SUPPLEMENTAL MATERIAL

Materials and Methods

Recombinant Adenoviral Expression Constructs: Viral constructs which express lacZ, β1A integrin and β1D integrin were as reported previously (1, 2). Adenovirus expressing full-length rat α7BX2 integrin under control of the CMV promoter was produced in the AdEasy system (Agilent). Adenovirus producing α5 integrin was kindly supplied by Drs. Clayton Buck (The Wistar Institute) and Lou Terracio (NYU School of Dentistry) (3).

Immunoblot analysis: Proteins extracted from isolated cardiomyocytes were subjected to Western blotting as described previously (4). Primary antibodies used are as listed below. Protein expression levels were evaluated with Image Lab software (MCM Design) based on the signal intensity of each protein using GAPDH as an internal control for protein loading. The amount of β1A integrin that co-immunoprecipitated with RyR2 was determined from immunoblots probed with an anti-β1D integrin antibody. Levels of RyR2-bound proteins were normalized to the total RyR2 immunoprecipitated.

Immunofluorescent Microscopy: Cells were fixed with ice-cold acetone followed by blocking and permeabilization with 2% BSA and 1% Triton X-100 in PBS, and then incubated with antibodies. Primary antibodies used and their dilutions are: β1D integrin (2) (1:1000), α5 integrin (5) (kindly supplied

by Dr. M. Valencik, U. Nevada) (1:1000), α7 integrin (6) (1:1000), JP-2 (1:100;Santa-Cruz; sc-51313), RyR2 (1:100; Sigma-Aldrich; 2554), WGA Alexa Fluor 488 conjugate (1:100; Invitrogen; W11261). The target proteins were visualized with the secondary antibodies (Alexa Fluor 488 and 568, 1:500; Invitrogen) and Hoechst nuclear stain. Fluorescence images were obtained using a Zeiss AxioObserver microscope and analyzed with Axiovision software. Colocalization was evaluated by Pearson correlation statistical analysis with Volocity 3D Image Analysis Software (Perkin Elmer).

Antibodies used for immunoblotting: β1D integrin (2), α5 integrin (Chemicon; AB1928), α7 integrin (B2 347 B15) (6), PyK2 (Upstate; 06-559), phospho-PyK2 (Biosource; 44-618G), FAK(Biosource; 44-624G), phospho-FAK(Biosource; 44-652G), ILK(Upstate; 05-575), Akt (Cell Signaling; 9272), phospho-Akt (Cell Signaling; 4058S), p38 (Cell Signaling; 9212), phospho-p38 (Cell Signaling; 9251S), Src (Cell Signaling; 2108), phospho-Src (Cell Signaling; 2105S), GAPDH (Santa-Cruz; sc-32233), iNOS (BD Transduction Labs; 610431), 12-LO (Cayman Chemical; 160304), EC-SOD (Upstate; 07-704), Cu/Zn-SOD (Stressgen; SOD-100), Mn-SOD (Stressgen; SOD-110), Cox2 (Santa-Cruz; sc-1747), NCX-1 (Abcam; ab6495-100), SERCA2a (Affinity Bio-Reagents; MA3-910), PLB (Affinity Bioreagents; MA3-922), phospho-PLB (Badrilla; A010-12), RyR2 (Sigma-Aldrich; 2554), phospho-RyR2 2808 (Badrilla; A010-30), phospho-RyR2 2814 (Badrilla; A010-31), LTCC (Abcam; ab2864-100).

Immunoelectron Microscopy: Samples were fixed in 4% paraformaldehyde in 1x PBS buffer (pH 7.4) for 20 minutes at room temperature. After placing them in blocking buffer (1x PBS plus 1% BSA, 3% normal goat serum, 0.1% - 0.2% Triton and 1% fish gelatin and 20 mM glycine) for 30min on ice, they were incubated in primary antibodies (β1D integrin (2); 1:1000 and RyR2; 1:1000 Sigma-Aldrich) in working buffer (10-fold dilution of blocking buffer in 1x PBS) overnight at 4ºC. Then, 6 and 10 nm gold-conjugated secondary antibodies (Electron Microscopy Sciences) diluted in working buffer, were applied to these samples for 4 hours at 4ºC, followed by post-fixing in 1% OsO4 (osmium tetroxide) + 0.8% potassium ferrocyanide in 0.1 M cacodylate buffer for 60min on ice. After polymerization at 60°C for 48h, sections (70 to 80 nm) were stained with lead citrate, and electron micrographs were recorded by using an electron microscope (1200EX; JEOL) operated at 80 kV.

Cell transfection: Full length human β1D integrin cDNA was cloned into pcDNA3.1. Then, the RyR2 plasmids were used to transfect human embryonic kidney (HEK) 293 cells along with β1D integrin plasmids, using Lipofectamine 2000 (Invitrogen). Following a 48h incubation, protein was isolated from these cells.

Generation of stable cell lines: Cells (5×10^5/well) were seeded on 24-well plates without antibiotics and cultured overnight. The next day, 0.8 μg of pcDNA3.1 and pcDNA-human β1D integrin (1-2412 bp)

plasmid were transfected into HEK293 cells using 2 μl of Lipofectamine 2000 (Invitrogen) in OptiMEM Reduced Serum Medium following the manufacturer's instructions. Transfected cells were selected with G418 at 800 μg/ml for 4 weeks, and the expression of β1D integrin protein in selected cell clones was determined by Western blotting.

Superoxide Anion Quantitation: Superoxide anions indicative of reactive oxygen species (ROS) were quantified using the dihydroethidium (HE, Sigma-Aldrich), which is freely permeable to cells and, once oxidized to ethidium, remains trapped inside cells by DNA intercalation. After hypoxia, ARVMs were washed with PBS and incubated in PBS containing 10 uM HE at 37°C for 45 minutes in the dark during reperfusion. Excess HE was removed by washing with PBS, and the oxidized product was detected using fluorescence (510-560 nm) microscopy. For fluorescence quantification, analyses of digital images were performed using ImageJ software. Single cell fluorescence was determined after correction for background intensity using cell-free fields of the same image.

Peptide arrays study: Peptide arrays were synthesized on nitrocellulose membranes using SPOT technology as previously described (7-9). Peptides 15 residues in length with a 2 residue skip were synthesized on the membrane, based on the region of RyR2 from 89 AA to 223 AA. The synthesis scale was roughly 30 nmol. Peptide arrays were immersed in ethanol for 1 min and pre-incubated with T-PBS

blocking buffer (PBS pH 7.4, 0.2% Tween 20, 3% BSA) for 1 h. After washing 3 times for 10min with PBS-T, the array was incubated in mouse heart protein lysate solution (1% Triton-X100, 150mM NaCl, 50mM HEPES pH 7.4, 1mM EDTA, 2mM DTT, Protease inhibitor cocktail - Roche) at a concentration of 0.8 mg/mL overnight at a temperature of 4°C. After that, the array was washed 3x each for 10 min with PBS-T and then a β1D antibody (2) (1:2000) was incubated on the array in blocking buffer for 1h at room temperature. The array was washed again 3 x 10 min with PBS-T at room temperature, incubated for 1h with the secondary antibody (1:10,000), followed by 3 additional 10 min washes in PBS-T buffer at room temperature. Detection was then performed using Lumigen TMA-6 (Lumigen Inc.).

FRET measurements: The cDNA construct $RyR2_{S437\text{-}YFP/S2367\text{-}CFP}$ is an intramolecular FRET sensor (YFP/CFP) that allows detection of interaction between the N-terminal and central regions of the RyR2 protein. It allows for study of stability of this protein and has been validated previously (10). HEK293 cells grown in 35-mm glass bottom culture dishes were maintained in Dulbecco's modified Eagle's for 24 hours. After subculture they were transfected with the RyR2S437-YFP/S2367-CFP plasmid alone, or this plasmid co-transfected with expression plasmids for α7, β1A or β1D integrin (molar ratio1:1), using Lipofectamine 2000 (Invitrogen). Transfected cells were continued in culture for an additional 24-48 hours, washed three times and examined on a Nikon TE300 inverted microscope equipped with a Dual-view image splitter (BioImaging Solutions Inc.) using a 63X/1.4 NA oil-immersion objective lens. During

examination, cells were kept at 37°C using a water-heated stage incubator. Simultaneous YFP/CFP images were captured under CFP excitation using a Dual-view image splitting filter system equipped with CFP and YFP excitation filters. MATLAB was then used to obtain average pixel intensity of defined regions of interest drawn by blinded operator. Average YFP/CFP intensity ratios were then calculated for each ROI.

SUPPLEMENTAL FIGURES

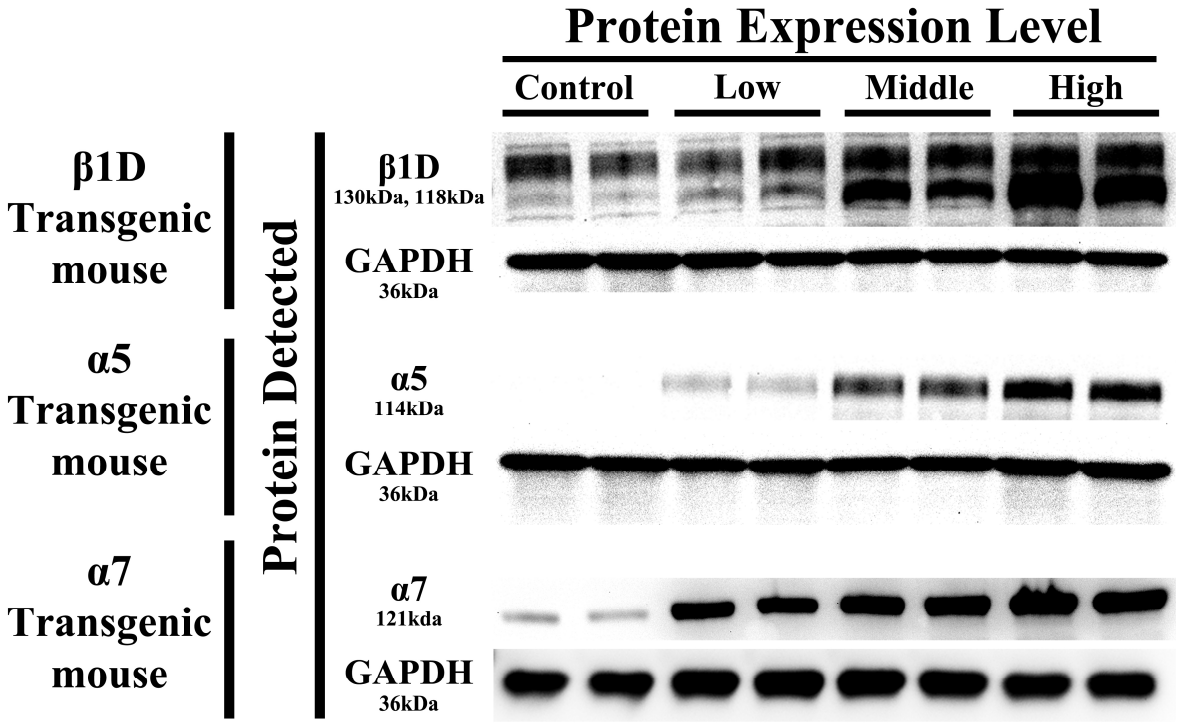

Figure S1: Representative western blot analyses of integrin expression levels in the cardiac myocyte-specific α5, α7 and β1D integrin transgenic mice. Expression in several lines is shown with varied levels of protein expression indicated as "high, middle and low." respectively.

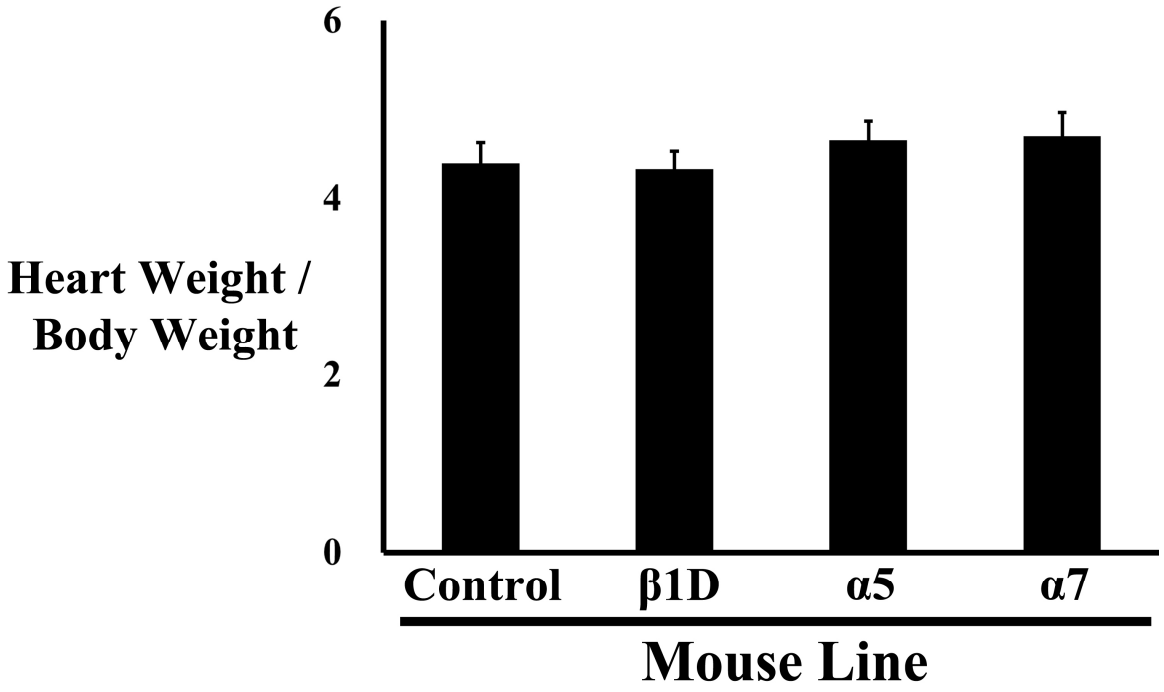

Figure S2: Morphological Analyses of Cardiomyocyte-specific, single subunit integrin transgenic mouse hearts. There was no significant difference in morphometry between controls and any of the single integrin subunit transgenic mice. Also, there were no significant histological differences noted between the specimens obtained from the various groups (Data not shown).

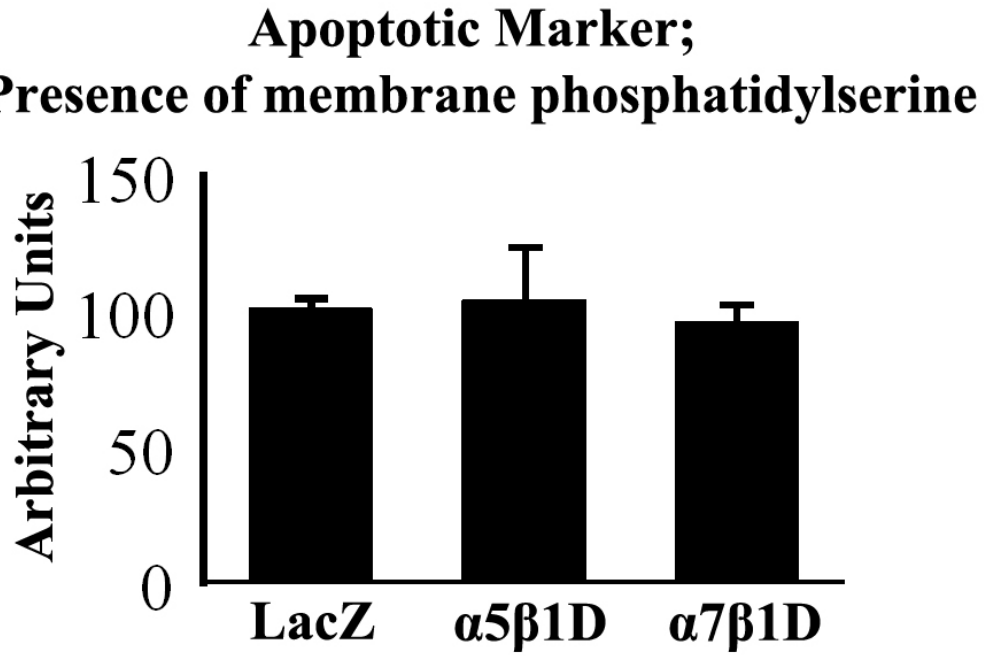

FIGURE S3 – Assessment of apoptosis in cardiomyocytes shows no difference between groups. A membrane phosphatidylserine dye-uptake bioassay was used to assess apoptosis in ARVMs infected with control (LacZ), α5β1D integrin and α7β1D integrin recombinant adenoviruses, and then subjected to hypoxia / reoxygenation. There were no significant differences between groups.

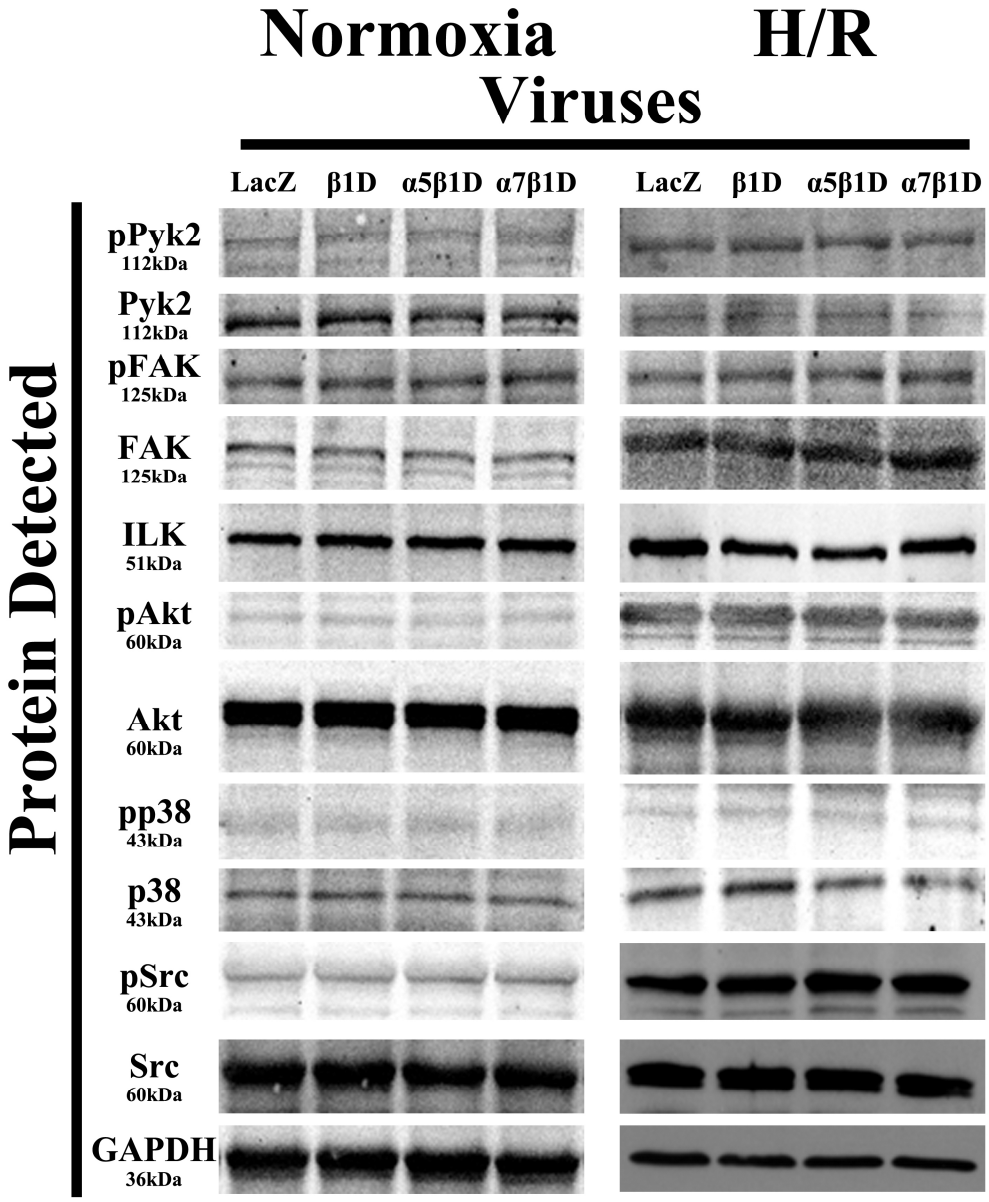

FIGURE S4 - Representative western blot analyses of integrin-related signaling molecules in control cells and ones with integrin overexpression, during normoxia and following H/R. (n = 8 each group.) ILK and all 'total' forms of the various other proteins were first normalized to GAPDH as an internal loading control. Then, phosphorylated forms of Pyk2, FAK, Akt, p38 and Src were normalized to the total protein / GAPDH of each respective phospho-protein. There were no significant differences between groups when analyzed by densitometry (Densitometric data not shown).

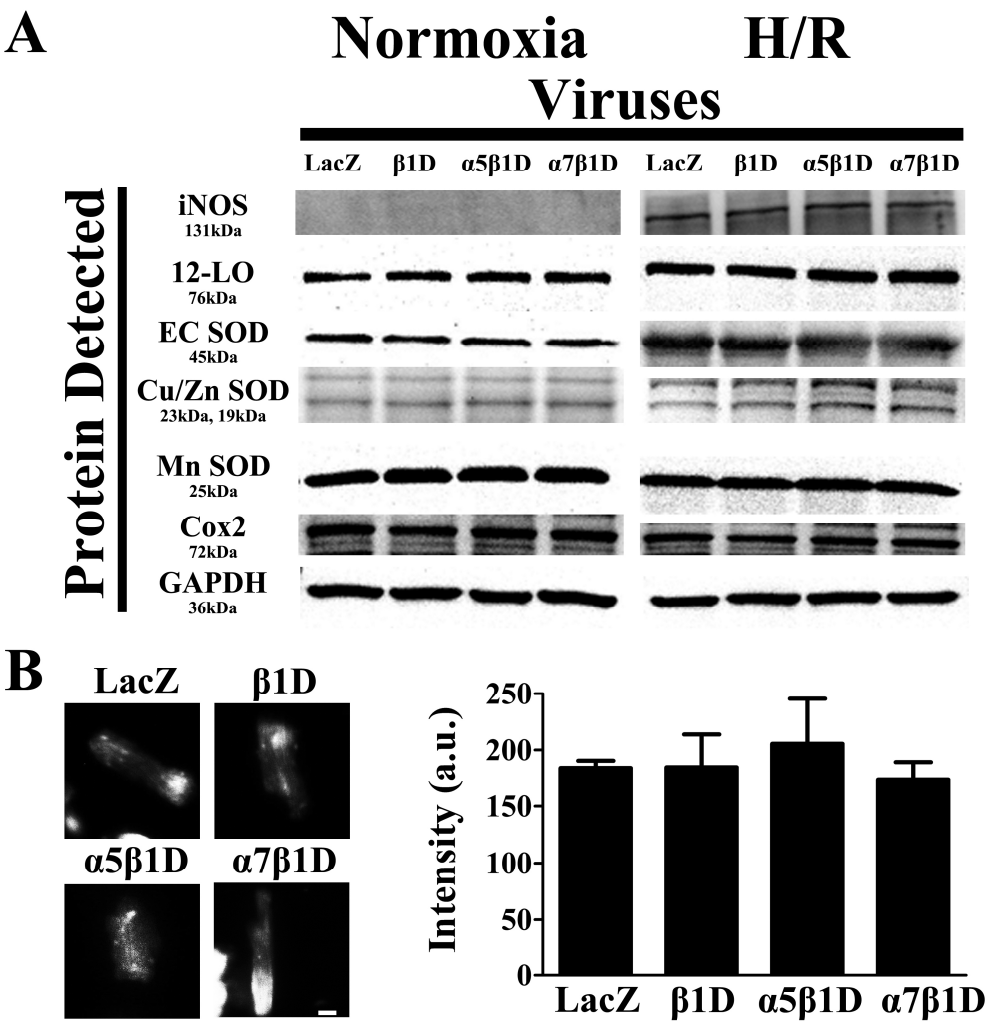

FIGURE S5 – Analysis of oxidative stress-related proteins and superoxide anions in cardiomyocytes subjected to H/R.

(A) Representative western blot analyses for oxidative stress related proteins in the basal state (normoxic) and also following H/R. (n = 8 each group.) There were no significant differences between iNos, 12-LO, EC SOD, Cu/Zn SOD, Mn SOD and Cox2, between groups (Densitometric data not shown).

(B) Left - Representative fluorescent photomicrographs of virally infected ARVMs probed for superoxide anions with dihydroethidium (HE) following H/R. Bar. 20um. Right - Densitometric quantification of superoxide anions in virally infected cells. There were no significant differences between groups.

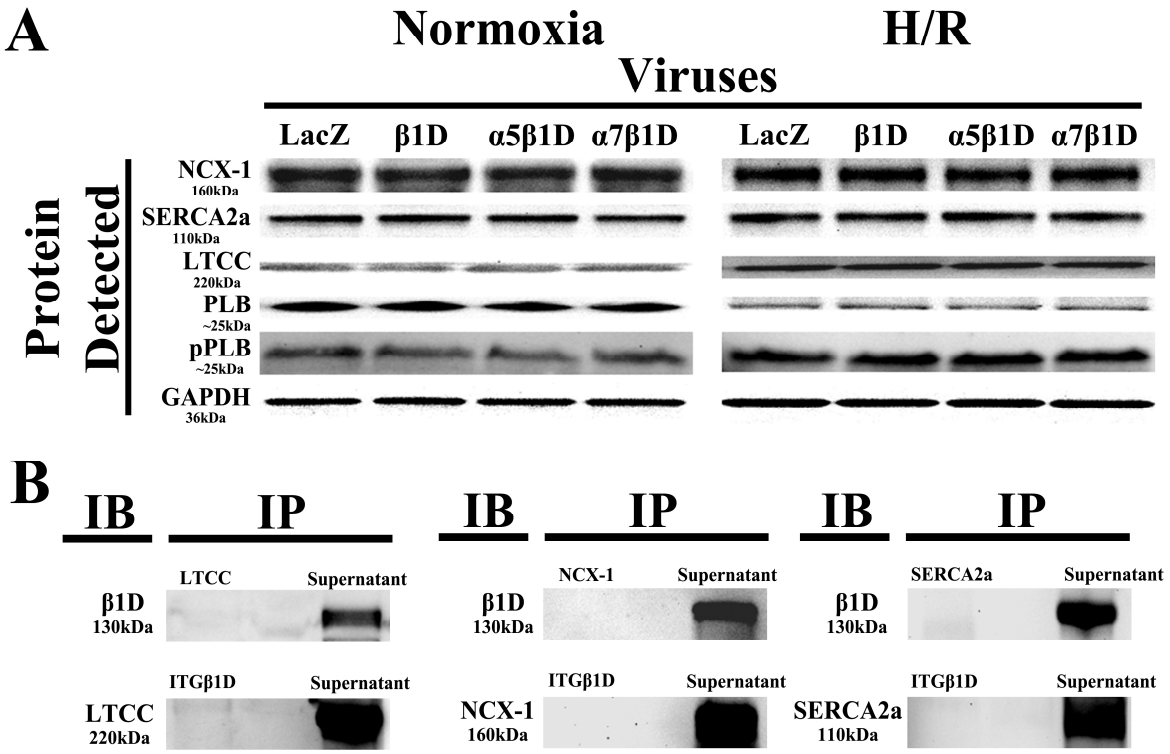

Figure S6 – Integrin overexpression does not modulate expression or interaction with several key myocyte Ca^{2+} handling proteins, nor cause changes in phospholamban phosphorylation.

(A) Western blot analyses shows that overexpression of integrins in cardiomyocytes during normoxia or H/R does not change expression of key Ca^{2+} regulatory proteins (NCX1, SERCA2A, LTCC, PLB) nor modulate PLB phosphorylation. (n = 8 each group.) There were no significant differences between groups.

(B) Integrin overexpression did not alter interactions between β1D integrin and key Ca^{2+} handling proteins in ARVMs. Protein extracts were immunoprecipitated using anti-β1D integrin and anti-LTCC antibodies (left panels), anti-β1D integrin and anti-NCX-1 (middle panel) or anti-β1D integrin and anti-SERCA2a antibodies (right panel). IgG was used as a negative control, Evaluation of protein expression in supernatant of IP buffer served as positive controls. β1D integrin did not immunoprecipitate with any of these proteins.

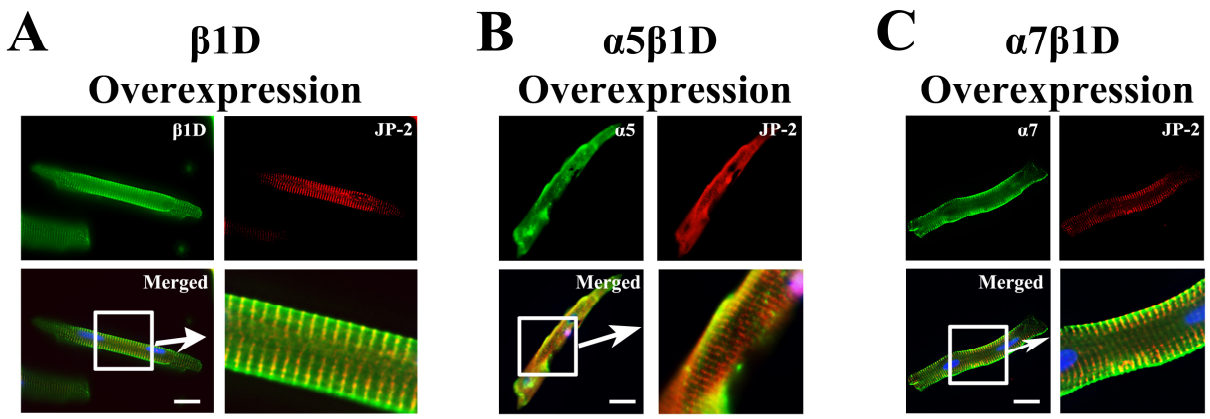

Figure S7 – T-tubule expression of integrins in cardiomyocytes.

Immunofluorescent microscopy shows that with overexpression of integrin β1D (Panel A), α5 (Panel B) or α7 (Panel C) all co-localize in T-tubules in cardiomyocytes. Integrin localization (Green) can be compared with the T-tubule marker, Junctophilin-2 (Red). Nuclei were stained with DAPI (blue). Bars = 20um

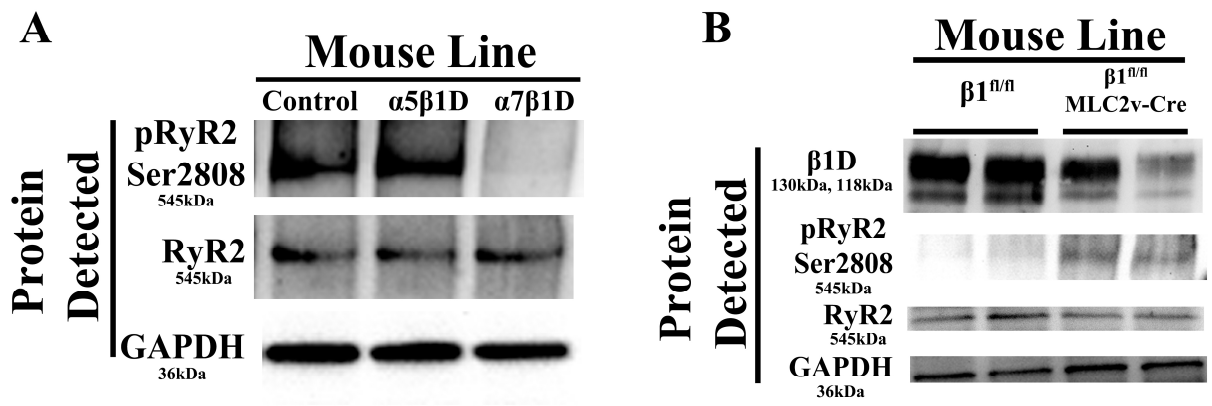

Figure S8: RyR phosphorylation *in vivo.*

Representative Western blot analyses of RyR2 Ser 2808 phosphorylation level in:

(A) Cardiac myocyte-specific α5, α7 or β1D integrin Tg mice and littermate controls,

(B) Cardiomyocyte-specific β1 integrin KO mice and littermate controls. RyR2 Ser-2808 phosphorylation was decreased in α7β1D Tg mice compared with control wild-type or α5β1D Tg mice, while RyR2 Ser-2808 phosphorylation was increased in β1 integrin deficient mice.

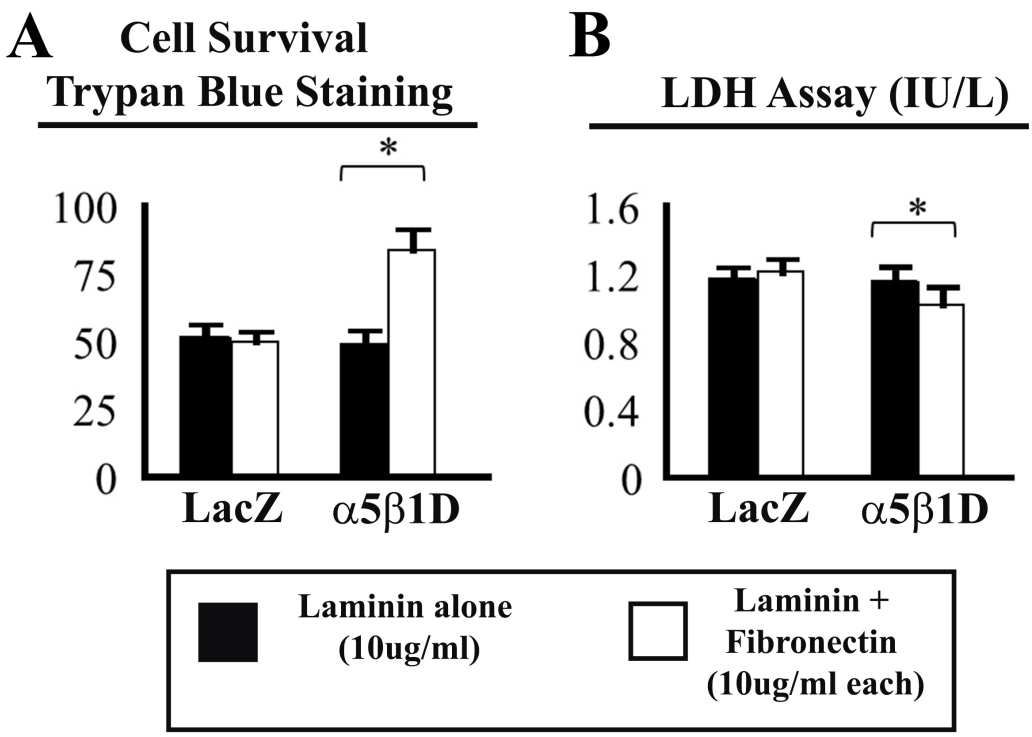

Figure S9 - Integrin α5β1D improved cell survival of cardiomyocytes when bound to ECM ligand.

Overexpression of integrin α5β1D improved cell survival of cardiomyocytes only when cells were plated on an extracellular matrix ligand (fibronectin) that could bind this integrin receptor. Thus, no improvement in survival was noted when the cells were plated only on laminin, but only when α5β1D cells were attached to a mixture of laminin and fibronectin was survival improved.

(A) Survival rate of cells assayed by Trypan-blue staining after H/R injury.

(B) Cell survival evaluated by LDH release after H/R. *P<0.05 vs. α5β1D on dish coated only with laminin (One-way ANOVA).

Fragment	Primer Sequence
1	F 5' CGAATTCGCCACCATGGCTGATGCGGGCGAAG 3'
	R 5' CGTCGACCTACAGGATGGATTTCCAAGACTCTC 3'
2	F 5' CGGATCCGTGCCACCATGTGCATCGACCGCCTGCATG 3'
	R 5' CGTCGACCTATTCTTGTAAGCCACAGATGG 3'
3	F 5' CGAATTCGCCACCATGGAAAATGCTCACAATGTGTGG 3'
	R 5' CGTCGACCTATTCGTTGTTCATCATGAGCCTGG 3'
4	F 5' CGAATTCGCCACCATGTTCATCGTCCCTATGACAGAGG 3'
	R 5' CGTCGACCTATGAAGCTGCGGCCACGTCT 3'
5	F 5' CGAATTCGCCACCATGGGTTCAACTCCTTTAGACGTGG 3'
	R 5' CGTCGACCTAAAAGTTCCCTTCAGAATCCATGG 3'
6	F 5' CGAATTCGCCACCATGCATGCAAAGATGCCTCTGAAG 3'
	R 5' CGTCGACCTACAAATCCTCTGCAGCATTGTC 3'
7	F 5' CGGATCCGCCACCATGGAGATGGTGACAAGCCTGTTC 3'
	R 5' CGTCGACCTAAATAAGTTGATGGAGGGGATCAAC 3'

SUPPLEMENTAL TABLE 1: Primers used for construction of RyR2 fragments

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