Supplemental Methods

Cloning

The open reading frame of $Ca_V1.2$ α1c subunit (NCBI X15539) was amplified by PCR and ligated into a modified pLenti6 plasmid (Invitrogen), pLentiNB, which had the blasticidin resistance gene and promoters of the pLenti6 plasmid removed to facilitate viral packaging. An extracellular hemaglutanin epitope was added to $Ca_V1.2$ by methods previously published[1]. The dihydropyridine resistance mutation (DHP^R, T1066Y) and TS mutation (G406R) were introduced by using the PCR method Quikchange (Stratagene) as per manufacturer's protocol.

HEK293 transfection

HEK293T cells were transfected with the pLentiNB $Ca_V1.2$ WT or TS with a pIRES eGFP β_{2a} subunit using Fugene6 (Roche) as described by the manufacturer. For electrophysiology experiments, transfected HEK293T cells were detected by expression of eGFP and confirmed by inward I_{Ca}. For immunofluorescence, transfected HEK293T cells were fixed with 2% PFA and stained as described under immunofluorescence methods.

Lenti virus

The transgene plasmid pLentiNB carrying the modified $Ca_V1.2$ was transfected (Qiagen, Effectene) with the Lenti viral packaging plasmids (Invitrogen's pLP1, pLP2 and pVSVG) into HEK293FT cells (Invitrogen). Media was collected and replaced at 24, 48 and 72 hours post-transfection. The viral containing media was concentrated by either ultrafiltration (Millipore Centricon Plus-70 30kDa) or

ultacentrifugation. Viral titer (transducing units per mL, TU/mL) was determined by serial dilution (10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, no virus) on HEK293 cells followed by immuno-staining (see Immunoflourescence methods) for the $Ca_V1.2$ HA epitope (anti-HA conjugated Alexa 488 Ig) and counting positively stained cells within each dilution. Viral titers achieved were between 10^5 and 10^6 TU/mL. Extracts from HEK293 cells used to produce virus were analyzed by SDS-PAGE and immuno-blotting with an affinity-purified HA Ig.

Ventricular myocyte isolation, culturing and viral transduction

Adult male Sprague-Dawley rats (250-300g) were anesthetized by Avertin (2.5%) with Heparin (55 units/mL) through IP injection (0.2mL/10g). Hearts were excised, perfused retro-aortically (Langendorff) and enzymatically digested with a mixture of Collogenase (Worthington, 250 units/mL), Hyaluronidase (Sigma, 0.01%) and Protease Type XIV (Sigma, 0.0025%) in a modified tyrodes solution (0.1mM $CaCl_2$, 10mM BDM). Dissociated cardiomyocytes were washed three times in Joklik MEM (Sigma M0518) with 1% Pen/Strep and 1X ITS (Sigma) with increasing Ca^{2+} (0.25mM, 0.5mM, 0.75mM). Ventricular myocytes were plated on glass cover slips (glass #1) coated with Geltrex (Invitrogen, thin layer) and allowed to attach for 1 hour. Cells were washed with a culture media consisting of a 50:50 mix of DMEM and F10 media with 1% Pen/Strep and 1X ITS. Attached cardiomyocytes were counted and the cell density was calculated. Lenti virus was added to the cells at a multiplicity of infection (MOI) of 1-3, and cells cultures were maintained for 24-36 hours. Cultured ventricular myocytes (WT, TS,

uninfected) extracts were analyzed by SDS-PAGE and immuno-blotting with a $Ca_V1.2$ Ig (ABR).

Electrophysiology

HEK293 I_{Ca} recordings for voltage dependence of inactivation (VDI) used a two step voltage clamp protocol (repeated 0.1 Hz, resting -80mV, 25°C) with an initial conditioning step (0.8s, -50mV to +60mV, Δ10mV) followed by a test pulse (300ms, +30mV). Bath solution was in mM; 130 NMDG, 10 HEPES, 5 KCl, 15 $CaCl_2$. Pipette solution was in mM; 120 Cs methanesulfonate, 5 $CaCl_2$, 1 $MgCl_2$, 2 MgATP, 10 HEPES, 10 EGTA. Available current observed each test pulse after a given conditioning pulse was accessed a percent of the maximum current observed.

Cardiomyocyte action potentials (AP) were stimulated (2ms, 1.5-2.5nA) in current clamp mode (0.5Hz, 25°C). Bath solution was in mM; 140 NaCl, 4 HEPES, 10 Glucose, 5.4 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$. Pipette solution was in mM; 120 K aspartate, 5 HEPES, 25 KCl, 4 Na_2ATP, 1 $MgCl_2$, 10 EGTA, 2 Na_2 phosphocreatine, 1 $CaCl_2$, 2 NaGTP. Recorded APs were analyzed using ClampFit's (Axon Instruments) event detection algorithm and statistics decay time (ms) algorithm.

Cardiomyocyte I_{Ba} recordings for VDI used a two step voltage clamp protocol (repeated 0.1 Hz, resting -80mV, 25°C) with an initial conditioning pulse (2.0s, -80mV to +30mV, Δ10mV) followed by a test pulse (300ms, 0mV). To record only VDI and prevent Ca^{2+} dependent inactivation, Ca^{2+} was tightly buffered through the use of Ba^{2+} as the charge carrier in the bath solution and

BAPTA with no Ca^{2+} in the pipette solution. Bath solution was in mM; 137 NMDG, 10 HEPES, 10 Glucose, 1.8 $BaCl_2$, 0.5 $MgCl_2$, 25 CsCl. Pipette solution was in mM; 120 CsCl, 10 TEA, 1 MgATP, 1 NaGTP, 5 phosphocreatine, 10 HEPES, 20 BAPTA. Available current observed each test pulse after a given conditioning pulse was accessed a percent of the maximum current observed.

Cardiomyocyte I_{Ca} facilitation was recorded using a single step (300ms, 0mV) voltage clamp protocol (repeated 0.5Hz, resting -80mV, 25°C). Bath solution was in mM; 137 NMDG, 10 HEPES, 10 Glucose, 1.8 $CaCl_2$, 0.5 $MgCl_2$, 25 CsCl. Pipette solution was in mM; 120 CsCl, 3 $CaCl_2$, 10 TEA, 1 MgATP, 1 NaGTP, 5 phosphocreatine, 10 HEPES, 10 EGTA. I_{Ca} facilitation was integrated using ClampFit's area statistics (pA*ms) algorithm and normalized to cell size (pF). Inactivation time constants were calculated using ClampFit.

Immunofluorescence

HEK293 cells, cultured on coverslips (glass #1), were gently washed with PBS and fixed for 20 minutes in 2% paraformaldyhyde (25°C). Cultured adult ventricular myocytes (WT, TS and uninfected) were paced by field stimulation (Ion Optix C-pace and C-dish, 1Hz, 35V, 2ms) for 5 minutes in Tyrodes (1.8mM $CaCl_2$, 37°C). Immediately following the pacing protocol, ventricular myocytes were fixed for 20 minutes in 2% parafomaldyhyde (25°C). Fixed cells were permeabilized for 10 minutes with PBS with 0.1% Triton X-100, 2 mg/mL BSA and 2% fish gelatin. Permeabilized cells were blocked with PBS with 2 mg/mL BSA and 2% fish gelatin. Cells were incubated overnight (4°C) in one of the following; anti-HA conjugated Alexa 488 Ig (Molecular Probes), HA Ig (Santa

Cruz), pCaMKII Thr^{286} Ig (ABR), CaMKII Ig (Bers Lab) and washed. The cells incubated with HA Ig were then incubated in donkey anti-rabbit Alexa 488 Ig (Molecular Probes) at 4°C. Cells incubated with pCamKII Thr286 Ig were then incubated in donkey anti-mouse 568 (Molecular Probes). Cells incubated with CaMKII Ig were then incubated in donkey anti-rabbit 568 (Molecular Probes). Ventricular myocytes were mounted with glass coverslips and Vectashield (with or without DAPI; Vector Laboratories).

Ventricular myocyte images were collected on a Zeiss 510 Meta confocal microscope (Carl Zeiss), under 40x magnification (oil, 1.30 NA lens), with a pinhole of 1.0 airy disc (Carl Zeiss), using the Zeiss image acquisition software. HEK293 images were taken at 40x magnification using both the FITC filter and DAPI filter. All images were exported to Photoshop (Adobe) for cropping and linear adjustment of contrast.

Calcium imaging

Myocytes were loaded with Fluo-3 AM (5μM, 20 min.) at room temperature. After de-esterification, the cells were perfused with normal Tyrode solution (1.8 mM Ca^{2+}). Confocal Ca^{2+} imaging was performed with a laser scanning confocal microscope (LSM 510 Meta, Carl Zeiss) equipped with a NA=1.35, 63x lens. Line scan measurement of Ca^{2+} transients, SR content and sparks were acquired at a sampling rate of 1.93 ms/line along the longitudinal axis of the myocytes. Sparks were measured under resting conditions. Steady state Ca^{2+} transients were achieved by a 30 sec pacing at 1 Hz. SR Ca^{2+} content

was measured as a global Ca^{2+} release induced by 10mM caffeine. All digital images were processed with IDL 6.0 (Research System Inc).

Mathematical modeling

Mathematical models of the WT and TS myocytes are based on the Luo-Rudy dynamic model of the mammalian ventricular action potential[2, 3]. For this study, we incorporated a revised formulation for Ca^{2+} release from the sarcoplasmic reticulum and regulation by CaMKII based on the model of Livshitz and Rudy[4]. Our model of SR Ca^{2+} release includes a formulation for spontaneous Ca^{2+} release from the sarcoplasmic reticulum, which occurs when the amount of Ca^{2+} bound to calsequestrin reaches threshold, as described in the original LRd model. Cells were paced to steady-state (over 15 min. pacing) at a cycle length of 700 ms using a conservative current stimulus[5]. Afterdepolarization events were monitored during a pause following steady-state pacing. Ordinary differential equations in the model were integrated numerically using the Forward Euler Method and an adaptive time step. Details on the mathematics involved in the model can be found in the Supplemental Equations.

Reference List

(1) Altier C, Dubel SJ, Barrere C et al. Trafficking of L-type calcium channels mediated by the postsynaptic scaffolding protein AKAP79. *J Biol Chem* 2002;277:33598-603.

(2) Faber GM, Rudy Y. Action Potential and Contractility Changes in [Na+]i Overloaded Cardiac Myocytes: A Simulation Study. *Biophys J* 2000;78:2392-404.

(3) Luo CH, Rudy Y. A dynamic model of the cardiac ventricular action potential. I. Simulations of ionic currents and concentration changes. *Circ Res* 1994;74:1071-96.

(4) Livshitz LM, Rudy Y. Regulation of Ca2+ and electrical alternans in cardiac myocytes: role of CAMKII and repolarizing currents. *Am J Physiol Heart Circ Physiol* 2007;292:H2854-H2866.

(5) Hund TJ, Kucera JP, Otani NF, Rudy Y. Ionic Charge Conservation and Long-Term Steady State in the Luo-Rudy Dynamic Cell Model. *Biophysical Journal* 2001;81:3324-31.

Supplemental Tables

Supplemental Table 1: Action potential data

				P value	
	WT	**TS**	**Uninfected**	**WT:TS**	**WT:Un**
Nifedipine (nM)	10	10	0		
Number of Cells (n)	10	10	12		
APD90% (ms)	46.35 ±8.02	112.00 ±23.47	68.04 ±12.54	**0.018**	0.17
Afterdepolarizations (#/Total)	0/10	5/10	0/11	**0.033**	1
Resting Potential (mV)	-62.60 ±3.69	-60.85 ±3.20	-67.10 ±1.02	0.497	0.966
Peak Amplitude (mV)	101.77 ±9.42	99.88 ±5.97	113.08 ±3.50	0.799	0.103

Supplemental Table 2: Voltage dependence of inactivation data

				P value	
	WT	**TS**	**Uninfected**	**WT:TS**	**WT:Un**
Nifedipine (nM)	10	10	0		
Number of Cells (n)	5	5	5		
VDI $V_{1/2}$ (mV)	-35.89 ±0.64	-30.75 ±0.76	-37.06 ±0.31	**0.008**	0.507
Peak (pA/pF)	21.30 ±1.45	21.71 ±2.48	19.40 ±3.56	0.880	0.583

Supplemental Table 3: Intracellular Ca^{2+} handling data

				P value	
	WT	**TS**	**Uninfected**	**WT:TS**	**WT:Un**
Number of Cells (n)	14	20	28		
Calcium Transient (F/F_0)	2.76 ±0.26	3.48 ±0.21	2.84 ±0.18	**0.042**	0.792
50% Decay Time (ms)	208.47 ±23.21	161.97 ±6.09	193.61 ±7.50	**0.047**	0.475
SR calcium content (F/F_0)	5.52 ±0.38	5.78 ±0.24	5.64 ±0.22	0.524	0.705
Spark Frequency (Sparks/ms/100μm)	2.28 ±0.58	4.52 ±0.50	2.50 ±0.51	**0.001**	0.354
Spark Intensity (F/F_0)	1.47 ±0.03	1.75 ±0.06	1.62 ±0.05	**0.002**	0.138
Spark Duration (FDHM)	2.27 ±0.06	2.22 ±0.07	2.28 ± 0.08	0.121	0.056
Spark Width (FWHM)	45.02 ±3.13	53.56 ±3.80	44.81 ±8.79	0.457	0.474

Supplemental Equations

Revised formulation for I_{rel}

$$I_{\mathrm{Re}l} = O \cdot \left([Ca^{2+}]_{Jsr} - [Ca^{2+}]_{SS}\right);$$

Where

$$\frac{dO}{dt} = -\frac{O_{\infty} + O}{\tau_{Irel}};$$

and

$$O_{\infty} = \frac{\alpha_{\mathrm{Re}l} \cdot I_{Ca(L)}}{1 + \left(K_{\mathrm{Re}l,\infty} / [Ca^{2+}]_{JSR}\right)^{h_{\mathrm{Re}l}}};$$

All other parameters and equations for I_{rel} are same as in original model published by Livshitz and Rudy.

Ca^{2+} release of JSR under Ca^{2+}-overload conditions.

If buffered [csqn] > $[csqn]_{th}$

$$O = O_{\infty} = 6.0;$$

$$\tau_{Irel} = 10.0;$$

Where [csqn]th = 7.0 as in original Luo-Rudy dynamic cell model.

Nonspecific Ca^{2+}-activated current

The nonspecific Ca^{2+}-activated current was used according to the formulation in the original Luo-Rudy cell model with a reduced permeability.

$$P_{Ns(Ca)} = 1.0 \times 10^{-7}\, cm/s;$$

Mathematical model of TS action potential.

L-type Ca^{2+} current

To simulate the effects of the Timothy Syndrome mutation on $I_{Ca(L)}$ channel gating, the following equation was used for steady-state voltage dependent inactivation:

$$f_{\infty} = \frac{1}{1+\exp\left(\frac{V_m + 28.86}{8.0}\right)} + \frac{0.6}{1+\exp\left(\frac{50.0 - V_m}{20.0}\right)};$$

To simulate the CaMKII-dependent effects on $I_{Ca(L)}$ facilitation, the following equation was used for the time constant of voltage-dependent inactivation:

$$\tau_f = \frac{1.35}{0.0197 \cdot \exp\left(-0.0337 \cdot \left(V_m + 10.0\right)^2\right) + 0.02};$$

Ca^{2+} leak from the SR

Increased leak from the SR due to CaMKII was simulated by increasing the conductance of I_{leak}.

$$I_{leak} = \frac{1.23 \cdot \overline{I_{up}}}{\overline{NSR}} \cdot \left[Ca^{2+}\right]_{NSR};$$

SR Ca^{2+} release

$\beta_{\tau} = 7.125$ ms;

$K_{Rel,\infty} = 0.65$ mM;

$[csqn]_{th}$ = 6.2 mM;

Supplemental Figure Legends

Supplemental Figure 1: (A) Immuno-blot for total $Ca_V1.2$ protein and from ventricular myocytes infected with $Ca_V1.2$ WT **(lane 1)**, $Ca_V1.2$ TS **(lane 3)** or uninfected **(lane 2)** as a control. The average increase in WT and TS $Ca_V1.2$ protein relative to uninfected was 31.9% (WT=36.4%, TS=27.4%) after correcting for total protein loading observed in the **(B)** Coomassie stained lanes.

Supplemental Figure 2: Adult ventricular myocytes **(A,B,C)** non-transduced, infected with **(D,E,F)** WT $Ca_V1.2$ virus or infected with **(G,H,I)** TS $Ca_V1.2$ virus (scale bar 10μm). TS and WT infected ventricular myocytes show overexpressed $Ca_V1.2$ **(D,G)** by HA immuno-staining, but no changes in total CaMKII protein **(E,H)** as compared to non-transduced ventricular myocytes **(B)**.

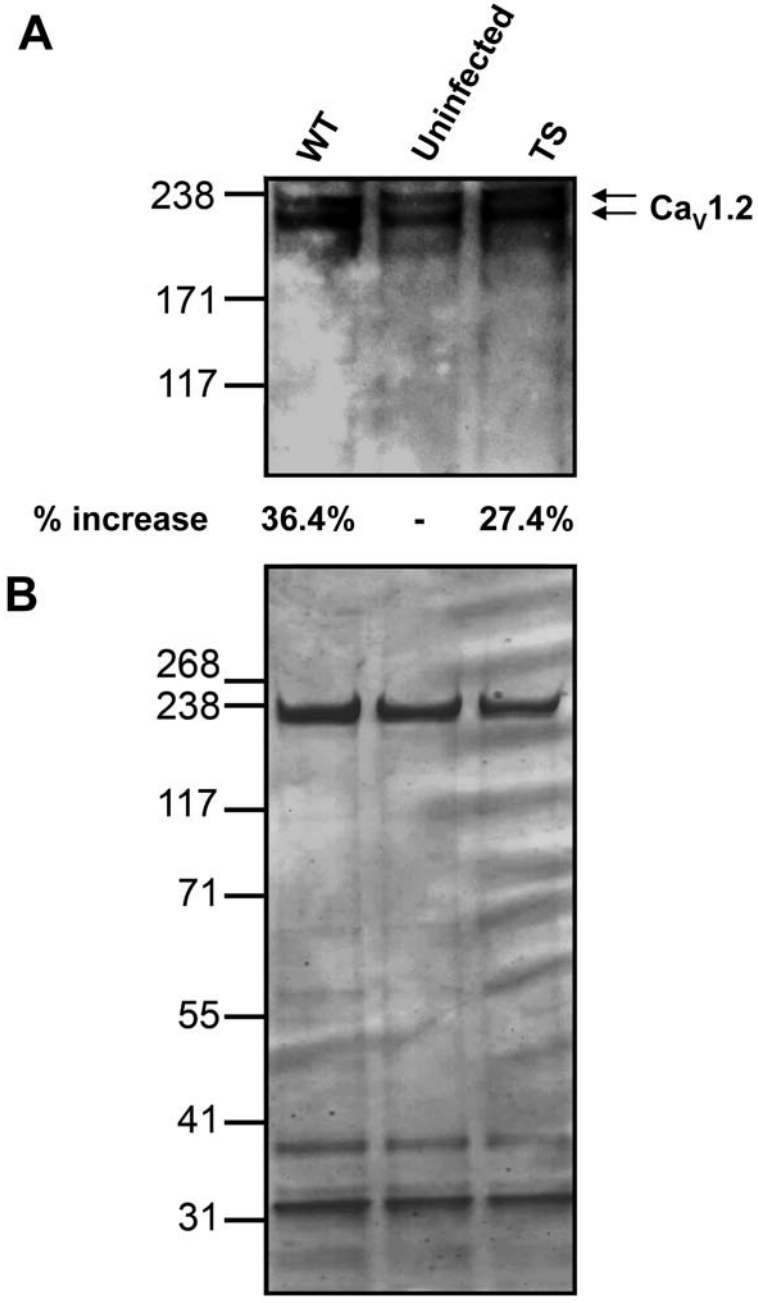
Supplemental Figure 3: TS ventricular myocyte I_{Ca} facilitation **(A)** TS ventricular myocytes show increased peak I_{Ca} during the first depolarizing voltage clamp command step (-80mV to 0mV, 300ms, 0.5Hz) over WT ventricular myocytes (N=6-7 cells/point, P=0.02). With the second depolarizing step WT ventricular myocytes match the peak I_{Ca} observed with TS ventricular myocytes (N=6-7 cells/point, P=0.46). The CaMKII inhibitory peptide, AC3-I, restores normal I_{Ca} facilitation to TS ventricular myocytes (N=5-6, P vs. WT=0.469), but not the control peptide AC3-C (N=5-6 cells/point, P vs. WT=0.038). **(B)** WT cardiomyocytes dialyzed with the CaMKII inhibitory peptide, AC3-I, loose the dynamic increase of integrated I_{Ca} and **(C)** the dynamic change of the fast time

constant (τ_{fast}) that are associated with facilitation (N=5 cells/point; integrated I_{Ca} WT AC3-C vs. WT AC3-I ANOVA P<0.001 ; τ_{fast} WT AC3-C vs. WT AC3-I ANOVA P<0.001). **(C and D)** The control peptide, AC3-C, has no effect on I_{Ca} facilitation (N=5 cells/point; integrated I_{Ca} WT AC3-C vs. WT ANOVA P=0.675; τ_{fast} WT AC3-C vs. WT ANOVA P=0.85).

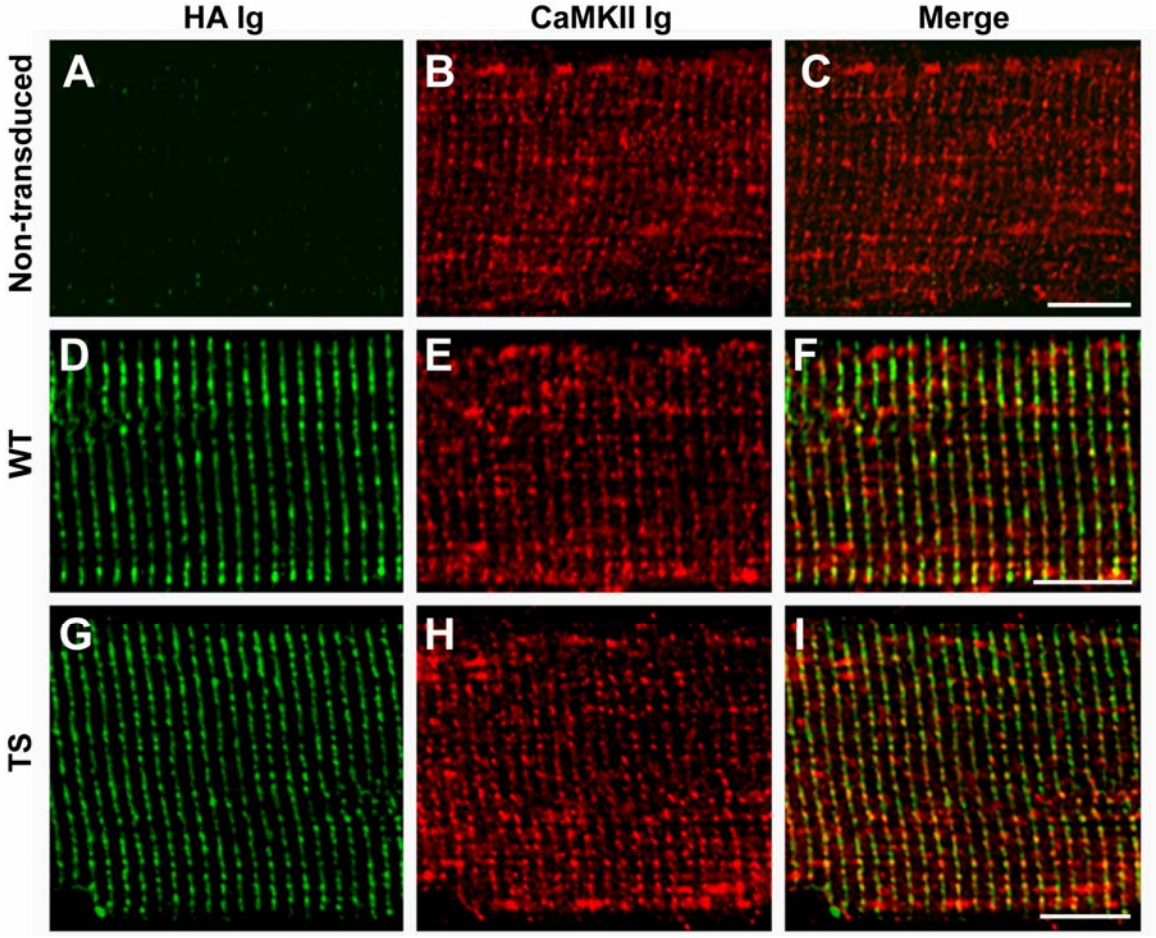
Supplemental Figure 4: Mathematical model of WT (black) and TS (red) myocytes. **(A)** Measured (*left*) and simulated (*right*) $I_{Ca(L)}$ steady-state voltage-dependent inactivation curves. **(B)** Simulated $I_{Ca(L)}$ current traces during a voltage pulse to 0 mV from a holding potential of -80 mV (*left*). Measured and simulated current integrals (*right*) are determined during the pulse duration (300 ms). In simulations and experiments, Ca^{2+} was buffered with 10 mM EGTA. **(C)** Measured and simulated Ca^{2+} transient amplitude (*left*) and SR Ca^{2+} content (*right*) after steady-state pacing.

Supplemental Figures

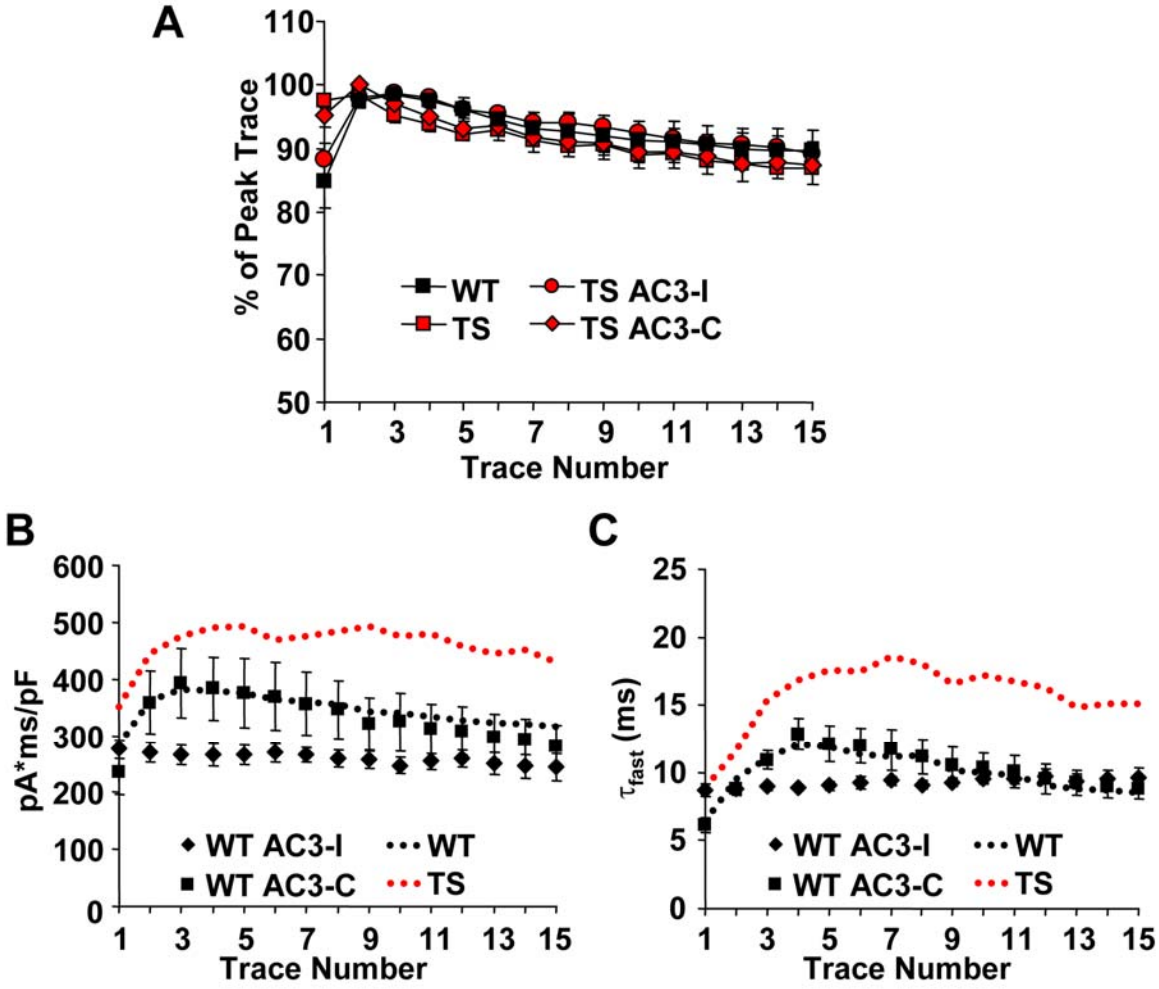
Supplemental Figure 1



Supplemental Figure 2



Supplemental Figure 3



Supplemental Figure 4