SUPPLEMENTARY FIGURES AND TABLES

Figure S1

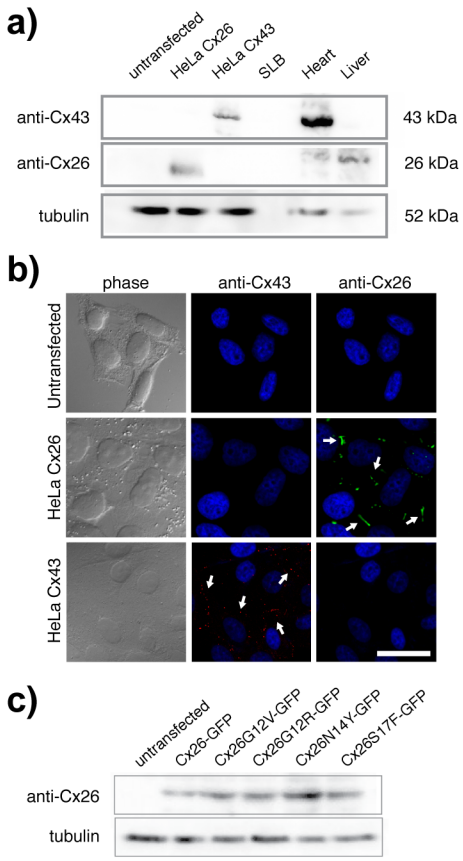

Figure S1: HeLa cells do not express Cx43 or Cx26. a) Western blot analysis was performed for Cx expression in HeLa cell lines untransfected or transfected with Cx26 or Cx43. Heart and liver samples were used as positive controls for Cx43 and Cx26 expression, respectively. Tubulin expression was used as gel loading control. ***b)*** Immunofluorescence analysis for Cx43 and Cx26 in HeLa cells untransfected or transfected with the respective Cxs. Bar= 30 μm. ***c)*** Immunoblot analysis of Cx26 expresssion from lysates of Hela cells expressing Cx26 or the N-terminus mutants.

Figure S2

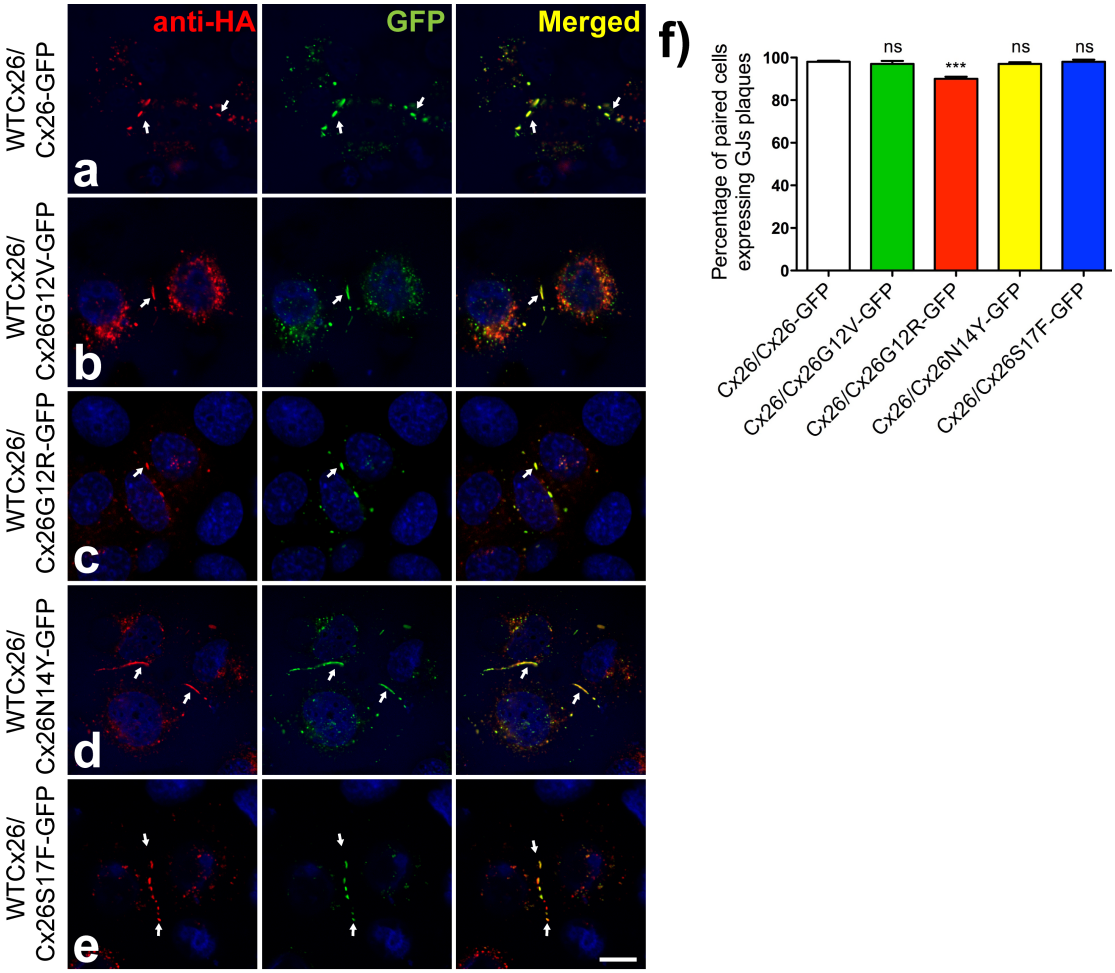

Figure S2: Co-expression of WT Cx26 with syndromic mutants recovers mutant expression in gap junction plaques. HeLa cells transfected with Cx26 were co-transfected with the respective Cx26 mutant. Arrows point to gap junction (GJ) plaques, and nuclei are stained with DAPI (blue). Bar= 30 μm. ***f)*** Graph depicted the quantification of the percentage of paired cells expressing GFP-labeled GJ plaques for each condition. The Student's t-test was used for unpaired data ($p<0.05$; n=5, 250 paired cells tested for each experiments).

Figure 3

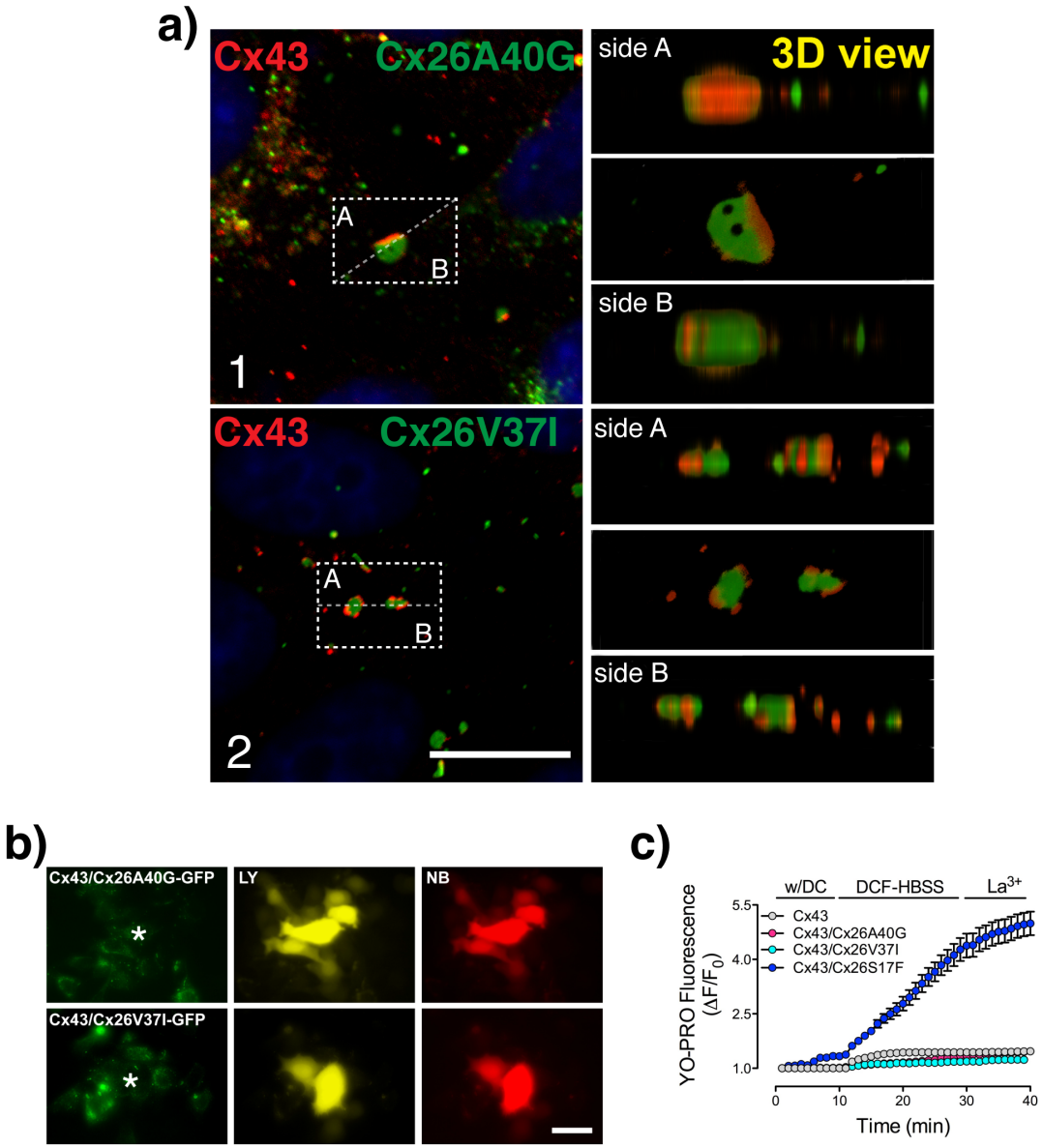

Figure S3: Non-syndromic mutants of the TM1/EL1 border do not interact with Cx43 nor do they affect the functional state of GJC and HC formed by Cx43. a) Confocal image of HeLa cells co-expressing Cx43 (red) with Cx26A40G-GFP or Cx26V37I-GFP. Dashed rectangles show regions of interest for 3D image projections of the GJ plaques. Right panels: different views of the same GJ plaques. Bar= 15 µM. ***b)*** Expression and sub-cellular localization of mutants when were co-transfected with Cx43. Asterisks indicate the cell that was microinjected with Lucifer Yellow (LY) and neurobiotin (NB). The tracers can diffuse to neighboring cells, indicating that GJCs are functional. ***c)*** The functional state of HCs determined in cells expressing Cx43 or co-expressing Cx43 with the respective mutant.

Figure S4

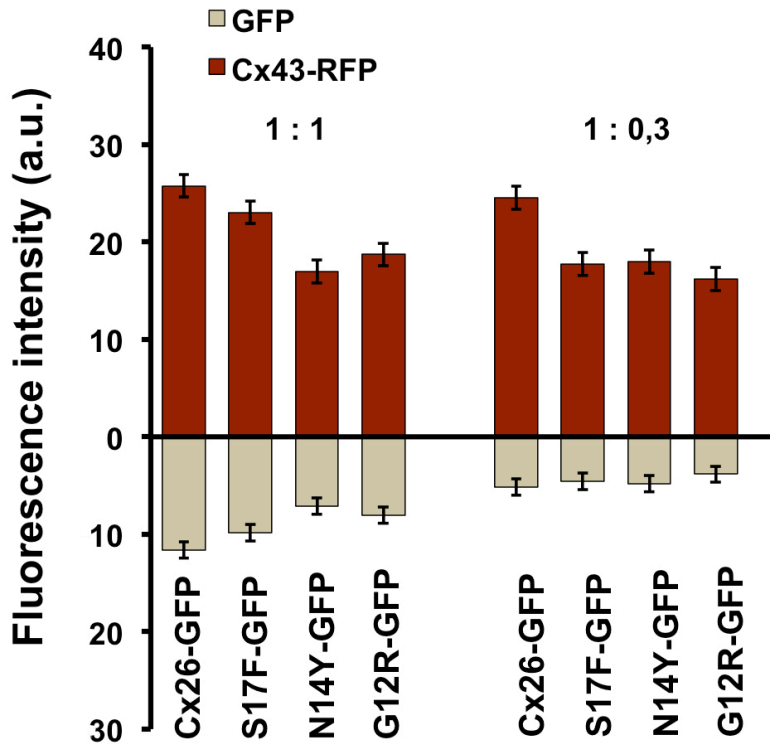

Figure S4: Different relative cellular expression of mutant Cx26-GFP co-transfected with Cx43-RFP at different plasmids ratios. Transient transfections were done using different ratios of plasmid cDNAs carrying Cx43-RFP and Cx26-GFP or its mutant variants (1:1, 1:0.3 ratios, respectively) and then quantified the fluorescence for each fluorescent protein. The graph depicted the comparison of the fluorescence intensity of the respective fluorescent protein. The reduction in the fluorescent intensity for Cx26-GFP or any of its mutant variants was clearly observed for the 1:0.3 ratio. These cells were used for dye coupling experiments shown in Table S3.

Figure S5

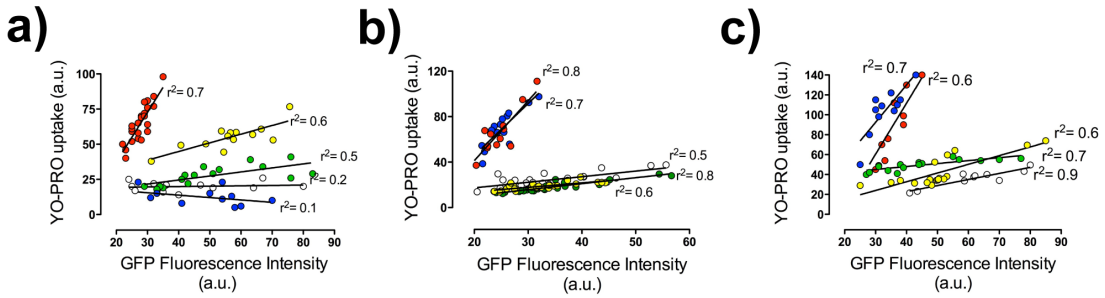

Figure S5: Relationship between levels of wild type or mutant Cx26 expression and the cellular uptake of YO-PRO under extracellular divalent cation free conditions. ***a)*** Activity of homomeric HCs related to the expression of Cx26-GFP or Cx26 deafness. Activity of heteromeric HCs formed by deafness mutants and Cx26 ***(b)*** or Cx43 ***(c)*** related to GFP expression. GFP fluorescent intensity at the beginning of the experiment was used as an estimation of corresponding protein levels for each condition. For each graph, every YO-PRO uptake point represents the fluorescent intensity observed 10 min after incubation in extracellular solution free of $Ca^{2+}$and Mg^{2+} (DCF-HBSS). Cx26GFP (white), Cx26G12V-GFP (green), Cx26G12R-GFP (red), 26N14Y-GFP (yellow), Cx26S17F (blue).

Figure S6

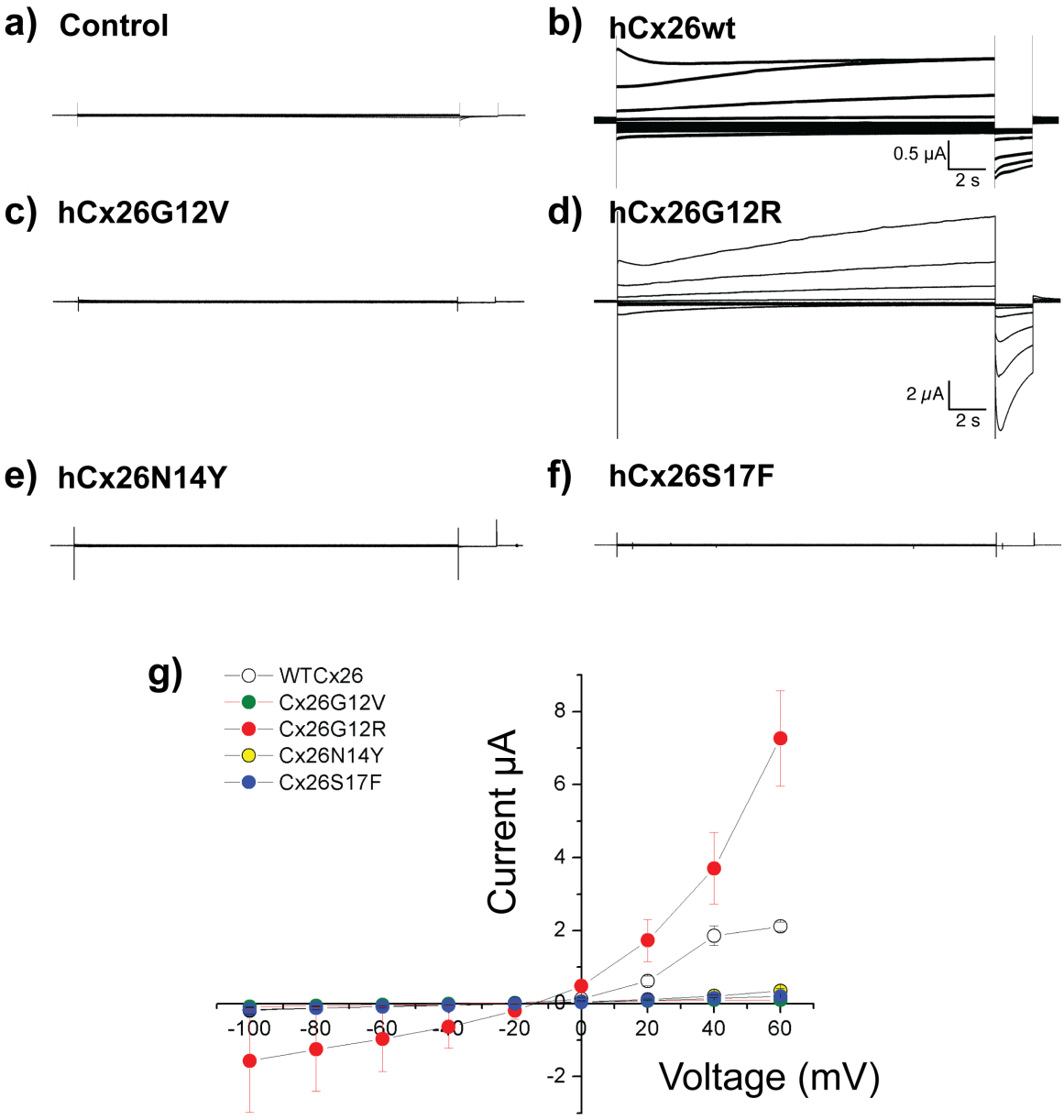

Figure S6: HCs currents in oocytes expressing WT Cx26 or syndromic mutant Cx26G12R: ***a-f)*** Current tracers developed in Oocytes that were injected the cRNA encoding for the respective Cx. All oocytes were injected with antisense nucleotide against endogenous Cx38. Traces show membrane currents recorded from oocytes in response to depolarizing voltage steps from a holding potential of -10 mV, and stepped in 20 mV increments from - 100 mV to + 60 mV. ***g)*** Graphic depicted the current voltage relationship of macroscopic HCs. The data represent mean ± SEM of at least ten independent experiments.

Figure S7

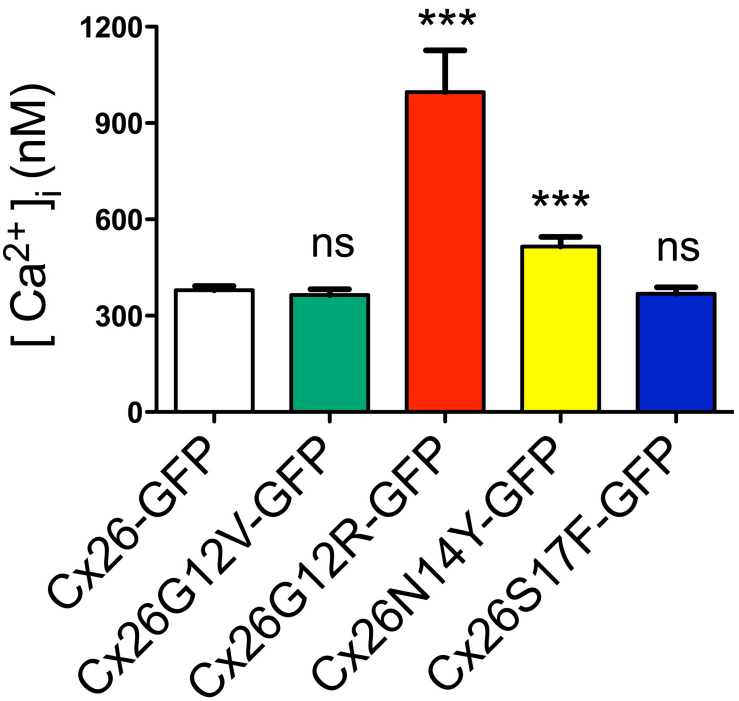

Figure S7: Cells expressing the syndromic mutants G12R and N14Y, but not S17F exhibit augmented levels of intracellular Ca^{2+} concentration: The graph depicted the intracellular Ca^{2+} levels determined using the ratiometric probe Indo-1 AM as described in methods. Cells expressing the non-syndromic mutant Cx26G12V and the syndromic Cx26S17F showed intracellular Ca^{2+} levels similar to cells expressing WT Cx26. However, intracellular Ca^{2+} level was higher in cells transfected with the syndromic mutant Cx26G12R, which almost triplicate the values obtained in cells transfected with WT Cx26; cells transfected with the mutant Cx26N14Y presented a moderate increment in intracellular Ca^{2+} concentration. Data is presented as average ± SEM (n=20 cells for each condition); *** $p<0.001$

Table S1

Effect of Cx26 deafness-related mutations on gap junction channel function and their effects on interactions between Cx26 mutants and wild type Cxs

Cell lines and Cx type expression	Incidence of coupling (n=total number of microinjected cell pairs)		Double patch clamp Conductance nS (Mean ± SEM)
	Lucifer yellow	Neurobiotin	
HeLa Parental	0 (12)	0 (12)	0.34 ± 0.13
Cx26-GFP	100 (35)	100 (35)	3.70 ± 0.77
Cx26G12V-GFP	0 (17)	0 (17)	0.24 ± 0.14
Cx26G12R-GFP	0 (17)	0 (17)	0.04 ± 001
Cx26N14Y-GFP	0 (25)	0 (25)	0.31 ± 0.77
Cx26S17F-GFP	0 (32)	0 (32)	0.74 ± 0.77
wtCx26/Cx26-GFP	100 (15)	100 (15)	ND
wtCx26/Cx26G12V-GFP	100 (12)	100 (12)	ND
wtCx26/Cx26G12R-GFP	100 (15)	100 (15)	ND
wtCx26/Cx26N14Y-GFP	15 (7)	100 (7)	ND
wtCx26/Cx26S17F-GFP	35 (22)	100 (22)	ND
wtCx43	100 (16)	100 (16)	ND
wtCx43/Cx26-GFP	100 (12)	100 (12)	ND
wtCx43/Cx26G12V-GFP	100 (24)	100 (24)	ND
wtCx43/Cx26G12R-GFP	0 (15)	0 (15)	ND
wtCx43/Cx26N14Y-GFP	0 (23)	0 (23)	ND
wtCx43/Cx26S17F-GFP	0 (30)	0 (30)	ND

Table S2

Summary of the primers used to generate the N-terminus mutants of Cx26

CONSTRUCT	PRIMERS	SEQUENCE
Cx26G12V	forward	5´-GAT CCT GGG GGT TGT GAA CAA ACA-3
	reverse	5-GTG TTT GTT CAC AAC CCC CAG GAT-3´
Cx26G12R	forward	5´-GAT CCT GGG GAG AGT GAA CAA ACA C -3´
	reverse	5´-GTG TTT GTT CAC TCT CCC CAG ATC-3’
Cx26N14Y	forward	5- AGC ATC CTC GGG GGT GTC TAC AAG CAC TCC ACCAGC ATT – 3´
	reverse	5`-AAT GCTGGTGGA GTGCTTGTA GACACCCCC GAGGATGCT– 3`
Cx26S17F	forward	5'-GAA CAA ACA CTT CAC CAG CAT TGG-3'
	reverse	5'-CCA ATG CTG GTG AAG TGT TTG TTC-3'

Table S3

Summary of dye coupling experiments in cells transfected with different ratios of Cx43-RFP / Cx26-GFP WT or it syndromic mutants variants

Cxs expression Cx43-RFP : Cx26-GFP WT or mutant	Incidence of coupling (n=total number of microinjected cell pairs)	
	Lucifer yellow	Neurobiotin
Ratio 1 : 1		
Cx43-RFP	100 (10)	100 (10)
Cx43-RFP/Cx26-GFP	100 (10)	100 (10)
Cx43-RFP /Cx26G12R-GFP	0 (10)	0 (10)
Cx43-RFP /Cx26N14Y-GFP	0 (10)	0 (10)
Cx43-RFP /Cx26S17F-GFP	0 (10)	0 (10)
Ratio 1 : 0,3		
Cx43-RFP/Cx26-GFP	100 (10)	100 (10)
Cx43-RFP /Cx26G12R-GFP	0 (11)	0 (11)
Cx43-RFP/Cx26N14Y-GFP	0 (8)	0 (8)
Cx43-RFP /Cx26S17F-GFP	0 (13)	0 (13)

Table S4

Summary of the effect of different mutation linked to non-syndromic or syndromic deafness, respect to oligomerization and GJCs and HCs activity in the homomeric and heteromeric conformation and their link to the skin phenotype in KID syndrome. Some results for non-syndromic mutations V37I and A40G were published before (Jara et al., 2012)

	Homomeric			With wild type Cx26			With wild type Cx43			
Connexin type	Oligomerization	GJC functional state	HC functional state in normal extracellular Ca^{2+}	Oligomerization	GJC functional state	HC functional state in normal extracellular Ca^{2+}	Oligomerization	GJC functional state	HC functional state in normal extracellular Ca^{2+}	Skin Phenotype
Cx26-GFP	+	+	-	+	+	-	-	+	-	-
Cx26A40G	+/-	-	-	+	-	-	-	+	-	-
Cx26V37I	+/-	+/-	-	+	+/-	-	-	+	-	-
Cx26G12V	+	-	-	+	+	-	-	+	-	-
Cx26G12R	+	-	+++	+	+	+++	+	-	+++	+
Cx26N14Y	+/-	-	+	+	+/-	-	+	-	+++	+
Cx26S17F	+/-	-	-	+	+/-	+++	+	-	+++	+

+, indicates relative normal condition; +/-, indicates partial or deficient condition; +++, indicates hyperactive condition; -, indicates completely deficient or null condition