-wide line across the cytosolic region of a single cell was repetitively scanned at 250 lines/s. All experiments were performed at room temperature.

Measurements of [Ca²⁺]_{mt}. H9c2 cells transfected by the mitochondria-targeted Ca²⁺ biosensor Mitycam were used for measurements of [Ca²⁺]_{mt} with confocal microscopy (40). Mitycam fluorescence was measured with excitation at 488 nm (the excitation peak is reported at 498 nm) and emission at 530 nm every 2 s. Mitycam fluorescence (F) was converted to 1 - (F/F₀), where F₀ is the initial fluorescence level (40), which represents the changes in [Ca²⁺]_{mt}.

Measurements of [ATP]_{mt}. H9c2 cells transfected by the mitochondria-targeted ATP biosensor Ateam were used for measurements of

[ATP]_{mt} with confocal microscopy. Ateam consists of variants of CFP (msecFP) and yellow fluorescent protein (cp173-mVenus) connected by the ϵ -subunit of *Bacillus subtilis* F₀F₁-ATP synthase (29). Cyan fluorescent protein (CFP) and fluorescence resonance energy transfer (FRET) images were obtained every 2 s through a 420- to 20-nm excitation filter, a 450-nm dichroic mirror, and a 475/40-nm emission filter (CFP) or 535/25-nm emission filter (FRET). The FRET-to-CFP emission ratio was calculated from CFP and FRET fluorescence images of individual cells, which indicates the basal ATP concentration in mitochondria.

Electron microscopy. Mitochondrial morphology at the ultrastructural level was observed in transmission electron microscopy (EM), as we have previously reported (55). Specimens were fixed in 2% glutaraldehyde in 0.1 M phosphate buffer, postfixed in 1% osmium tetroxide in the same buffer, dehydrated in ethanol, and embedded in epoxy resin. Thin sections were stained with uranyl acetate and lead citrate. All specimens were observed with a transmission EM (FEI Tecnai 12 TEM equipped with an XR111 11 megapixel charge-coupled device, Advanced Microscopy Techniques, Woburn, MA) at the EM Facility (Department of Pathology, Anatomy and Cell Biology, Thomas Jefferson University).

Data and statistical analysis. All results are shown as means $\pm$ SE. An unpaired Student's *t*-test was performed for two data sets. For multiple comparisons, one-way ANOVA followed by a post hoc Tukey test was performed. Statistical significance was set at *P* < 0.05.

Simple linear regression analysis (37) and Gaussian fitting in histograms were performed using Origin software (version 6.1, Northampton, MA).

RESULTS

Subcellular localizations of overexpressed RyR1 in cardiac H9c2 cells. It has been previously reported that cardiac H9c2 myoblasts derived from the embryonic rat heart do not possess detectable expression of RyR1 (19). Using a specific antibody against RyR1, we confirmed that H9c2 cells did not possess detectable protein levels of RyR1 (Fig. 1A). The isoform specificity of this antibody was confirmed using cytosolic/SR fractions obtained from mouse skeletal and cardiac muscles (Fig. 1B). To test the possibility of RyR1 trafficking/retention to cardiac mitochondria, we overexpressed RyR1 in H9c2 cells by DNA plasmid transfection. To deal with the potential issue

of low transfection efficiency by conventional transient plasmid transfection methods since the protein molecular weight of RyR1 is relatively large, we also established RyR1 stably overexpressing H9c2 cell lines (see MATERIALS AND METHODS), and the expression level was confirmed by Western blot analysis using the specific antibody against RyR1 (Fig. 1A). Because RyRs are mainly expressed in the SR or ER, where they mediate Ca²⁺ release (53), the purity of our mitochondria-enriched protein fractionation was evaluated by detection of VDAC and SERCA2 by Western blot analysis as mitochondrial and ER/SR markers, respectively (8). The mitochondria-enriched protein fractionation obtained from the RyR1-overexpressing cell line showed that RyR1 is dominantly found in the cytosolic/SR fraction but is also partially expressed in mitochondria-enriched protein fractionation (Fig. 1C). We next observed the subcellular localization of overexpressed GFP-tagged RyR1 by confocal microscopy using transient RyR1-overexpressing cells (Fig. 2A). mt-RFP was cotransfected with GFP-tagged RyR1 to detect the mitochondrial localization of RyR1 (83). GFP-tagged MCU, its regulatory protein MICU1, RyR2, and Letm1 were also overexpressed for comparison. MCU, MICU1, and Letm1 were localized predominantly in mitochondria, similar to the expression pattern of mt-GFP (Fig. 2, A and B). The subcellular localization of RyR1 and RyR2 showed “mesh-like” patterns indicative of mainly ER/SR localization (59), similar to the SR-GFP expression pattern (Fig. 1A). However, RyR1 but not RyR2 and SR-GFP was partially colocalized with mt-RFP, which corresponded with our data obtained from mitochondria-enriched protein fraction (Fig. 2A). Two-dimensional scatterplots of pixel intensities in red (mt-RFP) and green (GFP-RyR1) channels indicated that a small population of colocalized pixels (yellow) exists in RyR1-overexpressing cells (Fig. 2C). We next quantitatively assessed the mitochondrial localization efficiency of each GFP-tagged protein by calculating the values of Pearson's correlation (Fig. 2D). GFP-tagged MCU, MICU1, and Letm1 showed values close to 1, representing that these proteins were highly mitochondria-targeted proteins, as previously reported (4, 17, 67). The Pearson's value for RyR1 was smaller than those of MCU,

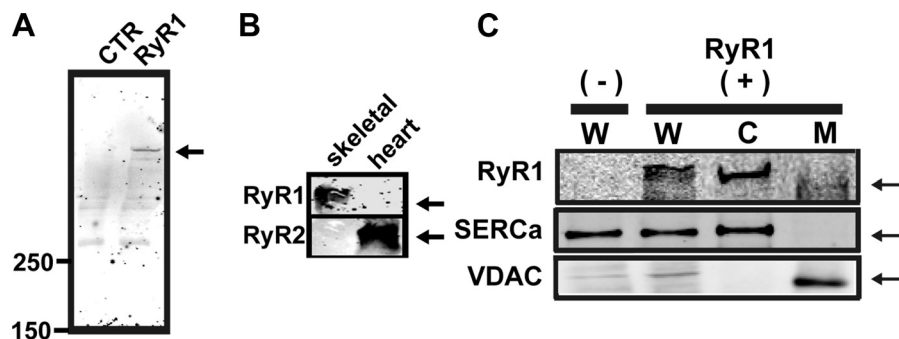
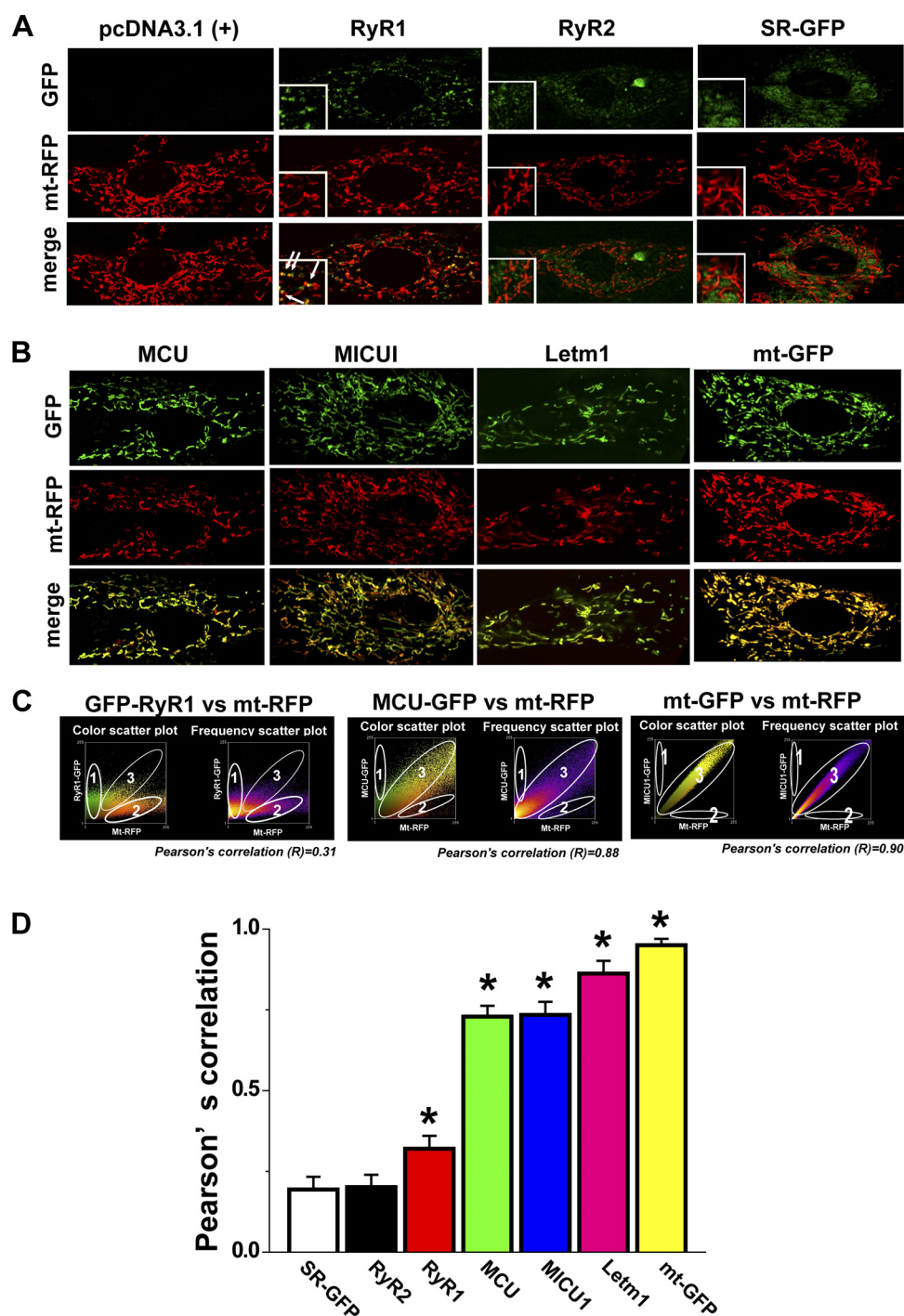


Fig. 1. Overexpressed ryanodine receptor (RyR) type 1 (RyR1) is partially localized in mitochondria in cardiac H9c2 cells. A: detection of overexpressed RyR1 (arrows) from H9c2 cells stably overexpressing multifunctional green fluorescent protein (mGFP)-RyR1 in whole cell lysates (25 μ g/well) using a specific antibody against RyR1 (rabbit polyclonal antibody, Alomone) using 4% SDS-PAGE (see MATERIALS AND METHODS). The control (CTR) was lysate from nontransfected cells. B: specificity of the antibodies against RyR1 and RyR2. Cytosolic/sarcolemmal reticulum (SR) proteins obtained from mouse skeletal (left lane) and cardiac (right lane) muscles were separated using 4% SDS-PAGE (20 μ g/well). The isoform specificities of the antibody against RyR1 (rabbit polyclonal antibody, Alomone) and RyR2 (mouse monoclonal antibody, Sigma) were confirmed in the same blot. C: detection of overexpressed RyR1 from H9c2 cells stably overexpressing mGFP-RyR1 in the whole cell lysate (W; 25 μ g/well), cytosolic (C) fraction (including the SR; 25 μ g/well), and mitochondrial (M) fraction (25 μ g/well). Whole cell lysates from nontransfected H9c2 cells were used as the control (left side). Sarco(endo)plasmic reticulum Ca²⁺-ATPase 2 (SERCA2; Abcam) or voltage-dependent anion channel (VDAC; Cell Signaling) detection was used as confirmation of the purity of the cytosolic (including the SR) or mitochondrial fractions, respectively.

Fig. 2. Overexpressed RyR1 (but not RyR2) is partially localized in mitochondria in cardiac H9c2 cells. **A**: representative images obtained from H9c2 cells coexpressing mitochondria-targeted red fluorescence protein (mt-RFP) and GFP-tagged RyR1 and RyR2 using confocal microscopy. Arrows in the *inset* show the mitochondrial localization of RyR1. A GFP-nontransfected cell [transfected by empty vector pcDNA3.1(+)] is shown for comparison to demonstrate background fluorescence. Cells coexpressing SR-targeted GFP (SR-GFP) and mt-RFP are also shown for comparison. **B**: representative pictures obtained from H9c2 cells coexpressing mt-RFP and GFP-tagged mitochondrial Ca^{2+} uniporter (MCU), mitochondrial Ca^{2+} uptake 1 (MICU1), and leucine zipper-EF hand-containing transmembrane protein 1 (Letm1) using confocal microscopy. Cells overexpressing mitochondrial matrix-targeted (mt)-GFP and mt-RFP were used positive controls for the colocalization analysis (*right*). **C**: color scatterplots (*left*) and frequency scatterplots (*right*) obtained from the representative images in **A** and **B**. Plots obtained from cells overexpressing mt-GFP and mt-RFP are shown as positive controls for the colocalization analysis. *Region 1* and *2* pixels represent signals in *channels 1* (green, GFP) or *2* (red, RFP) only, respectively, and *region 3* represents colocalized pixels. **D**: colocalization coefficient analysis using Pearson's correlation coefficient (see MATERIALS AND METHODS). Values for Pearson's correlation coefficient ranged from 1 to -1 . A value of 1 represents perfect correlation, -1 represents perfect exclusion, and 0 represents random localization. * $P < 0.05$ compared with SR-GFP.



MICU1, and Letm1 but was significantly higher than RyR2 and SR-GFP, suggesting that RyR1 has a higher probability of mitochondrial localization compared with RyR2.

RyR1 overexpression induces mitochondrial fragmentation. The above results show that RyR1 has a high possibility of mitochondrial retention compared with RyR2, which is consistent with our previous report (8) using native mitochondria obtained from adult rat cardiomyocytes. Next, to evaluate the mitochondrial morphological changes after RyR overexpression, we analyzed the mitochondrial shapes (mt-RFP signal) obtained from cells expressing GFP-tagged proteins. We only

used cells successfully transfected with both mt-RFP and GFP-tagged RyR1 for this analysis. In GFP-RyR1-overexpressing cells, RyR1-overexpressing mitochondria showed a remarkably circular and fragmented morphology (see Fig. 2A, *inset*), whereas there were no obvious changes in mitochondrial morphology in RyR2- (Figs. 2A and 3A), MCU-, MICU1-, and Letm1-overexpressing cells (Fig. 2C) (84). We quantitatively evaluated mitochondrial morphology after RyR, MCU, MICU1, and Letm1 overexpression by the values of FF (the reciprocal of circularity value) and AR (major axis/minor axis) obtained from mt-RFP fluorescent images in single cells

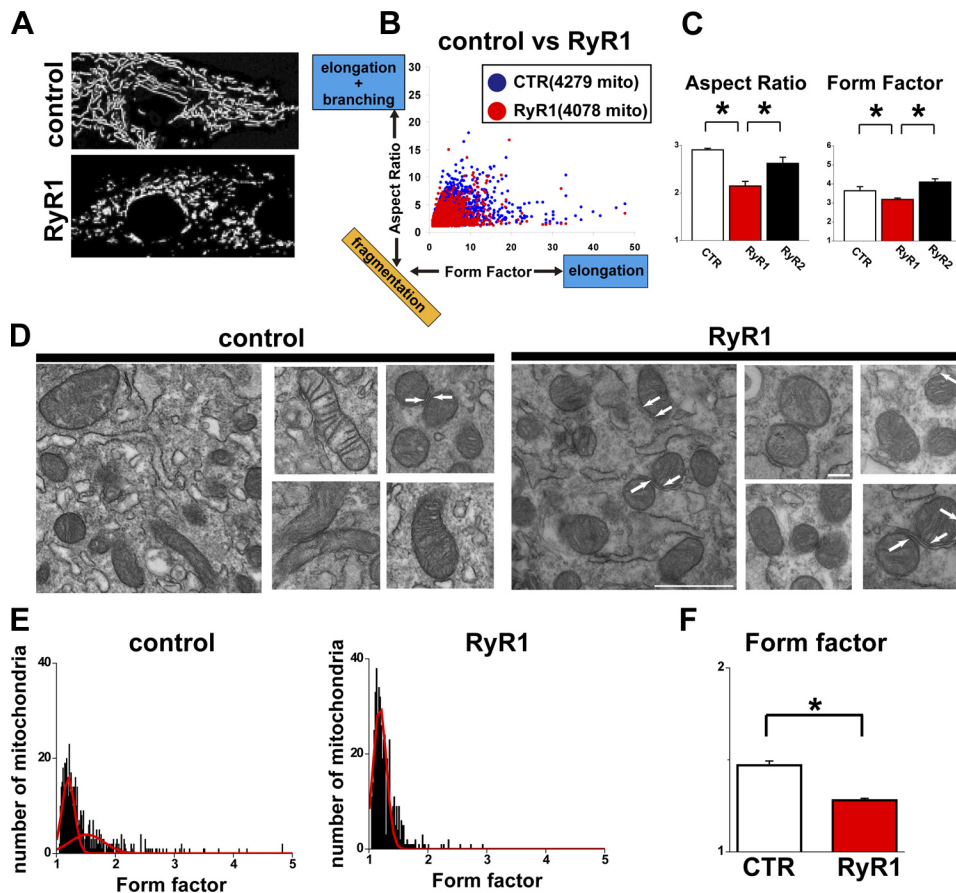


Fig. 3. RyR1 (but not RyR2) overexpression induces mitochondrial fragmentation in cardiac H9c2 cells. **A:** representative mitochondrial morphology in GFP-RyR1-overexpressing (bottom) and control (top) H9c2 cells. Mitochondrial morphology was monitored by cotransfection of mt-RFP. Confocal images of mt-RFP using a convolve filter are shown and were used for the calculation of the aspect ratio (AR) and form factor (FF) from each mitochondrion (see MATERIALS AND METHODS). **B:** summary data of merged AR/FF plots obtained from multiple cells of control and GFP-RyR1-overexpressing cells. **C:** summary data of AR/FF from RyR1-overexpressing cells. AR/FF from RyR2-overexpressing cells is shown for comparison. * $P < 0.05$ compared with control cells. **D:** representative mitochondrial morphology in mfGFP-RyR1-overexpressing (right) and control (left) H9c2 cells observed using electron microscopy (EM). Scale bar in high magnification = 500 nm; scale bar in low magnification = 100 nm. Arrows indicate the location of endoplasmic reticulum (ER)/SR-mitochondria contact sites. **E:** summary data of FF histograms obtained from EM images of control (left) and mfGFP-RyR1-overexpressing (right) cells. Red solid lines show FF distribution patterns in each histogram by Gaussian fitting (one or two peaks). **F:** summary data of averaged FFs obtained from EM images of control and GFP-RyR1-overexpressing cells. * $P < 0.05$ compared with control cells.

(Figs. 2 and 3) (26, 27). The values of FF and AR represent the mitochondrial shape (fragmentation or elongation) and network (branching; see MATERIALS AND METHODS; Fig. 3B). MCU, MICU1, and Letm1 overexpression did not change either FF or AR (only AR data was shown), confirming that these two proteins do not change mitochondrial morphology in this cell line (Fig. 4A). RyR2, which is not expressed in mitochondria, also did not change these two parameters (Fig. 3C). Recently, coenzyme Q₁₀ has been identified as an additional Ca²⁺ uptake pathway (10). However, coenzyme Q₁₀ treatment did not show significant changes in mitochondrial morphology in this cell line (Fig. 4B). Only RyR1 overexpression significantly de-

creased both FF and AR, indicating that there was significant mitochondrial fragmentation (Fig. 3, B and C).

The changes in mitochondrial morphology induced by RyR1 overexpression were also observed by EM. In control cells, mitochondrial morphology consisted of a mix of both tubular and round mitochondria, but cells that stably overexpressed mfGFP-RyR1 contained mostly small and round mitochondria (Fig. 3D). Interestingly, we also found that ER/SR-mitochondrial contact sites were more frequently observed around round mitochondria after overexpression of RyR1 compared with control cells (Fig. 3D, white arrows). We calculated FF from each mitochondrion observed in EM images and generated a

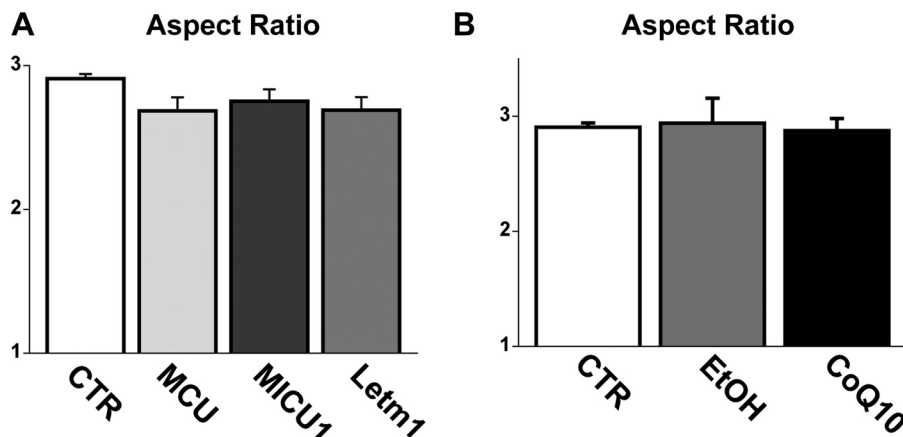


Fig. 4. MCU, MICU1, Letm1, and coenzyme Q₁₀ (CoQ10) do not change mitochondrial morphology in cardiac H9c2 cells. **A:** summary data of ARs obtained from GFP-tagged MCU-, MICU1-, and Letm1-overexpressing cells (see also Fig. 2). Mitochondrial morphology was monitored by cotransfection of mt-RFP. **B:** summary data of ARs obtained after treatment of 10 μ M coenzyme Q₁₀ for 72 h. More than 24 h of treatment of 10 μ M coenzyme Q₁₀ from extracellular solution can significantly increase the mitochondrial coenzyme Q₁₀ concentration in H9c2 cells (6). Since coenzyme Q₁₀ stock solution was made in ethanol (EtOH), data from control (no treatment) and vehicle (EtOH)-treated cells are also shown for comparison. Mitochondrial morphology was monitored by cotransfection of mt-RFP.

histogram for FF distribution in each group (Fig. 3E). In control cells, there was one peak distribution near 1 (round mitochondria), but the FF values are widely spread in the range of from 1 to 5 (tubular mitochondria), showing as a two-peak Gaussian fitting (peak values of 1.20 and 1.51). In cells that stably overexpressed mfGFP-RyR1, the distribution pattern of FF lost diversity, and we observed one sharp peak near 1 (round mitochondria), showing as a single-peak Gaussian fitting (peak value of 1.19). In addition, the mean FF value in cells that stably overexpressed mfGFP-RyR1 obtained from EM was significantly decreased compared with control cells, which is consistent with our observations using confocal microscopy (Fig. 3F).

Next, we addressed the question of whether these changes in mitochondrial morphology are derived from overexpressed RyR1 channel function. Indeed, mitochondrial fragmentation was inhibited by treatment with dantrolene, a RyR1 blocker (8, 85), whereas treatment with dantrolene itself did not alter any mitochondrial morphology in RyR2-overexpressing or control cells (Fig. 5A). To definitively confirm that the mitochondrial fragmentation was RyR1 mediated, we tested the effect of RyR1 knockdown on mitochondrial morphology in H9c2 cells that stably overexpressed mfGFP-RyR1. We used the transfection vector encoding siRNA against GFP (63) since mfGFP-RyR1 contains the GFP sequence. The knockdown efficiency using this siRNA was first confirmed in H9c2 cells transiently with GFP (Fig. 5B). The mitochondrial fragmentation in cells that stably overexpressed mfGFP-RyR1 was inhibited by siRNA transfection concomitant with significant knock down of mfGFP-RyR1 (Fig. 5C), indicating that this effect was mediated through overexpression of RyR1. In contrast, transfection of GFP-siRNA vector in control cells did not show any morphological changes (Fig. 5C). Because H9c2 cardiac myoblasts do not possess detectable RyR1 before differentiation (see Fig. 1A) and skeletal myoblasts do (19), we next used the mouse skeletal myoblast cell line (C2C12 cells) as a template of RyR1 knockout and compared those cells with the immortalized RyR1-dyspedic mouse myoblast cell line (1B5 cells;

Fig. 5D) (20). In C2C12 cells, mitochondrial morphology consisted of a mix of both tubular and round (and small) mitochondria, but 1B5 cells contained only tubular mitochondria. Quantitative analysis of the values of FF and AR also revealed significant differences in mitochondrial shape between C2C12 and 1B5 cells (Fig. 4D; only AR values are shown). The mitochondrial elongation by stable knockdown of RyR1 in 1B5 cells was countered by GFP-RyR1 knockin (Fig. 5D), suggesting that the RyR1 expression level is critical for mitochondrial morphology.

RyR1 overexpression induces mitochondrial fragmentation through the fission process. The next question is how RyR1 overexpression induces mitochondrial fragmentation. It has been well demonstrated that cardiac cells undergo mitochondrial fission in response to Ca^{2+} elevation and ROS accumulation (26, 27), which leads to Ψ_m depolarization (5) and finally to decreased ATP production and increased activation of apoptotic cell death signaling (62). Since the majority of RyR1 is expressed in the ER/SR after overexpression, we first investigated whether there was an elevation or oscillation of $[\text{Ca}^{2+}]_c$ in H9c2 cells overexpressing RyR1 due to the increased amount of SR Ca^{2+} leak through overexpressed RyR1 at the SR. Resting $[\text{Ca}^{2+}]_c$ was measured with a double-indicator ratiometric procedure by loading cells with fluo-3 and fura red (Fig. 6) (30, 31, 38). RyR1 overexpression did not change resting $[\text{Ca}^{2+}]_c$, whereas RyR2 overexpression significantly increased resting $[\text{Ca}^{2+}]_c$, compared with control cells (Fig. 6, A and B). In addition, cells that overexpressed RyR1 did not show $[\text{Ca}^{2+}]_c$ oscillation at rest, but cells that overexpressed RyR2 did, which is consistent with results we have previously shown in human embryonic kidney-293 cells (Fig. 6C) (58). We also found that RyR1 overexpression did not change basal ROS levels in mitochondria measured by the mitochondrial superoxide indicator MitoSox (Fig. 7A). In addition, there was no significant change in Ψ_m as measured by the Ψ_m -sensitive probe TMRE (Fig. 7B). These data indicate that the mitochondrial fragmentation by RyR1 overexpression

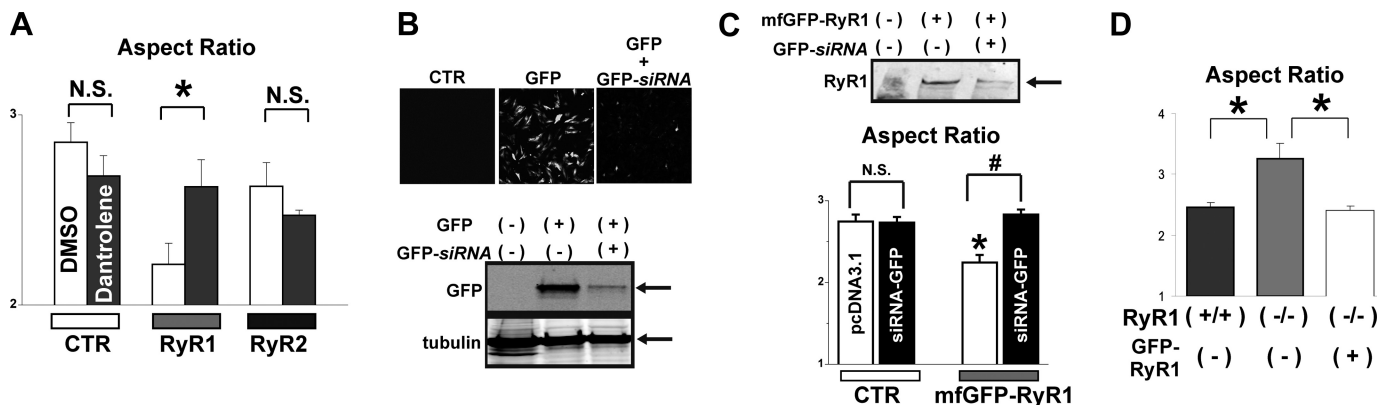


Fig. 5. RyR1 inhibition/knockdown attenuates mitochondrial fragmentation. *A*: effect of RyR blocker (1 μM dantrolene) on mitochondrial morphology in RyR1- or RyR2-overexpressing cells. Cells were treated with either 1 μM dantrolene or DMSO (as a control) overnight and were used for morphological analysis. Mitochondrial morphology was monitored by transfection of mt-RFP. *B*: suppression of GFP expression by cotransfection of GFP-small interfering (si)RNA in H9c2 cells. Expression of GFP by pEGFP-C1 vector in H9c2 cells was examined by cotransfection with or without GFP-siRNA in pSP108 vector using confocal microscopy and Western blot analysis (whole cell lysates, 25 $\mu\text{g}/\text{well}$). *C*: suppression of mfGFP-RyR1 expression by cotransfection of GFP-siRNA vector in H9c2 cells stably overexpressing mfGFP-RyR1 (whole cell lysates, 25 $\mu\text{g}/\text{well}$). Mitochondrial morphology was monitored by transfection of mt-RFP. *D*: effect of RyR knockin on mitochondrial morphology in RyR1-dyspedic mouse myoblasts (1B5 cells, RyR1^{-/-}). GFP-RyR1 was transfected in 1B5 cells, and mitochondrial morphology was observed 48 h after transfection. The average AR from C2C12 cells (RyR1^{+/+}) is shown as a control. Mitochondrial morphology was monitored by cotransfection of mt-RFP. N.S., not significant.

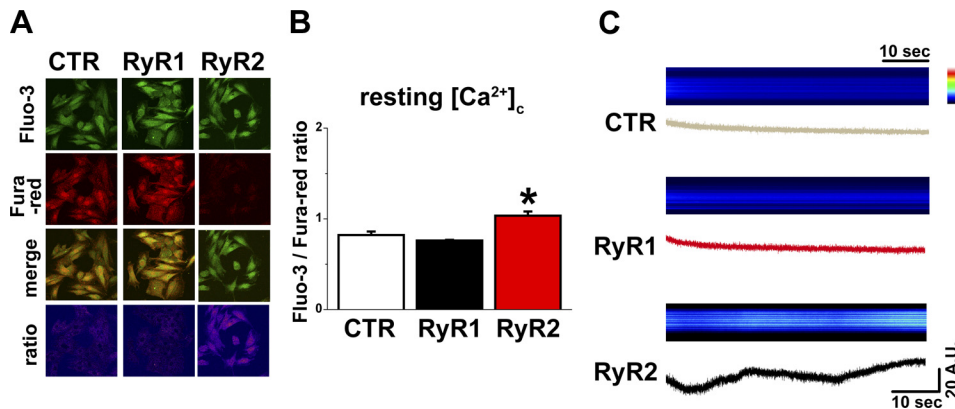


Fig. 6. Overexpression of RyR1 does not show resting cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) elevation/oscillation in H9c2 cells. **A**: representative images of cells after fluo-3 (green images) and fura red (red images). Hot map images showing the fluo-3-to-fura red ratio calculated from the original images are also shown (*bottom*). **B**: summary data from the images in **A**. * $P < 0.05$ compared with control cells. **C**: representative line-scan images obtained from H9c2 cells overexpressing RyR1 or RyR2 loaded with fluo-3. The time-dependent movement of fluo-3 fluorescence intensity calculated from each line-scan image is also shown (*bottom*). A.U., arbitrary units.

is not likely due to cytosolic Ca^{2+} elevation, mitochondrial ROS elevation, and/or Ψ_m depolarization.

We (34) have recently reported that glucose stimulation induces mitochondrial fragmentation, ATP production, and insulin secretion without Ψ_m depolarization through the activity of the fission protein DLP1 in an insulinoma cell line (INS-1E cells). This led us to question whether RyR1 overexpression might cause mitochondrial fragmentation through increased fission machinery activation without Ψ_m depolarization in H9c2 cells. First, we performed protein fractionation using cells that stably overexpressed mfGFP-RyR1 and observed the amount of DLP1 in the mitochondria-enriched protein fraction. Interestingly, the mitochondria-enriched protein fraction from mfGFP-RyR1-overexpressing cells contained more DLP1 compared with control cells, indicating that DLP1 activity in mitochondria from mfGFP-RyR1-overexpressing cells is higher than that from control cells (Fig. 8A). To confirm that the mitochondrial fragmentation by RyR1 overexpression is mediated through DLP1 activity, we transfected a dominant negative mutant of DLP1 (DLP1-K38A) to cells that stably overexpressed mfGFP-RyR1 (83). Indeed, inhibition of mitochondrial fission by DLP1-K38A eliminated the RyR1 overexpression-induced change in mitochondrial morphology in this stable cell line (Fig. 8B). These data indicate that RyR1 overexpression induces mitochondrial fragmentation through an acceleration of the fission process (translocation of DLP1 to mitochondria) without cytosolic Ca^{2+}

elevation, mitochondrial ROS elevation, and Ψ_m depolarization.

Fragmented mitochondria show prolonged elevation of $[Ca^{2+}]_{mt}$ in response to a cytosolic Ca^{2+} transient. Increasing evidence indicates that changes in mitochondrial dynamics/morphology are intricately related to mitochondrial function, including ATP production and cellular function, suggesting that mitochondrial form and function are linked (25). However, little is known about the relationship between mitochondrial form and mitochondrial Ca^{2+} -handling profiles. Since our data showed that RyR1 overexpression can change mitochondrial morphology, we next addressed the question of whether the change in mitochondrial morphology is associated with a change in $[Ca^{2+}]_{mt}$ profiles in H9c2 cells. We used a mitochondrial matrix-targeted Ca^{2+} -sensitive inverse pericam named Mitycam (40) as a mitochondrial Ca^{2+} probe and measured the changes in $[Ca^{2+}]_{mt}$ in both global and individual mitochondria (Fig. 9). First, we confirmed that this Ca^{2+} sensor was expressed in mitochondria in H9c2 cells after transfection (Fig. 9A). Surface membrane depolarization by a high- K^+ (60 mM) solution (to evoke $[Ca^{2+}]_c$ elevation) elicited global $[Ca^{2+}]_{mt}$ increases that returned to resting levels 60 s after stimulation (Fig. 9, B and C). We also successfully measured $[Ca^{2+}]_{mt}$ changes by 20 nM endothelin (ET)-1 and 3 μ M thapsigargin (Fig. 9C). Preincubation with the uncoupler 5 μ M FCCP (40) significantly inhibited ET-1-induced mitochondrial Ca^{2+} accumulation (Fig. 9C). Next, we plotted basal

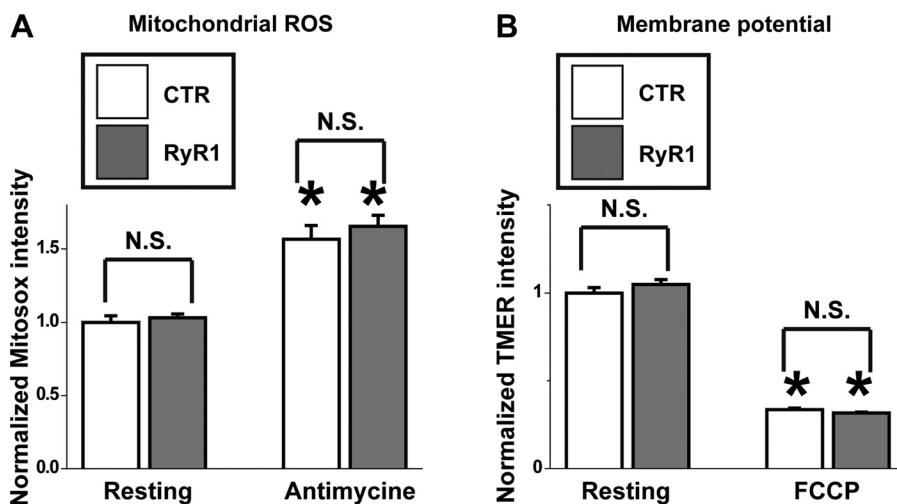


Fig. 7. Overexpression of RyR1 does not change the oxidative level at mitochondria and mitochondrial membrane potential (Ψ_m) in H9c2 cells. **A**: mitochondrial superoxide levels in control cells and cells stably overexpressing mfGFP-RyR1 measured by MitoSOX red. A complex III inhibitor, 10 μ M antimycin A (10 min), was used to confirm that fluorescence intensity does not saturate at rest in each cell group. * $P < 0.05$ compared with control cells at rest. **B**: Ψ_m in control cells and cells stably overexpressing mfGFP-RyR1 measured by the Ψ_m -sensitive probe tetramethyl rhodamine ester (TMRE). FCCP (10 μ M, 10 min) was applied to confirm that there was no significant difference in Ψ_m -sensitive fluorescence intensity between the two groups. * $P < 0.05$ compared with control cells at rest.

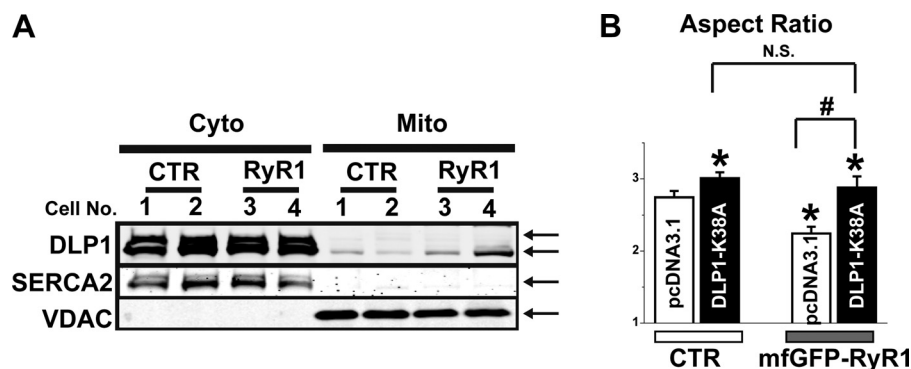


Fig. 8. Inhibition of mitochondrial fission eliminates the RyR1 overexpression-induced change in mitochondrial morphology in H9c2 cells. *A*: detection of dynamin-like protein 1 (DLP1) from H9c2 cells stably expressing mfGFP-RyR1 (cells 3 and 4) or control cells (nontransfected cells; cells 1 and 2) in the cytosolic (Cyto) fraction (including the SR, 25 μ g/well) and mitochondrial (Mito) fraction (50 μ g/well). SERCA2 (Cell Signaling) or VDAC (Cell Signaling) detection was used as a confirmation of the purity of the cytosolic fraction (including the SR) or mitochondrial fraction, respectively. *B*: effect of dominant negative DLP1 (DLP1-K38A) on mitochondrial morphology in H9c2 cells stably overexpressing mfGFP-RyR1 or control cells. Mitochondrial morphology was monitored by cotransfection of mt-RFP. * $P < 0.05$ compared with control cells transfected with empty vector [pcDNA3.1(+)].

$[Ca^{2+}]_{mt}$ (Fig. 9D) and changes of $[Ca^{2+}]_{mt}$ (Fig. 9E) in each mitochondrion separately. Basal $[Ca^{2+}]_{mt}$ (estimated by the fluorescence intensity of Mitycam before stimulation) of each mitochondrion was not significantly associated with either mitochondrial shape (FF and AR) or mitochondrial volume (size; Fig. 9D). Mitochondria that were tubular and networked showed $[Ca^{2+}]_{mt}$ peaks similar to average global mitochondrial $[Ca^{2+}]_{mt}$ increases with faster recovery kinetics (Fig. 9, E and F). In contrast, fragmented mitochondria (or isolated mitochondria that were not networked) showed larger and sustained $[Ca^{2+}]_{mt}$ increases compared with averaged global $[Ca^{2+}]_{mt}$ increases (Fig. 9, E and F). These observations suggest that mitochondrial form (morphology) and the Ca^{2+} -handling profile are linked in H9c2 cells.

RyR1 overexpression induces Ca^{2+} -dependent mitochondrial ATP production. Our data clearly showed that RyR1 overexpression induces mitochondrial fragmentation (Figs. 2, 3, and 5). Interestingly, fragmented mitochondria in H9c2 cells elicited a sustained $[Ca^{2+}]_{mt}$ elevation in response to a cytosolic Ca^{2+} transient (Fig. 9). Since ATP generation is known to be a Ca^{2+} -dependent process, the next question we examined was how the changes in mitochondrial morphology (fragmentation) by RyR1 and the alteration in $[Ca^{2+}]_{mt}$ handling influence mitochondrial metabolism. To test whether RyR1 overexpression can affect ATP production, $[ATP]_{mt}$ was measured using the mitochondrial matrix-targeted FRET-based ATP sensor Ateam by confocal microscopy (see MATERIALS AND METHODS; Fig. 10A) (29, 61, 79). We confirmed that this ATP sensor was expressed in mitochondria in H9c2 cells after transfection (Fig. 10A). RyR1-overexpressing cells had higher $[ATP]_{mt}$ under basal conditions, as observed using Ateam (Fig. 10B). Next, we addressed whether cells that overexpressed RyR1 could produce ATP in response to a cytosolic Ca^{2+} elevation. Because overexpressed RyR1 was also expressed at the SR, we stimulated cells with ET-1 and mobilized inositol 1,4,5-trisphosphate (IP_3) receptor-based SR Ca^{2+} release (45) to match the magnitude of a cytosolic Ca^{2+} transient in control and RyR1-transfected cells. We confirmed that ET-1 treatment was able to evoke a $[Ca^{2+}]_{mt}$ transient immediately after stimulation (Fig. 9C). Cells were also treated with 10 μ g/ml oligomycin A to determine the range of the FRET ratio derived from basal ATP levels in the mitochondrial matrix (Fig. 10C). ET-1-

induced $[Ca^{2+}]_c$ elevation caused a rapid increase of ATP production in RyR1-overexpressing cells compared with control cells (Fig. 10, C and D). These results strongly support our hypothesis that Ca^{2+} influx via mRyR1 is critical in the regulation of mitochondrial morphology (form) and ATP generation (function).

DISCUSSION

In this study, we used RyR1-overexpressing cardiac myoblasts and showed that 1) overexpressed RyR1 is partially expressed in mitochondria but RyR2 is not (Figs. 1 and 2); 2) overexpression of RyR1 but not RyR2, MCU, MICU1, or Letm1 or treatment of coenzyme Q_{10} leads to mitochondrial fragmentation (Figs. 3 and 4), which can be blocked by pharmacological inhibition or genetic knockdown of RyR1; 3) RyR1 overexpression induces mitochondrial fragmentation through the fission process but not by $[Ca^{2+}]_c$ elevation, mitochondrial ROS elevation, and Ψ_m depolarization (Figs. 6–8); 4) fragmented and interconnected tubular mitochondria have a different $[Ca^{2+}]_{mt}$ profile in response to high- K^+ -induced $[Ca^{2+}]_c$ increases and, especially, fragmented mitochondria have a larger and sustained $[Ca^{2+}]_{mt}$ increase (Fig. 9); and 5) cells that overexpress RyR1 have higher $[ATP]_{mt}$ under basal conditions and accelerated $[Ca^{2+}]_c$ -dependent ATP production (Fig. 10). These results show that mRyR1 plays an important role in the regulation of both mitochondrial morphology and Ca^{2+} -dependent ATP production, suggesting distinctive roles of mRyR1 and MCU in the regulation of mitochondrial form and function in cardiac cells (Fig. 11).

Molecular mechanism underlying mitochondrial localizations of RyR1 in cardiac H9c2 cells. First, we found that overexpressed RyR1 can partially target and localized to mitochondria, but overexpressed RyR2 cannot, which is consistent with our previous observations demonstrating that a small amount of RyR1 (~5% of total RyR), but not RyR2, is expressed in native cardiomyocytes (8). Here, the important question arises of how RyR1 but not RyR2 is localized in mitochondria.

The precursor forms of mitochondrial nuclear-encoded proteins contain targeting and sorting signals, presented as an NH_2 -terminal signal sequence (e.g., Ref. 32) or within other

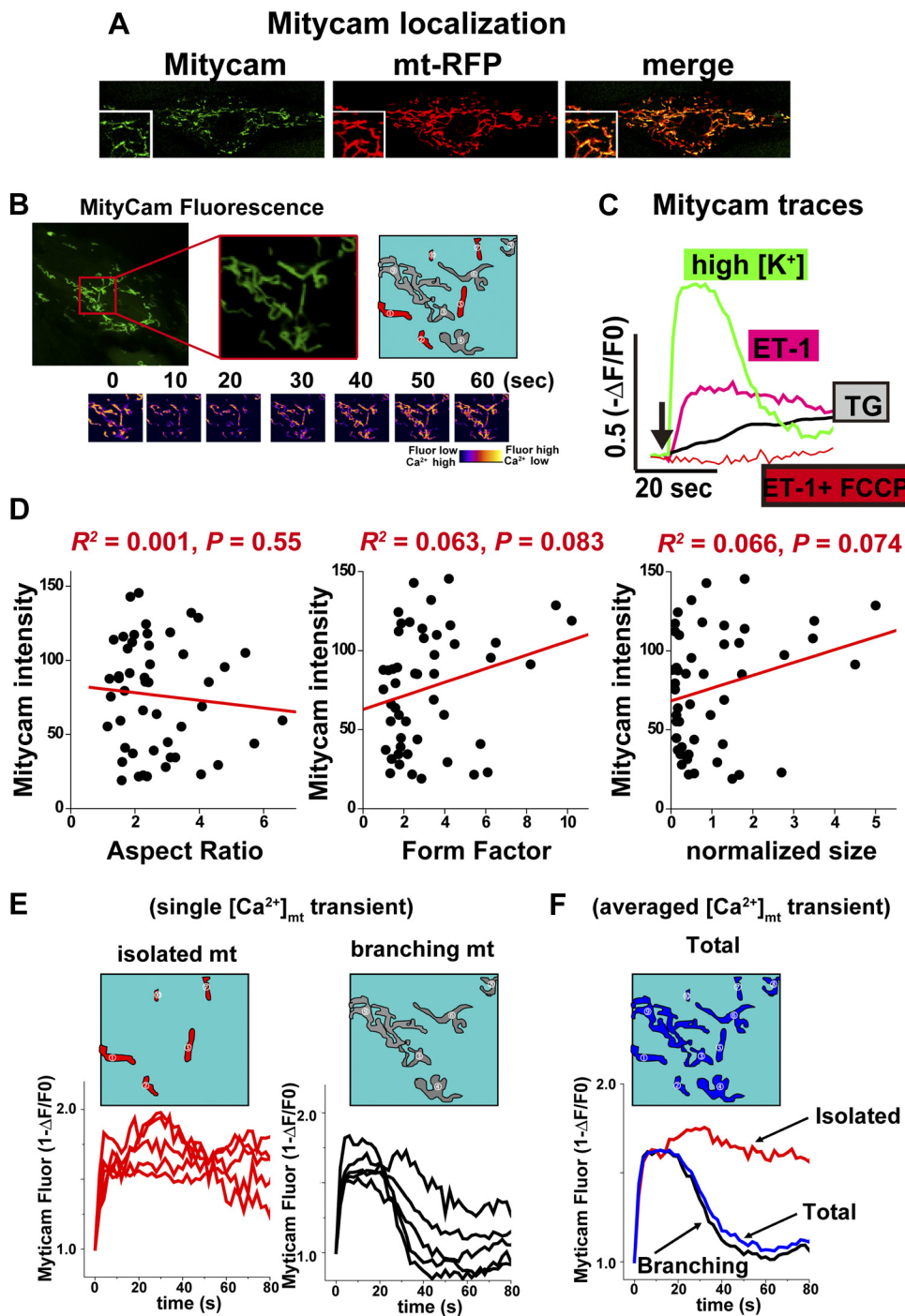
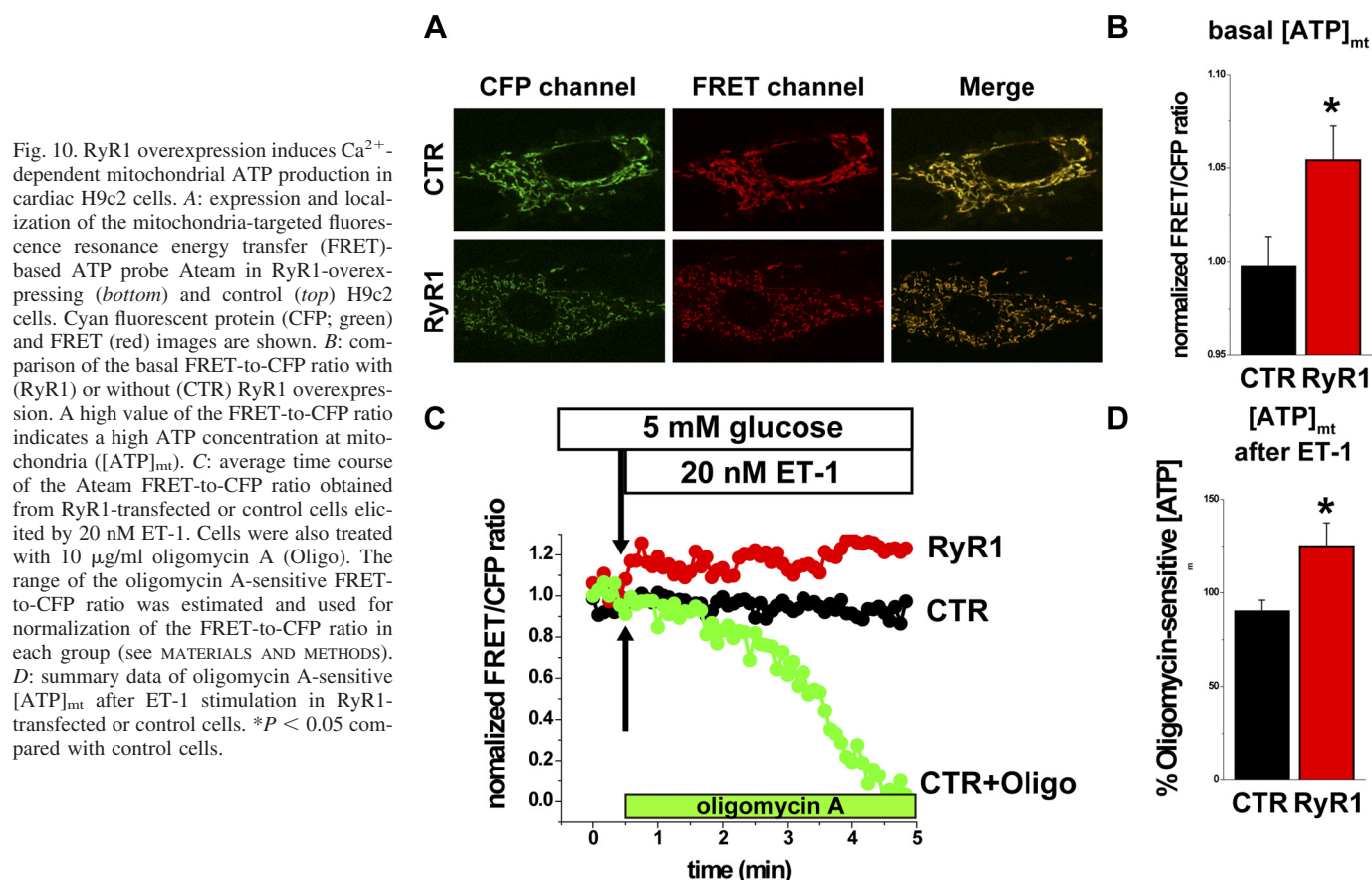


Fig. 9. Fragmented mitochondria show prolonged elevation of the matrix Ca^{2+} concentration in response to a cytosolic Ca^{2+} transient in cardiac H9c2 cells. **A**: expression and localization of the mitochondria-targeted Ca^{2+} -sensitive probe Mitycam in H9c2 cells. The location of mitochondria was labeled by mt-RFP expression. **B**: time course of Mitycam fluorescence images after depolarization by high- K^+ (60 mM) application. A line trace of mitochondria shapes is also shown (right). Mitochondria that were tubular and in the network are shown in gray (branching mitochondria), and those that were fragmented and isolated from the network are shown in red (isolated mitochondria). **C**: time course of changes in intramitochondrial Ca^{2+} concentration ($[\text{Ca}^{2+}]_{\text{mt}}$) stimulated by high K^+ (60 mM), 20 nM endothelin (ET)-1, and 3 μM thapsigargin (TG). The arrow shows the timing of application of those stimulants. The time course of changes in $[\text{Ca}^{2+}]_{\text{mt}}$ stimulated by ET-1 after 10 min of pretreatment with 5 μM FCCP is also shown as a control. Fluorescence values are expressed as $-\Delta F/F_0$ (see MATERIALS AND METHODS). **D**: simple linear regression analysis (37) between basal fluorescence in each mitochondrion and AR (left), FF (middle), or mitochondrial size (right) in each mitochondrion. Each size was normalized to the averaged mitochondrial size calculated from all mitochondria observed in the whole cell area in **B**. **E**: intramitochondrial Ca^{2+} transient ($[\text{Ca}^{2+}]_{\text{mt}}$) traces obtained from single mitochondria in **B**. **F**: averaged $[\text{Ca}^{2+}]_{\text{mt}}$ from isolated or branching mitochondria in **B**. The average $[\text{Ca}^{2+}]_{\text{mt}}$ from all mitochondria in the field is also shown (total).

parts of precursor proteins [e.g., the COOH-term(47)] (for a review, see Ref. 41), which are essential to direct them to mitochondria. However, the existence of NH_2 -terminal signal sequences is not always indispensable for mitochondrial transport and retention. We can predict the existence of NH_2 -terminal signal sequences and their cleavage sites in these proteins using a computational prediction program named Mitoprot (12). Indeed, MCU and MICU1, used in this study, possess typical NH_2 -terminal signal sequences (Table 1). Conversely, other major mitochondrial proteins, such as cyclophilin D and adenine nucleotide translocator, do not possess predict-

able motifs (Table 1). RyRs also do not have any predictable NH_2 -terminal signal sequences. Much like cyclophilin D and adenine nucleotide translocator, Mitoprot does not show a high probability of RyR import to mitochondria (Table 1).

Previous studies have reported that the ER retention signal is likely present within the COOH-terminal portion of RyR1 (9, 53, 78) or RyR2 (22), which contains transmembrane domains (TMDs). Bhat and Ma (9) reported that amino acids 4918–4943 of RyR1, which are within TMD6 and have a consensus sequence with the IP_3 receptor and RyR2, contain a strong ER retention signal (Fig. 12). Taylor's group (53) showed that



TMD1 has a stronger ER localization signal compared with TMD6. Interestingly, they also found that the peptide corresponding to TMD3–TMD4 has the potential to target to mitochondria (53). Because the sequences of TMD3 in RyR1 and RyR2 have less similarity (60%; Fig. 12A), the difference in the potential mitochondrial target sequence within the RyR proteins might explain the isoform-specific subcellular localization in cardiomyocytes. Because overexpressed RyR1 in the mitochondrial fraction has a slightly smaller molecular weight than that found in the SR fraction (see Fig. 1C), it is conceivable that RyR1 in cardiac cells might undergo posttranslational modification (e.g., truncation in the COOH-term portion) and thereby enhance the mitochondrial retention signal located at TMD3–TMD4. Future studies will address the detailed mechanism of RyR1 trafficking to mitochondria, including the clarification of the full sequence of the mRyR1 protein using mass spectrometry.

Molecular mechanism underlying the regulation of mitochondrial form and function by RyR1 in cardiac H9c2 cells: distinct roles of mRyR1 and MCU in the regulation of mitochondrial form and function. The next important finding in this study was that overexpression of RyR1, but not MCU, leads to mitochondrial fragmentation (Figs. 2 and 3). This fragmentation is mediated through mitochondrial Ca^{2+} sequestration via mRyR1 as it is reversed by dantrolene or knockdown of this channel (Fig. 5). This observation is consistent with our previous finding that an increase in $[\text{Ca}^{2+}]_{\text{mt}}$ induces mitochondrial fragmentation in cardiac cells (27). We also found that cells that overexpress RyR1 have a higher $[\text{ATP}]_{\text{mt}}$ under basal

conditions and accelerated $[\text{Ca}^{2+}]_{\text{c}}$ -dependent ATP production. This observation is consistent with our previous finding in RyR1 knockout mice that the respiratory control index is very low (≈ 1) and $[\text{Ca}^{2+}]_{\text{c}}$ does not stimulate O_2 consumption. Our previous reports and present data strongly support our hypothesis that Ca^{2+} influx via mRyR1 is critical in the regulation of form (mitochondrial morphology) and function (ATP generation).

Interestingly, recent ground-breaking studies have shown that mitochondrial respiration, cell morphology, and cell viability surprisingly remain normal in MCU or MICU1 knockout/overexpressing cells even though Ca^{2+} influx is well known to be critical in the regulation of these functions (4, 17, 67). Our present study using H9c2 cells also confirmed these previous observations demonstrating that overexpression of MCU or MICU1 does not significantly change mitochondrial morphology (Figs. 2 and 4). An important question arises as why only RyR1 expression (but not MCU) induces mitochondrial fragmentation and ATP generation (Fig. 7). There are several possibilities that can explain the unique roles of MCU and mRyR1.

First, the Ca^{2+} sensitivity and Ca^{2+} -dependent activation/inactivation profiles of mRyR1 and MCU are quite different (for reviews, see Refs. 64 and 71). mRyR1 starts to open in response to $\approx 10^{-7}$ M $[\text{Ca}^{2+}]_{\text{c}}$ (the half-maximal activation ($K_{0.5}$) is between 10^{-6} and 10^{-5} M (7)) and can respond to rapid Ca^{2+} pulses (73). On the other hand, the MCU current observed in mitoplast patch clamp requires rather high $[\text{Ca}^{2+}]_{\text{c}}$ to activate, with $K_{0.5} \approx 20$

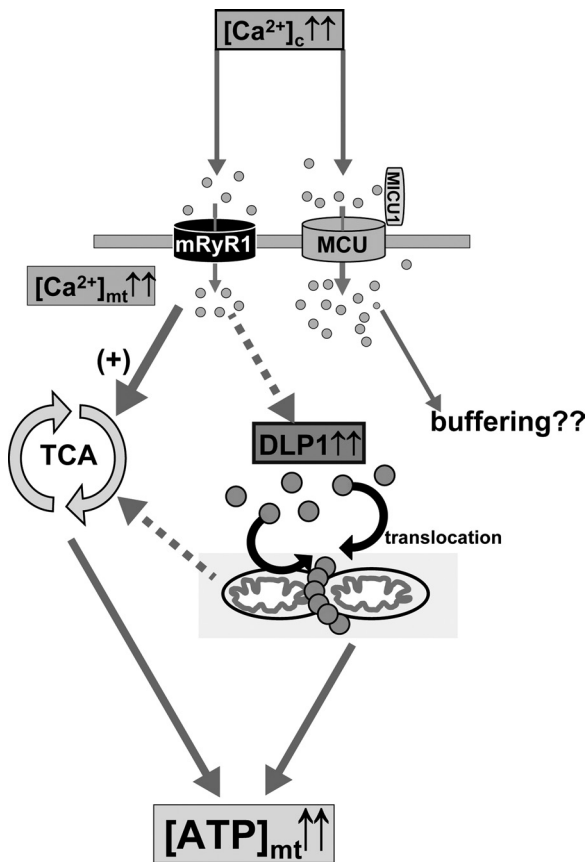


Fig. 11. Ca^{2+} influx through mRyR1 (but not MCU) mediates mitochondrial fragmentation and ATP production in cardiac cells.

mM (42). A recent mathematical model has shown that there is almost no MCU current at $1 \mu\text{M}$ $[\text{Ca}^{2+}]_c$ but that there is limited MCU current activation at $20 \mu\text{M}$ $[\text{Ca}^{2+}]_c$ ($\sim 1/3$ the potential to extract Ca^{2+} from the cytosol compared with SERCA) (81). Therefore, it is likely that MCU does not participate in Ca^{2+} uptake at resting $[\text{Ca}^{2+}]_c$ (or at small spontaneous $[\text{Ca}^{2+}]_c$ oscillations under nonstimulated conditions) but that it can be activated by slower and higher Ca^{2+} elevations at the ER/SR contact site (approximately up to $10 \mu\text{M}$ $[\text{Ca}^{2+}]_c$) (14, 15, 70), either by IP_3 receptor-mediated Ca^{2+} release in physiological conditions or during the cytosolic Ca^{2+} overload frequently observed in pathophysiological conditions.

Second, the localization of mRyR1 and MCU in mitochondria might be different. Growing evidence shows that the structural proximity of mitochondria and ER/SR Ca^{2+} release sites has an important role in the regulation of cellular Ca^{2+} dynamics and metabolism (16, 76). Hajnóczky's group (82) recently demonstrated that an increase in the expression levels of RyRs, by either increasing endogenous expression levels of RyRs via differentiation or overexpression of RyR1 by transfection, can enhance ER/SR-mitochondrial Ca^{2+} signaling in H9c2 myoblasts. They also speculated that RyRs are an important component for the formation of ER/SR-mitochondrial Ca^{2+} coupling. In this study, we found that not only the number of fragmented (round) mitochondria increases after overexpression of RyR1 in EM but also that the ER/SR-mitochondrial contact sites are frequently observed (see Fig. 3D). Taken together, it is likely that mRyR1 is one of the determining components for mitochondrial morphology and also the formation of the ER/SR-mitochondrial Ca^{2+} coupling unit, probably due to overexpressed mRyR1 being preferentially located at mitochondria-SR/ER contact sites. Since MCU is shown to have relatively uniform distribution over the mitochondrion (17, 50), it is probable that mRyR1 can more efficiently import ER/SR Ca^{2+} release to increase $[\text{Ca}^{2+}]_{mt}$ and induce Ca^{2+} -dependent mitochondrial fragmentation compared with MCU due to its localization and its role in the formation of the ER/SR-mitochondrial Ca^{2+} coupling unit. However, there has been no report showing that RyRs themselves can work as the anchoring or tethering protein between the ER/SR and mitochondria. One possibility is that mRyR1 expression or Ca^{2+} influx via mRyR1 can regulate local Ca^{2+} and/or ROS signaling and modulate the activity or subcellular localization of the fission protein DLP1 since we found that DLP1 significantly translocates to mitochondria after RyR1 overexpression (see Fig. 8). Further studies will be required to identify the detailed mechanism of how RyR1 overexpression determines DLP1 translocation to mitochondria, especially focusing on the posttranslational modification of DLP1 by Ca^{2+} and/or ROS signaling (e.g., phosphorylation).

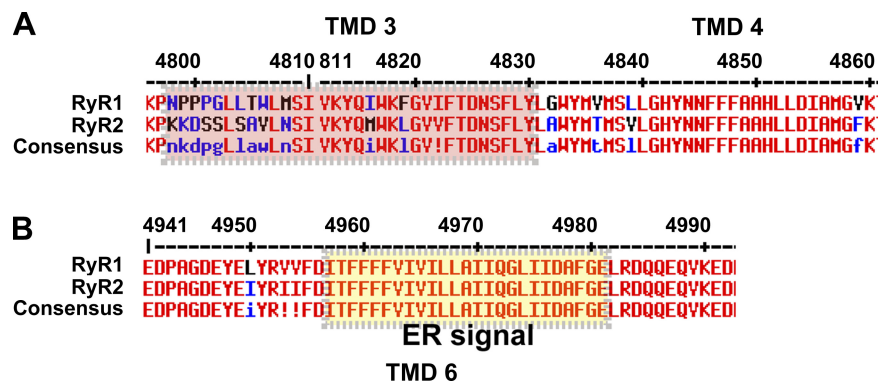
The formation of ER/SR and mitochondria contacts could also explain why the fragmented mitochondria have more robust Ca^{2+} sequestration. ER and mitochondrial networks are intricately intertwined, and it is estimated that 5–20% of the surface of the mitochondrial network are close to the ER surface; therefore, ER/SR-mitochondrial Ca^{2+} coupling is formed at those contact sites (70). Rizzuto and colleagues (77) showed that division of the mitochondrial network by overex-

Table 1. Prediction of mitochondrial targeting sequences and cleavage sites

	Sequence Length, amino acids	Cleavage Site	Cleaved Sequence	Probability of Export to Mitochondria
Mitochondrial Ca^{2+} uniporter	351	56	MAAAGRSLLLLSSRGGGG GAGGGALTAGCFPLGVSRHRQQQHRTVHQRI	0.9759
Mitochondrial Ca^{2+} uptake 1	478	56	MFRLNLSALAEAVGSRWYH GGSQPIQIRRLMMVAFLGASAVTASTGLWKRA	0.8223
Adenine nucleotide translocator	298		Not predictable	0.2422
Cyclophilin D	370		Not predictable	0.0531
Ryanodine receptor type 1	5,038		Not predictable	0.0002
Ryanodine receptor type 2	4,967		Not predictable	0.0002

The probability of protein export to mitochondria was computationally calculated by MitoProt software based on the existence of the NH_2 -terminal mitochondrial targeting sequence and the whole protein sequence (see Ref. 12).

Fig. 12. Alignment of the amino acid sequences between human RyR1 and RyR2. Multiple sequence alignment was performed by Multalin (version 5.4) (13). *A*: alignment of the amino acid sequences around transmembrane domain (TMD)3–TMD4 in RyR1 and RyR2. TMD3 in RyR1 corresponding to the rabbit RyR1 sequence (4770–4882) is shown in pink. *B*: alignment of the amino acid sequences around TMD6 in RyR1 and RyR2. The ER retention signal in RyR1 corresponding to the rabbit RyR1 sequence (4918–4943) is shown in yellow.



pression of the mitochondrial fission factor DLP1 (which shows global mitochondrial fragmentation) creates heterogeneity of Ca^{2+} uptake/release kinetics in each mitochondrion and blocks the propagation of Ca^{2+} waves in mitochondrial networks in HeLa cells. Indeed, we show here that there is a heterogeneity of mitochondrial Ca^{2+} handling profiles in tubular/in-network mitochondria and fragmented/out-of-network mitochondria in H9c2 cells (Fig. 8). It is a reasonable to consider that fragmented mitochondria can sustain $[\text{Ca}^{2+}]_{\text{mt}}$ increases without propagation of Ca^{2+} to the neighboring mitochondria, which can activate the TCA cycle to increase ATP generation (Fig. 11). Growing evidence also suggests that the physiological activation of the mitochondrial fission process itself can enhance the efficiency of electron transport chain activity, possibly via the facilitation of forming super-complexes of the respiratory chain (48, 74). We (34) have also previously reported that fragmented mitochondria show efficient ATP production without Ψ_m depolarization during glucose stimulation in an insulinoma cell line. Interestingly, the expression pattern of mRyR1 was heterogeneous between each mitochondrion, whereas MCU, MICU1, and Letm1 were homogeneously expressed in all mitochondria (Fig. 2, *A* and *B*). In addition, RyR1-overexpressing mitochondria observed in confocal microscopy showed remarkably more round and fragmented morphology (Fig. 2A, *inset*). Collectively, this evidence and our present data strongly support our hypothesis that the expression of mRyR1 modulates mitochondrial morphology (fragmentation) and their Ca^{2+} -handling profiles, thereby accelerating Ca^{2+} -dependent ATP production at the matrix (Fig. 11).

There are several reports that have demonstrated that RyR1 expression is increased in diseased cardiomyocytes. In human heart failure, RyR1 expression increases approximately two-fold (57). In an electrical remodeling model, cultured atrial cells showed an increase of ~ 27 -fold RyR1 mRNA (51). In the present study, we overexpressed RyR1 in H9c2 cells and confirmed that RyR1 increases from undetectable levels to detectable levels in Western blot analysis (see Fig. 1) and functionally increase ATP production (see Fig. 10). However, we did not carefully quantified how much RyR1 expression is required to increase ATP generation efficiency in this cell line. A future study needs to address whether an increase in RyR1 expression in the diseased heart can alter ATP production.

Taken together, it is likely that Ca^{2+} influx kinetics via mRyR1 and/or the role of mRyR1 for the formation of ER/SR-mitochondria coupling units enable maximum ATP pro-

duction efficiency with a minimum amount of net Ca^{2+} transport. In contrast, the relatively slower kinetics and lower Ca^{2+} affinity make MCU an ideal candidate for cytosolic Ca^{2+} buffering when $[\text{Ca}^{2+}]_c$ increases at ER/SR contact sites under physiological or pathophysiological conditions.

Limitations of this study. As shown in Figs. 1C and 2A, overexpressed RyR1 is partially localized in mitochondria, although the majority of overexpressed RyR1 is localized in the ER/SR. As discussed above, the detailed mechanism of how RyR1 can target to mitochondria is still unclear, and, currently, there is no tool to control the location of overexpressed RyR1. Therefore, we still need to take into account that the changes in mitochondrial morphology and function after RyR1 overexpression might be a secondary effect from the ER/SR-located RyR1, although we could not see any significant effect of RyR1 overexpression on $[\text{Ca}^{2+}]_c$ (Fig. 6) and ER/SR-overexpressed RyR2 on mitochondrial morphology (Figs. 2 and 3). Future work is needed to address a physiological role of mRyR1 in the adult cardiomyocyte by knockdown of this channel in situ.

In addition, since it is practically difficult to obtain a 100% pure mitochondria membrane preparation for biochemistry, a future study will further confirm the localization of overexpressed mRyR1 using immunoelectron microscopy, as we have previously reported (7).

Conclusions. Taken together, our results show the molecular and functional roles of mRyR1 using a RyR1 overexpression system in H9c2 cardiac myoblasts, which clearly distinguish its role from classical MCU. mRyR1 may uniquely work to sequester Ca^{2+} to generate ATP in myoblasts. Elucidation of the unique biophysical characteristics and physiological functions of the Ca^{2+} influx mechanism via both MCU and mRyR1 in cardiac myoblasts is significant for our understanding of mitochondrial Ca^{2+} signaling in the regulation of cardiac physiology and pathophysiology.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: J.O.-U. and S.-S.S. conception and design of research; J.O.-U., B.S.J., S.H., S.B., P.G., and M.C. performed experiments; J.O.-U., B.S.J., S.H., and S.-S.S. analyzed data; J.O.-U., B.S.J., S.K., J.P., H.O., G.L.S., T.M., and S.-S.S. interpreted results of experiments; J.O.-U. prepared figures; J.O.-U. and S.-S.S. drafted manuscript; J.O.-U., B.S.J., S.H., H.O., T.M., and S.-S.S. edited and revised manuscript; J.O.-U., B.S.J., S.H., S.B., P.G., M.C., S.K., J.P., H.O., G.L.S., T.M., and S.-S.S. approved final version of manuscript.

REFERENCES

- Altschaff BA, Beutner G, Sharma VK, Sheu SS, Valdivia HH. The mitochondrial ryanodine receptor in rat heart: a pharmacokinetic profile. *Biochim Biophys Acta* 1768: 1784–1795, 2007.
- Bannai H, Inoue T, Nakayama T, Hattori M, Mikoshiba K. Kinesin dependent, rapid, bi-directional transport of ER sub-compartment in dendrites of hippocampal neurons. *J Cell Sci* 117: 163–175, 2004.
- Barshesht A, Goldenberg I, O-Uchi J, Moss AJ, Jons C, Shimizu W, Wilde AA, McNitt S, Peterson DR, Zareba W, Robinson JL, Ackerman MJ, Cypress M, Gray DA, Hofman N, Kanters JK, Kaufman ES, Platonov PG, Qi M, Towbin JA, Vincent GM, Lopes CM. Mutations in cytoplasmic loops of the KCNQ1 channel and the risk of life-threatening events: implications for mutation-specific response to β -blocker therapy in type-1 long QT syndrome. *Circulation* 125: 1988–1996, 2012.
- Baughman JM, Perocchi F, Girgis HS, Plovanich M, Belcher-Timme CA, Sancak Y, Bao XR, Strittmatter L, Goldberger O, Bogorad RL, Kotliansky V, Mootha VK. Integrative genomics identifies MCU as an essential component of the mitochondrial calcium uniporter. *Nature* 476: 341–345, 2011.
- Benard G, Rossignol R. Ultrastructure of the mitochondrion and its bearing on function and bioenergetics. *Antioxid Redox Signal* 10: 1313–1342, 2008.
- Bergamini C, Moruzzi N, Sblendido A, Lenaz G, Fato R. A water soluble CoQ10 formulation improves intracellular distribution and promotes mitochondrial respiration in cultured cells. *PLoS One* 7: e33712, 2012.
- Beutner G, Sharma VK, Giovannucci DR, Yule DI, Sheu SS. Identification of a ryanodine receptor in rat heart mitochondria. *J Biol Chem* 276: 21482–21488, 2001.
- Beutner G, Sharma VK, Lin L, Ryu SY, Dirksen RT, Sheu SS. Type 1 ryanodine receptor in cardiac mitochondria: transducer of excitation-metabolism coupling. *Biochim Biophys Acta* 1717: 1–10, 2005.
- Bhat MB, Ma J. The transmembrane segment of ryanodine receptor contains an intracellular membrane retention signal for Ca^{2+} release channel. *J Biol Chem* 277: 8597–8601, 2002.
- Bogeski I, Gulaboski R, Kappl R, Mirceski V, Stefova M, Petreska J, Hoth M. Calcium binding and transport by coenzyme Q. *J Am Chem Soc* 133: 9293–9303, 2011.
- Brookes PS, Yoon Y, Robotham JL, Anders MW, Sheu SS. Calcium, ATP, and ROS: a mitochondrial love-hate triangle. *Am J Physiol Cell Physiol* 287: C817–C833, 2004.
- Claros MG, Vincens P. Computational method to predict mitochondrially imported proteins and their targeting sequences. *Eur J Biochem* 241: 779–786, 1996.
- Corpet F. Multiple sequence alignment with hierarchical clustering. *Nucleic Acids Res* 16: 10881–10890, 1988.
- Csordas G, Thomas AP, Hajnoczky G. Quasi-synaptic calcium signal transmission between endoplasmic reticulum and mitochondria. *EMBO J* 18: 96–108, 1999.
- Csordas G, Varnai P, Golenar T, Roy S, Purkins G, Schneider TG, Balla T, Hajnoczky G. Imaging interorganelle contacts and local calcium dynamics at the ER-mitochondrial interface. *Mol Cell* 39: 121–132, 2010.
- Csordas G, Varnai P, Golenar T, Sheu SS, Hajnoczky G. Calcium transport across the inner mitochondrial membrane: molecular mechanisms and pharmacology. *Mol Cell Endocrinol* 353: 109–113, 2012.
- De Stefani D, Raffaello A, Teardo E, Szabo I, Rizzuto R. A forty-kilodalton protein of the inner membrane is the mitochondrial calcium uniporter. *Nature* 476: 336–340, 2011.
- Dedkova EN, Blatter LA. Mitochondrial Ca^{2+} and the heart. *Cell Calcium* 44: 77–91, 2008.
- Ebbinghaus-Kintscher U, Lummen P, Raming K, Masaki T, Yasokawa N. Flubendiamide, the first insecticide with a novel mode of action on insect ryanodine receptors. *Pflanzenschutz-Nachricht Bayer* 60: 117–140, 2007.
- Estrada M, Cardenas C, Liberona JL, Carrasco MA, Mignery GA, Allen PD, Jaimovich E. Calcium transients in 1B5 myotubes lacking ryanodine receptors are related to inositol trisphosphate receptors. *J Biol Chem* 276: 22868–22874, 2001.
- Fieni F, Kirichok Y. Patch-clamp analysis of mitochondrial Ca^{2+} uniporter in different tissues. *J Gen Physiol* 137: 1317, 2011.
- George CH, Jundi H, Thomas NL, Scoote M, Walters N, Williams AJ, Lai FA. Ryanodine receptor regulation by intramolecular interaction between cytoplasmic and transmembrane domains. *Mol Biol Cell* 15: 2627–2638, 2004.
- Glancy B, Balaban RS. Role of mitochondrial Ca^{2+} in the regulation of cellular energetics. *Biochemistry* 51: 2959–2973, 2012.
- Gunter TE, Sheu SS. Characteristics and possible functions of mitochondrial Ca^{2+} transport mechanisms. *Biochim Biophys Acta* 1787: 1291–1308, 2009.
- Hom J, Sheu SS. Morphological dynamics of mitochondria—a special emphasis on cardiac muscle cells. *J Mol Cell Cardiol* 46: 811–820, 2009.
- Hom J, Yu T, Yoon Y, Porter G, Sheu SS. Regulation of mitochondrial fission by intracellular Ca^{2+} in rat ventricular myocytes. *Biochim Biophys Acta* 1797: 913–921, 2010.
- Hom JR, Gewandter JS, Michael L, Sheu SS, Yoon Y. Thapsigargin induces biphasic fragmentation of mitochondria through calcium-mediated mitochondrial fission and apoptosis. *J Cell Physiol* 212: 498–508, 2007.
- Hom JR, Quintanilla RA, Hoffman DL, de Mesy Bentley KL, Molken JD, Sheu SS, Porter GA, Jr. The permeability transition pore controls cardiac mitochondrial maturation and myocyte differentiation. *Dev Cell* 21: 469–478, 2011.
- Imamura H, Nhat KP, Togawa H, Saito K, Iino R, Kato-Yamada Y, Nagai T, Noji H. Visualization of ATP levels inside single living cells with fluorescence resonance energy transfer-based genetically encoded indicators. *Proc Natl Acad Sci USA* 106: 15651–15656, 2009.
- Ingalls CP, Warren GL, Armstrong RB. Intracellular Ca^{2+} transients in mouse soleus muscle after hindlimb unloading and reloading. *J Appl Physiol* 87: 386–390, 1999.
- Ingalls CP, Warren GL, Williams JH, Ward CW, Armstrong RB. E-C coupling failure in mouse EDL muscle after in vivo eccentric contractions. *J Appl Physiol* 85: 58–67, 1998.
- Isenmann S, Khew-Goodall Y, Gamble J, Vadas M, Wattenberg BW. A splice-isoform of vesicle-associated membrane protein-1 (VAMP-1) contains a mitochondrial targeting signal. *Mol Biol Cell* 9: 1649–1660, 1998.
- Jeong EM, Liu M, Sturdy M, Gao G, Varghese ST, Sovari AA, Dudley SC Jr. Metabolic stress, reactive oxygen species, and arrhythmia. *J Mol Cell Cardiol* 52: 454–463, 2012.
- Jhun BS, Lee H, Jin ZG, Yoon Y. Glucose stimulation induces dynamic change of mitochondrial morphology to promote insulin secretion in the insulinoma cell line INS-1E. *PLoS One* 8: e60810, 2013.
- Jhun BS, O-Uchi J, Wang W, Ha CH, Zhao J, Kim JY, Wong C, Dirksen RT, Lopes CM, Jin ZG. Adrenergic signaling controls Rgk-dependent trafficking of cardiac voltage-gated L-type Ca^{2+} channels through PKD1. *Circ Res* 110: 59–70, 2012.
- Jiang D, Zhao L, Clapham DE. Genome-wide RNAi screen identifies Letm1 as a mitochondrial $\text{Ca}^{2+}/\text{H}^{+}$ antiporter. *Science* 326: 144–147, 2009.
- Jons C, O-Uchi J, Moss AJ, Reumann M, Rice JJ, Goldenberg I, Zareba W, Wilde AA, Shimizu W, Kanters JK, McNitt S, Hofman N, Robinson JL, Lopes CM. Use of mutant-specific ion channel characteristics for risk stratification of long QT syndrome patients. *Sci Transl Med* 3: 76ra28, 2011.

38. Jung C, Martins AS, Niggli E, Shirokova N. Dystrophic cardiomyopathy: amplification of cellular damage by Ca^{2+} signalling and reactive oxygen species-generating pathways. *Cardiovasc Res* 77: 766–773, 2008.
39. Kashour T, Burton T, Dibrov A, Amara F. Myogenic signaling by lithium in cardiomyoblasts is Akt independent but requires activation of the β -catenin-Tcf/Lef pathway. *J Mol Cell Cardiol* 35: 937–951, 2003.
40. Kettlewell S, Cabrero P, Nicklin SA, Dow JA, Davies S, Smith GL. Changes of intra-mitochondrial Ca^{2+} in adult ventricular cardiomyocytes examined using a novel fluorescent Ca^{2+} indicator targeted to mitochondria. *J Mol Cell Cardiol* 46: 891–901, 2009.
41. Kiebler M, Becker K, Pfanner N, Neupert W. Mitochondrial protein import: specific recognition and membrane translocation of preproteins. *J Membr Biol* 135: 191–207, 1993.
42. Kirichok Y, Krapivinsky G, Clapham DE. The mitochondrial calcium uniporter is a highly selective ion channel. *Nature* 427: 360–364, 2004.
43. Kobayashi T, Morone N, Kashiwata T, Oyamada H, Kurebayashi N, Murayama T. Engineering a novel multifunctional green fluorescent protein tag for a wide variety of protein research. *PLoS One* 3: e3822, 2008.
44. Lanner JT, Georgiou DK, Joshi AD, Hamilton SL. Ryanodine receptors: structure, expression, molecular details, and function in calcium release. *Cold Spring Harb Perspect Biol* 2: a003996, 2010.
45. Lax A, Soler F, Fernandez-Belda F. Intracellular Ca^{2+} pools and fluxes in cardiac muscle-derived H9c2 cells. *J Bioenerg Biomembr* 37: 249–259, 2005.
46. Lefebvre R, Legrand C, Gonzalez-Rodriguez E, Groom L, Dirksen RT, Jacquemond V. Defects in Ca^{2+} release associated with local expression of pathological ryanodine receptors in mouse muscle fibres. *J Physiol* 589: 5361–5382, 2011.
47. Li M, Zhong Z, Zhu J, Xiang D, Dai N, Cao X, Qing Y, Yang Z, Xie J, Li Z, Baugh L, Wang G, Wang D. Identification and characterization of mitochondrial targeting sequence of human apurinic/aprimidinic endonuclease 1. *J Biol Chem* 285: 14871–14881, 2010.
48. Liu W, Acin-Perez R, Geghman KD, Manfredi G, Lu B, Li C. Pink1 regulates the oxidative phosphorylation machinery via mitochondrial fission. *Proc Natl Acad Sci USA* 108: 12920–12924, 2011.
49. Liu Z, Zhang J, Li P, Chen SR, Wagenknecht T. Three-dimensional reconstruction of the recombinant type 2 ryanodine receptor and localization of its divergent region 1. *J Biol Chem* 277: 46712–46719, 2002.
50. Lu X, Ginsburg KS, Kettlewell S, Bossuyt J, Smith GL, Bers DM. Measuring local gradients of intra-mitochondrial $[\text{Ca}]$ in cardiac myocytes during SR Ca release. *Circ Res* 112: 424–431, 2012.
51. Mace LC, Yermalitskaya LV, Yi Y, Yang Z, Morgan AM, Murray KT. Transcriptional remodeling of rapidly stimulated HL-1 atrial myocytes exhibits concordance with human atrial fibrillation. *J Mol Cell Cardiol* 47: 485–492, 2009.
52. Mallilankaraman K, Cardenas C, Doonan PJ, Chandramoorthy HC, Irrinki KM, Golenar T, Csordas G, Madireddi P, Yang J, Muller M, Miller R, Kolesar JE, Molgo J, Kaufman B, Hajnoczky G, Foscett JK, Madesh M. MCUR1 is an essential component of mitochondrial Ca^{2+} uptake that regulates cellular metabolism. *Nat Cell Biol* 14: 1336–1343, 2012.
53. Meur G, Parker AK, Gergely FV, Taylor CW. Targeting and retention of type 1 ryanodine receptors to the endoplasmic reticulum. *J Biol Chem* 282: 23096–23103, 2007.
54. Moore RA, Nguyen H, Galceran J, Pessah IN, Allen PD. A transgenic myogenic cell line lacking ryanodine receptor protein for homologous expression studies: reconstitution of Ry1R protein and function. *J Cell Biol* 140: 843–851, 1998.
55. Morimoto S, O-Uchi J, Kawai M, Hoshina T, Kusakari Y, Komukai K, Sasaki H, Hongo K, Kurihara S. Protein kinase A-dependent phosphorylation of ryanodine receptors increases Ca^{2+} leak in mouse heart. *Biochem Biophys Res Commun* 390: 87–92, 2009.
56. Mughal W, Kirshenbaum LA. Cell death signalling mechanisms in heart failure. *Exp Clin Cardiol* 16: 102–108, 2011.
57. Munch G, Bolck B, Sugar A, Schwinger RH. Isoform expression of the sarcoplasmic reticulum Ca^{2+} release channel (ryanodine channel) in human myocardium. *J Mol Med* 78: 352–360, 2000.
58. Murayama T, Kurebayashi N. Two ryanodine receptor isoforms in nonmammalian vertebrate skeletal muscle: possible roles in excitation-contraction coupling and other processes. *Prog Biophys Mol Biol* 105: 134–144, 2011.
59. Murayama T, Kurebayashi N, Oba T, Oyamada H, Oguchi K, Sakurai T, Ogawa Y. Role of amino-terminal half of the S4-S5 linker in type 1 ryanodine receptor (RyR1) channel gating. *J Biol Chem* 286: 35571–35577, 2011.
60. Nakai J, Dirksen RT, Nguyen HT, Pessah IN, Beam KG, Allen PD. Enhanced dihydropyridine receptor channel activity in the presence of ryanodine receptor. *Nature* 380: 72–75, 1996.
61. Nakano M, Imamura H, Nagai T, Noji H. Ca^{2+} regulation of mitochondrial ATP synthesis visualized at the single cell level. *ACS Chem Biol* 6: 709–715, 2011.
62. Ong SB, Hausenloy DJ. Mitochondrial morphology and cardiovascular disease. *Cardiovasc Res* 88: 16–29, 2010.
63. Orimo A, Gupta PB, Sgroi DC, Arenzana-Seisdedos F, Delaunay T, Naem R, Carey VJ, Richardson AL, Weinberg RA. Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. *Cell* 121: 335–348, 2005.
64. O-Uchi J, Pan S, Sheu SS. Perspectives on: SGP Symposium on Mitochondrial Physiology and Medicine: Molecular identities of mitochondrial Ca^{2+} influx mechanism: updated passwords for accessing mitochondrial Ca^{2+} -linked health and disease. *J Gen Physiol* 139: 435–443, 2012.
65. Pagano M, Naviglio S, Spina A, Chiosi E, Castoria G, Romano M, Sorrentino A, Illiano F, Illiano G. Differentiation of H9c2 cardiomyoblasts: the role of adenylate cyclase system. *J Cell Physiol* 198: 408–416, 2004.
66. Pereira SL, Ramalho-Santos J, Branco AF, Sardao VA, Oliveira PJ, Carvalho RA. Metabolic remodeling during H9c2 myoblast differentiation: relevance for in vitro toxicity studies. *Cardiovasc Toxicol* 11: 180–190, 2011.
67. Perocchi F, Gohil VM, Girgis HS, Bao XR, McCombs JE, Palmer AE, Mootha VK. MICU1 encodes a mitochondrial EF hand protein required for Ca^{2+} uptake. *Nature* 467: 291–296, 2010.
68. Piao L, Li Y, Kim SJ, Byun HS, Huang SM, Hwang SK, Yang KJ, Park KA, Won M, Hong J, Hur GM, Seok JH, Shong M, Cho MH, Brazil DP, Hemmings BA, Park J. Association of LETM1 and MRPL36 contributes to the regulation of mitochondrial ATP production and necrotic cell death. *Cancer Res* 69: 3397–3404, 2009.
69. Pizzo P, Drago I, Filadi R, Pozzan T. Mitochondrial Ca^{2+} homeostasis: mechanism, role, and tissue specificities. *Pflügers Arch* 464: 3–17, 2012.
70. Rizzuto R, Pinton P, Carrington W, Fay FS, Fogarty KE, Lifshitz LM, Tuft RA, Pozzan T. Close contacts with the endoplasmic reticulum as determinants of mitochondrial Ca^{2+} responses. *Science* 280: 1763–1766, 1998.
71. Ryu SY, Beutner G, Dirksen RT, Kinnally KW, Sheu SS. Mitochondrial ryanodine receptors and other mitochondrial Ca^{2+} permeable channels. *FEBS Lett* 584: 1948–1955, 2010.
72. Ryu SY, Beutner G, Kinnally KW, Dirksen RT, Sheu SS. Single channel characterization of the mitochondrial ryanodine receptor in heart mitochondria. *J Biol Chem* 286: 21324–21329, 2011.
73. Sharma VK, Ramesh V, Franzini-Armstrong C, Sheu SS. Transport of Ca^{2+} from sarcoplasmic reticulum to mitochondria in rat ventricular myocytes. *J Bioenerg Biomembr* 32: 97–104, 2000.
74. Soubannier V, McBride HM. Positioning mitochondrial plasticity within cellular signaling cascades. *Biochim Biophys Acta* 1793: 154–170, 2009.
75. Sparagna GC, Gunter KK, Sheu SS, Gunter TE. Mitochondrial calcium uptake from physiological-type pulses of calcium. A description of the rapid uptake mode. *J Biol Chem* 270: 27510–27515, 1995.
76. Szabadkai G, Simoni AM, Bianchi K, De Stefani D, Leo S, Wieckowski MR, Rizzuto R. Mitochondrial dynamics and Ca^{2+} signaling. *Biochim Biophys Acta* 1763: 442–449, 2006.
77. Szabadkai G, Simoni AM, Chami M, Wieckowski MR, Youle RJ, Rizzuto R. Drp-1-dependent division of the mitochondrial network blocks intraorganellar Ca^{2+} waves and protects against Ca^{2+} -mediated apoptosis. *Mol Cell* 16: 59–68, 2004.
78. Takeshima H, Nishimura S, Nishi M, Ikeda M, Sugimoto T. A brain-specific transcript from the 3'-terminal region of the skeletal muscle ryanodine receptor gene. *FEBS Lett* 322: 105–110, 1993.
79. Waldeck-Weiermair M, Jean-Quartier C, Rost R, Khan MJ, Vishnu N, Bondarenko AI, Imamura H, Malli R, Graier WF. Leucine zipper EF hand-containing transmembrane protein 1 (Letm1) and uncoupling proteins 2 and 3 (UCP2/3) contribute to two distinct mitochondrial Ca^{2+} uptake pathways. *J Biol Chem* 286: 28444–28455, 2011.
80. Wei AC, Liu T, Winslow RL, O'Rourke B. Dynamics of matrix-free Ca^{2+} in cardiac mitochondria: two components of Ca^{2+} uptake and role of phosphate buffering. *J Gen Physiol* 139: 465–478, 2012.

81. Williams GS, Boyman L, Chikando AC, Khairallah RJ, Lederer WJ. Mitochondrial calcium uptake. *Proc Natl Acad Sci USA* 110: 10479–10486, 2013.
82. Yi M, Weaver D, Eisner V, Varnai P, Hunyady L, Ma J, Csordas G, Hajnoczky G. Switch from ER-mitochondrial to SR-mitochondrial calcium coupling during muscle differentiation. *Cell Calcium* 52: 355–365, 2012.
83. Yoon Y, Krueger EW, Oswald BJ, McNiven MA. The mitochondrial protein hFis1 regulates mitochondrial fission in mammalian cells through an interaction with the dynamin-like protein DLP1. *Mol Cell Biol* 23: 5409–5420, 2003.
84. Yu T, Sheu SS, Robotham JL, Yoon Y. Mitochondrial fission mediates high glucose-induced cell death through elevated production of reactive oxygen species. *Cardiovasc Res* 79: 341–351, 2008.
85. Zhao F, Li P, Chen SR, Louis CF, Fruen BR. Dantrolene inhibition of ryanodine receptor Ca^{2+} release channels. Molecular mechanism and isoform selectivity. *J Biol Chem* 276: 13810–13816, 2001.
86. Zinchuk V, Zinchuk O, Okada T. Quantitative colocalization analysis of multicolor confocal immunofluorescence microscopy images: pushing pixels to explore biological phenomena. *Acta Histochem Cytochem* 40: 101–111, 2007.

