Deletion of the last five C-terminal amino acid residues of Connexin43 leads to lethal ventricular arrhythmias in mice without affecting coupling of gap junction channels

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Short title: Lethal cardiac arrhythmias in Cx43D378stop mice

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Methods

Construction of the conditional Cx43D378stop-vector

The Cx43D378stop mutation (ablation of the last five amino acid residues of the Cx43 protein and insertion of a stop codon) was generated by PCR mutagenesis and cloned into the vector pBluescriptCx43WT (10 kb genomic mouse fragment from a phage clone in pBluescript) which contained the coding sequence of mCx43 together with the corresponding 3´ homologous region. In this step the Cx43 wildtype gene was exchanged by the Cx43D378stop sequence using XbaI/ClaI digestions. Downstream of the Cx43D378stop coding region we inserted the IRES-eGFP construct from the HeLa cell expression vector (see below) by partial AflII and AflII/XbaI digestions, respectively, resulting in the intermediate construct pBluescriptCx43D378stop-IRES-eGFP. In parallel, we cloned the Cx43 wildtype gene together with the 3´ untranslated region followed by the inverted neomycin resistance gene driven by a PGK (phosphoglycerate kinase) promoter and flanked by frt (Flp recognition target) sites [5] (constructed in our laboratory) into the vector 5´HRinpHWlox by XhoI/NotI and EcoRI digestions, respectively. The vector 5´HRinpHWlox already included the corresponding 5´ homologous region of Cx43 followed by loxP (locus of crossing over P1) sites flanking a HPRT minigene (constructed in our laboratory). In the final step we exchanged the Cx43D378stop-IRES-eGFP sequence followed by the corresponding 3´homologous region from the vector pBluescriptCx43D378stop-IRES-eGFP by PspOMI and NotI/XhoI digestions, respectively, with the HPRT minigene. The final conditional Cx43D378stop-vector was analyzed by restriction mapping and partial sequencing (AGOWA, Berlin, Germany). The functionality of frt and loxP sites was tested by transformation of the targeting vector into Flp- or Cre-recombinase expressing Escherichia coli bacteria [2].

Screening of ES cell clones

For transfection of HM1 ES cells [14] via electroporation (0,8 kV, 3 µF), 300 µg of the conditional Cx43D378stop-vector were linearized by NotI digestion. Screening of positively transfected ES cells was carried out with 350 µg/µl G418-neomycin (Invitrogen, Darmstadt, Germany). Surviving ES cell clones were tested by two different PCR analysis. PCR1 amplifies the 3´ homologous region (GFP_rev: CAT GGA CGA GCT GTA CAA GTA AAG CG and Cx43_3′HR: CAC TTG ATA GTC CAC TCT AAG CAA CC) and PCR2 the first loxP site (Cx43/31for: GCA CTT GGT AGG TAG AGC CTG TCA GGT C and Cx43/31rev: CTC CAC GGG TCT GTA CCC ACT GAC CTC). ES cell clones positive in both PCRs were further characterized by Southern blot analyses for correct recombination at the Cx43 locus (external probe) as well as for single integration of the vector construct (internal probe). DNA extracted from PCR positive clones was digested with HindIII (external probe) and HincII or BamHI (internal probe). After electrophoresis in an agarose gel the digested DNA was transferred on Hybond-N+-membranes (Amersham Biosciences, Buck UK) and fixed to the membrane via cross-linking by exposure to UV-light. The external and internal probes were radioactively labeled with α-32P- dCTP (Amersham Biosciences, Buck UK). Hybridization with the membranes was performed in Quick-Hyb solution (Stratagene, La Jolla, CA, USA) at 68 °C for 2h. The external probe consisted of a 550 bp AvaI-fragment from the 3´untranslated region of Cx43 and the internal probe of a 550 bp HincII-fragment from the coding region of Cx43.

Generation of Cx43D378stop mice

Two homologously recombined ES cell clones were injected into C57BL/6 blastocysts [23]. Resulting high-extent fur coloured chimeras were bred with C57BL/6 mice and agouti colored offspring were genotyped by PCR analyses of isolated tail DNA. Heterozygous Cx43 +/floxD378stop mice were backcrossed several times to increase the C57BL/6 genetic background to at least 87.5%. Additionally, Cx43 +/floxD78stop mice were mated to Cre-recombinase (αMyHC- [1] and αMyHC-Cre-ER(T2) [22]) expressing mice to delete the wildtype Cx43 cDNA sequence and the neomycin resistance gene via the Cre/loxP system. After Cre mediated deletion of the wildtype Cx43 region the truncated Cx43D378stop sequence is expressed. To verify correct homologous recombination in floxed Cx43D78stop transgenic mice by Southern blot analyses, DNA was extracted from hearts of mice with different genotypes and digested with BglII. Analyses with an internal probe (814 bp HincII/ClaI fragment from the coding region of Cx43) were performed as described for screening of ES cell clones. For PCR genotyping two different primers flanking the first loxP site (before_loxP: GCA CTT GGT AGG TAG AGC CTG TCA GGT C and in_Cx43: GCT TCC CCA AGG CGC TCC AGT CAC CC) resulted in a 350 bp fragment for the Cx43 wildtype allele and a 400 bp fragment for the Cx43D378stop allele.

Treatment of mice

All animal experiments were performed according to the Guidelines for the Care and Use of Laboratory Animals published by the US National Institutes of Health and were approved by the local ethics committee. Mice were kept under standard housing conditions with a 12-h/12-h dark/light cycle, and with food and water ad libitum. Intraperitoneal injection of tamoxifen (Sigma Aldrich, Steinheim, Germany) dissolved in sterile peanut oil with 10 % ethanol was performed on four consecutive days in a dosis of 3 mg each day. As control mice in the following experiments we used either heterozygous or homozygous Cx43floxD378stop mice. Adult control mice were injected with sterile peanut oil without tamoxifen.

Northern blot analyses

Hearts from newborn as well as adult Cx43D378stop and control mice were shock frozen in liquid nitrogen. Total RNA was extracted using TRIZOL regent (Invitrogen, Darmstadt, Germany), following the instructions of the manufacturer. After electrophoresis of 10 µg RNA in an agarose gel the RNA was transferred on Hybond-N-membranes (Amersham Biosciences, Buck, UK) and fixed to the membrane via cross-linking by exposure to UV-light [7]. The internal probe was radioactively labeled with α-32P- dCTP (Amersham Biosciences, Buck, UK). Hybridization with the membranes was performed in Quick-Hyb solution (Stratagene, La Jolla, CA, USA) at 68 °C for 2h. The internal probe consisted of a 814 bp HincII/ClaI fragment from the coding region of Cx43.

Histological analyses

Hearts from neonatal Cx43D378stop:αMyHC-Cre and adult Cx43D378stop:αMyHC-Cre-ER(T2) as well as control mice were dissected from cervically dislocated animals, shock frozen in liquid nitrogen and cryosectioned (12 µm). For paraffin sections hearts were directly fixed in 4% paraformaldehyde after dissection. After dehydration in graded series of ethanol, hearts were embedded in Paraplast plus (Sherwood Medical Co., St Louis, MO) and sectioned (5 µm). Staining of the hearts was performed with hematoxylin and eosin.

Surface ECG recordings

ECG recordings on Cx43 D378stop/D378stop;αMyHC-Cre and control mice were performed at ED 16.5. Embryos together with the placenta were removed from the uterus, placed in a recording chamber and superfused with oxygenated tyrode solution at 33±2°C. Bipolar ECG was recorded by two silver chloride electrodes placed at the right and left chest wall with a bioamplifier recording system (PowerLab 8/35, AD instruments). Analysis of the PQ and QRS duration was performed semi-automatically using the ECG analysis plugin of Chart (AD instruments).

Holter ECG: Chip implantation and longterm ECG recording

For detection of spontaneous arrhythmias, telemetric ECG recordings were performed in conscious tamoxifen-injected Cx43D378stop:αMyHC-Cre-ER(T2) and control mice. Holter ECG chips with two subcutaneous electrodes (Data Science International, St. Paul, USA) were implanted under inhalative anaesthesia with 1.0 - 1.2 vol% isoflurane in 70% N_2O/30% O_2. Chips were implanted under sterile conditions on the upper back of the mouse after medial incision and subcutaneous preparation. ECG leads were implanted subcutaneously in the left-lateral and anterior part of the thorax, and connected to the telemetric chip device. After that, skin was sutured. No mice died during the procedure, 2 mice had to be excluded from further studies and sacrificed due to inflammation of the wound. Postoperative analgesia was performed for seven days, using subcutaneous injection of 5 mg/kg Carprofen.

Ten days after final tamoxifen injection, chip implantation was performed. Recordings were conducted and performed until the death of the mutant mice (n=4) or, in case of uninduced controls (n=4), for 14 days. All mice showed normal behaviour after implantation of the telemetric chips. Recordings were performed for 24h at normal day and night circle. In each mouse, electrograms were recorded continuously during this period and sampled and stored digitally using standard mouse Holter equipment (PowerLab™ System, ADInstruments, Milford, MA, USA). All recordings were manually screened for spontaneous tachy- and bradyarrhythmias and alterations of the QRS-complex.

Langendorff-perfused hearts and epicardial mapping

For analyses of myocardial conduction velocities and characteristics, Cx43D378stop:αMyHC-Cre-ER(T2) and control hearts were Langendorff-perfused, and epicardial activation mapping (EAM) using a 128-electrode array was performed 12 days after last tamoxifen injection as described before [20]. Hearts were excorporated and dissected from surrounding tissue in ice-cold Krebs-Henseleit buffer. After cannulation of the aorta, hearts were retrogradely perfused in a Langendorff-apparatus (Radnoti Technologies Inc., Monrovia, CA, USA) at constant pressure perfusion (80 mm Hg). The perfusate composition was (in mM): NaCl 110, KCl 4.6, $MgSO_4$ 1.2, CaCl 2, NaH_2PO_4 2, $NaHCO_3$ 25, glucose 8.3, Na-pyruvate 2 and gassed with carbogen (O_2 95%, CO_2 5%), pH 7.35 – 7.45. A perfusate temperature of 37 °C was continuously maintained. The heart was immersed in a water-jacketed chamber and further fixed on a moisturized support. All hearts started beating spontaneously. Unipolar extracellular electrograms (128) were recorded from the epicardial surface with regard to a reference electrode positioned in the water-bath, using a custom-built electrode array (interelectrode distance: 300 ± 7 µm). Fixed-rate stimulation (S1S1: 120 ms) was performed using two adjacent electrodes of the array. Electrograms were recorded using a 128-channel, computer-assisted recording system (Multi Channel Systems, Reutlingen, Germany) with a sampling rate of 25 kHz (25.000 samples per second). Data were bandpass filtered (50 Hz), digitized with 12 bit and a signal range of 20 mV. Activation maps were calculated from these

data using custom-programmed software (Labview 7.1, National Instruments, Austin, TX, USA). To obtain an index of local conduction slowing for each electrode, the activation time differences to neighbouring points were normalised to interelectrode distance. The largest difference at each site was defined as local phase delay. The variation coefficient of these phase delays, calculated for every single electrode compared to all adjacent electrodes of the array, was used as heterogeneity index for inhomogeneity in global conduction, as described before [13, 20].

Microinjection analyses of cardiomyocytes

Embryonic cardiomyocytes were isolated from Cx43D378stop:αMyHC-Cre and Cx43+/floxD378stop (control) ventricles of embryonic hearts on ED 16.5, as described previously [8]. Each heart was dissociated and plated separately until confluency and the corresponding genotype was determined by PCR analysis of the embryonic tissue. During the microinjection of neurobiotin by iontophoresis (20 nA, 2-15 sec) the cells were kept at 37°C [6]. The glass microinjection capillaries had a diameter of 1 – 2 µm and were loaded upright with neurobiotin during a 5 min incubation period in neurobiotin solution (6 % neurobiotin [287 Da, net charge +1], 0.4 % rhodamine 3-isothiocyanate dextran, 0.1 M Tris, pH 7,6). Subsequently, the capillary was filled with 1 M KCl solution and placed into a microelectrode holder with an AgCl-electrode on the micromanipulator. After impalement of the cell with the filled capillary, the injection was started by applying a positive current of 20 nA for approx. 2-10 sec. The cardiomyocytes were injected at the periphery of beating clusters. For examination of the injection process the neurobiotin solution contained rhodamine 3-isothiocyanate dextran, which cannot pass gap junction channels due to its size. Using appropriate fluorescence filters the injection process could be visualized.

For control and Cx43D378stop:αMyHC-Cre cardiomyocytes at least 20 injections were performed and analyzed. After the last injection the cells were fixed with 1 % glutaraldehyde in PBS for 5 min at room temperature, washed three times with PBS and permeabilized with 2 % Triton X-100 in PBS at 4°C over night. On the next day, the cells were washed twice with PBS and incubated in 0.1 % horseradish peroxidase-conjugated Avidin D (Vector Laboratories, Burlingame, USA) solution for 90 min at room temperature. After three washing steps with PBS, staining of neurobiotin containing cells was performed using the HistoGreen substrate kit (Linaris, Wertheim – Bettingen, Germany) according to the manufacturer's instructions until blue colored cells appeared. The staining reaction was stopped by washing three times in PBS and areas of dye transfer microscopically evaluated.

Generation of Cx43D378stop expressing HeLa cells

Coupling-deficient HeLa cells were stably transfected by lipofection with a vector construct containing the Cx43D378stop-IRES-eGFP coding sequence under the control of a CMV (cytomegalovirus) promoter. Furthermore the vector included puromycin resistance cDNA driven by a SV40 (Simian virus 40) promoter for the selection of successfully transfected clones. For generation of the Cx43D378stop expression vector we cloned the Cx43D378stop sequence into the vector pMJ-Cx23-IRES-eGFP [21] by XhoI/SpeI digestion. This resulted in the exchange of the Cx23 coding region by the Cx43D378stop coding sequence.

Cell-to-cell dye transfer studies in Cx43D378stop HeLa cells

The major goal of this study was to determine permeability of the Cx43D378stop gap junction channels to dyes differing in the molecular mass or the net charge. To assess the single channel permeability (P_γ) of Cx43D378stop gap junctions to fluorescent dyes, we combined

dye transfer studies with junctional conductance measurements in HeLaCx43D378stop cell pairs, as described before [18]. Dyes used include (molecular mass of the fluorescent ion, valence): Lucifer yellow (LY) (443, -2), Alexa Fluor-350 (AF350) (326, -1), EAM-1 (MW 266, +1) and EAM-2 (MW 310, +1). All dyes with the exception of EAM-1 and EAM-2 (Macrocyclics Company, Dallas, Texas, USA) were purchased from Invitrogen (Eugene, Oregon, USA).

Electrophysiological recordings of cell-to-cell coupling in Cx43D378stop HeLa cells

Junctional conductance (gj) was measured in selected HeLaCx43D378stop cell pairs by dual whole-cell voltage clamp method, as described previously [17].

Myocyte isolation and cell electrophysiology of Cx43D378stop cardiomyocytes

Adult mouse ventricular myocytes were obtained from control and tamoxifen-injected Cx43D378stop mice by enzymatic dissociation following standard procedures [16]. Briefly, after thoracotomy, hearts were placed in a Langendorf column and perfused sequentially with low calcium, and with a collagenase-containing (Worthington) solution. Ventricles were cut into small pieces, and gently minced with a Pasteur pipette. Ca^{2+} concentration was then increased gradually to normal values. Cells were used for electrophysiological recording within 8 hours after isolation.

Electrophysiological recordings of Cx43D378stop cardiomyocytes

All electrophysiological recordings were conducted in the whole-cell configuration at room temperature. Pipette resistance was maintained within the range of 1.5 to 1.8 MΩ. For sodium current recordings [19] recording pipettes were filled with a solution containing (in mmol/l): NaCl 5, CsF 135, EGTA 10, MgATP 5 and HEPES 5, pH 7.2 with CsOH. Cells were maintained in a solution containing (in mM): NaCl 5, CsCl 132.5, $CaCl_2$ 1, MgCl2 1, $CdCl_2$ 0.1, HEPES 20 and Glucose 11, pH 7.35 with CsOH. Voltage clamp protocols were as follows: for determination of peak current voltage relation, 200 msec voltage pulses were applied to Vm -90 mV to +30 mV in 5 mV voltage steps, from a holding potential of Vm = -120 mV. Interval between voltage steps was 3 sec. To calculate the time constants of Na+ current deactivation, currents at -40 mV were fitted with single exponential function. Steady state inactivation was determined by stepping Vm from -130 mV to -40 mV, followed by a 30 msec test pulse to Vm = -40mV to elicit I_{Na}. The steady state voltage-dependent inactivation curves were fitted to Boltzmann's functions. Recovery from inactivation was studied by applying paired voltage clamp steps. Two 20-msec test pulses (S1,S2) to Vm = -40mV (holding potential = -120 mV) were separated by increasing increments of 2 msec to a maximum S1-S2 interval of 100 msec. The S1-S1 interval was kept constant at 3 sec. The time-dependent recovery from inactivation curves were fit with exponential functions. Late Na+ current was measured using an external solution [15] containing (in mmol/L): 135 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 10 Hepes, 10 glucose, 0.2 $CdCl_2$, pH 7.4 (NaOH). The late tetrodotoxin-sensitive Na+ current density was measured at the end of a 500ms test pulse to - 30mV and 20 sweeps before and after tetrodotoxin (20μM) application were averaged and subtracted. Currents were normalized to the cell capacitance and expressed in pA/pF. To enable that the recorded currents were flowing through potassium channels, our recordings were obtained in isotonic conditions, thus preventing activation of volume-sensitive outward rectifying anion currents (see, e.g., [24]). Furthermore, our recording conditions included a high concentration of EGTA (10 mM) in the recording pipette solution and the presence of

$CdCl_2$ 0.2 in the extracellular bath. Therefore, the probability of open volume- or calcium-activated chloride-permeable channels was kept near zero.

To measure whole cell depolarization-activated outward K (K_v) currents and inwardly rectifying potassium currents I_{K1} [3], recording pipette solution contained (in mmol/l): KCl 135, $MgCl_2$ 1, EGTA 10, HEPES 10, and glucose 5, pH 7.2 with KOH. The bath solution contained (in mmol/l): NaCl 136, KCl 4, $CaCl_2$ 1, $MgCl_2$ 2, $CdCl_2$ 0.2, HEPES 10, tetrodotoxin 0.04 and glucose 10, pH 7.4 with NaOH. For current clamp experiments, the tetrodotoxin and $CdCl_2$ were omitted from the bath. K_v currents were recorded in response to 4.5 s voltage steps to potentials between -40 and +40 mV from a holding potential (HP) of -70 mV; voltage steps were presented in 10-mV increments at 15-s intervals. Peak K_v currents at each test potential were defined as the maximal outward current recorded during the 4.5 s voltage steps. The decay phases of currents were described by the sum of two exponentials: y(t) = A1 * exp(-t/t1) + A2 exp(-t/t2) + B, where t is time, t1 and t2 are the decay time constants, A1 and A2 are the amplitudes of the inactivating current components ($I_{to,fast}$ or $I_{to,f}$ and $I_{K,slow}$), and B is the amplitude of the noninactivating current component, I_{ss}. Fitting residuals and correlation co-efficients were determined to assess the quality of fits. I_{K1} was recorded in response to 350 ms voltage steps to test potentials between -40 and -120 mV (in 10-mV increments) from the same HP [3]. I_{K1} densities were determined from the amplitudes of the currents measured at the end of 350 ms hyperpolarizing voltage steps from a HP of -70 mV.

For current clamp analyses, the numeric value (action potential threshold, width, etc.) was taken from the first action potential initiated on current injection. Action potential thresholds were determined from phase plane plots [15]. Resting membrane potentials, action potential amplitudes, and action potential durations at 50% (APD50), 70% (APD70) and 90% (APD90) repolarization were measured. Data were acquired at a sampling rate of 10-20 kHz (voltage clamp) or 5 kHz (current clamp), filtered at 1 KHz, digitized with the Patchmaster software (HEKA) and analyzed offline using the Fitmaster (HEKA) or the Labchart software (AD Instruments). AP parameters were analyzed with the cardiac action potential analysis module of Labchart.

Statistical tests were performed using unpaired Student's t-Test for all data. A P-value of <0.05 was considered significant. All data is expressed as mean ± SEM.

Immunohistochemical analyses for Cx43, ZO-1, N-Cadherin and $Na_V1.5$

Cryosections from adult Cx43D378stop:αMyHC-Cre-ER(T2) and control hearts (14 days after last tamoxifen injection) were fixed in 4 % PFA for 5 min or ethanol at -20°C for 10 min (ZO-1 antibody), washed three times in PBS with 0.1 % Triton X-100 and blocked with 5 % BSA, 0.1 % Triton X-100 in PBS for 1 h at room temperature. Rabbit anti-Cx43 (1:500 [25]), rat anti-ZO-1 (1:100, Millipore, Temecula, CA, USA), rabbit anti-N-Cadherin (1:500, Santa Cruz Biotechnoloy, CA, USA), mouse anti-N-Cadherin (1:100, BD Transduction Laboratories, San Jose, CA, USA) or rabbit anti-$Na_V1.5$ (1:100 [10]) antibodies were diluted in blocking solution and incubated with the cryosections at 4 °C overnight. The rabbit anti-Nav1.5 antibodies were generated against an epitope of the DI-DII cytoplasmic loop specific for $Na_V1.5$. Further details can be found in [9, 11]. On the next day, the sections were washed three times in PBS with 0.1 % Triton X-100 and incubated with Alexa Fluor 488 goat anti-rabbit and goat anti-rat as well as Alexa Fluor 594 goat anti-rabbit and goat anti-mouse secondary antibodies (Invitrogen, Darmstadt, Germany) for 1 h at room temperature, diluted 1:1000 in blocking solution. After three times washing in PBS with 0.1 % Triton X-100 the cryosections were mounted with Glycergel mounting medium (Dako, Glostrup, Denmark) and viewed with a Laser Scanning Microscope (Zeiss, Jena, Germany).

Co-localization was defined by calculation of Pearson coefficient (plus/minus SEM) as described before [4]. Staining took place on two separate days, and visualization also took place on two separate days. For each day, two different slides from each heart were stained. Regions of interest were chosen on each slide and the extent of co-localization was determined. Statistical analysis for the data on each experimental day was significant. In order to combine the data of the separate experiments, the value of the Pearson coefficient for each region of interest measured in a D378stop sample was measured relative to the average value of the Pearson coefficient of all regions of interest from the control sample, analyzed on that same day. The data were normalized in such a way that the control value was set to the unit. Therefore, there is no scattering of the control data (every event is set to a value of 1). A one sample t-test yielded a highly significant value of the combined data.

Immunoblot analyses for Cx43, ZO-1 and $Na_V1.5$

Total proteins were extracted from hearts of neonatal Cx43D378stop:αMyHC-Cre and adult Cx43D378stop:αMyHC-Cre-ER(T2) (14 days after last tamoxifen injection) as well as control mice and homogenized with a precellys tissue homogenizer (Peqlab, Erlangen, Germany) in lysis buffer (2 × Complete [Roche, Mannheim, Germany], 1 % Triton X-100, 0.5 % Nonidet P40, 50 mM NaCl, 30mM $Na_4P_2O_7$ x 10 H_2O, 1 mM Na_3VO_4, 50mM NaF, 1mM PMSF, 20mM HEPES), three times for 25 sec and 5000 rpm. Laemmli buffer [12] was added to all samples. Proteins (100 µg) were separated by electrophoresis on 8 or 12% sodium dodecyl sulfate polyacrylamide gels and transferred to a Hybond ECL membrane (Amersham Biosciences, Buck, UK). After blocking with 5% milk powder in TBST (10mM Tris, 150 mM NaCl, 0.1% Tween-20, pH 7.5) for 1 h at room temperature, the membranes were incubated overnight at 4°C with rabbit anti-Cx43 (diluted 1:500 [25]), rat anti-ZO-1 (diluted 1:200, Millipore, Temecula, CA, USA) or rabbit anti-$Na_V1.5$ (diluted 1:200, Alomone labs, Jerusalem, Israel). On the next day the membranes were washed with TBST three times at room temperature and incubated for 1 h at room temperature with goat anti-rabbit, donkey anti-rat or goat anti-mouse horseradish peroxidase-conjugated antibodies (diluted 1:10000, Dianova, Hamburg, Germany). Finally, the membranes were incubated with enhanced chemiluminescence reagents (Pierce, Rockford, IL, USA) and developed on X-ray films (Super RX, FUJIFILM Europe, Düsseldorf, Germany). Loading controls were performed with mouse anti-actin (diluted 1:500, Sigma, Munich, Germany) or mouse anti-GAPDH (diluted 1:10000, Millipore, Temecula, CA, USA) antibodies.

Statistical data of the different immunoblots (n≥3), performed on different days were assessed on each experimental day separately. To combine the data we defined the expression level of the protein of interest relative to the control mice on the same membrane. Then the data were normalized in such a way that the control value was set to the unit (see above, analysis of co-localization).

Quantitative Real-Time-PCR

Hearts from adult Cx43D378stop:αMyHC-Cre-ER(T2) and control littermates were shock frozen in liquid nitrogen 12 days after last tamoxifen injection. Total RNA was extracted using TRIZOL reagent (Invitrogen, Darmstadt, Germany), following the instructions of the manufacturer. 800 ng RNA was reverse transcribed into cDNA with the SuperScript VILO cDNA Synthesis Kit (Invitrogen). Quantitative PCR was performed in triplicates using Tagman Assays (Mm00451971_m1 for $Na_V1.5$ and Mn99999915_g1 for GAPDH) and Gene Expression Master Mix (Applied Biosystems) according to manufacturer's instructions on a Rotorgene 6000 realtime PCR machine (Corbett). Cycle threshold (C_T) was calculated during the exponential phase at identical threshold values for all runs. C_T values of $Na_V1.5$ were subtracted by the C_T value of the housekeeping gene GAPDH to obtain ΔC_T and relative

expression was calculated as $2^{\Delta CT}$. Relative expression values were normalized to the mean of controls and expressed in %±SD.

Triton X-100 Fractionation Assay [based on the method of Musil and Goodenough (1991)]

Stable Cx43 and Cx43D378stop expressing HeLa cells were lysed with 1 % Triton X-100, 100 µM PMSF in 1× Complete [Roche, Mannheim, Germany] at 4 °C for 1 h. After centrifugation at 100.000 x g for 50 min at 4°C, the supernatant (Triton X-100 soluble fraction) was carefully removed. The pellet (Triton X-100 insoluble fraction) was resuspended in lysis buffer. Laemmli buffer [12] was added to all samples. Equal amounts of whole, Triton X-100 soluble, and insoluble lysate were separated by 10 % sodium dodecyl sulfate polyacrylamide gels and transferred to a Hybond ECL membrane (Amersham Biosciences, Buck, UK). Detection of Cx43 was performed as described for immunoblot analyses.

Co-Immunoprecipitation of ZO-1, $Na_v1.5$ and Cx43

Co-Immunoprecipitation (Co-IP) was performed using a Co-IP kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Thousand µg protein lysate from adult Cx43D378stop:αMyHC-Cre-ER(T2) (14 days after last tamoxifen injection) and control hearts for each reaction were prepared as described for immunoblot analyses. For pull-down of ZO-1 or $Na_v1.5$ and their interaction partners 10 µg of ZO-1 antibodies (rabbit anti ZO-1, Invitrogen, Darmstadt, Germany) or $Na_v1.5$ antibodies (rabbit anti $Na_v1.5$, Alomone labs, Jerusalem, Israel) were applied. As a negative control, 10 µg of rabbit IgG antibodies (normal rabbit IgG, Santa Cruz Biotechnology, CA, USA) were used for pull-down. Samples were separated by electrophoresis on 10% sodium dodecyl sulfate polyacrylamide gels and transferred to a Hybond ECL membrane (Amersham Biosciences, Buck, UK). Detection of Cx43, ZO-1 and $Na_v1.5$ was performed as described for immunoblot analyses.

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