Supplemental Data

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RyR1 *S*-Nitrosylation Underlies Environmental Heat Stroke and Sudden Death in Y522S RyR1 Knockin Mice

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Supplemental Experimental Procedures

Reagents. Etomidate was purchased from Bedford Laboratories. *N*-acetylcysteine (NAC), N(Ω)-nitro-L-arginine (L-NNA), N(Ω)-nitro-L- arginine methyl ester (L-NAME), Carbonylcyanide-4-(trifluoromethoxy)-phenylhydrazone (FCCP), GSH ethyl ester (GSHEE), 2-thiobarbituric acid, phenylmethylsulfonyl fluoride (PMSF), aminobenzamidine, sodium orthovanadate, NaF, β-phosphoglycerate, 5,5'-dithiobis-2-nitrobenzene, β-NADP glucose-6-phosphate, glucose-6-phosphate dehydrogenase (bovine liver), and glutathione reductase (yeast) were purchased from Sigma Aldrich. Pepstatin A and aprotinin were purchased from USB Corporation. Leupeptin was purchased from Millipore. Ryanodine and N-Acetyl-Leu-Leu-Met-CHO (ALLM) were obtained from Biomol International. [^{3}H]ryanodine was purchased from GE Healthcare. Indo-1 AM, fura-2 AM, tetramethyl rhodamine ethylester (TMRE), 2',7'-difluorofluorescein diacetate (DCF-DA), and 5-carboxy-2',7'-dichlorodihydrofluorescein methyl (DAF-DA) were purchased from Invitrogen.

Mice. RyR1$^{Y522S/wt}$ mice were generated as previously described (Chelu, 2006). Because these mice are meant to be a model for the human disease, we use the numbering of the mutant amino acid in humans (Y522S) to allow for easy

comparison to the human disease. The mutation in the mouse skeletal muscle ryanodine receptor is Y524S. Wild-type and RyR1$^{Y522S/wt}$ mice were maintained on a 12:12 h light:dark cycle. Unless otherwise stated, mice were killed by cervical dislocation after anesthetizing them with etomidate (30mg/kg i.p.).

Indirect calorimetry. The Oxymax (Columbus Instruments, Columbus, Ohio) indirect calorimetry system was used to measure O_2 and CO_2. Mice were studied at an ambient temperature of 31-32ºC, at which the mice spend the majority of their time resting quietly and cold- and activity-induced thermogenesis are minimized.

Isolation of microsomes from mouse skeletal muscle. All steps were performed either on ice or at ≤ 4°C. Muscle (1.0 to 1.5 g) was homogenized in 15ml of 0.5mM EGTA, 300mM sucrose, 20mM Tris-maleate at pH 7.0, 1mg/L aprotinin, 1mg/L pepstatin A, 1mg/L leupeptin, 0.2mM aminobenzamidine, 0.1mM PMSF, 2mM sodium orthovanadate, 50mM NaF, 1mM β-phosphoglycerate, and 0.5μM ALLM (Buffer A), using a Tissue Tearor 3x10s at medium speed. An aliquot (0.4ml) was deproteinized with 1 volume of 10% metaphosphoric acid and reserved for glutathione assays. The remaining homogenate was centrifuged at 10,000xg for 10min at 4ºC. Pellets were resuspended in 15ml of Buffer A using a Tissue Tearor 1x20s at high speed and re-centrifuged at 10,000xg for 10min at 4ºC. Supernatants were pooled and centrifuged at 100,000xg for 45min at 4ºC. Pellets were resuspended in 0.2ml of a buffer containing 0.3M sucrose, 0.5mM EGTA, 0.1M KCl, 10mM MOPS-NaOH

pH 7.4, 1mg/L leupeptin, 1mg/L aprotinin, 1mg/ml pepstatin A, 0.2mM aminobenzamidine, 0.1mM PMSF, and 0.5μM ALLM by 20X homogenization in a hand held glass homogenizer. Protein was determined by the Lowry method (Lowry, 1951).

Isolation of mitochondrial enriched samples. All procedures were performed at 4°C. *Gastrocnemius* muscle from 2 or 12 months-old mice, chronically treated with or without NAC (≥ 2 months), were finely minced with scissors and homogenized in 50 volumes of 0.2mM sodium citrate in 50mM Tris-HCl at pH 7.4, using a Tenbroeck glass-glass homogenizer (50 strokes). Homogenates were centrifuged at 800*xg* for 10 min and supernatants were further centrifuged at 20,000*xg* for 10min. Pellets were resuspended in 0.2mM sodium citrate and incubated with 2% CHAPS to disrupt mitochondrial membranes. Succinate dehydrogenase activity was measured in these samples, as previously described (Green, 1980), and homogenates to confirm mitochondrial enrichment.

Primary cultures and fluorescent microscopy. After 4-7 days of myotube differentiation, cultures were loaded with 50nM TMRE in Tyrodes buffer (in mM): NaCl (129), KCl (5), $CaCl_2$ (2), $MgCl_2$ (1), HEPES (20), glucose (20), glycine (0.1), pH 7.4 at room temperature for 30-50min. Myotubes were then perfused continuously with Tyrode's buffer containing 50nM TMRE during imaging. Temperature was controlled by continuous perfusion (~2mL/min) through an in-line solution heater (SF28; Warner Instruments). Images were taken with a Zeiss (Oberkochen, Germany) LSM 510 Meta system with a Plan Apochromat 63X oil

(1.4NA) or Plan Apochromat 40X oil (1.0NA) immersion objectives. TMRE was excited at 543nm and detected through a 560nm longpass filter. High resolution (1.4NA objective) 12-bit 512x512 images were collected at 0.7μm depth intervals through a 0.8μm pinhole. Where indicated, 20μM ryanodine was added 30minutes prior to experimentation. FCCP was used at 20μM to demonstrate the loss of TMRE fluorescence with mitochondrial depolarization. ROS and RNS were measured using 10μM DCF-DA or DAF-DA, respectively, dissolved in DMSO/10% F127 for 60minutes. After washing, cultures were imaged in Tyrode's solution. Z-stacked (1μm interval) images were taken through a wide pinhole for low intensity excitation light. Bath temperature was controlled with a Warner Instruments Series20 platform. Both dyes were excited at 488nm and detected through a 505nm longpass filter. Imaging conditions used produced <5% signal loss or detector saturation. Z-stacks were combined as single maximum projections and quantified with ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997-2007), while representative images of single myotubes are from a single plane.

Intracellular Ca^{2+} and EC coupling measurements in intact myotubes. Myoblasts from newborn mice were used to generate primary cultures of skeletal myotubes as described previously (Chelu, 2006). L-type Ca^{2+} currents (L-currents) and intracellular Ca^{2+} transients were simultaneously recorded under conditions described in Chelu et al. (Chelu, 2006). Myotubes were loaded by incubation in 0.5ml Tyrode's solution containing 6μM of the fluorescent Ca^{2+}

indicator Indo-1 AM or Fura-2 AM for 1 hr at room temperature. Cells were washed twice with Tyrodes solution and maintained in Tyrode's solution. GSHEE (5mM), L-NNA (50μM) or L-NAME (50μM) in Tyrode's solution were added during the loading and experimental periods. Coverslips were mounted on an upright fluorescence microscope (Nikon Eclipse E600FN) equipped with a Cascade 512B CCD camera (Photometrics). For Fura-2 measurements, fluorescence emission at 340 and 380nm, excited at 510nm, was monitored using a 40x/0.8, water immersion objective. Images were captured and analyzed with Metafluor (Molecular Devices, PA, USA).

Resting indo-1 fluorescence ratios ($R=F_{405}/F_{485}$) were converted to free Ca^{2+} concentrations using an *in situ* calibration approach and the following equation (Grynkiewicz, 1985): $[Ca^{2+}]_I = K_d \cdot \beta \cdot [(R-R_{min})/(R_{max}-R)]$, where K_d is the *in situ* Ca^{2+} affinity of indo-1, β is the ratio of the 485nm emission recorded under Ca^{2+} free and Ca^{2+} saturating conditions, R_{min} is the emission ratio under Ca^{2+} free conditions, and R_{max} is the emission ratio under Ca^{2+} saturating conditions. The values of β, R_{min}, and R_{max} were determined experimentally. The K_d used was taken from the *in situ* calibration of indo-1 in cardiac myocytes (844nM; Bassani, 1995) and was assumed to be independent of temperature in intact cells where pH is not under experimental control (Wang, 1999).

[^{3}H]ryanodine binding. Equilibrium binding at saturating Ca^{2+} was performed in a buffer containing 50mM NaCl, 1.2mM $CaCl_2$, 1mM EGTA, 0.1mg/ml BSA, 0.1% CHAPS and 50mM MOPS-NaOH at pH 7.4. Ca^{2+} titration of binding was performed in a buffer containing 100mM KCl, 0.1mg/ml BSA, 0.1% CHAPS and

30mM MOPS-NaOH at pH 7.2. Kinetic assays were measured in a buffer containing 50mM NaCl, 5μM $CaCl_2$, 0.1mg/ml BSA, 0.1% CHAPS and 50mM MOPS-NaOH at pH 7.4. All buffers were supplemented with the following protease inhibitors: 1mg/L aprotinin, 1mg/L pepstatin A, 1mg/L leupeptin, 0.2mM aminobenzamidine and 0.1mM PMSF.

Preparation and analysis of samples for electron microscopy (EM). Mice were sacrificed at either 2-3 months or 1 year of age. The skin was quickly removed from the lower limbs, FDB and Soleus muscles dissected and immediately fixed at room temperature with 3.5% glutaraldehyde in 0.1 M NaCaCo buffer (pH 7.2). Small bundles of fixed fibers were embedded, sectioned and examined as previously described (Paolini, 2007). The average minimum diameter of mitochondrial arrays was measured in micrographs taken at 17,700X magnification using the Soft Imaging System (Germany). In each fiber, 4 to 5 micrographs were randomly collected from longitudinal sections. For FDB fibers, 4 to 6 fibers were analyzed for each muscle (2 $RyR1^{wt/wt}$, 4 $RyR1^{Y522S/wt}$). The number of severely disrupted mitochondria was counted and their number is reported as % control. In addition, the diameter of normal and abnormal mitochondria presenting a translucent matrix, but with an apparently intact external membrane, was measured in the same micrographs (excluding the severely disrupted ones). Only mitochondria which were entirely visualized in the micrograph were measured. A total of 643 to 1069 mitochondria were measured in each group and the average diameter (in nm) ± S.D. is reported. Mitochondria with any or several of the following unltrastructural alterations were classified as

severely disrupted: a) mitochondria with clear disruption of the external membrane; b) severe vacuolization and disruption of the mitochondria internal cristae; c) mitochondria containing myelin figures.

Statistical analyses. Fluorescence data for Ca^{2+} measurements, and *in vivo* heating responses were fit using non-linear regression. Ca^{2+} currents were analyzed by one-way ANOVA with Scheffe post tests. Differences between groups of fit data were analyzed using F-tests. Differences in EC coupling parameters and oxygen consumption were analyzed by one-way ANOVA with Tukey post-hoc tests. Differences in developed stress between groups were analyzed using a two-way ANOVA with Bonferroni post-tests. Differences between groups for ryanodine binding parameters, *S*-nitrosylation, *S*-glutathionylation, Indo-1 fluorescence, mitochondrial membrane potential and mitochondrial morphology were analyzed using Student's t-tests. Values of $p<0.05$ (95% confidence) were considered significant.

Supplemental References

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Supplemental Figures

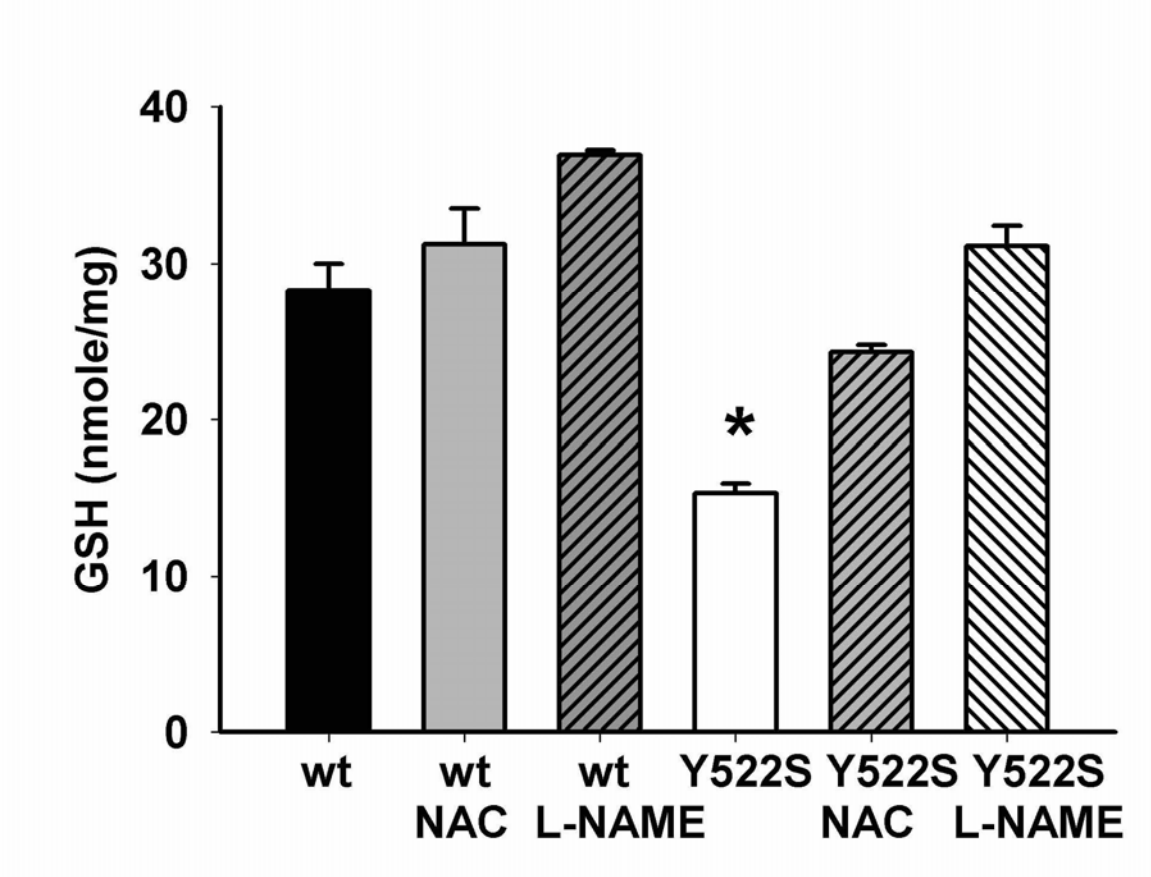

Figure S1. GSH content is decreased in muscles from RyR1$^{Y522S/wt}$. GSH content was determined as described in Experimental Procedures. Data are presented as mean ± SEM of 3-4 independent determinations. *p<0.05 and compared to RyR1$^{wt/wt}$.

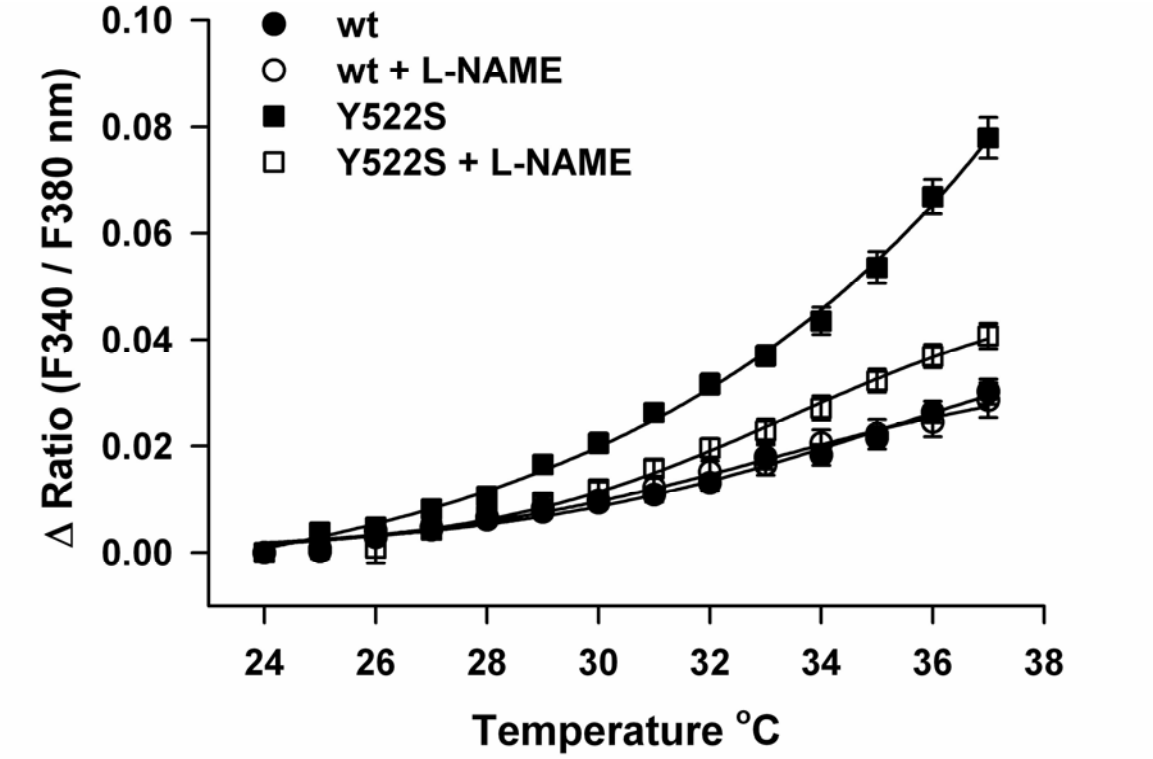

Figure S2. Effect of L-NAME on temperature dependent increases in cytosolic Ca^{2+}. Temperature dependent increases in cytosolic Ca^{2+} levels were measured with Fura-2. Myotubes loaded with Fura-2 AM were warmed to the indicated temperatures in the presence or absence of L-NAME (50μM). Data shown are mean ± SEM using at least three separate cultures. Numbers of myotubes are 23 for wt, 27 for wt plus L-NAME, 25 for Y522S and Y522S plus L-NAME, separately.

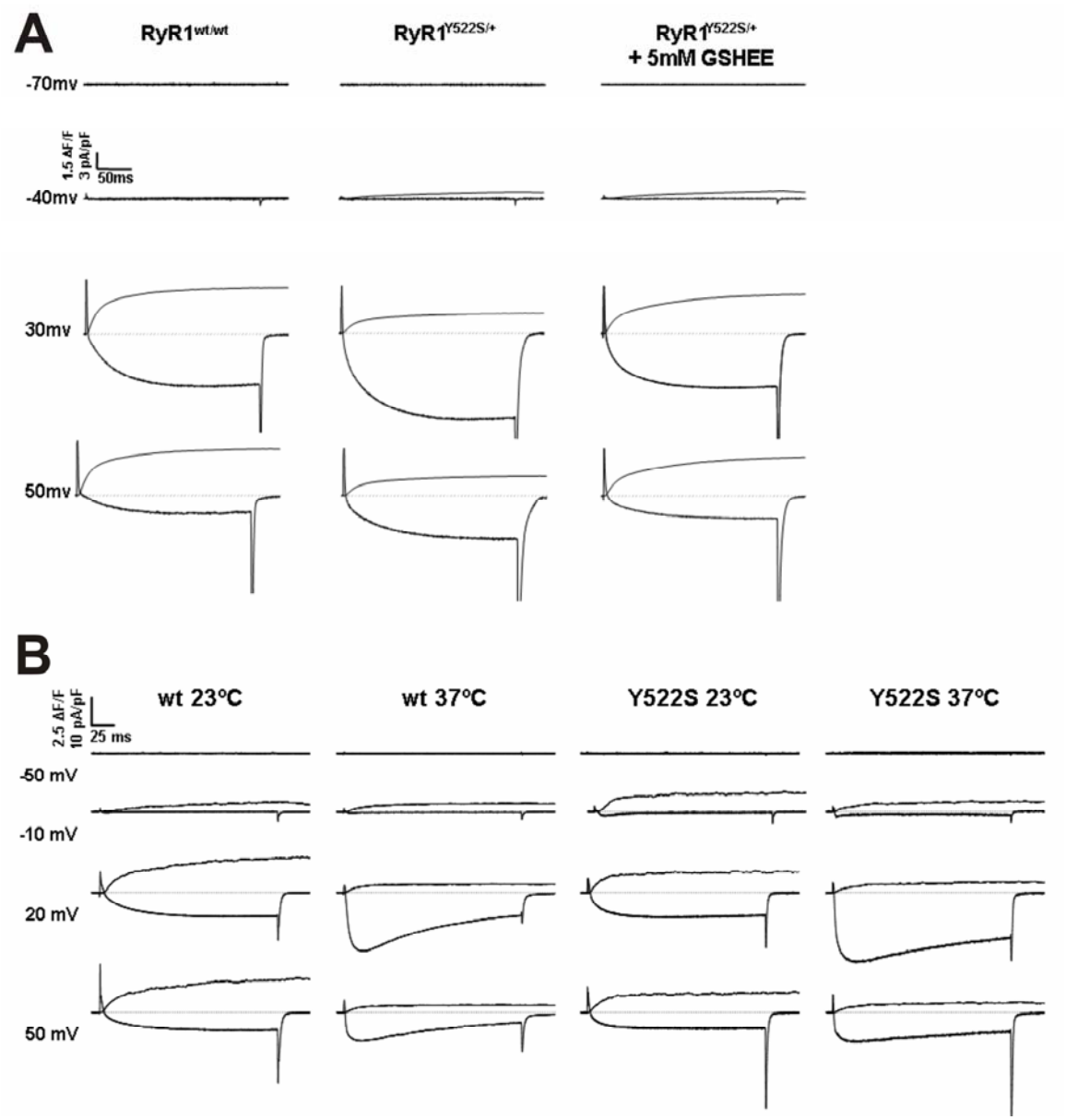

Figure S3. Representative L-currents and intracellular Ca^{2+} transients in RyR1$^{wt/wt}$ and RyR1$^{Y522S/wt}$ myotubes. A. Representative L-currents (lower traces) and intracellular Ca^{2+} transients (upper traces) at room temperature recorded in response to 200 ms depolarizations to the indicated potentials in RyR1$^{wt/wt}$ myotubes (left), RyR1$^{Y522S/wt}$ myotubes (middle), and RyR1$^{Y522S/wt}$ myotubes preincubated with 5 mM GSHEE (right). **B.** Temperature dependence of depolarization-induced L-type Ca^{2+} currents and intracellular Ca^{2+} transients in myotubes from RyR1$^{wt/wt}$ and RyR1$^{Y522S/wt}$ mice. Representative L-type Ca^{2+} currents (lower traces) and intracellular Ca^{2+} transients (upper traces) recorded

at 23°C and 37°C in response to 200 ms depolarizations to the indicated potentials (left) in RyR1$^{wt/wt}$ and RyR1$^{Y522S/wt}$ myotubes.

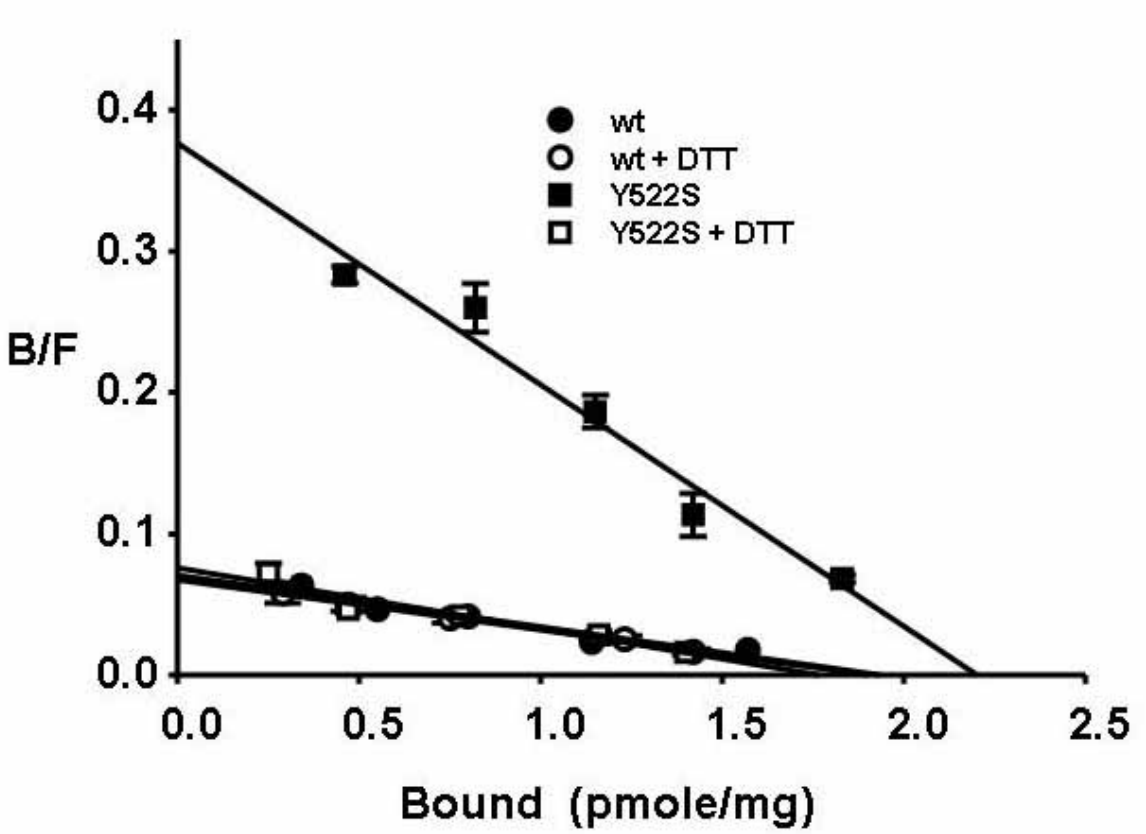

Figure S4. Representative Scatchard plots of [^{3}H]ryanodine binding. Equilibrium [^{3}H]ryanodine binding (1.56 to 50 nM) of microsomal preparations were performed in a buffer containing 50mM NaCl, 1.2mM $CaCl_2$, 1mM EGTA, 0.1mg/ml BSA, 0.1% CHAPS and 50mM MOPS-NaOH at pH 7.4, in the presence or absence of 1mM DTT for 16 hours at 22°C (room temperature). Assays were performed as described in Supplemental Procedures. Free [^{3}H]ryanodine concentrations were calculated for each point from experimental total and bound [^{3}H]ryanodine. Representative Scatchard plots (mean of 3-4 replicates ± SD) are shown for: RyR1$^{Y522S/wt}$ (■), RyR1$^{Y522S/wt}$ + DTT (□), RyR1$^{wt/wt}$ (●) and RyR1$^{wt/wt}$ + DTT (○).

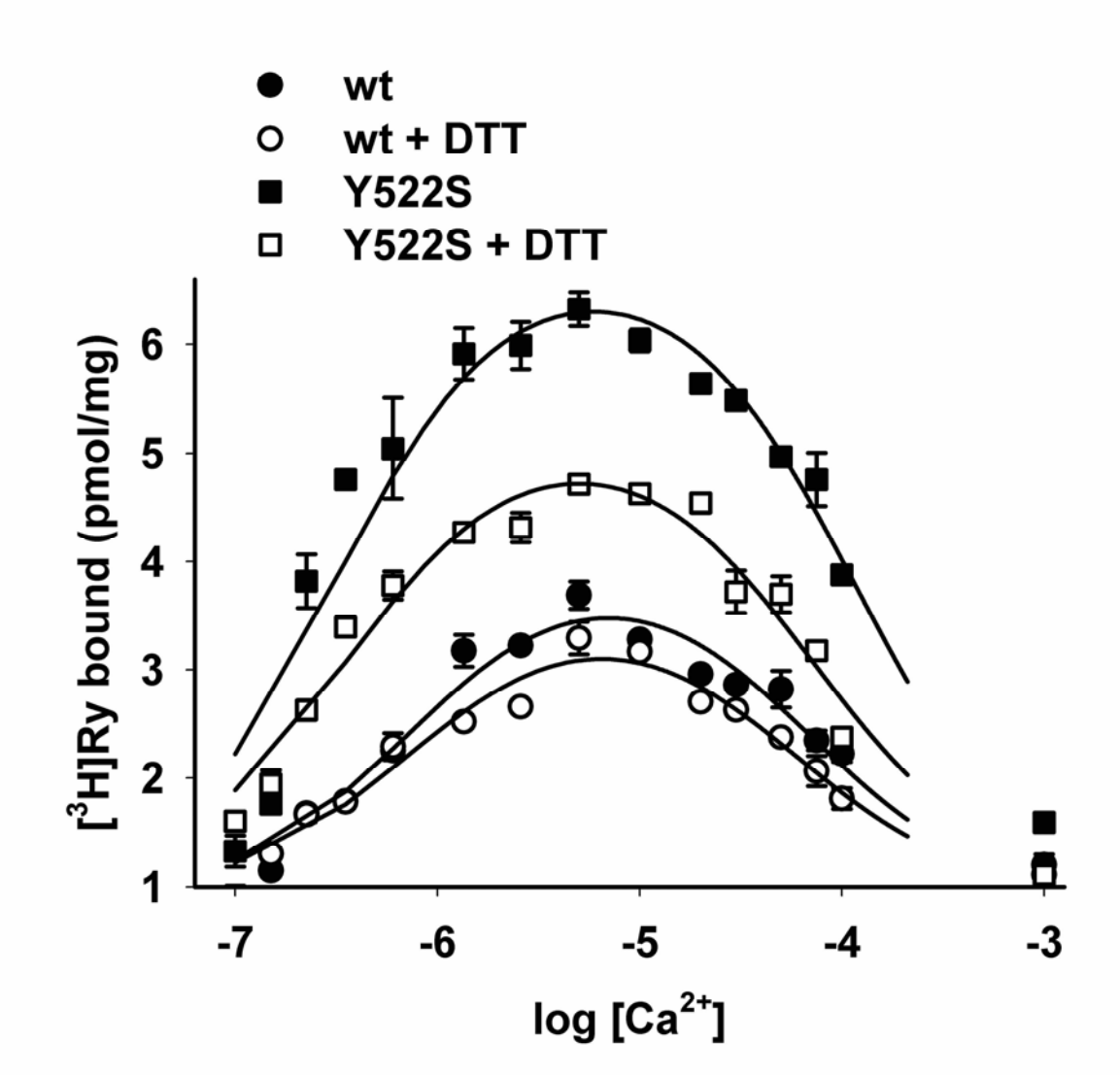

Figure S5. Ca^{2+} dependence of [^{3}H]ryanodine binding. Assays were performed by incubating microsomal preparations with 5nM [^{3}H]ryanodine in calibrated Ca^{2+} buffers (17nM to 1mM) containing 100mM KCl and 50mM MOPS-NaOH at pH 7.4 (Invitrogen), supplemented with 0.1mg/ml BSA and 0.1% CHAPS, in the presence or absence of 1mM DTT for 16 hours at 22°C. Representative plots (mean of 3-4 replicates ± SEM) are shown for: $RyR1^{Y522S/wt}$ (■), $RyR1^{Y522S/wt}$ + DTT (□), $RyR1^{wt/wt}$ (●) and $RyR1^{wt/wt}$ + DTT (○).

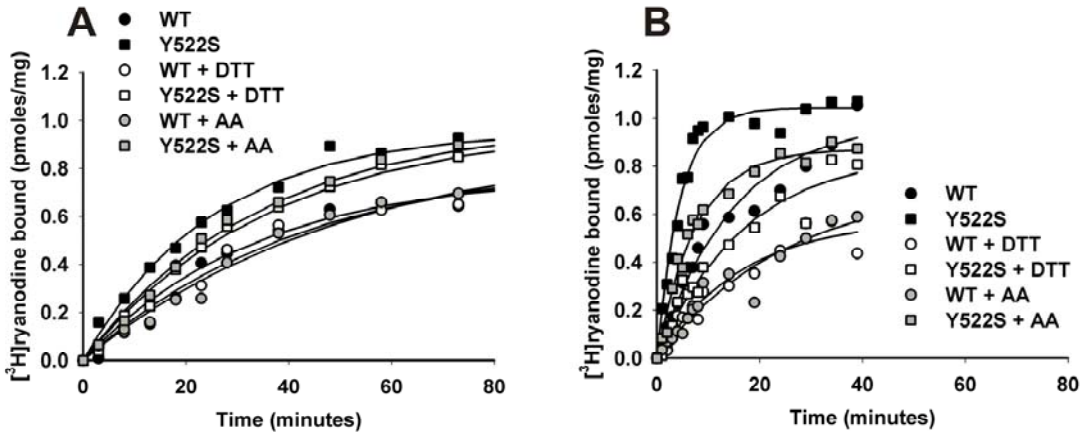

Figure S6. Temperature dependence of the association kinetics of [^{3}H]ryanodine binding in microsomes from RyR1$^{wt/wt}$ and RyR1$^{Y522S/wt}$ mice. Microsomes from untreated mice were pre-incubated *in vitro* with buffer (untreated), 1mM AA or 2mM DTT for 30min in ice. [^{3}H]ryanodine binding was assessed at different time points (1-90min), as described in Experimental Procedures. Shown are representative experiments for 23°C (left panel) and 37°C (right panel). Data were fitted to single exponential curves to calculate apparent k_{obs} values.

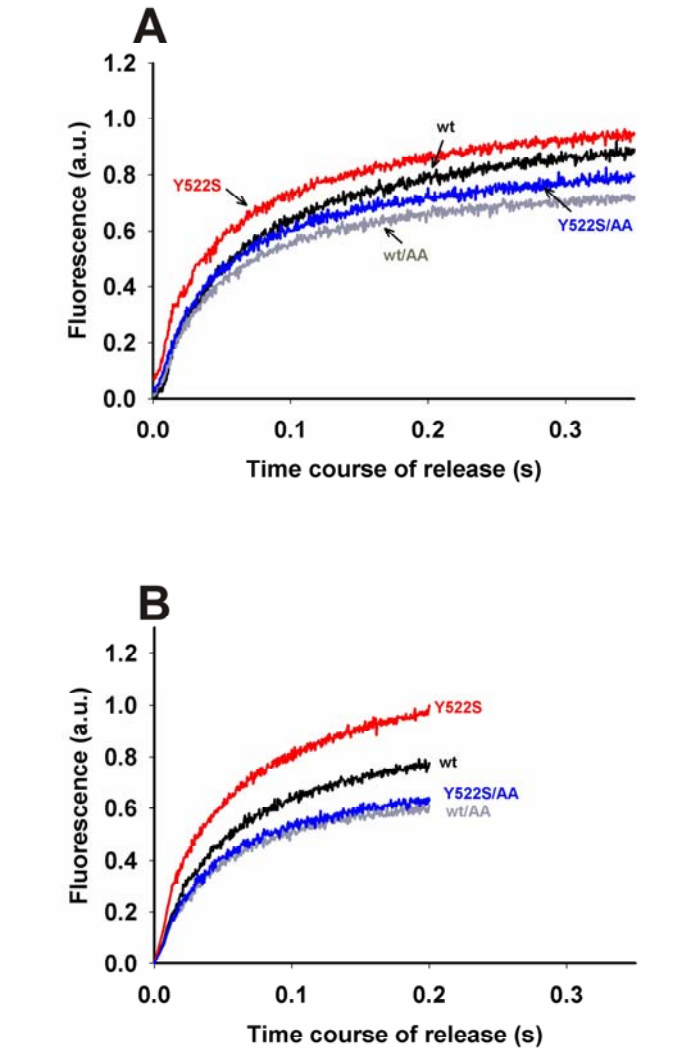

Figure S7. Rate of Ca^{2+} Efflux from SR vesicles. Ca^{2+}-induced Ca^{2+} release was measured in the presence of 1mM free ATP and 10μM free Ca^{2+} using stopped-flow spectrofluorometry and extravesicular Calcium Green-5N as a Ca^{2+} probe. Representative traces of release are shown for microsomes (treated or not with 1mM AA for 30min in ice) at 25°C (top panel) or 37°C (bottom panel).

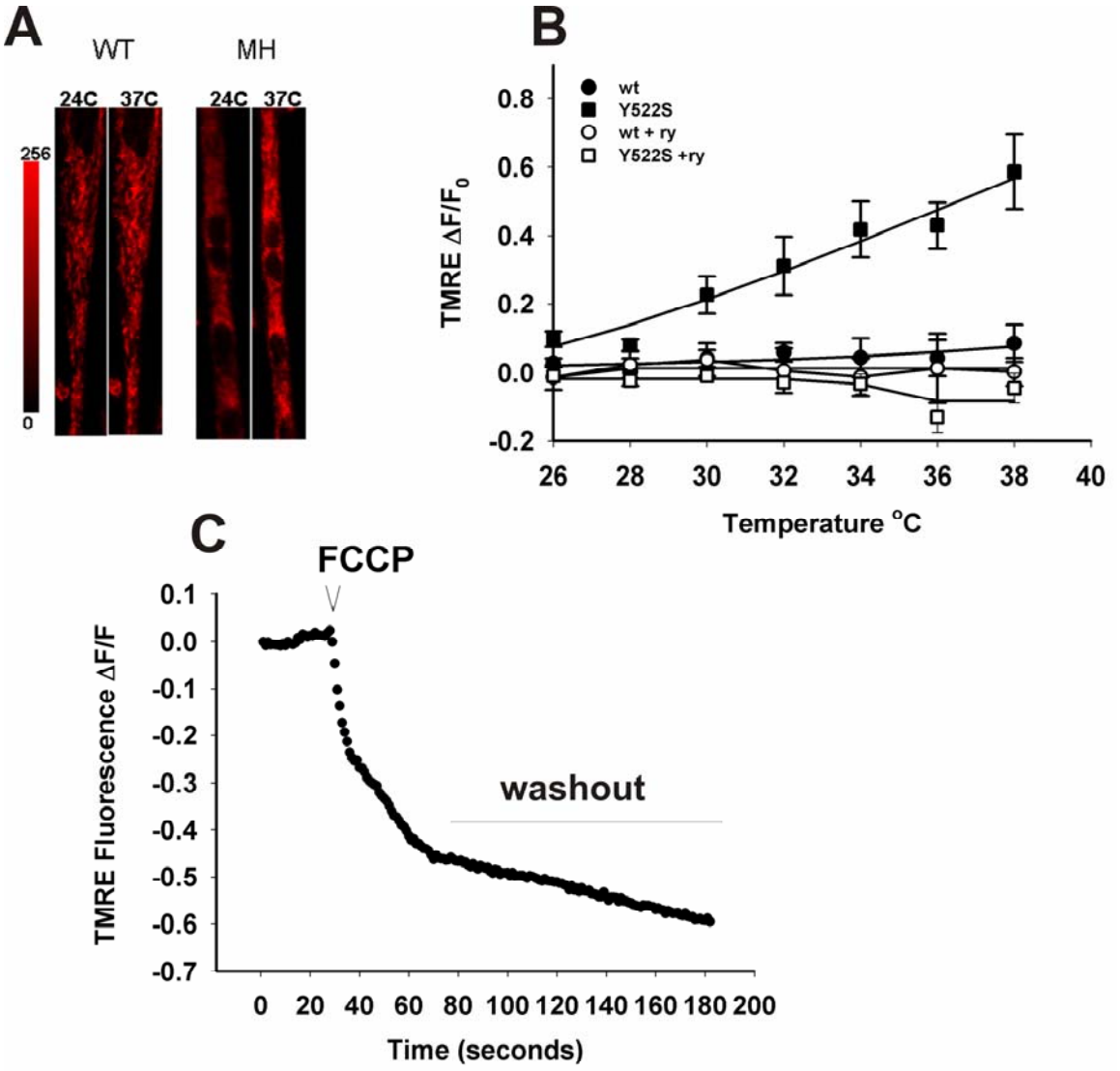

Figure S8. Mitochondrial membrane potential measurements with TMRE. Cells were equilibrated with perfusing medium containing TMRE (50nM), as detailed in Supplemental Experimental Procedures. **A.** Mitochondrial visualization with TMRE at room temperature (24°C) and 37°C. **B.** Mitochondrial membrane potential was measured, as TMRE fluorescence normalized to 24°C (F_0), in myotubes from RyR1$^{Y522S/wt}$ (closed squares) and RyR1$^{wt/wt}$ mice (closed circles) either in the presence (open symbols) or absence (closed symbols) of pre-incubation with 20μM ryanodine (open symbols). Data are presented as mean ± SEM. **C.** TMRE-loaded myotubes were imaged for in control media, following addition of 4μM FCCP, and followed return to normal control media (washout). A representative trace of TMRE fluorescence for one such experiment conducted in a RyR1$^{wt/wt}$ myotube.