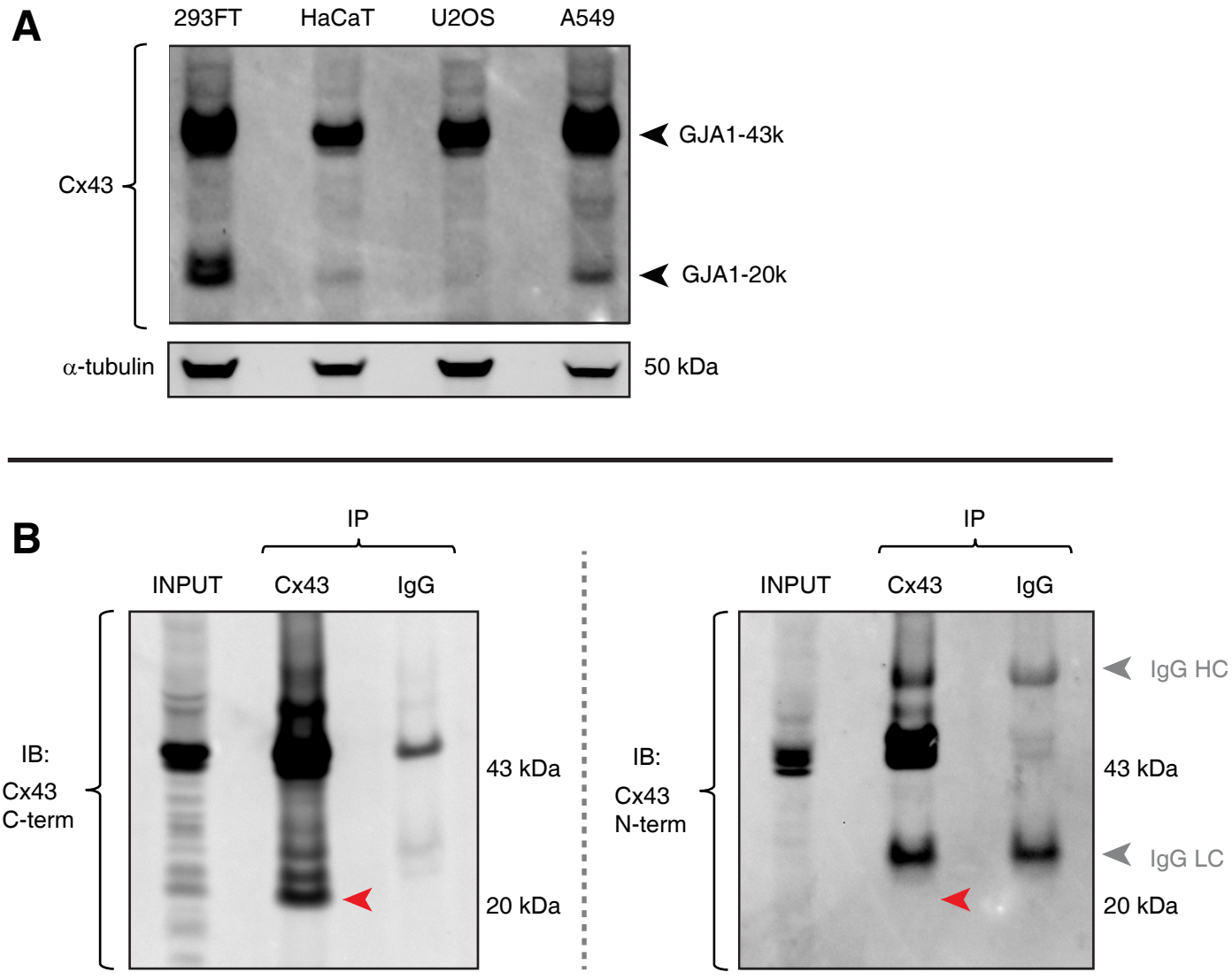

Figure S1. Truncated isoforms of connexin 43 correspond to N-terminally truncated polypeptides, related to Figure 1.

(**A**) Western blot of human cell lines probed for Cx43 and α-tubulin. (**B**) Immunoprecipitations of exogenously expressed Cx43 in 293T cells. Western blots probed with monoclonal antibodies against Cx43 C-terminus (left panel) or N-terminus (right panel). Red arrows: point of major GJA-20k isoform migration.

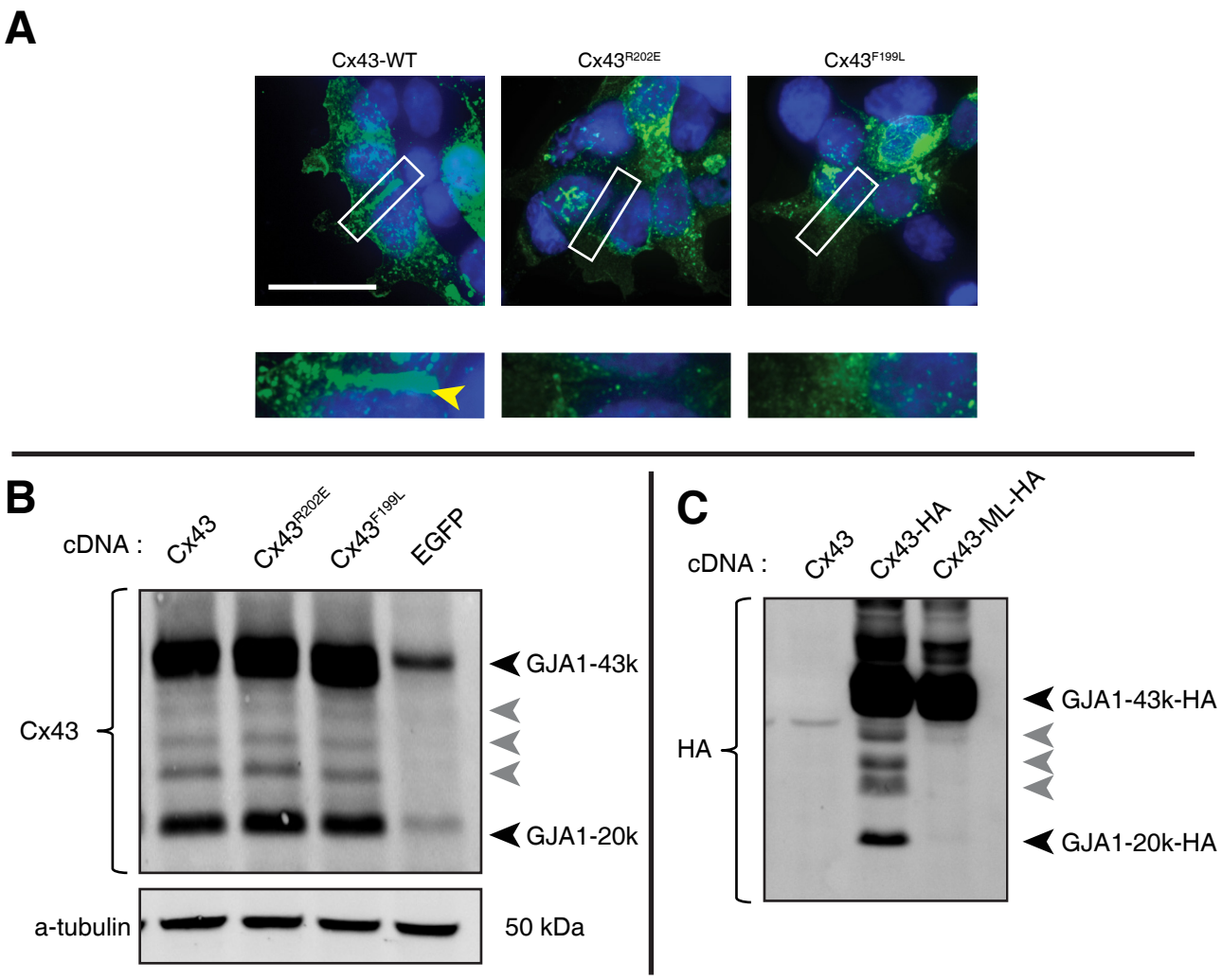

Figure S2. Truncated Cx43 isoform expression occurs in trafficking defective Cx43 mutants and mutation of all in frame internal methionine (AUG) codons ablates expression of truncated isoforms related to Figures 1 and 2.

(**A**) Fixed-cell immunofluorescence of 293T cells transfected with Cx43, Cx43^{R202E}, and Cx43^{F199L}. Original magnification x 100. Scale bar: 25 µm. (**B**) Western blot of transfections in (**A**). (**C**) Western blot of 293T cells transfected with Cx43 C-terminal HA fusion proteins. Cx43-ML-HA has all in frame methionine (AUG) codons mutated to leucine (CUG).

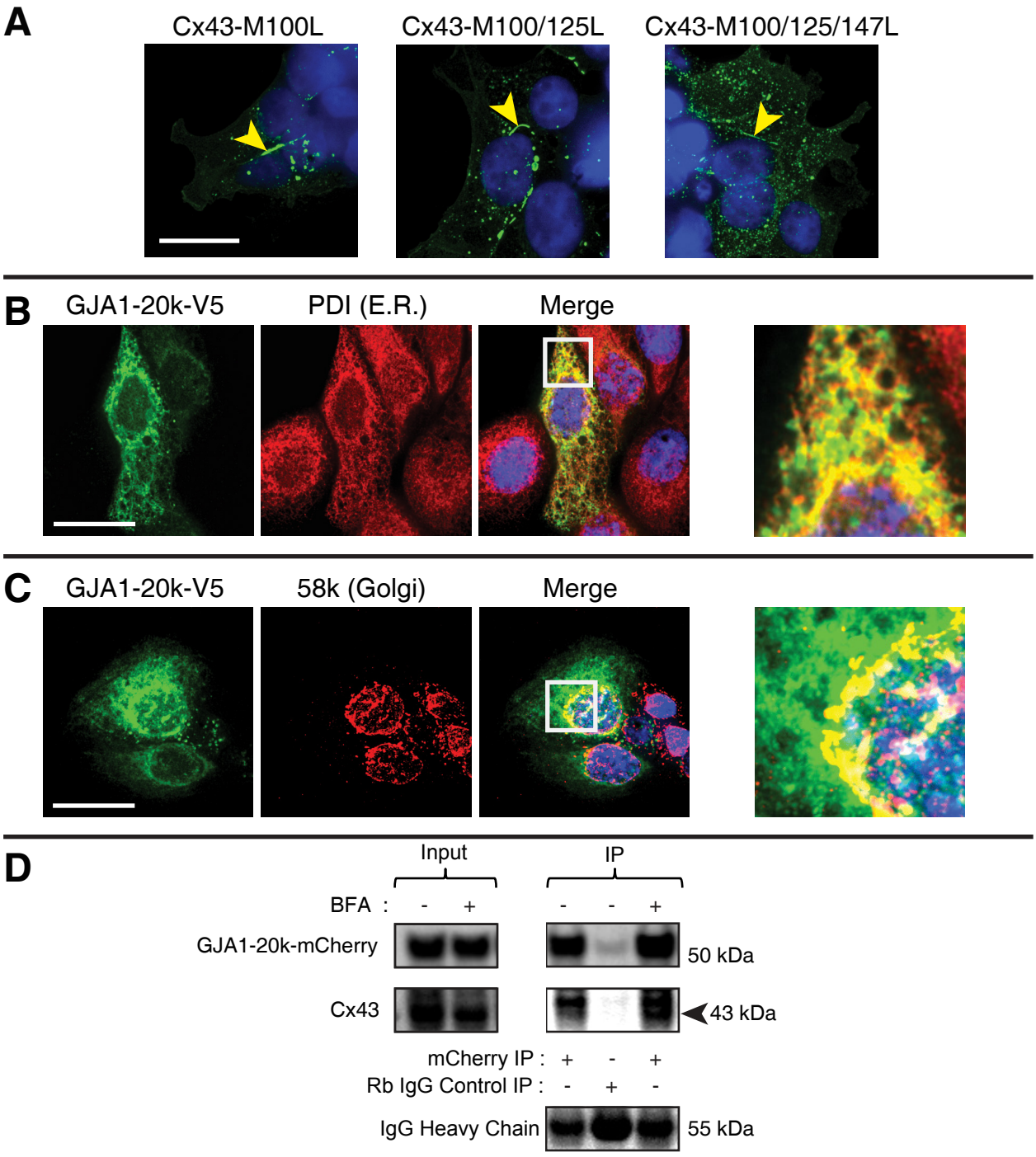

Figure S3. Truncated Cx43 isoforms localize to the ER/Golgi and are necessary for trafficking of full-length Cx43, related to Figure 3.

(**A**) Fixed-cell immunofluorescence of 293T cells expressing siRNA-resistant Cx43 (green) with knockdown of endogenous Cx43. Nuclei detected with DAPI (blue), arrow: gap junction plaque at cell-cell border. Cx43 constructs containing additive AUG to CUG mutations for internal methionines M100, M125, and M147. (**B**) 293T cells expressing GJA1-20k initiating at M213 as a V5 fusion protein GJA1-20k-V5 immunostained for V5 (green) and for ER marker PDI (red). (**C**) Fixed-cell immunofluorescence of 293T cells expressing GJA1-20k-V5 (green) and the Golgi apparatus resident 58K protein (red). (**D**) Co-immunoprecipitation of Cx43 GJA1-43k (arrow) with GJA1-20k-mCherry in 293T cells in the presence or absence of 2.5 μg/ml Brefeldin A (BFA). Original magnification of images x 100, scale bar: 25 μm.

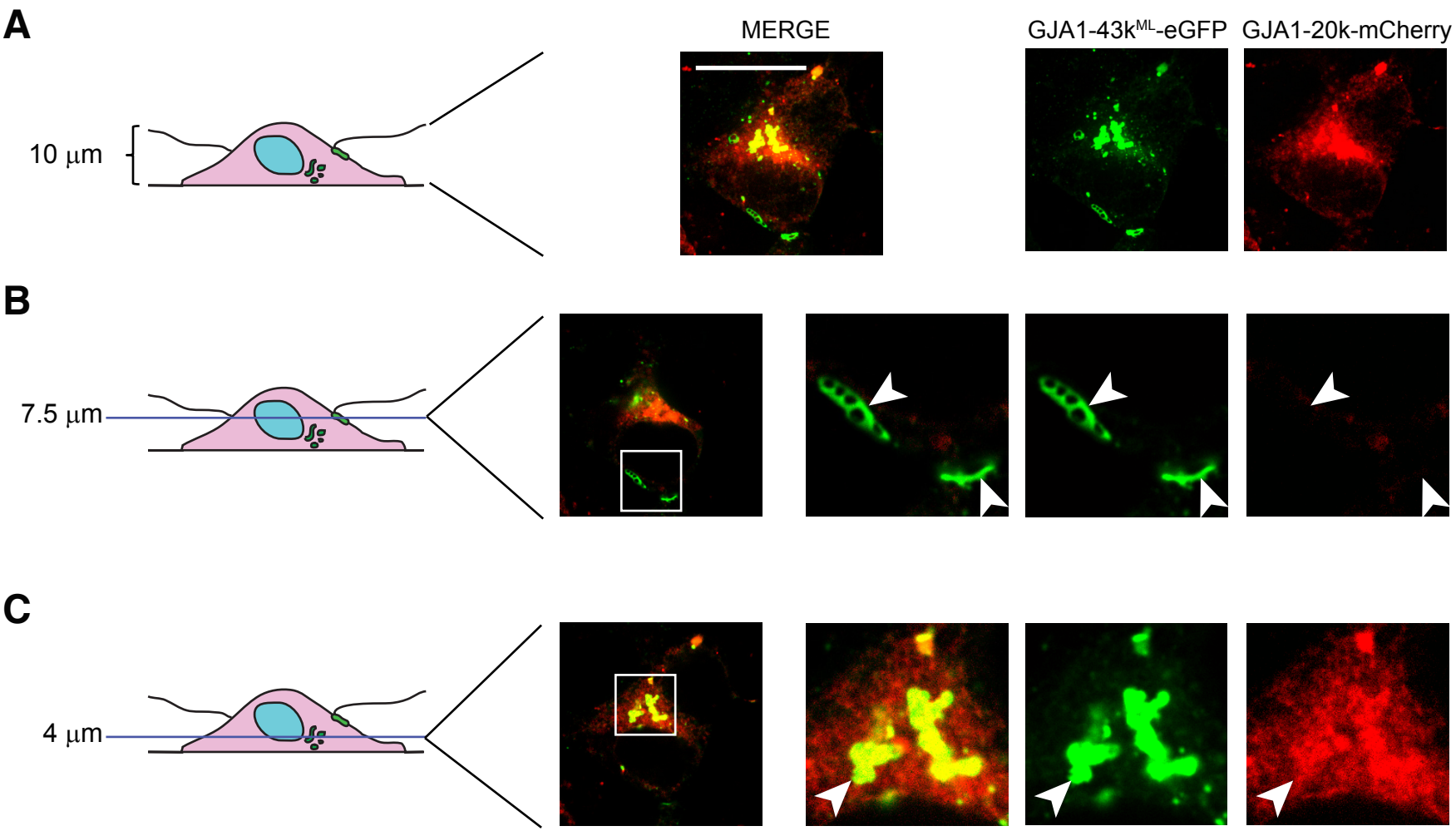

Figure S4. GJA1-20k colocalizes with GJA1-43k in the ER/Golgi but is not recruited to gap junction plaques, related to Figure 3.

Confocal imaging of 293T cells transfected with constructs encoding GJA1-43k^{ML}-eGFP (green) and GJA1-20k-mCherry (red). (**A**) Maximum intensity projection of entire 10 μm Z-stack, eGFP and mCHerry channels are merged on left with individual images displayed on right. (**B**) 0.5 μm Z-slice visualizing gap junction plaque at cell-cell border (arrows). (**C**) 0.5 μm Z-slice visualizing colocalization of GJA1-43k^{ML}-eGFP and GJA1-20k-mCherry (yellow) in the perinuclear Golgi compartment (arrows). Original magnification of images x 100, scale bar: 25 μm.

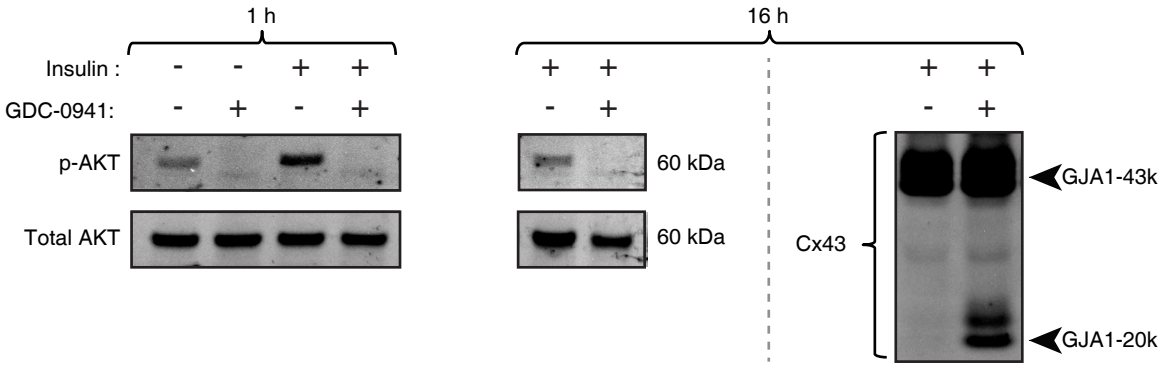

Figure S5. Expression of Cx43 truncated isoforms is regulated by the PI3K/AKT pathway

Primary neonatal mouse ventricular cardiomyocytes (NMVMs) cultured in the presence or absence of the PI3K inhibitor GDC-0941 for 1 h (left panels) and 16 h (right panels). Total AKT, p-AKT-Ser473, and Cx43 detected by Western blot.

Supplementary Information

Extended Experimental Procedures

Cell culture and transfection

All cell lines were obtained from ATCC with the exception of 293T (Life Technologies). Dulbecco's Modified Eagle medium supplemented with 10% FBS, non-essential amino acids, sodium pyruvate (Life Technologies) and Mycozap-CL (Lonza). Transient transfections were performed using Lipofectamine 2000 (Life Technologies) according to manufacturer's instructions and analyzed by biochemistry or immunofluorescence 24 h post transfection. For siRNA transfections cells were reverse transfected using RNAiMAX (Life Technologies) and siRNA duplexes at a final concentration of 2.5 nM. siRNA-resistant constructs were transfected 24 h post siRNA transfection.

Co-immunoprecipitation

293T cells co-transfected with Cx43 and GJA1-20k-mCherry were incubated with 2.5 μg/ml Brefeldin A or vehicle for 8 h. Cells were lysed in 05% Triton co-IP buffer (50 mM Hepes pH 7.4, 150 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM NaF, 100 μM Na3VO4, 0.5% Triton X-100) incubated at 4°C with rotation for 1 hour before centrifugationat 10,000 x *g* for 20 minutes to remove insoluble debris. Following protein normalization, co-immunoprecipitation was performed as previously described (Smyth *et al*., 2010) using rabbit anti-RFP (Abcam, 2 μg per reaction) or rabbit anti-GST (SantaCruz Biotechnology) as isotype control.

Oligonucleotides:

Gateway Cloning:

Human Cx43 Fwd:
GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGGGTGACTGGAGCGCC

Human Cx43 Rev:
GGGGACCACTTTGTACAAGAAAGCTGGGTACTAGATCTCCAGGTCATCAGGC

Human Cx43 nostop Rev:
GGGGACCACTTTGTACAAGAAAGCTGGGTAGATCTCCAGGTCATCAGGCCGA

GJA1-20k Fwd:
GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGCTGGTGGTGTCCTTG

siRNA sequences:

GJA1 siRNA i: GCGCCUUAGGCAAACUCCUUGACAA

GJA1 siRNA ii: GGGAGAUGAGCAGUCUGCCUUUCGU

GJA1 siRNA iii: GGGUUGCUGCGAACCUACAUCAUCA

Mutagenesis:

GJA1 siRNA II Resist:

GTTGAGTCAGCCTGGGG**C**GA**C**GA**A**CA**A**TC**C**GC**A**TT**C**CG**C**TGTAACACTCAGCAA

Cx43 M1I

AAAGCAGGCTTCACCAT**A**GGTGACTGGAGCGCC

Cx43 M100L

CATGTGTTCTATGTG**C**TGCGAAAGGAAGAGAAA

Cx43 M125L

GGTGTCAATGTGGAC**C**TGCACTTGAAGCAGATT

Cx43 M147L

CATGGTAAGGTGAAA**C**TGCGAGGGGGGTTGCTG

Cx43 M213L

ATCTTCATCATCTTC**C**TGCTGGTGGTGTCCTTG

Cx43 M281L

GCTCCCCTCTCGCCT**C**TGTCTCCTCCTGGGTAC

Cx43 M320L

GCAGAACAAAATCGA**C**TGGGGCAGGCGGGAAGC

Cx43 180 STOP

TACATCTATGGATTC**TAGTAATGA**GCTGTTTACACTTGC

References

Smyth, J., Hong, T., Gao, D., Vogan, J., Jensen, B., Fong, T., Simpson, P., Stainier, D., Chi, N., and Shaw, R. (2010). Limited forward trafficking of connexin 43 reduces cell-cell coupling in stressed human and mouse myocardium. The Journal of Clinical Investigation *120*, 266-279.