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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Suppl. Fig. 1 Generation of conditional Cx43D378stop mice. **a** Homologous recombination of the conditional Cx43D378stop-vector with the genomic locus of the *Cx43* gene leads to the recombined allele mCx43floxD378stop. Besides the 5´ and 3´ homologous regions the vector consists of a loxP (locus of crossing over P1) site flanked region containing the Cx43WT sequence, its 3´ untranslated region and an inverted neomycin resistance cDNA driven by a PGK (phosphoglycerate kinase) promoter and flanked by frt (Flp recognition target) sites. Furthermore, the vector contains the mutated Cx43D378stop sequence followed by an IRES-eGFP construct. Cre-mediated recombination leads to the deletion of the Cx43WT sequence, its 3´untranslated region, neomycin resistance gene and results in the genotype mCx43D378stop. **b** Southern blot analysis with an internal probe on *Bgl*II digested heart DNA from different genotypes verified homologous recombination in Cx43floxD378stop mice. Detection of a 7.5 kb fragment indicates the Cx43 wild-type allele and a 3.9 kb as well as a 10.3 kb fragment the Cx43floxD378stop allele. **c** Northern blot analysis with an internal probe on total RNA from neonatal hearts of different genotypes verifies the transcriptional expression of the Cx43D378stop mutation after Cre-mediated recombination. Detection of a 3.1 kb fragment indicates the Cx43 wild-type mRNA and a 4.4 kb fragment the Cx43D378stop-IRES-eGFP bicistronic mRNA. **d** PCR genotyping of Cx43D378stop mice results in a 350 bp fragment for the Cx43 wildtype allele and a 400 bp fragment for the Cx43floxD378stop allele

Suppl. Fig. 2. Voltage gating of Cx43D378stop GJ channels. **a** Junctional current (I_j) in response to a transjunctional voltage (V_j) step of -95 mV, followed by repeated V_j ramps of small aplitude in a HeLaCx43D378stop cell pair. The insert shows that I_j does not decay substantially during ~0.5 s. This result supports the notion that the minor, short lasting differences in membrane potential between two neighboring myocytes during propagation cannot cause a substantial decay of junctional conductance (g_j). **b** Normalized g_j-V_j plot of Cx43D378stop channels (in black) averaged from four g_j-V_j plots measured using V_j ramps changing over 30 s from 0 to 105 mV. The data demonstrate that Cx43D378stop gap junctions are in fact less dependent on Vj than the wild-type control (grey line; from [1]). These results exclude the possibility that the enhanced arrhythmogenicity observed in Cx43D378stop hearts results from an enhanced dynamic reduction in junctional conductance [2] associated with the Vj gating of the channel.

Suppl. Fig. 3 Late sodium current densities recorded from adult cardiac myocytes isolated from Cx43D378stop mice. There is no change of late sodium current density in Cx43D378stop cells

Suppl. Fig. 4 Sodium current properties recorded from adult cardiac myocytes isolated from wild-type mice injected with tamoxifen. **a,b,c:** Average peak sodium current density as a function of voltage command, Voltage dependence of I_{Na} steady-state inactivation curves and Time course of I_{Na} recovery from inactivation. n=6 for each group (pNS)

Suppl. Fig. 5 Average I_{K1} current density as a function of voltage command. There is no difference of I_{K1} current densities at both -120 mV and -50 mV between Cx43D378stop and control cells. n=14 for control and 15 for Cx43D378stop

Suppl. Fig. 6 Transcriptional expression of the $Na_V1.5$ gene in adult Cx43D378stop hearts. Quantitative Real-Time-PCR analyses of $Na_V1.5$ expression revealed no significant differences between control and Cx43D378stop hearts. n=4 for both groups

Suppl. Fig. 7 Triton X-100 fractionation assay with Cx43 and Cx43D378stop stable expressing HeLa cells. Immuno blot analyses reveal presence of Cx43D378stop protein in both Triton-insoluble and Triton-soluble fractions. Similar to the Cx43 wild-type protein, the Cx43D378stop protein can be found in junctional and non-junctional connexin channels. n=3 for both groups

Suppl. Fig. 8 Quantification of ZO-1 and Nav1.5 protein expression in neonatal and adult Cx43D378stop hearts. Statistical analyses revealed no significant differences in the expression levels of ZO-1 and Nav1.5 protein between neonatal (**a**) or adult (**b**) control and Cx43D378stop hearts. n=3 for both groups

Suppl. Fig. 9 Localization of N-Cadherin and cardiac actin in the mutated Cx43D378stop adult heart. Immunological staining of N-Cadherin (green), cardiac actin (red) and nuclei (blue) in sections of control (**a**) and Cx43D378stop (**b**) hearts verifies localization of N-Cadherin at intercalated discs between ventricular cardiomyocytes (merged magnifications). Staining of cardiac actin reveals no obvious differences in cytoskeleton organization between control and Cx43D378stop hearts. n=4 for both groups. Bar: 20 µm

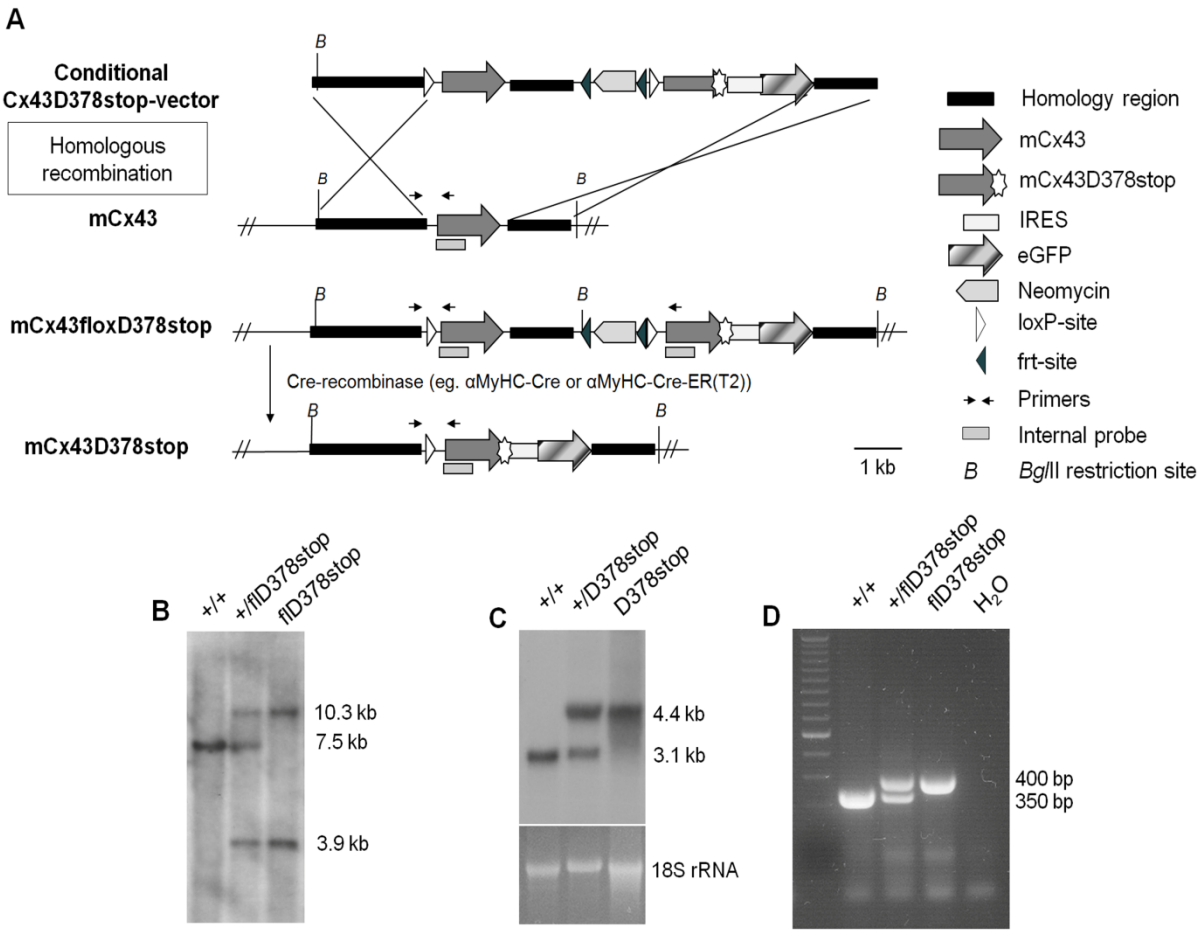
Suppl. Fig. 10 Co-immunoprecipitation analysis (Co-IP) of Cx43D378stop, ZO-1 and $Na_v1.5$ in adult hearts. **a** Immuno blots of pulled-downed fractions performed on lysates from control and Cx43D378stop hearts revealed immuno precipitation of wildtype and mutated Cx43D378stop protein by ZO-1 or $Na_v1.5$. After Co-IP, membranes were probed with polyclonal Cx43 antibodies (upper panel) and re-probed with polyclonal ZO-1 or $Na_v1.5$ antibodies (middle panel) to confirm immuno precipitation. As an input control, 80 µg of flow-through lysate were probed with polyclonal Cx43 antibodies (lower panel). n=3 for both groups. **b** As a negative control, polyclonal rabbit IgG antibodies were used to pull-down fractions of control (left panel) and Cx43D378stop (right panel) lysates. Immunoblots with polyclonal Cx43 antibodies revealed no unspecific pull-down of Cx43 or Cx43D378stop protein by the IgG antibody-coupled beads. As an input control, 80 µg of flow-through lysate were used.

References

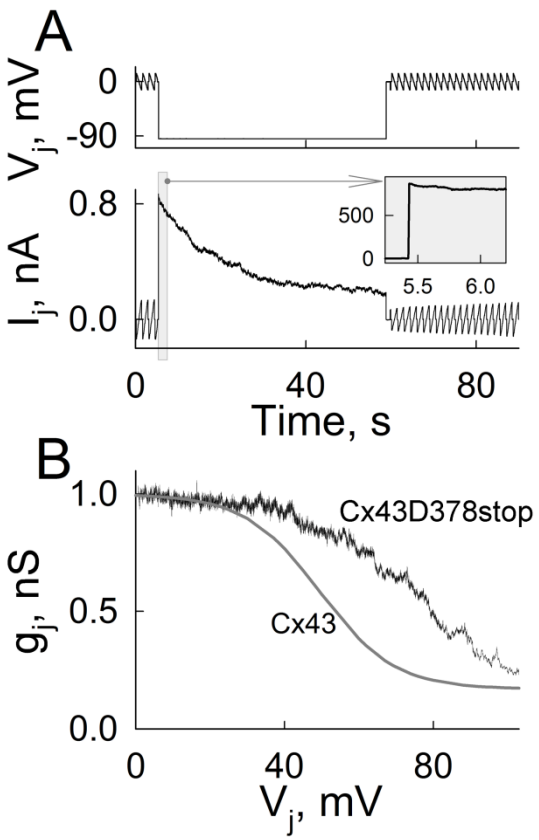
1. Bukauskas FF, Bukauskiene A, Bennett MV, Verselis VK (2001) Gating properties of gap junction channels assembled from connexin43 and connexin43 fused with green fluorescent protein. Biophys J 81:137-152 doi:10.1016/S0006-3495(01)75687-1

2. Lin X, Gemel J, Beyer EC, Veenstra RD (2005) Dynamic model for ventricular junctional conductance during the cardiac action potential. Am J Physiol Heart Circ Physiol 288:H1113-1123 doi:10.1152/ajpheart.00882.2004

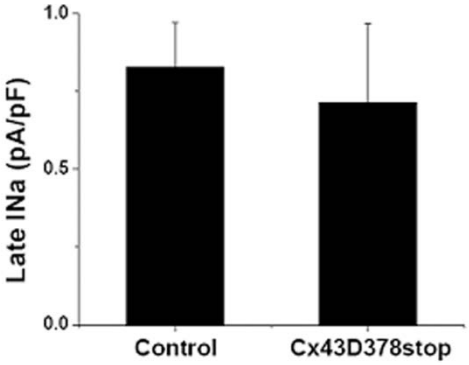
Suppl. Figs.



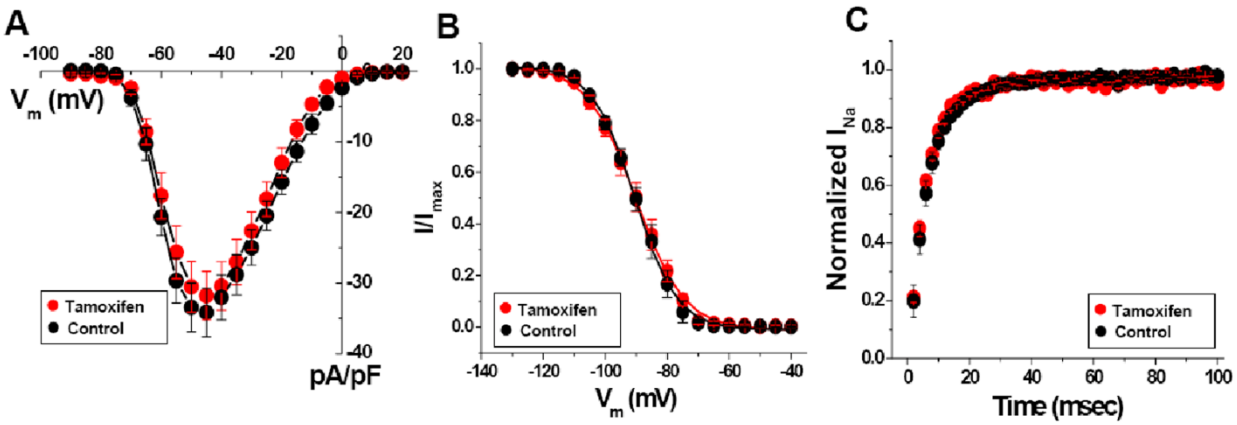
Suppl. Fig. 1


A
0
-90
V_j, mV
0.8
0.0
I_j, nA
500
0
5.5
6.0
0
40
80
Time, s
B
1.0
0.5
0.0
g_j, nS
Cx43D378stop
Cx43
0
40
80
V_j, mV

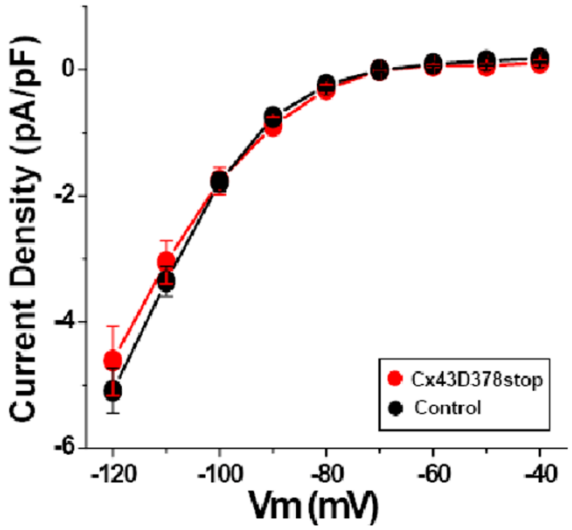
Suppl. Fig. 2



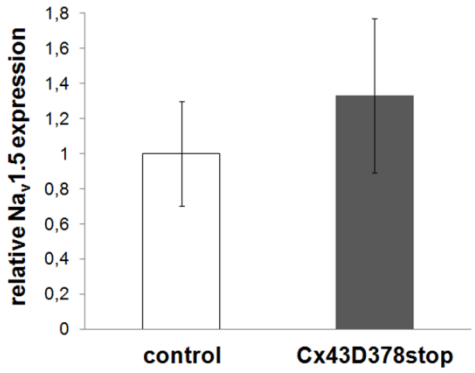
Suppl. Fig. 3


A
-100
-80
-60
-40
-20
0
20
V_m (mV)
-10
-20
-30
-40
pA/pF
Tamoxifen
Control
B
1.0
0.8
0.6
0.4
0.2
0.0
I/I_max
-140
-120
-100
-80
-60
-40
V_m (mV)
Tamoxifen
Control
C
1.0
0.8
0.6
0.4
0.2
0.0
Normalized I_Na
0
20
40
60
80
100
Time (msec)
Tamoxifen
Control

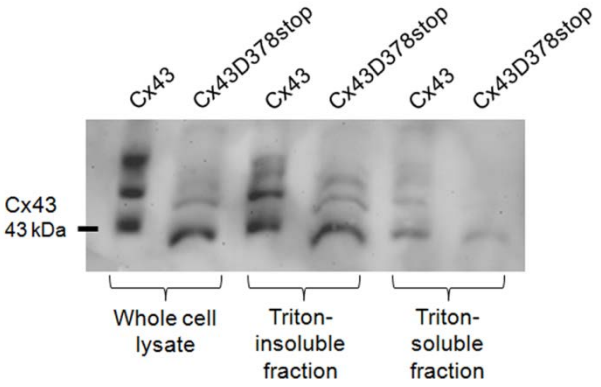
Suppl. Fig. 4


Current Density (pA/pF)
0
-2
-4
-6
-120
-100
-80
-60
-40
Vm (mV)
Cx43D378stop
Control

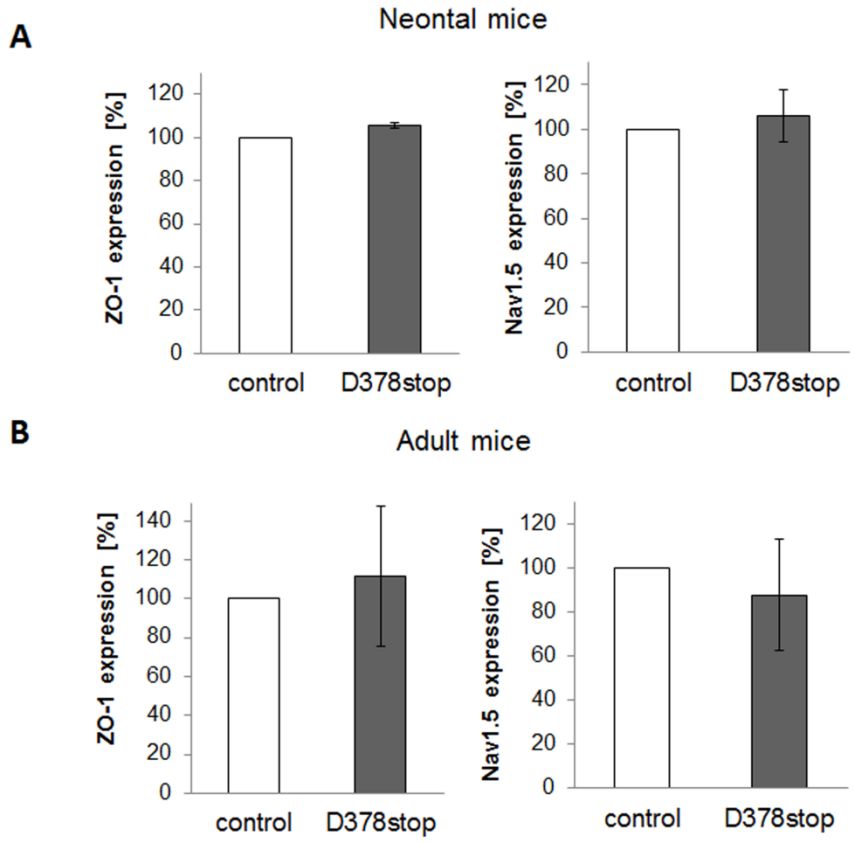
Suppl. Fig. 5


relative Na$_v$1.5 expression
1,8
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
control
Cx43D378stop

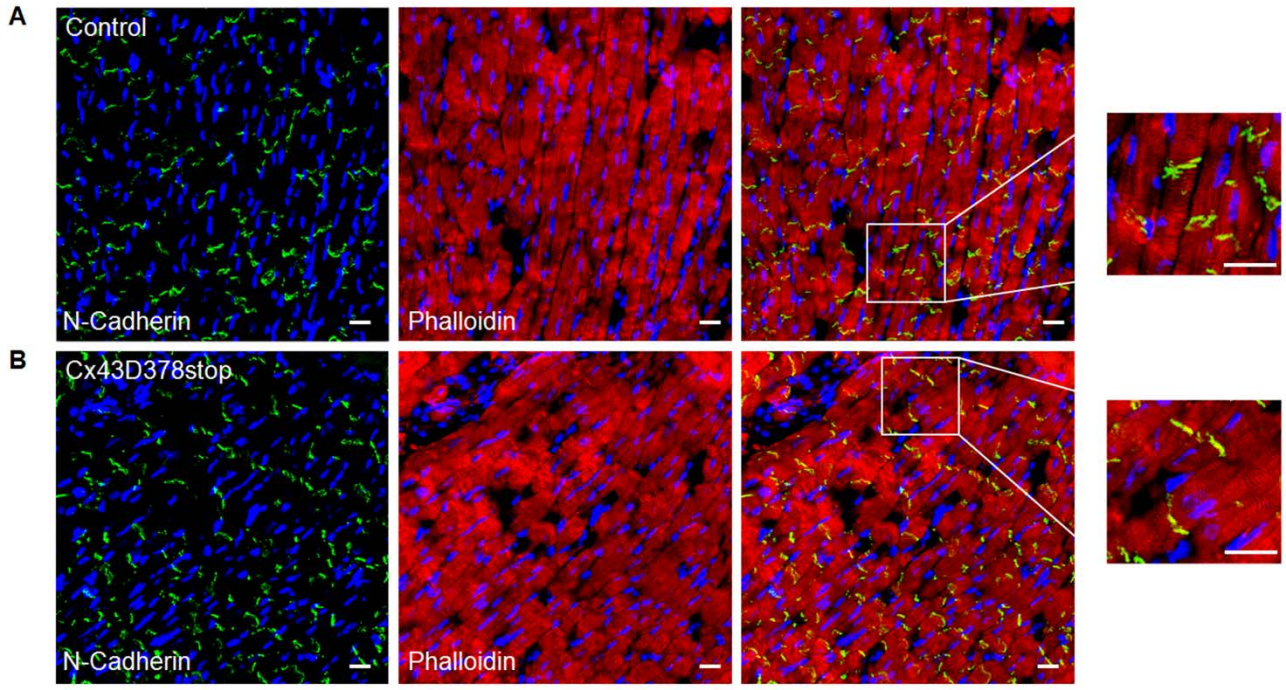
Suppl. Fig. 6


Cx43
Cx43D378stop
Cx43
Cx43D378stop
Cx43
Cx43D378stop
Cx43
43 kDa
Whole cell lysate
Triton-insoluble fraction
Triton-soluble fraction

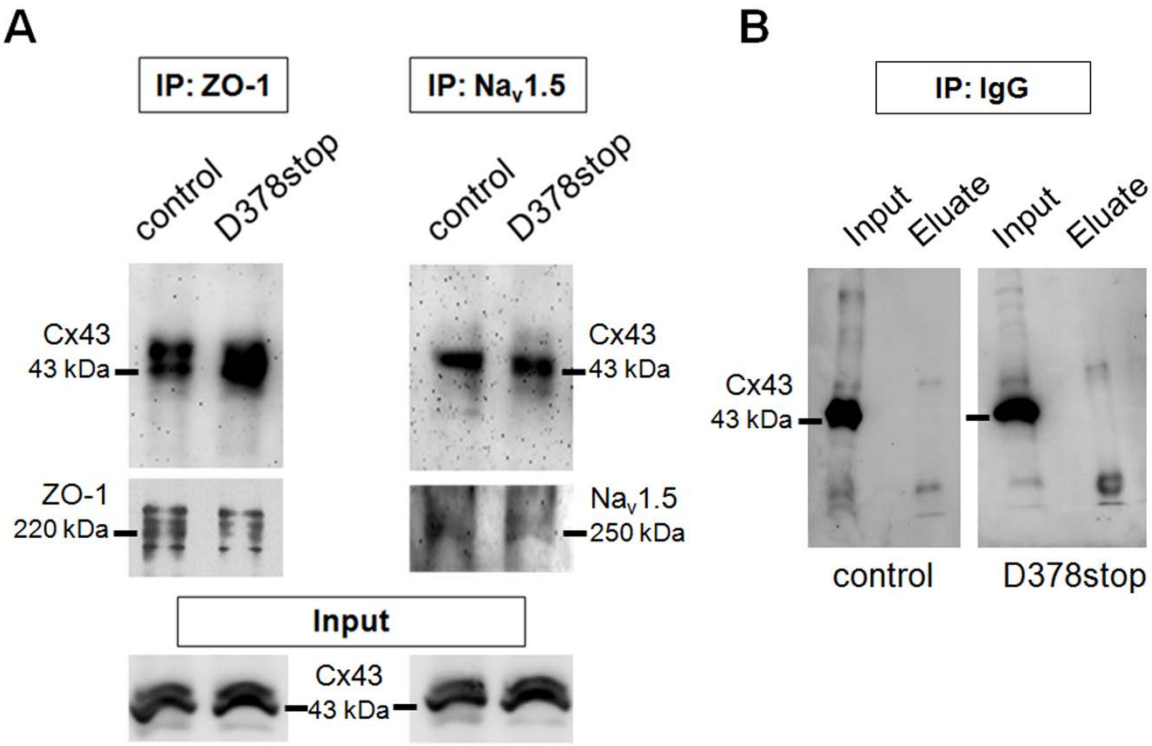
Suppl. Fig. 7


A
Neontal mice
ZO-1 expression [%]
120
100
80
60
40
20
0
control
D378stop
Nav1.5 expression [%]
120
100
80
60
40
20
0
control
D378stop
B
Adult mice
ZO-1 expression [%]
140
120
100
80
60
40
20
0
control
D378stop
Nav1.5 expression [%]
120
100
80
60
40
20
0
control
D378stop

Suppl. Fig. 8


A
Control
N-Cadherin
Phalloidin
B
Cx43D378stop
N-Cadherin
Phalloidin

Suppl. Fig. 9


A
IP: ZO-1
IP: Nav1.5
control
D378stop
control
D378stop
Cx43
43 kDa
ZO-1
220 kDa
Cx43
43 kDa
Nav1.5
250 kDa
Input
Cx43
43 kDa
B
IP: IgG
Input
Eluate
Input
Eluate
Cx43
43 kDa
control
D378stop

Suppl. Fig. 10

Suppl. Table 1 Parameters of heart rate, PQ interval and QRS amplitude of ECG recordings from fetal Cx43D378stop mice

	Heart rate (bpm)	**PQ interval (msec)**	**QRS amplitude (µV)**
Control	108 ± 13	94 ± 9	119 ± 40
Cx43D378stop	117 ± 22	107 ± 12	130 ± 75

Suppl. Table 2 Parameters of heart rate and PQ interval of ECG recordings from adult Cx43D378stop mice

	Heart rate (bpm)	**PQ interval (msec)**
Control	573 ± 23	40 ± 0.6
Cx43D378stop	558 ± 33	43 ± 4

Suppl. Table 3 Fitting parameters of K_v currents at 40 mV recorded from Cx43D378stop cells

	A_1	t_1	A_2	t_2	A_0
Control	24 ± 3.3	57 ± 3.0	11 ± 0.9	1108 ± 97	6 ± 0.5
Cx43D378stop	16 ± 1.2*	62 ± 4.6	11 ± 1.6	1049 ± 56	5 ± 0.6

*P < 0.05.

Suppl. Table 4 Parameters of action potentials recorded from Cx43D378stop cells

	APD90 (msec)	**APD70 (msec)**	**APD50 (msec)**	**RP (mV)**	**APA (mV)**
Control	25 ± 4.5	5 ± 0.3	2 ± 0.1	-76 ± 0.5	124 ± 2
Cx43D378stop	26 ± 3.1	6 ± 0.3*	3 ± 0.2*	-78 ± 1	127 ± 2

*P < 0.05. n = 9 for control and 10 for Cx43D378stop.