SUPPLEMENTARY MATERIALS

Prevention of Connexin43 remodeling protects against Duchenne muscular dystrophy cardiomyopathy.

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Supplemental Methods

Western Blotting

Snap frozen mouse and human ventricular tissues were homogenized in RIPA buffer (50mM Tris-HCl pH 7.4, 150mM NaCl, 1% Triton X-100, 0.5% Sodium Deoxycholate, 0.1% SDS, 1mM EDTA, 1mM PMSF) supplemented with protease and phosphatase inhibitor cocktails (Roche). Whole cell lysates (WCL) were centrifuged at 16,000 g for 20 minutes to remove debris. 15µg WCL samples were prepared in 4x Laemlli buffer with ß-MEtOH and heated for 5 minutes at 99ºC. Mouse WCL and triton solubility assay samples were separated on 8% SDS-PAGE gels (Bio-Rad) and human WCL samples were run on 10% SDS-PAGE gels. Gels were then transferred to 0.45µm nitrocellulose membranes and blocked for 1 hour at room temperature in 5% non-fat dry milk (Bio-Rad) in TBSt wash buffer (TBS + 0.1% Tween 20). Membranes were probed overnight at 4ºC with the following antibodies: Cx43 (Sigma C6219; 1:10000, rabbit), pS325/S328/S330-Cx43 (custom made by Dr. Paul Lampe, 1:1000, mouse), pS-365-Cx43 (custom made by Dr. Paul Lampe, 1:1000, rabbit), Vinculin (Sigma V9131; 1:2000, mouse), N-Cadherin (Invitrogen 33-3900; 1:2000, mouse), α-Tubulin (Abcam ab7291; 1:2000,

mouse), β-tubulin (Sigma T8328, 1:1000, mouse), gp91-phox (Santa Cruz sc-130543; 1:1000, mouse), CaMKIIδ (GeneTex GTX111401; 1:1000, rabbit) and oxidized-CaMKII (Met281/282, Millipore 07-1387; 1:1000, rabbit). Membranes were subsequently washed 3x in TBSt, probed for either goat anti-rabbit or mouse HRP-conjugated secondary antibodies (Bio-Rad) in blocking buffer (1:10000), washed 3x, and images were obtained using the ChemiDoc detection system (Bio-Rad). Densitometry analyses were performed using Fiji gel analysis (NIH).

Mass Spectrometry

Snap frozen ventricular tissues were homogenized in modified RIPA buffer (50mM Tris-HCL 7.4, 150mM NaCl, 1% Nonidet P40, 0.5% Sodium Deoxycholate, 0.1% SDS, 4M Urea, 5% Glycerol) supplemented with protease and phosphatase inhibitor cocktails. After centrifugation to remove debris, immunoprecipitation was performed on heart samples to enrich for Cx43 utilizing a Dynabeads Protein G Immunoprecipitation Kit (Invitrogen) with a rabbit Cx43 antibody (Sigma C6219). 50µl of Dynabeads were incubated with 5µg of antibody for 30 minutes at room temperature with rotation to form the Dynabeads-antibody complex. Supernatant was removed, and Dynabeads-antibody complexes were incubated with 1mL of each tissue homogenate for 1 hour at room temperature with rotation. Enriched samples were eluted from bead complexes utilizing an Elution buffer according to the manufacturer's protocol. Samples were combined with 4x Laemmli buffer and run on a 12% SDS-PAGE gel (Bio-Rad) at 120V to achieve adequate separation. Coomassie blue staining was performed on SDS-PAGE gels, and bands between 37kDa and 50kDa molecular weights were excised and used for mass spectrometry with the Rutgers New Jersey Medical School Center for Advanced Proteomics Research core facility. In-gel trypsin digestion was performed, and the resultant peptides were C18 desalted and directly analyzed by LC-MS/MS on Q Exactive instrument. The resulting

MS/MS spectra were searched against a Swiss-Prot mouse database using MASCOT (V2.3) search engines on Proteome Discoverer (V1.4) platform with the false discovery rate less than 1%. The spectra for phosphorylation site mapping were manually validated. The phosphopeptides quantitation was calculated based on MS1 peak area using Skyline software (V. 3.1). Since the peptides containing S325, T326, S328, or S330 have the same precursor mass, and the fragment ions are not enough to pinpoint the phosphorylation sites among those four, the percentage of phosphorylation was calculated as following: (Sum of the peak area of the phosphopeptides/Sum of the peak area of the same peptide with phosphorylation and non-phosphorylation) × 100%.

Triton Solubility Assay

Method adapted from (1) with modifications. Snap frozen ventricular tissues were homogenized in 1% Triton X-100 buffer (50mM Tris-HCL pH 7.4, 150mM NaCl, 2mM EDTA, 1mM PMSF, 1mM NaF, 1% Triton X-100) supplemented with protease and phosphatase inhibitor cocktails (Roche, cOmplete ULTRA Mini and PhosSTOP) at 100mg tissue/ml. Samples were incubated for 1 hour at 4ºC. Subsequently, samples were centrifuged (4ºC) at 500 g for 2 minutes to remove cellular debris. Next, 50-200µl of supernatant was added to an equal volume of 1% Triton X-100 buffer containing 8M urea (Sigma) the solubilize junctional proteins and create the total protein fraction. The remaining lysate was centrifuged at 16,000 g for 20 minutes in pre-weighed tubes. The supernatant was removed and added to an equal volume of 1% Triton X-100 buffer with 8M urea to generate the soluble fraction. The pellets were weighed and suspended in 1% Triton X-100 buffer containing 4M urea to a final concentration of 50 mg/ml. Insoluble pellets were either dissolved by a motorized pestle homogenizer (Argos Technologies) or by sonication. Protein concentration was determined by the Bio-Rad DC

Protein Assay. Prior to electrophoresis, 5µg of protein fractions were prepared in 4x Laemlli buffer with ß-MEtOH and heated for 30 minutes at 37ºC. After band quantifications, all Cx43 values were normalized to its corresponding loading control (Vinculin). Subsequently, insoluble fraction values were divided by total fraction values to quantify relative amount of insoluble Cx43.

Intracellular Ca^{2+}responses to hypo-osmotic shock

Intact cardiomyocytes were loaded with fluo-4AM (5µM, Invitrogen), de-esterified and imaged as described in (2). 140 images were acquired; 40 images were taken in isotonic Normal Tyrode (NT) solution that contained (in mM): 140 NaCl, 5.4 KCl, 1.1 $MgCl_2$, 1 NaH_2PO_4, 5 HEPES, 1.8mM $CaCl_2$ and 10 glucose. Osmolarity and pH were adjusted to 310 mOsm and 7.4 respectively. Then 40 images were recorded in hypo-osmotic solutions with contained 70 mM NaCl (instead of 140) and consequent reduced osmolarity of 170 mOsM and the remaining 60 images were recorded back in isotonic solution. Images were exported and processed for quantification in Fiji. Fluorescent data acquired during the 60 seconds following osmotic shock were then averaged (F_{ave}) and normalized to the average intensity during the first 40 seconds of exposure before osmotic shock (F_0) and quantified as F_{ave}/F_0.

Tissue Immunofluorescence

Mouse ventricular tissue were frozen in O.C.T (Tissue-Tek) using liquid nitrogen cooled isopentane. Cryosections were cut at 6µm, fixed in ice cold acetone (-20ºC) for 10 minutes and blocked for 1 hour at room temperature with 10% normal goat serum in PBSt wash buffer (PBS + 0.1% Tween20). Sections were then incubated with either Cx43 (Sigma C6219, 1:2000, rabbit) or pS325/S328/S330-Cx43 (see above, 1:1000) and N-Cadherin (Invitrogen 33-3900,

1:300, mouse) antibodies in blocking buffer overnight at 4ºC. Following 3 washes in PBSt, sections were incubated for an hour at room temperature with Alexa Fluor secondary antibodies (Invitrogen) in blocking buffer (1:250). Slides were subsequently washed in PBSt and coverslips were mounted using ProLong gold antifade reagent containing DAPI. Sections were imaged on a Nikon Eclipse T1 (40x objective) for standard immunofluorescence microscopy. Sections analyzed for colocalization were imaged on an Olympus Fluoview 1000 Confocal Laser Scanning Microscope. Confocal Z-stacks of 0.5µm thickness were acquired at 60x magnification using the Fluoview software. The images were then separated into their separate channels and processed as maximum intensity z-stack projections in Fiji prior to analysis.

Quantification of Cx43 Localization at the Intercalated Disc

Method adapted from (3) with modifications. All the following image modifications and analyses were performed using Fiji ImageJ software (NIH). To determine amount of detectable Cx43 at the intercalated discs, maximum intensity projections of 5µm confocal z-stacks were generated in both Cx43 (green) and N-Cadherin (red) channels. To isolate intercalated disc (ID) regions, background signal was subtracted from N-Cadherin images, converted to 8-bit pixel depth, and were equally thresholded to generate binary masks. In the masks, all positive signal (ID) had a value of 1 and all non-ID regions had a value of 0. These masks were then image multiplied by the corresponding 8-bit Cx43 image (background subtracted) to generate images with Cx43 signal only at IDs and mean fluorescent intensity was measured. Then, mean fluorescent intensity was measured in the original (background-subtracted, 8-bit) Cx43 image. The Cx43 co-localization index was quantified by dividing the mean intensity values of the ID-Cx43 image by the original Cx43 image. For each sample, 6 images with comparable numbers of intercalated discs were quantified and analyzed in a blinded fashion.

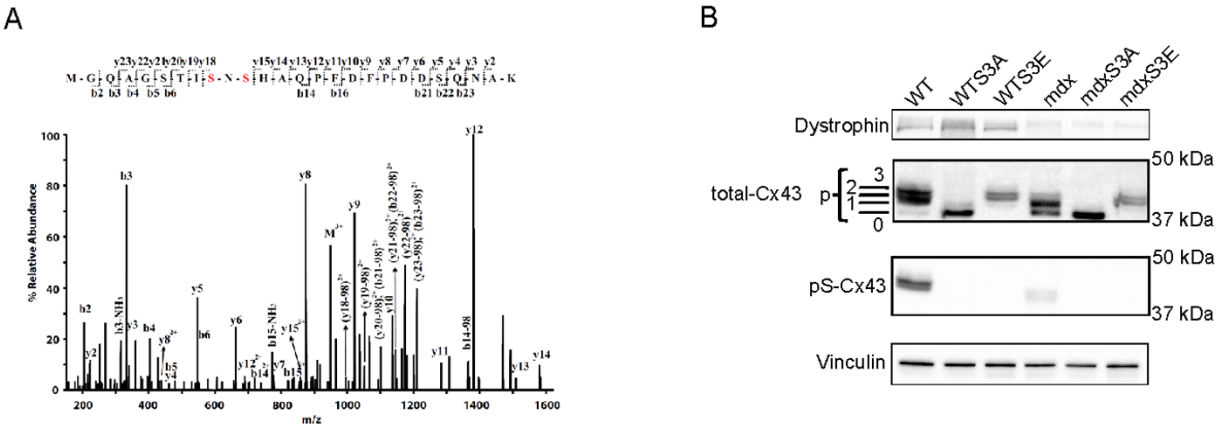
Fibrosis Staining and Quantification

Masson Trichrome staining was performed using a Masson trichrome staining kit (Sigma) following the manufacturer's established protocol on paraffin embedded, 6µm thick heart sections from aged 14-18 month-old heart sections. Images were taken using an Olympus BX51 microscope at a 4x magnification. The Masson trichrome color deconvolution plugin in Fiji was used to determine areas of fibrosis and muscle. Percentage of fibrosis was quantified as fibrotic area divided by whole muscle area. Four representative images per heart and diaphragm were analyzed in a blinded fashion.

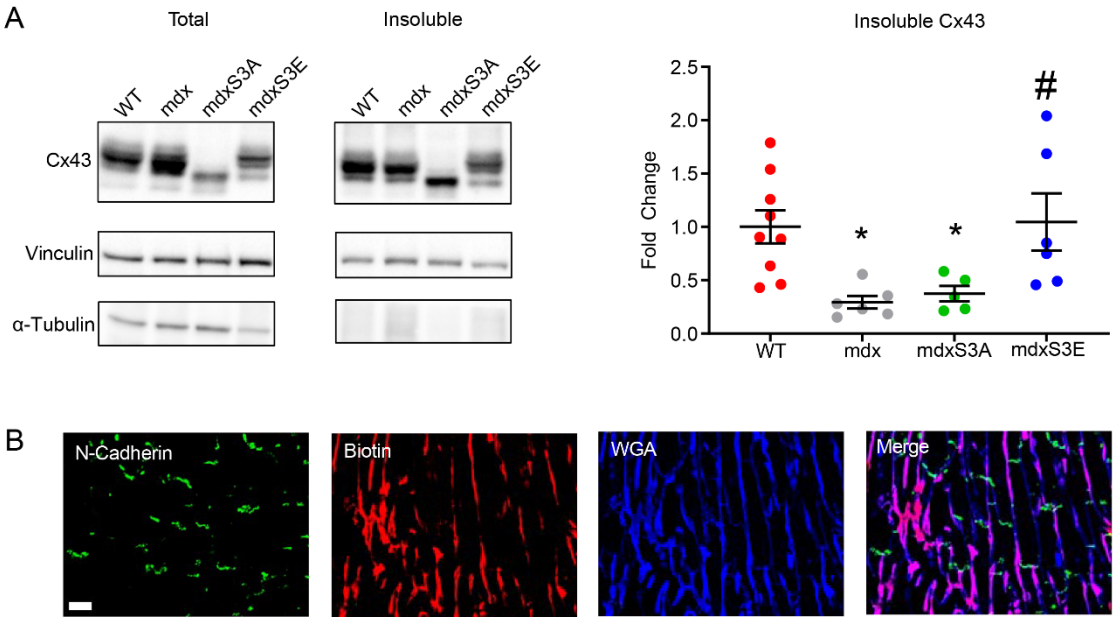
Immunofluorescence of Isolated Cardiomyocytes

Freshly isolated cardiomyocytes were plated on laminin-coated (10µg/mL) chamber slides and allowed to adhere for 1-2 hours at room temperature. Cells were fixed in 4% paraformaldehyde for 15 minutes at room temperature, washed 3 x 5 minutes in PBS, and permeabilized with 0.5% Triton X-100 in PBS for 20 minutes. Following 3 x 5 minute washes in PBS, cells were incubated in blocking buffer (2% NGS, 2% BSA, 0.3M glycine in PBS) for 1 hour at room temperature. Cells were then incubated with β-tubulin (Sigma T8328, 1:1000, mouse) in blocking buffer overnight at 4ºC. Following 3 washes in PBS, cells were incubated with Following 3 washes in PBS, sections were incubated for an hour at room temperature with Alexa Fluor secondary antibodies (Invitrogen) in blocking buffer (1:250). Slides were subsequently washed in PBS and coverslips were mounted using ProLong gold antifade reagent containing DAPI. Confocal images were collected as described above. Images were processed as average intensity z-stacks in Fiji for display.

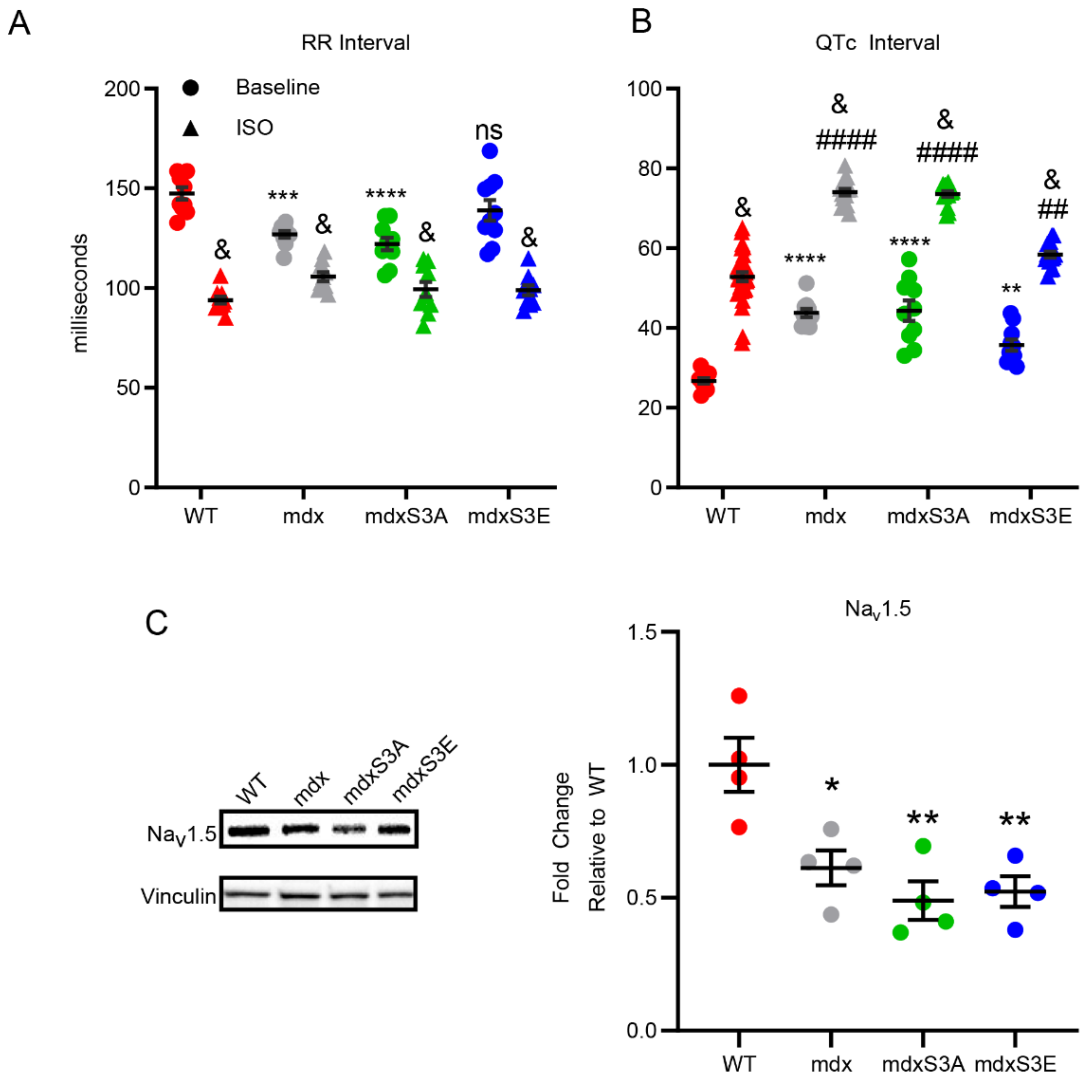
Supplemental Figures



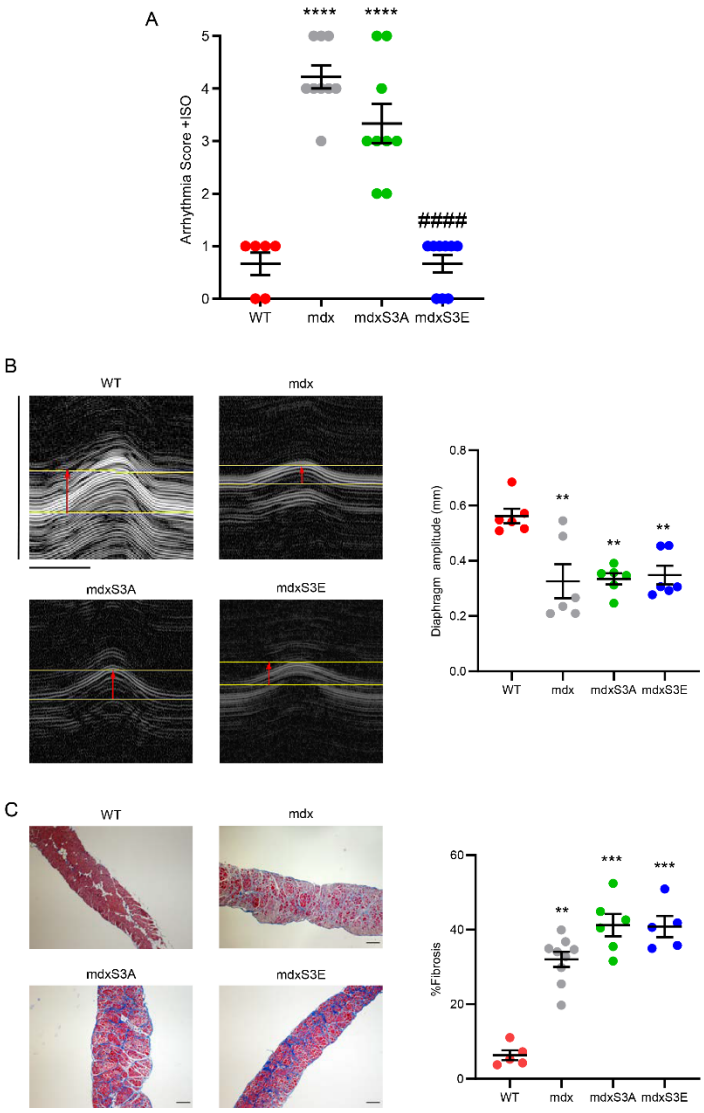
Supplementary Figure 1. Generation and validation of Cx43 phospho-mutant WT and mdx mice. (**A**) A representative MS/MS spectrum of a triply-charged ion (*m/z* 949.39) is matched to the peptide sequence of ^{320}MGQAGSTISNSHAQPFDFPDDSQNAK^{345} with one phosphorylation modification at either S^{328} or S^{330}. The observed *y*- and *b*-ion series confirmed the peptide sequence and pinpointed a phosphorylation site on S^{328} or S^{330} (highlighted in red). **(B)** Representative western blot analyses performed on WT, WTS3A, WTS3E, mdx, mdxS3A, mdxS3E ventricular whole cell lysates. Immunoblotting for dystrophin (top panel, negative control for mdx mice), vinculin (loading control, 2nd panel), pan-Cx43 (total Cx43, 3rd panel) and pS325/S328/S330 (pS-Cx43, bottom panel, positive control for WT/mdx, negative control for phospho-mutant mice). Note the differential Cx43 migration patterns indicated by the phospho-isoforms P3, P2, P1 and P0 in Cx43-mutant lysates.



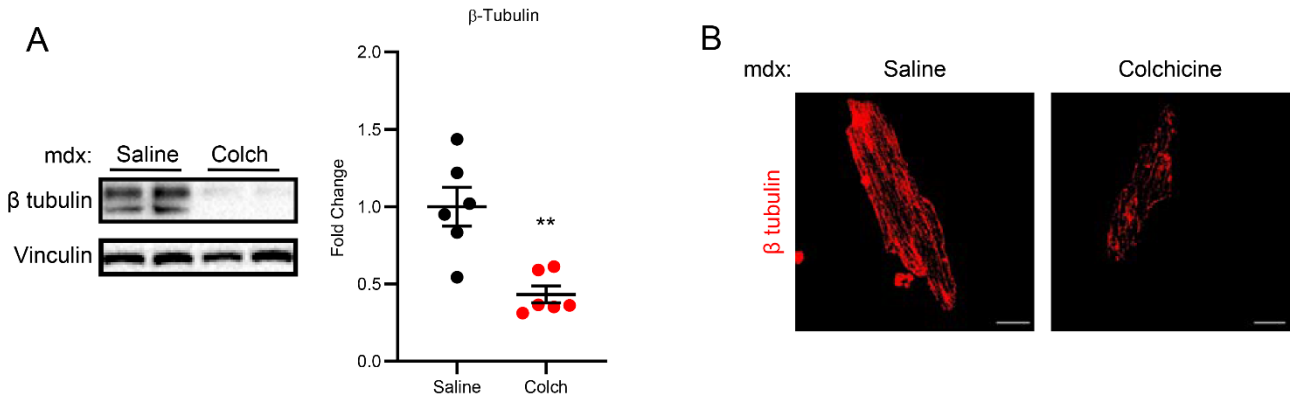
Supplementary Figure 2. MdxS3E mice display increased junctional and Cx43 protein levels**.** (**A**) Representative western blots (top) and quantification (right) of Cx43 (top panels) from 4-6 month old hearts subject to Triton X-100 based tissue fractioning of insoluble (junctional, right) and total cell (total, left) lysates. Vinculin (middle panels) was used as a loading control for both fractions. α –tubulin (bottom panels) was used as a negative control for insoluble fraction. Insoluble Cx43 protein levels were normalized to corresponding total Cx43 levels and then expressed as fold change relative to WT mean. * $p < 0.05$ versus WT; # $p < 0.05$ versus mdx. N =9 (WT), N = 6 (mdx), N = 5 (mdxS3A), N =6 (mdxS3E). Data are presented as means ± SEM. Statistical significance was determined by 1-way ANOVA followed by Tukey post-hoc test. (**B**) Representative immunofluorescence images of a Biotin-perfused mdx heart stained for N-Cadherin (green), Biotin (red), WGA (blue) along with merged image. Note that biotin is only positively stained along the lateral borders of cardiomyocytes and not at IDs. Scale bars, 20μm



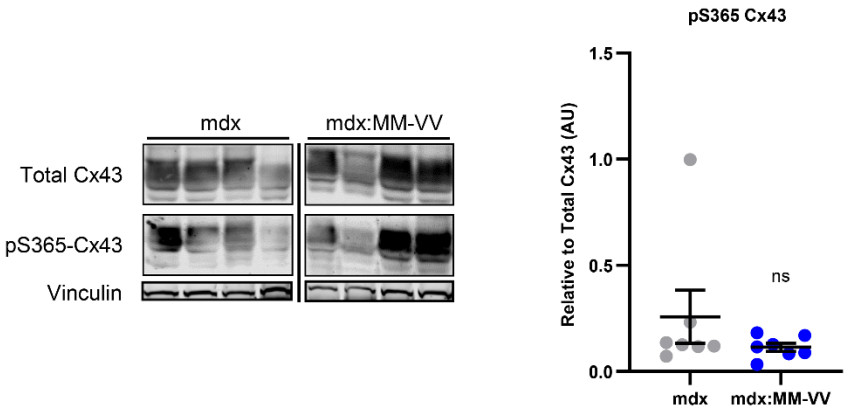
Supplementary Figure 3. MdxS3E mice exhibit improved ECG parameters with no upregulation of cardiac sodium channel levels. (**A**) Quantification of cardiac frequency (RR Interval) in anesthetized WT, mdx, mdxS3A and mdxS3E mice at baseline (circle) and following Iso challenge (triangle). No statistical differences were detected between groups after Iso treatment. (**B**) Quantification of corrected QT interval (QT_C) at baseline and following Iso challenge. **** p <0.0001, ** p <0.005, * p <0.05, ns = not significant versus WT at baseline; #### p <0.0001, ## p <0.005, versus WT +Iso; & p < 0.01 versus baseline per genotype. N = 10 (WT, mdx, mdxS3A, mdxS3E) for both (a-b). (**c**) Representative western blot (left) and quantification (right) of $Na_v1.5$ protein levels in ventricular lysates. Vinculin was used as a loading control. ** = p <0.01, * = p <0.05 versus WT. N=4 per each genotype. Data are presented as means ± SEM. Statistical significance was determined by 2-way ANOVA (**A**-**B**) or 1-way ANOVA (**C**) followed by Tukey post-hoc test.



Supplementary Figure 4. Aged mdxS3E mice are protected against Iso induced arrhythmias in vivo, but display dystrophic phenotype in diaphragm muscle**.** (**A**) Arrhythmia scores of 14-18-month-old WT, mdx, mdxS3A and mdxS3E mice following Iso (5mg/kg, IP) injection based on pre-determined scale as mentioned in Figure 3. N = 5 (WT), N = 7 (mdx), N = 7 (mdxS3A), N = 8 (mdxS3E). ****p<0.0001 versus WT; #### p<0.0001 versus mdx. (**B**) Representative diaphragm ultrasonography from 14-18-month-old WT, mdx, mdxS3A and mdxS3E. The baseline (bottom yellow line per image), contraction peak (top yellow line per image) and amplitude (red arrow) for each trace are shown. Right graph illustrates pooled mean amplitude data per genotype (in mm). Vertical scale bar, 2.00mm; horizontal scale bar, 100ms. N = 6 for all genotypes. **(C)** Representative 14-18-month-old WT, mdx, mdxS3A and mdxS3E diaphragms stained with Masson's trichrome (left) and quantification of fibrosis (right). N = 5 (WT), N = 9 (mdx), N = 6 (mdxS3A), N = 5 (mdxS3E). **** p<0.0001, *** p <0.005, ** p <0.01 versus WT; #### p<0.0001 versus mdx. Data are presented as means ± SEM. Statistical significance determined by 1-way ANOVA followed by Tukey's post hoc test (a-c).



Supplementary Figure 5. Confirmation of colchicine effect in vivo and in vitro. **(A)** Representative western blot (left) and quantification (right) for β tubulin and Vinculin (loading control) in lysates from either Saline or Colchicine treated mdx hearts. N=6 (WT, mdx). ** $p < 0.01$ versus mdx Saline. Data are presented as means ± SEM. Statistical significance determined by 2-sided t-test. **(B)** Representative confocal images of β tubulin (red) in isolated cardiomyocytes derived from mdx mice treated with either Saline or Colchicine. Scale bar, 20μm.



Supplementary Figure 6. Cx43-S365 phosphorylation unchanged in mdx:MM-VV hearts. Representative western blot (left panel) and quantification (right panel) of total Cx43 (top blot), pS365-Cx43 (middle blot) and Vinculin (bottom blot, loading control) in mdx (gray) and mdx:MM-VV (blue) whole cell lysates. N = 7 for both genotypes. Data are presented as means ± SEM. Statistical significance determined by 2-sided t-test. mdx and mdx:MM-VV samples were run on the same gel but were noncontiguous, as indicated with black line between samples.

Supplemental References:

1. Smyth JW, Hong T-T, Gao D, Vogan JM, Jensen BC, Fong TS, et al. Limited forward trafficking of connexin 43 reduces cell-cell coupling in stressed human and mouse myocardium. *The Journal of Clinical Investigation.* 2010;120(1):266-79.
2. Gonzalez JP, Ramachandran J, Himelman E, Badr MA, Kang C, Nouet J, et al. Normalization of connexin 43 protein levels prevents cellular and functional signs of dystrophic cardiomyopathy in mice. *Neuromuscular Disorders.* 2018;28(4):361-72.
3. Basheer WA, Xiao S, Epifantseva I, Fu Y, Kleber AG, Hong T, et al. GJA1-20k Arranges Actin to Guide Cx43 Delivery to Cardiac Intercalated Discs. *Circulation research.* 2017;121(9):1069-80.