The Connexin31 F137L mutant mouse as a model for the human skin disease *Erythrokeratodermia variabilis* (EKV)

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*Erythrokeratodermia variabilis* (EKV) is a rare autosomal dominant human genodermatosis. Its clinical appearance varies from transient, fast moving erythemas to persistent brown hyperkeratoses. So far, several mutations in the Cx31 or Cx30.3 gene have been reported to cause EKV in humans. We have generated a conditional mouse mutant that carries the human F137L mutation in the Cx31 gene which was described to act in a transdominant negative manner. The phenylalanine residue at position 137 is highly conserved in several human and mouse connexin genes. Mouse embryonic stem (ES) cells expressing one allele of the Cx31F137L mutation were stable but showed ~30% decreased transfer of neurobiotin. This is probably due to dominant negative effects of the Cx31F137L protein on wild type Cx31 and Cx43 protein expressed in ES cells. Surprisingly, the healing process of tail incision wounds in Cx31¹/F137L mice was shortened by 1 day, i.e. very similar as previously reported for mice with decreased expression of Cx43 in the epidermis. This suggests again that Cx31 and Cx43 proteins functionally interact, possibly by forming heteromeric channels in the epidermis. Heterozygous Cx31¹+/F137L mice are viable and fertile, in contrast to homozygous Cx31¹/F137L/F137L mice that die around ED 7.5. In Cx31¹+/F137L mice, the epidermal expression pattern and level of Cx26, Cx30, Cx30.3 and Cx43 proteins were not altered compared with wild-type mice. No erythemas were detected in young C31¹+/F137L mice before 2 weeks of age. In contrast to human EKV patients, hyperproliferation of the stratum germinativum was found in only 5% of the analyzed skin area.

INTRODUCTION

Connexins (Cx) are subunit proteins of gap junction channels which allow the intercellular diffusion of ions and metabolites below a molecular mass of about 1.500 Dalton. Two hemichannels, also called connexons, in contacting plasma membranes can dock to each other to form a gap junction channel. In heterotypic gap junction channels, each hemichannel is composed of a different connexin isoform. Heteromeric hemichannels express more than one type of connexins (1). Different connexin channels can have distinct molecular permeabilities (2).

During recent years, several human genodermatoses have been shown to be caused by mutations in Cx26, Cx30, Cx30.3 or Cx31 (3). Among these inherited diseases are *Erythrokeratodermia variabilis* (EKV) which is characterized by an unspecific appearance of transient erythemas and locally defined or general hyperkeratosis. Sometimes, one of these features prevails or is completely absent. The variability of the erythematous patches regarding number, size and shape is indicated by the name of the disease. So far, connexin mutations in EKV patients were described in 18 unrelated families, mainly from northern Europe. Seven mutations in the Cx31 gene and five mutations in the Cx30.3 gene were found to cause EKV. In one case, the appearance of EKV could not be correlated with mutations in connexin genes (4). The clinical symptoms caused by these different connexin mutations are generally not distinguishable. All mutations alter amino acid residues highly conserved in the beta sub-group of connexins, but up to now only mono-allelic mutations have been found. Dominant and transdominant...
negative effects were discussed with Cx30.3 and Cx31 (5,6). A recessive variant of EKV was also reported (7).

The F137L mutation was found both in the Cx31 and the Cx30.3 gene of EKV patients. This mutation was caused by a single point mutation of T to C at position 409 which led to an altered base triplet coding for leucine (L) instead of phenylalanine (F). The phenylalanine residue at position 137 is highly conserved throughout different connexins and different species. Patients suffering from EKV caused by the Cx31F137L mutation showed more pronounced phenotypic alterations of the skin (6).

Expression of the Cx31F137L mutated form in HeLa cells led to the death of transfected cells (8). In contrast, no effect of the Cx30.3F137L mutation was found after transfection, although this mutation was also discussed to have transdominant negative effects on Cx31 (5,8). Cx31 and Cx30.3 proteins can build up heteromeric hemichannels which were shown to be more stable and functional more efficient in dye coupling than corresponding homomeric channels (5).

Previously, we had found that Cx31 deficient embryos showed a reduced survival probability in utero associated with placental dysmorphogenesis but adult Cx31 deficient mice did not exhibit any epidermal abnormalities (9). In order to investigate the effects of the Cx31F137L mutation in living animals, we generated transgenic mice that express the Cx31F137L mutation. The mutation was generated by overlapping PCRs and the mutated Cx31 DNA was inserted into a targeting vector which contained a 5′- (about 2 kb) and a 3′-untranslated region (about 5 kb) of the Cx31 locus flanking the endogenous Cx31 coding region (CR), a Neomycin (Neo) selection cassette and the mutated Cx31 coding region (F137L). Since the Neo cassette was flanked by frt sites, it could be removed by Flp recombinase activity. The wild-type Cx31 coding region and the frt flanked Neo cassette were enclosed by loxP sites and thus could be deleted by Cre recombinase activity. Thus, our cloning strategies allowed conditional expression of the Cx31F137L mutation if the corresponding embryos turned out to be lethal.

RESULTS

Figure 1A illustrates the location of the F137L mutation in the third transmembrane region of the Cx31 protein. This mutation, which was identified in a human patient suffering from EKV, was inserted into the corresponding mouse Cx31 gene. Mouse and human Cx31 coding DNAs show 83% sequence identity. As shown in Figure 1B, the phenylalanine residue at position 137 is highly conserved among several connexins of the beta subgroup.

Figure 2A illustrates schematically the construction of the targeting vector and the recombinant Cx31 locus after introduction of the Cx31F137L mutation. The mutation was generated by overlapping PCRs and the mutated Cx31 DNA was inserted into a targeting vector which contained a 5′- (about 2 kb) and a 3′-untranslated region (about 5 kb) of the Cx31 locus flanking the endogenous Cx31 coding region (CR), a Neomycin (Neo) selection cassette and the mutated Cx31 coding region (F137L). Since the Neo cassette was flanked by frt sites, it could be removed by Flp recombinase activity. The wild-type Cx31 coding region and the frt flanked Neo cassette were enclosed by loxP sites and thus could be deleted by Cre recombinase activity. Thus, our cloning strategies allowed conditional expression of the Cx31F137L mutation if the corresponding embryos turned out to be lethal.

Using the targeting vector for electroporation of mouse embryonic stem (ES) cells (11), recombinant clones were isolated at a frequency of ~15%. ES cells from these clones were...
We chose the human Cx31F137L mutation to introduce it into the Cx31 gene of a transgenic mouse, in order to generate a mouse model for the human genodermatosis EKV. The same point mutation had been found in the Cx30.3 gene of a patient who also suffered from EKV. In order to explain the autosomal dominant inheritance of these mutations, it had been suggested that the mutated Cx31 protein (or the

Table 1. Genotype frequencies of born mice. Wild type and Cx31+/F137L animals were born at the expected Mendelian ratios, but no Cx31/F137/F137L mice were found.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>+/+</th>
<th>+/F137L</th>
<th>F137L/F137L</th>
</tr>
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<tbody>
<tr>
<td>Number of animals</td>
<td>35</td>
<td>69</td>
<td>0</td>
</tr>
<tr>
<td>Expected ratio</td>
<td>1/4</td>
<td>2/4</td>
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DISCUSSION

We chose the human Cx31F137L mutation to introduce it into the Cx31 gene of a transgenic mouse, in order to generate a mouse model for the human genodermatosis EKV. The same point mutation had been found in the Cx30.3 gene of a patient who also suffered from EKV. In order to explain the autosomal dominant inheritance of these mutations, it had been suggested that the mutated Cx31 protein (or the
mutated Cx30.3 protein) interacts with and functionally inhibits wild type Cx31 and/or Cx43 protein (6). When human Cx31 EKV mutations were attempted to be expressed in HeLa cells, no transfectants carrying the point mutation could be isolated (12,14). It was suggested that the mutated Cx31 protein can form hemichannels in transfected HeLa cells, leading to extracellular leakage of ions and metabolites and finally to lysis of the affected cells. Obviously, this result contrasted to the situation in the skin of EKV patients. By generating a mouse model of EKV, we wanted to study the Cx31F137L mutation in ES cells and in epidermal keratinocytes of transgenic mice.

Our results with heteromeric mouse ES cells, which carry one allele of the Cx31F137L mutation, indicated that the cells are viable under cell culture conditions. They can contribute to the germ line when introduced via manipulated blastocysts into transgenic mice. Perhaps other connexins (i.e. Cx31, Cx43 and Cx45), which are also expressed in ES cells, may prevent the lytic effect of the single Cx31F137L allele found in HeLa cells. All HeLa cells were transfected with vectors containing strong viral promoters (i.e. Cytomegalovirus or Simian Virus 40) so that the expression level might have been higher than with the corresponding endogenous Cx31 promoter. Interestingly, Cx31+/+ F137L ES cells show ~30% less neurobiotin transfer to neighboring cells than control ES cells (i.e. Cx31+/lox).

No difference in ATP release, indicating hemichannel activity, could be observed between the wild-type ES cells or cells heterozygously expressing Cx31F137L besides Cx43 and Cx45. Thus, we think it unlikely that phenotypic abnormalities observed in Cx31F137L expressing mice can be explained by altered hemichannel activity. We suggest that the Cx31F137L mutated protein likely interacts with wild-type Cx31 and Cx43 protein, probably leading to closed
homomeric and heteromeric channels, respectively. Alternatively, a trafficking defect of Cx31F137L containing connexons appears possible (12,14) which could lead to the observed decrease in neurobiotin transfer. This conclusion is in accordance with our observations that Cx31+/F137L ES cells do not show increased secretion of ATP (through hemichannels) in Ca2+ free extracellular medium relative to wild-type ES cells (Supplementary Material, Figure S1).

Previously, Elfgang et al. (15) had reported that mouse Cx31 did not form heterotypic gap junction channels with Cx43 when expressed in transfected HeLa cells. However, the formation of heteromeric channels was not investigated in this study. Plantard et al. (5) have demonstrated that Cx31 and Cx30.3 protein can stabilize each other in heteromeric channels of HeLa cell transfectants. In order to exclude that wild-type Cx31 protein can stabilize the Cx31F137L protein, we raised Cx31+/F137L mice. These mice were viable and fertile. Interestingly, homozygous Cx31F137L/F137L mice died around ED 7.5. Since Cx31+/F137L mice survived to adulthood, it cannot be the wild-type Cx31 protein that stabilizes the Cx31F137L allele, but presumably other connexin isoform(s) which are expressed in the epidermis. The molecular reasons for the early embryonic death of Cx31F137L/F137L mice are not known. We speculate that the increased dosage of the mutated protein in homozygous Cx31F137L/F137L embryos may lead to more extensive functional inhibition of heteromeric channels.

Figure 6. Closure of tail wounds in Cx31+/F137L mice (A), in comparison to wild-type Cx31+/+ mice (B). On day 3, the wound area in Cx31+/F137L skin was already multilayered with punctate Cx31 immunosignals in the reformed epidermis. In contrast, the stratification of the wounded epidermis in wild-type skin had not yet occurred on day 3, and Cx31 immunosignals were sparse. In Cx31+/F137L mice, wound healing occurred about 1 day faster, i.e. on day 3 it was equivalent to Cx31+/+ wounds on day 4 after wounding. Nuclei and coagulum in the wound were stained with propidium iodide in red. Bar: 20 μm.

Figure 7. Hyperproliferation of keratinocytes was observed in mutated (Cx31+/F137L) tail skin (A), but not in wild-type skin (B). The stratum germinativum was extended in ~5% of tail skin areas in Cx31+/F137L mice. Micrographs show immunofluorescence analyses using anti-Cx31 (green) and propidium iodide staining of nuclei (red). Bar: 20 μm.
Cx31F137L containing heteromeric gap junction channels is affected, but not their intracellular transport and localization or that of connexons composed of other epidermal connexins (16).

Previously, a transgenic mouse line had been described (17) that expressed a mutant connexin26 (D66H). The corresponding mutation in the human Cx26 gene causes a dominant genodermatosis, the Vohwinkel syndrome. This disease and the phenotype of the transgenic mouse model are characterized by thickening of the epidermal cornified layers and accumulation of the mutated Cx26 protein together with wild-type Cx30 protein in the cytoplasm of keratinocytes. Furthermore, increased apoptosis was found in suprabasal keratinocytes of the mouse mutant. In Cx31+/F137L mice of this study, no generalized thickening of the stratum corneum was seen, although this is commonly found in patients suffering from erythrodermatoma variabilis. The Cx31+/F137L mouse mutant should be genetically very similar as the corresponding human patients, since the transgene is expressed at the homologous genomic position from the endogenous Cx31 promoter, like the Cx31+/−/F137L mutation in patients. Thus, the relatively weak keratodermic abnormalities in Cx31+/F137L mice may reflect a difference in the function of the Cx31 protein in humans and mice. Furthermore, it is possible that the Cx31+/F137L mutation shows stronger keratodermic effects in other mouse strains.

Surprisingly, we found that the healing process of incision wounds in tail skin was shortened by 1 day in Cx31+/F137L mice when compared with wild-type mice. This result is reminiscent to recent findings that induced downregulation of Cx43 (10) or decreased expression of Cx43 due to localized application of antisense RNA to the skin of wild-type mice (18) resulted in a premature closure of the epidermal wound, compared to non-treated control mice. Our data suggest that the interaction of Cx31 and Cx43 may lead to this effect. Kretz et al. (10) showed that downregulation of the Cx31 protein occurred at about the same time as the decreased Cx43 protein levels, i.e. 1 day after wounding. The transdominant Cx31F137L mutation may have the same inhibitory effect on wild-type Cx31 as downregulation of Cx43 by induced ablation (10). None of the other epidermal connexins was altered in its expression level in Cx31+/F137L mice (Fig. 5). Dye transfer in the epidermis of Cx31−/− mice was hardly altered, in contrast to Cx43 ablated mouse epidermis where it was strongly reduced (10). Which of the different steps of wound healing is affected by transdominant interaction of Cx31 and Cx43 or by the decrease of Cx43 in mouse epidermis needs to be clarified by further investigations.

MATERIALS AND METHODS

Construction of the Cx31F137L targeting vector

The blunt-end EcoRI–BamHI fragment (2202 bp) from the mouse Cx31 gene locus (19, Fig. 1) was inserted into the NotI restriction site of the vector pBSK:lox–HPRT-lox (5693 bp), (20, modified in our laboratory) resulting in vector A1 (7905 bp). Then, another BamHI-Hind III fragment (2369 bp) from the Cx31 gene locus (19, Fig. 1) was cloned into pBluescript (2961 bps, Stratagene, La Jolla, USA) at the identical restriction sites. This vector B1 was cut by SalI, followed by insertion of a SalI fragment from the vector pBSK:frt–neo–frt (4796 bp; 13), containing the frt flanked neomycin resistance cassette, in order to generate vector B2 (7214 bp). Cutting out the HPRT minigene of vector A with EcoRI and inserting a NotI–Xhol fragment of vector B2 led to vector AB which now contained the 5′-homologous region, the wild-type Cx31 coding region and the frt-flanked Neomycin resistance cassette flanked by loxP sites. The BamHI-Hyal F137L fragment (2677 bp), generated via PCR using DNA of the Cx31 gene locus (19, Fig. 1), contained the Cx31 coding region with the point mutation T to C leading to the amino acid shift of F to L. For construction of the mutated coding region, we used the following PCR using primers Eco_mCx31_F (5′-G GAA TTC GGC ACC ATG GAC TGG TGG AAG AAG CTC-3′) and mCx31F137LP2 (5′-GAT GAG CTT GAG GTG AAT GGT CAG GCT GGG CGC TGA-3′) and mCx31F137LP3 (5′-AGC CTC ATC CTC AAG CTC ATC-3′). The two PCR fragments were fused together via a PCR using primers Eco_mCx31_F (5′-G GAA TTC GGC ACC ATG GAC TGG TGG AAG AAG CTC-3′) and Xba_mCx31_R (5′-TGC TCT AGA AAT GGG GGT CAG GCT GGG CGC TGA-3′) and Xba_mCx31_R (5′-TGC TCT AGA AAT GGG CAG GCT GGG CGC TGA-3′). Conditions for all three PCRs were: 5 min at 94°C, 30 cycles of 1 min at 94°C, 1 min at 55°C, 1 min at 72°C, followed by 10 min at 72°C. PCRs were carried out in 25 μl Taq buffer, containing 1.5 mM MgCl2, 1.75 units of Taq polymerase (Promega, Germany), 0.2 mM dNTPs and 20 pmol of each primer. After the PCR reaction, the resulting fragment was digested with BamHI as well as Xbal and cloned into the pBSK vector (2961 bp, Stratagene) which was cut with the same enzymes, resulting in vector C1 (5626 bp). DNA of the Gjb3 locus (9, Fig. 1) was cut with Xbal and the resulting fragment (4075 bp) was used as 3′-homologous region, inserted into Xbal-cut C1 DNA, resulting in vector C2 (9701 bp). The vector AB was digested with Xhol, the vector C2 with ClaI and NotI. Afterwards both fragments were blunted and ligated to the final Cx31F137L targeting vector (16394 bp).

Screening of ES cell clones

ES cell culture, transfection and analyses were performed as described (11,21). G418 resistant ES cell clones were screened for homologous recombination by PCR and were subsequently confirmed by Southern blot hybridization.

The PCR with extracts from Cx31F137L ES cells indicated homologous recombination using the primers Cx31_hom_For3 (5′-CGA AGA TTC TGG CAG TAA TCC A-3′) and Cx31_hom_Rev (5′-TAG TTC TAG AGC GCC CAA TTC-3′). PCR conditions were: 3 min at 94°C, 35 cycles of 1 min at 94°C, 3 min at 66°C, 1 min at 72°C, followed by 10 min at 72°C. PCR was carried out in 50 μl RedTaq-Mix (Sigma, Germany), containing 50 pmol of each primer and 25 μl water.

The Cx31F137L positive ES cell clones were further analyzed by Southern blot analysis of DNA from lysates of about 15 million cells. Digestion of the PCR amplicon was performed by NdeI and AgeI. The probes were generated via PCR using the vector Gx2 (19) for the internal and for the
external probe. The internal probe was generated using the
primers probe_int_for (5'-CCA CGT GGT TGC TAG TAT
TGG-3') and probe_int_rev (5'-GAG AAT CTG ATA ATC
AAA CGA C-3'), the external probe using primers pro-
be_ext_for (5'-GTT GTC TCT AGA TCT ATA ACC-3')
and probe_ext_rev (5'-GTT TTC TAG AAT GAA TGA
AGA C-3'). PCR conditions were: 5 min at 94°C, 30 cycles
of 1 min at 94°C, 1 min at 55°C, 1 min at 72°C, followed
by 10 min at 72°C. PCR was carried out in 25 µl Taq buffer,
containing 1.5 mM MgCl₂, 1.75 units of Taq polymerase
(Promega, Germany), 0.2 mM dNTPs and 20 pmol of each
primer.

Characterization of Cx31+/floxF137L ES cell clones
The homologously recombined ES cells were cultivated on
gelatinized cell culture dishes (Falcon, USA) and the transient
transfection with the vector pCre-Pac was carried out by elec-
troporation as described by Eckardt et al. (22).

Neurobiotin (N-2 [aminoethyl]-biotinamide hydrochloride;
Vector Laboratories, Burlingame, USA) was iontophoresically
applied for 2–4 s in 0.1 M Tris buffer, pH 7.6, using positive
current of 20 nA (Ionophoresis Programmer model 160, World
precision instruments inc., New Haven, USA). During injec-
tion, the cell culture dishes were kept on a heated block at
37°C. Five minutes after injection, cells were washed twice
with PBS, fixed for 10 min in paraformaldehyde (4%)/0.2%
picrinic acid in sodium phosphate buffer (0.15 mM, pH 7.4),
washed twice with PBS, incubated in 0.4% Triton X-100,
washed with PBS, incubated with horseradish peroxidase-
avidin D (Vector Laboratories) for 2 h, washed with PBS,
icubated in 0.05% diaminobenzidine, 0.003% hydrogen
peroxide solution for 15 min and examined using an inverted
microscope (IM35, Zeiss, Oberkochem, Germany).

No difference in morphology was noticed during each series
of microinjections with neurobiotin (up to about 1 h) in ES-
cell transfectants.

STATISTICS
All data are represented as mean ± standard deviation (SD).
Each of the samples was tested for normality using the
Gauss grid method. Samples were subsequently compared
using Student’s t-test. Samples were considered to be signifi-
cantly different if P < 0.05.

Extracellular ATP measurements
The ES cells were cultivated in gelatinized 35 mm plates to a
confluence of 40–50%, washed twice with PBS and incubated
with 500 µl of Hank’s balanced salt solution (HBSS) with
1 mM EGTA for 25 min at 37°C and 5% CO₂. After
this stimulation, 100 µl of the supernatant were collected and
incubated for 20 min with 100 µl of the nucleotide releasing
reagent from the ViaLight™ HS kit (Cambrex, East Rutherford,
USA). Finally, the ATP concentration was measured for 10 s
with the Berthold Microplate LB96V luminometer which
automatically added 20 µl of the ATP monitoring reagent.
The ATP release could be stimulated by the addition of
HBSS with 1 mM EGTA and could be decreased to a basic
level by washing cells with PBS- and incubating them in ES
cell medium. All results of ATP release were normalized
after determination of the total protein amount. Results were
expressed as a mean ± standard error of the mean (SEM),
of relative luciferase activity.

Animals
Mice were kept in accordance with local governmental and
institutional instructions for animal care and maintained
under a 12/12 hours light/dark cycle. All transgenic mice
were of mixed genetic background (at least 75% C57BL/6
and at most 25% 129ola). In order to minimize the possible
influence of the genetic background, we performed all ana-
lyses with littermates of heterozygote breedings, including
control animals that lacked the Cre allele.

Genotyping of transgenic mice
Genotyping of Cre mediated deletion of the floxed region was
performed as described previously (21). Cx31F137L, Cx31floxF137L
and Cx31 alleles were detected by the Cx31F137L PCR,
using primers GenotypCreFor (5'-CTC AAA GCT AGT
CTG AGA TGC-3'), Cx31hmrev (5'-TAG TTC TAG AGC
GCC CAA TTC-3') and GenotypCreRev (5'-GCA TCA
CAA GGC TCC TAA GAA-3'). PCR conditions were:
5 min at 94°C, 30 cycles of 1 min at 94°C, 1 min at 60°C,
1 min at 72°C, followed by 10 min at 72°C. PCR was
carried out in 25 µl Taq buffer, containing 1.5 mM MgCl₂,
1.75 units Taq polymerase (Promega, Germany), 0.2 mM
dNTPs and 20 pmol of each primer. The F137L mutation
was detected by the Cx31CR-PCR, using primers
Cx31CR_For (5'-GGT CCC TCA GGT GGG CAC AGC-3')
and Cx31CR_Rev (5'-CGG CTT CAC CCC TTC TCT
AGC-3') followed by SmlI digestion. PCR conditions were:
4 min at 94°C, 35 cycles of 30 s at 94°C, 1 min at 55°C,
1 min at 72°C, followed by 10 min at 72°C. PCR was
carried out in 50 µl RedTaq-Mix (Sigma, Germany), contain-
ing 25 pmol of each primer and 25 µl water. After completing
the PCR 5 µl ‘buffer 4’, 1 µl of SmlI and 5 µl 10-fold concen-
trated BSA (all from New England Biolabs, Ipswich, USA)
are added and the complete mix was incubated for 2 h at 55°C.
The PCR product was demonstrated as an 809 bp ampiclon,
the presence of the mutation was proven by two additional
bands at 408 bp and 401 bp.

The Cx31floxF137L allele was detected by the
Cx31F137L-NEO PCR, using primers Neo For neu (5'-CTG
ATA TCC TTG ACA TAT CCA-3'), Neo Rev 1 (5'-GAA
GT TTT CCT GTC ATA CTT-3') and Neo Rev 2 (5'-TTC
GAG TGC GAC TCC ACC GGC-3'). PCR conditions were:
5 min at 94°C, 30 cycles of 1 min at 94°C, 1 min at 60°C,
1 min at 72°C, followed by 10 min at 72°C. PCR was
carried out in 25 µl Taq buffer, containing 1.5 mM MgCl₂,
1.75 units Taq polymerase (Promega, Germany), 0.2 mM
dNTPs and 20 pmol of each primer.

Southern blot analysis of the Cx31 alleles
was performed after NdeI and A gel digestion of liver
DNA and hybridization with a probe spanning the complete coding region of the Cx31 gene.

**Immunoblot analyses**

Wounded or uninjured pieces of tail skin were dissected on ice and immediately rinsed in liquid nitrogen. Homogenized tissue was taken up in protein lysis buffer [60 mM Tris HCl, pH 7.4, and 3% sodium dodecyl sulfate (SDS)], supplemented with proteinase inhibitor ‘Complete’ (Roche, Mannheim, Germany) and sonicated three times for 20 s on ice. Protein concentration was determined using the assay with bichinonic acid (Sigma, Taufrichingen, Germany). For electrophoresis, 50 μg of protein were separated on 10% SDS-polyacrylamide gels. Proteins were electrophoretically separated on nitrocellulose membranes (Hybond ECL, Biosciences, Bucks, UK) for 2 h at 100 V. Membranes were blocked for 1 h with blocking solution [20 mM Tris, HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20 (TBS-Tween)] and 5% skim milk powder (w/v) and afterwards incubated with primary antibodies overnight at 4°C. The rabbit antibodies were diluted in blocking solution [anti-Cx43: 1:500 (23); anti-Cx31: 1:250 (24); anti-Cx30.3: 1:500 (Zymed); anti-Cx30; 1:250 (Zymed, Berlin, Germany); anti-Cx26: 1:500 (Zymed)]. After washing for 30 min in blocking solution, membranes were incubated with anti-rabbit horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h (Dianova, Hamburg, Germany), diluted 1:20000 for anti-Cx43 and 1:5000 for the other antibodies in blocking solution. Afterwards, membranes were washed for 1 h in TBS-Tween and incubated with an ECL chemiluminescence detection system (Amersham Biosciences). In order to check equal loading in all lanes of the immunoblot, we performed Ponceau staining of the blotting membranes, followed by densitometric analyses of the stained protein. Lysates of HeLa cells stably transfected with the corresponding connexin expression vector or sections of connexin expressing tissues were used as positive controls.

**Preparation of skin sections and histochemistry**

Mice were killed by cervical dislocation. The tail skin was dissected, immediately frozen in liquid nitrogen and stored at −70°C. Tail epidermis was cut lengthwise (10 μm sections) with a cryostat.

**Wounding and isolation of mouse tail skin**

Incision wounds into mouse tail were cut with a scalpel, as previously described for rat tail (25). Six to eight transverse sections through the tail skin with a length of 1 cm were performed per mouse. Two of them were processed for immunofluorescence, two for histological and four were used for immunoblot analyses. At each time point after wounding, mutated and control mice were analyzed.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG Online.

**ACKNOWLEDGEMENTS**

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**Conflict of Interest statement.** None declared.

**REFERENCES**


