Supplemental Material

Expanded Materials and Methods

Isolation of primary neonatal ventricular cardiomyocytes

Primary mouse neonatal cardiomyocytes were isolated from p1 C57BL/6 mice. Briefly, pups were decapitated and hearts excised and washed in ice-cold Hanks Balanced salts solution without calcium and magnesium (HBSS; Invitrogen). Atria were removed and remaining ventricular tissue was quartered, placed in 5 ml warm HBSS containing 1 mg/ml collagenase II (Worthington Biochemical), and stirred for 10 min at 37 °C. Solution was gently titurated twenty times using a disposable transfer pipette and allowed to settle for 2 min. Supernatant was then carefully removed and added to 5 ml FBS on ice, leaving tissue behind. An additional 5 ml warm collagenase/HBSS was added to remaining tissue and this process repeated a maximum of three times or until most of the tissue was digested. The FBS/cell solution was then centrifuged for 10 min at 300 x *g* and supernatant discarded. The cell pellet was gently resuspended in 10 ml F12/DMEM 50/50 (Invitrogen) supplemented with 5 % FBS, insulin-transferrin-sodium selenite media supplement, 10 μM BrdU, 20 μM cytosine β-d-arabinofuranoside (Sigma), and Mycozap-PR (Lonza). Cells were strained through a 70 μm nylon cell strained (BD Falcon) and pre-plated for 30 min at 37 °C in 100 mm Primera dishes (BD Falcon) to remove fibroblasts and enrich cardiomyocyte population. Any adherent cells were discarded with dishes and the cell suspension plated onto dishes which had been previously coated with 1% gelatin and 0.01 mg/ml Fibronectin (Sigma). After overnight incubation at 37 °C medium was replaced with F12/DMEM 50/50 supplemented with 2 % FBS, insulin–transferrin–sodium selenite media supplement, 10 μM BrdU, 20 μM cytosine β-d-arabinofuranoside, and Mycozap-PR.

Isolation of adult mouse ventricular cardiomyocytes

Adult mouse ventricular cardiomyocytes were isolated from 6 to 8 week old C57BL/6 mice as previously described[1]. Briefly, mice were anesthetized with 3% isoflurane and 100% O2 (0.5 L/min) in an anesthesia chamber and injected with 0.5 ml heparin (100 IU/ml, i.p.). Cervical dislocation was performed, and hearts removed rapidly, immersed in ice-cold calcium-free perfusion buffer containing (in mmol/L) NaCl 120.4, KCl 14.7, KH2PO4 0.6, Na2HPO4 0.6, 5 MgSO4-7H2O 1.2, Na-HEPES 10, NaHCO3 4.6, taurine 30, butanedione monoxime (BDM) 10, glucose 5.5. Extraneous tissue was then removed, and the aorta cannulated under a dissection scope. Hearts were retrograde perfused through the aorta using a Langendorff perfusion apparatus with calcium-free perfusion buffer (3ml/min) for 4 min, then switched to calcium-free digestion buffer (perfusion buffer containing collagenase II [2mg/ml] from Worthington Biochemical) for 10 min. This was followed by perfusion with digestion buffer containing 100 μmol/L CaCl2 for another 8-10 min. Hearts were removed from the perfusion apparatus and placed in a 10 cm Petri dish containing 2 ml digestion buffer and 3 ml of stop buffer (perfusion buffer supplemented with 10% FBS). The atria were removed and the ventricles were pulled into 10-12 equally sized pieces. Tissue was then gently dispersed into cell suspension using plastic transfer pipettes. The cell suspension was collected in a 15 ml falcon tube, brought to 10 ml with stop buffer and centrifuged at 40 x *g* for 3 min. Damaged myocytes and non-myocytes were removed by a series of washes in 10 ml stop buffer containing, sequentially, 100, 400, or 900 μmol/L $CaCl_2$. Cardiomyocytes were pelleted by centrifugation at 40 x *g* for 3 min after each wash. After the final wash, cardiomyocytes were resuspended in 10 ml cardiomyocyte culture medium (ScienCell) and plated onto dishes previously coated with 10 μg/ml Laminin (BD Biosciences).

Generation of pLenti6.3-Cx43 clonal HaCaT cell line.

HaCaT cells stably overexpressing Cx43 were generated as previously described for HeLa cells[2]. Lentivirus was produced from pLenti6.3-Cx43 according to the manufacturer's (Invitrogen) instructions and used to infect HaCaT cells previously plated in 6-well dishes in the presence of 4 μg/ml hexadimethrine bromide (Sigma-Aldrich). Medium was changed the following morning, and cells were split into 100-mm dishes at a dilution of 1:40 in the presence of blasticidin (10 μg/ml). Medium was changed every 2 days and healthy colonies were picked using trypsin cloning cylinders (Scienceware), expanded, and screened for expression by Western blotting and immunofluorescence.

Langendorff-perfused mouse heart preparation

C57BL/6 mice were anesthetized with isoflurane and injected with heparin (50 IU i.p.). After cervical dislocation, hearts were removed quickly by a midsternal incision and placed into ice-cold modified Krebs-Henseleit (K-H) solution. Under a dissecting microscope, the aortic opening was immediately cannulated and tied on a 23-gauge stainless steel blunt needle. The heart was attached to a Langendorff apparatus (ADInstruments) and perfused through the aorta at a constant rate of 4 ml/min with a modified pH 7.4 K-H buffer of the following composition (in mM): NaCl 118, KCl 4.7, CaCl2.H2O 2.5, MgCl2.7H2O 1.2, NaHCO3 24, KH2PO4 1.2, glucose 11, EDTA 0.5. The K-H solution was prefiltered by a microfilter (0.2-μm diameter; Nalgene) and constantly gassed with 95% O2/5% CO2. Perfusion medium was passed through water-jacketed tubing and cylinders, and the temperature was maintained at 37°C with a temperature-controlled circulating water bath. Normal perfusion was maintained for 30 min followed by 15 min of perfusion with 1 μM Latrunculin A (Sigma) or DMSO. Hearts were then either perfused for a further 30 min for control conditions or perfusion was terminated and hearts exposed to ischemia for 30 min. Control hearts were perfused continuously throughout the protocol. During no-flow ischemia, the heart was immersed in warm K-H buffer in order to maintain warmth and moisture. Immediately after Langendorff procedure, hearts were placed in cryovials and snap-frozen in liquid N2 for biochemical studies. For cryosectioning, hearts were embedded in OCT (Sakura Finotek) and snap-frozen by immersing in liquid N2-chilled isopentane to snap-freeze before storage at –80°C.

Immunofluorescence

Cells were fixed for 30 minutes at in 4% paraformaldehyde in PBS. Three PBS washes were performed, and cells were permeabilized for 5 minutes using 0.1% Triton X-100 (Sigma-Aldrich) in PBS. Cells were then washed and blocked for 1 hour at room temperature in 5% normal goat serum (NGS; Invitrogen) before addition of primary antibodies. Cells were incubated with primary antibodies diluted in 5% NGS in PBS for 1 hour at room temperature. Following several PBS washes, cells were incubated for an additional hour with goat secondary antibodies conjugated to AlexaFluor fluorophores (Invitrogen). Cells were washed with PBS, washed briefly with dH2O, and coverslips were mounted using ProLong gold antifade reagent (Invitrogen). Slides were allowed to dry overnight and imaged using a Nikon T*i* microscope with a ×60/1.49 Apo TIRF objective, Yokogowa CSU-X1 spinning disk confocal unit with 486-, 561-, and 647-nm DPSS laser source, and Coolsnap HQ2 camera controlled by NIS Elements software (Nikon). Additional image processing and analysis was performed using ImageJ (NIH).

Western Blotting

After heating to 70°C for 10 minutes, samples were cooled to room temperature and subjected to SDS-PAGE electrophoresis using NuPAGE Bis-Tris gels and MES (Invitrogen) buffer according to the manufacturer's instructions. Gels were transferred to FluoroTrans PVDF membranes (Pall), which were subsequently fixed by soaking in methanol and air drying before rewetting with methanol and blocking for 1 hour at room temperature in 5% nonfat milk (Carnation) in TNT buffer (0.1% Tween 20, 150 mM NaCl, 50 mM Tris pH 8.0). Membranes were then incubated overnight with primary antibodies diluted in 5% milk in TNT. Primary antibodies used were rabbit anti-Cx43 (1:4,000; Sigma-Aldrich), mouse anti-α-tubulin (1:2,000; BD Biosciences), mouse anti-HA (1:2,000; Sigma-Aldrich), mouse anti–β-actin (1:3,000; Sigma-Aldrich). Membranes were washed 5 times before incubation for 1 hour at room temperature with secondary antibodies diluted in 5% milk TNT. Goat secondary antibodies conjugated to Alexa Fluor 555 and 646 were used at a dilution of 1:1,000. Following incubation with secondary antibody, membranes were washed 5 times, soaked in methanol, and allowed to air dry. Membranes were imaged using the Versadoc MP 4000 fluorescent western detection system (BioRad).

Co-immunoprecipitation of Cx43/β-actin from primary adult mouse cardiomyocytes and mouse heart tissue.

To enrich cytoplasmic Cx43 pools, a low-detergent co-immunoprecipitation lysis buffer was prepared (0.1% Nonidet P-40 (Roche), 50 mM Hepes pH 7.4, 150 mM KCl, 1mM EDTA, 1mM EGTA, 1mM DTT, 1mM NaF, 0.1 mM Na_3VO_4) supplemented with HALT protease and phosphatase inhibitors (Pierce). Immunoprecipitation performed as previously described [2] as follows. Tissue was weighed and homogenized for 10 seconds in lysis buffer and incubated at 4°C with rotation for 1 hour before centrifugation at 10,000 *g* for 20 minutes to remove insoluble debris. Following protein normalization, lysates were precleared using Dynabeads protein G (Invitrogen) for 30 minutes at 4°C with rotation. Beads were discarded, and 1 mg of precleared lysate was used per reaction. For pulldowns, 2 μg of antibody was employed (mouse anti-β-actin (Sigma); mouse anti-GST as IgG control (SantaCruz). Dynabeads protein G (15 μl) was added to each reaction, and tubes were rotated for a further 45 minutes at 4°C. Beads were washed 4 times for 5 minutes each time with 1 ml lysis buffer on ice using a Dynamag-2 magnet (Invitrogen). Proteins were eluted from beads in 10 μl 2X NuPAGE sample buffer and subjected to SDS-PAGE electrophoresis and Western blotting as described above.

Freshly isolated primary adult mouse cardiomyocytes were plated and maintained in the presence or absence of 1 μM Latrunculin A for 2 h prior to lysis by scraping into low-detergent co-immunoprcipitation lysis buffer (1 ml/dish). Immunoprecipitation was performed as described above.

Quantification of Western Blotting

All blots were imaged using the Versadoc 4000 MP (BioRad). Flat-fielding was used, and Quantity One (BioRad) analysis software was used to quantify individual bands. Samples were normalized to α-tubulin or β-actin. Graphs were plotted and statistics performed using Prism 5 software (GraphPad).

Statistics

All quantitative data were analyzed using Prism 5 software (GraphPad) and expressed as mean ± SEM. A 2-tailed unpaired Student's *t* test was used to analyze data containing 2 groups, and a 1-way ANOVA

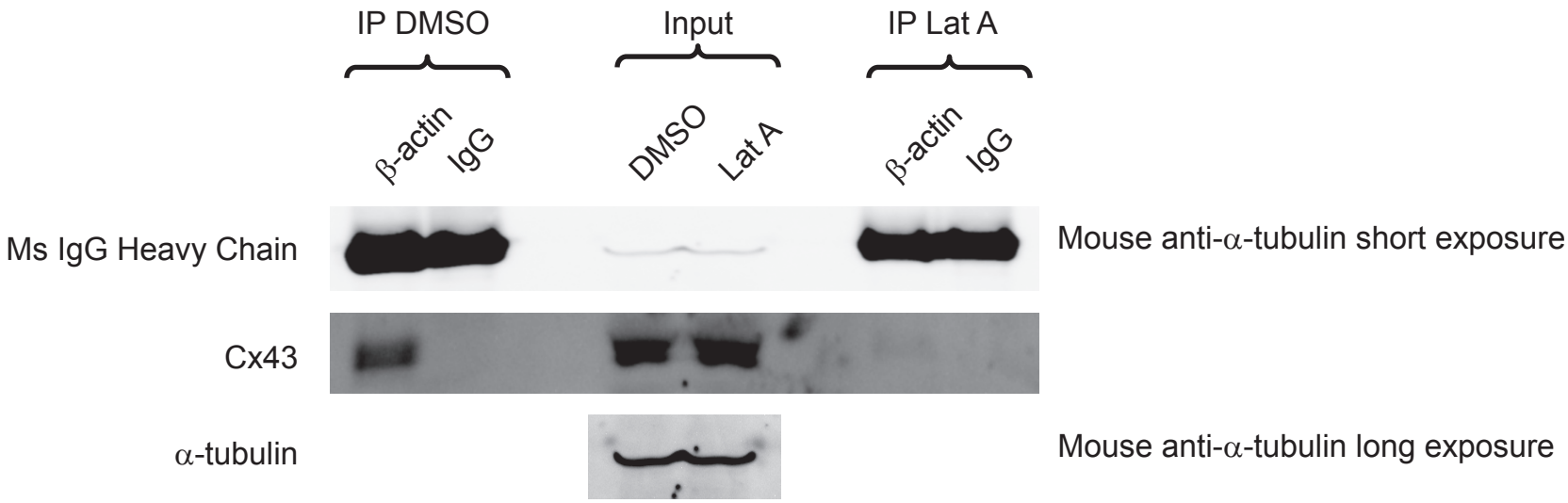
with Bonferroni post-test was used to analyze data with 3 or more groups. In both cases, a *P* value less than 0.05 was deemed statistically significant.

References

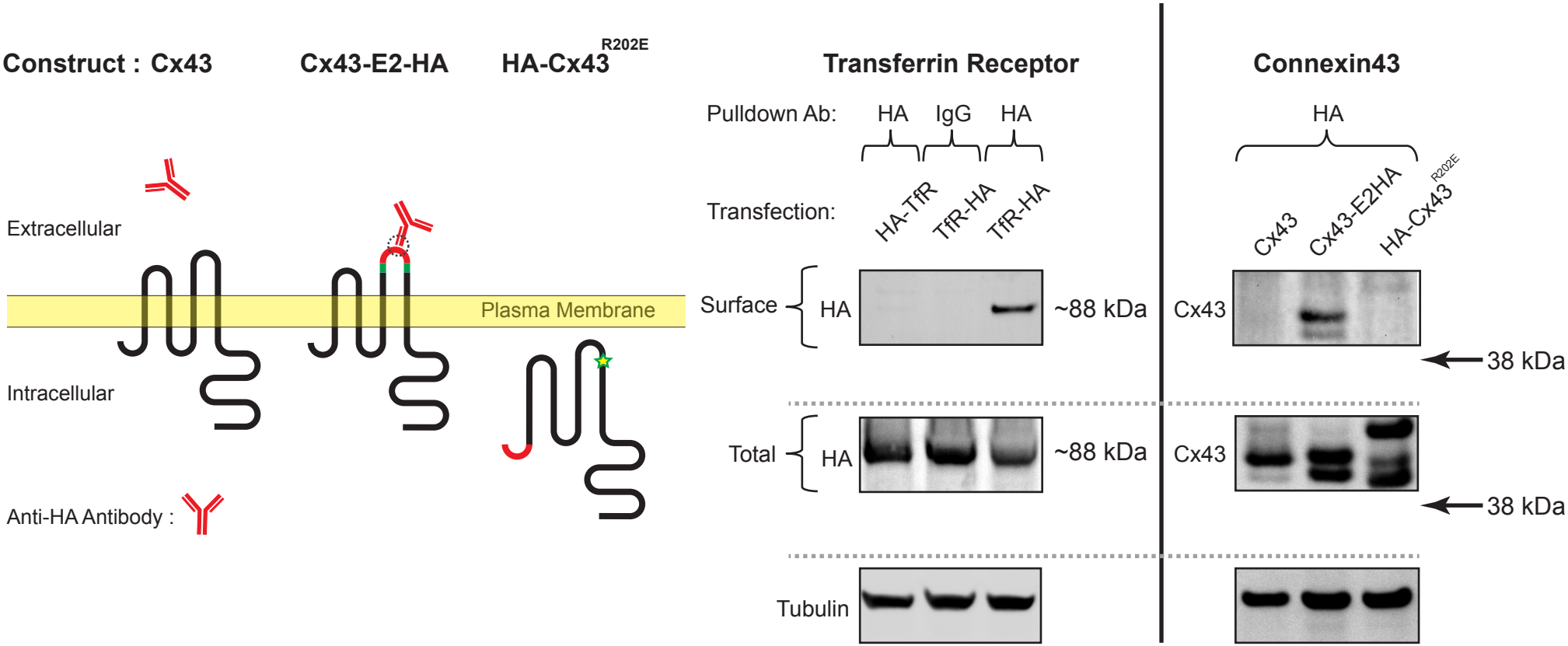
1. Chen S, Law CS, Grigsby CL, Olsen K, Hong TT, Zhang Y, Yeghiazarians Y, Gardner DG. Cardiomyocyte-specific deletion of the vitamin d receptor gene results in cardiac hypertrophy. *Circulation*. 2011;124:1838-1847
2. Smyth JW, Hong TT, Gao D, Vogan JM, Jensen BC, Fong TS, Simpson PC, Stainier DY, Chi NC, Shaw RM. Limited forward trafficking of connexin 43 reduces cell-cell coupling in stressed human and mouse myocardium. *J Clin Invest.* 2010;120:266-279

Supplemental Movie I: 3D reconstruction of adult mouse cardiomyocyte immunolabeled for Cx43 and F-actin.
Freshly isolated adult mouse cardiomyocytes immunolabeled for Cx43 (green), filamentous actin (red). 3D reconstruction from a 12.5 μm confocal z-stack of 25 0.5 μm slices (x100).

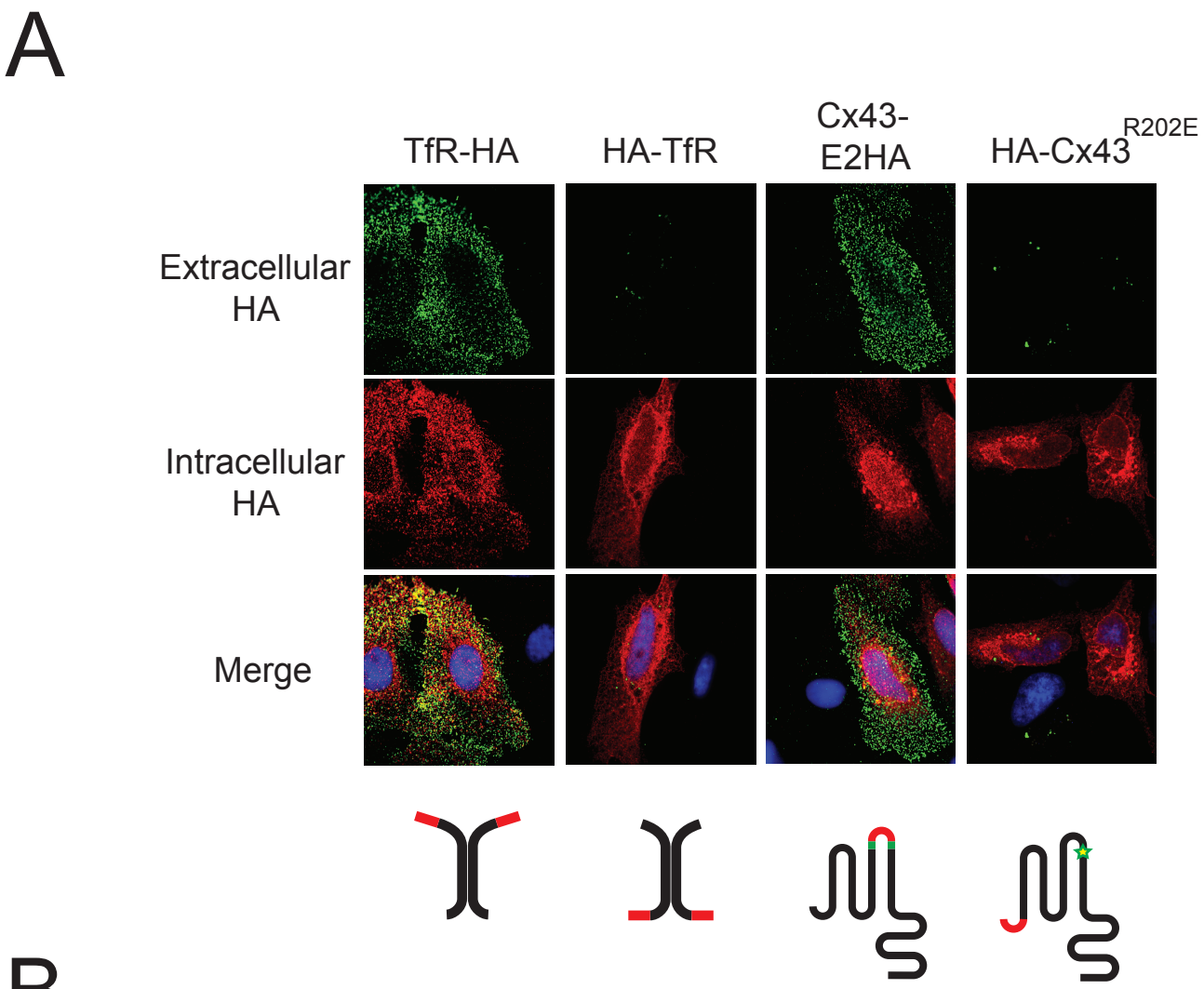
Supplemental Movie II: Cx43-eGFP vesicle (green) and LifeAct-mCherry (red) dynamics.
Images were acquired every 2.5 seconds for a total of 3 minutes. Vesicles were tracked using the MTrackJ plugin for ImageJ. Original magnification: x100.



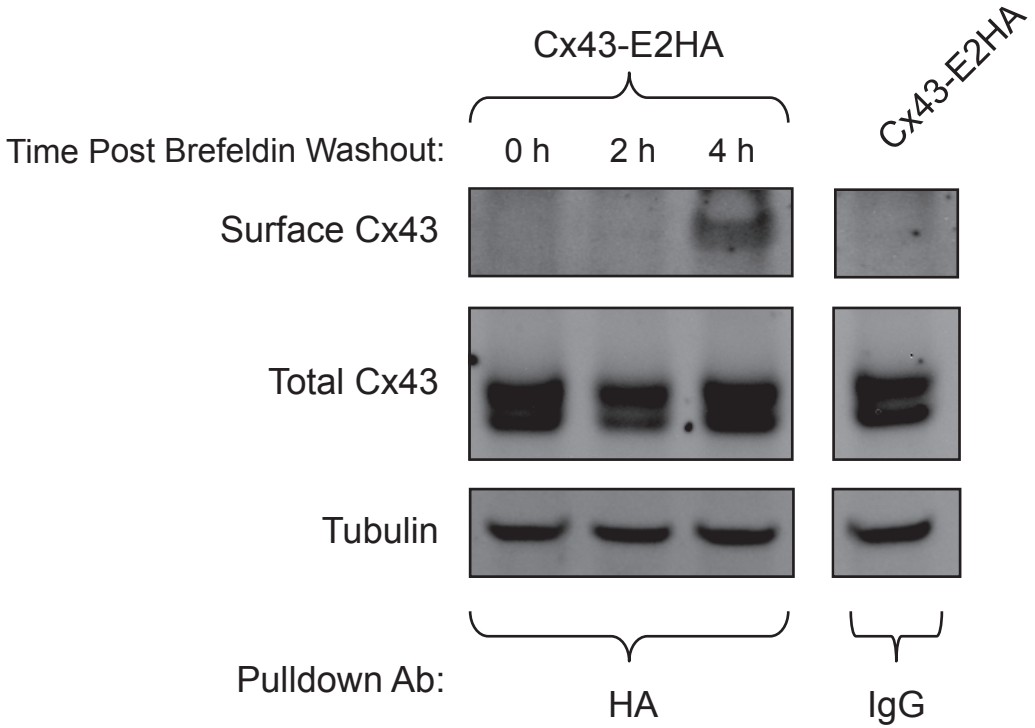
Supplemental Figure I: Cx43 co-immunoprecipitates with β-actin in adult mouse cardiomyocytes. Adult mouse cardiomyocytes were isolated from 6- to 8-week-old C57BL/6 mice and cultured in the presence or absence of 1 μM Latrunculin A for 2 h prior to lysis and co-immunprecipitation.



Supplemental Figure II: Cx43-E2HA trafficks to the cell surface in HeLa cells as determined by surface immunoprecipitation. Cx43-E2HA was transfected into HeLa cells and surface expression confirmed by incubation of live cells with anti-HA antibody and immunoprecipitation. HA-tagged transferrin receptor was included as a control.



Supplemental Figure III: Surface immunofluorescence of Cx43-E2HA in HeLa cells. Hela cells were transfected with Cx43-E2HA and immunofluorescence for surface HA (green) and total HA (red) performed. Nuclei were counterstained with ToPro3 (blue, **A**). **B**. Ratiometric analysis of surface HA expression in **A**.



Supplemental Figure IV: Cx43-E2HA reaches the plasma membrane of HaCaT cells within 4 h of release from Brefeldin A transport block.
HaCaT cells were transfected with Cx43-E2HA and subjected to Brefeldin A vesicular transport block. Cells were washed and surface immunoprecipitation against the HA epitope performed at 0, 2, and 4 hours post release.