Induction of cell death and gain-of-function properties of connexin26 mutants predict severity of skin disorders and hearing loss

Eric R. Press, Qing Shao, John J. Kelly, Katrina Chin, Anton Alaga, and Dale W. Laird

From the Departments of Anatomy and Cell Biology and Physiology and Pharmacology, University of Western Ontario, London, Ontario N6A 5C1, Canada

Edited by Xiao-Fan Wang

Connexin26 (Cx26) is a gap junction protein that oligomerizes in the cell to form hexameric transmembrane channels called connexons. Cell surface connexons dock between adjacent cells to allow for gap junctional intercellular communication. Numerous autosomal dominant mutations in the Cx26-encoding GJB2 gene lead to many skin disorders and sensorineural hearing loss. Although some insights have been gained into the pathogenesis of these diseases, it is not fully understood how distinct GJB2 mutations result in hearing loss alone or in skin pathologies with comorbid hearing loss. Here we investigated five autosomal dominant Cx26 mutants (N14K, D50N, N54K, M163V, and S183F) linked to various syndromic or nonsyndromic diseases to uncover the molecular mechanisms underpinning these disease links. We demonstrated that when gap junction-deficient HeLa cells expressed the N14K and D50N mutants, they undergo cell death. The N54K mutant was retained primarily within intracellular compartments and displayed dominant or transdominant properties on wild-type Cx26 and coexpressed Cx30 and Cx43. The S183F mutant formed some gap junction plaques but was largely retained within the cell and exhibited only a mild transdominant reduction in gap junctional communication when co-expressed with Cx30. The M163V mutant, which causes only hearing loss, exhibited impaired gap junction function and showed no transdominant interactions. These findings suggest that Cx26 mutations that promote cell death or exert transdominant effects on other connexins in keratinocytes will lead to skin diseases and hearing loss, whereas mutants having reduced channel function but exhibiting no aberrant effects on coexpressed connexins cause only hearing loss. Moreover, cell death-inducing GJB2 mutations lead to more severe syndromic disease.

The GJB2 gene encoding connexin26 (Cx26) has an estimated mutation prevalence of 3% in the general population (1).

Globally, an estimated 17.3% of hearing loss cases are linked to bi-allelic GJB2 mutations, highlighting the importance of Cx26 in hearing (1). In addition, numerous syndromic diseases exhibiting hearing deficits and a variety of skin abnormalities are linked to GJB2 missense mutations with autosomal dominant inheritance (2). Interestingly, some speculate that the perversiveness of GJB2 mutations may result from a selective heterozygote advantage (1) conferred by subclinical epidermal thickening and a stronger cutaneous barrier (3). In humans, Cx26 is expressed in a variety of tissues and, not surprisingly, in several cell types in the cochlea (4) and in keratinocytes of the epidermis (5). Within these tissues, several other members of the connexin family are expressed, most notably Cx30 and Cx43, wherein mutations in their respective genes have also been implicated in syndromic diseases sharing some similar features (2, 5, 6).

Cx26 is a gap junction protein that oligomerizes in the cell to form hexameric transmembrane channels called connexons (7). Connexons that span the plasma membrane are called hemichannels and may allow a cell to pass small signaling molecules between the cytosol and the extracellular environment (7). However, when hemichannels from adjacent cells dock together, they form a single conduit called a gap junction channel, which connects the cytosol of these cells and facilitates gap junctional intercellular communication (GJIC) (7). ATP, inositol trisphosphate, and cations frequently pass through Cx26 gap junction channels and have been shown to play important roles in regulating cell proliferation and differentiation as well as maintaining ionic homeostasis within tissues (8, 9).

The Cx26 polypeptide has four transmembrane domains, two extracellular loops, an intracellular loop, and cytosolic N and C termini. The N-terminal domain (amino acid residues 1–20) is suggested to play a major role in voltage sensing and channel gating (10). The extracellular loops (E1 and E2) (amino acid residues 41–75 and 155–192, respectively) are thought to be key domains for oligomerization and interchannel docking (10). Disease-causing point mutations have been documented in nearly every domain of the Cx26 polypeptide, and depending on the mutation and the motif that harbors the altered residue, variations can occur in connexin folding and trafficking, channel assembly, channel gating, half-life, degradation, and/or

This research was supported by Canadian Institutes of Health Research Grant 123228 (to D. W. L.). The authors declare that they have no conflicts of interest with the contents of this article.

1 To whom correspondence should be addressed: Dept. of Anatomy and Cell Biology, University of Western Ontario, 00077 Dental Sciences Bldg., 1151 Richmond St., London, Ontario N6A 5C1, Canada. Tel.: 519-661-2111 (ext. 86827); E-mail: dale.laird@schulich.uwo.ca.

2 The abbreviations used are: Cx26, connexin26; Cx30, connexin30; Cx43, connexin43; KIDS, keratitis-ichthyosis-deafness syndrome; CC3, cleaved caspase 3; REK, rat epidermal keratinocyte; ECS, extracellular solution; PPK, palmoplantar keratodermia; PDI, protein-disulfide isomerase; Pl, protein.

© 2017 by The American Society for Biochemistry and Molecular Biology, Inc. Published in the U.S.A.

J. Biol. Chem. (2017) 292(23) 9721–9732 9721
interactions between other co-expressed connexins (11). Some mutations have been shown to disrupt several connexin life-cycle characteristics (12), increasing the complexity of delineating how \textit{GJB2} point mutations cause diseases that affect one or more organs with varying severity.

In this study, we selected five autosomal dominant \textit{GJB2} missense mutations that result in single amino acid substitutions in various domains of the Cx26 polypeptide and are linked to an array of auditory and skin pathologies. The N14K mutation causes a disease that shares symptoms with Clouston syndrome and keratitis-ichthyosis-deafness syndrome (KIDS) (13), the D50N mutation leads to KIDS (14), the N54K mutation results in Bart-Pumphrey syndrome (15), and the S183F mutation causes palmoplantar keratoderma (PPK) and hearing loss (16). Finally, the M163V mutation is linked to moderate hearing loss only (17). Considering the pleiotropic nature of \textit{GJB2} mutations, we proposed that Cx26 mutants that give rise to similar clinical presentations would share common mechanisms of action.

Here we found that the N14K and D50N mutants leading to widespread erythrokeratoderma and severe hearing loss caused cell death, the N54K and S183F mutants leading to PPK and hearing loss had trafficking defects and reduced channel function, and the M183V mutant leading to hearing loss alone had reduced channel function. Last, all mutants linked to syndromic disease had transdominant effects on co-expressed connexins.

Results

\textbf{N14K and D50N mutants induce cell death in vitro}

To assess the impact of Cx26 mutants on cellular health, specific Cx26 mutants were expressed in GJIC-deficient HeLa cells. HeLa cells expressing the GFP-tagged N14K and D50N mutants appeared unhealthy as early as 24 h post-transfection; therefore, we immunolabeled cleaved caspase-3 (CC3) to determine whether cells expressing these mutants were undergoing apoptosis (Fig. 1A). HeLa cells expressing the GFP-tagged N14K and D50N mutants were more extensively labeled for CC3 (Fig. 1B), similar to cells treated with staurosporine, suggesting that the expression of these mutants in HeLa cells triggers apoptosis \textit{in vitro}. Expression of untagged N14K and D50N mutants also triggered apoptosis in HeLa cells (data not shown), eliminating the possibility that the GFP tag was responsible for the induction of cell death. Consequently, cells expressing the N14K or D50N mutants were deemed not sufficiently healthy for further mutant localization or functional studies.

\textbf{N54K and S183F mutants have trafficking defects and impaired dye-transfer ability}

Cx26 mutants associated with hearing loss and various skin diseases were expressed in HeLa cells to examine how a point mutation in Cx26 may affect its trafficking, cellular localization, and channel function. Cx26 and the M163V mutant formed abundant gap junction plaques at cell-cell interfaces (Fig. 2, A and B). The N54K and S183F mutants were retained primarily in intracellular compartments (Fig. 2C), partially colocalized with protein-disulfide isomerase (PDI) labeling of the endoplasmic reticulum (Fig. 2A), and formed fewer gap junction plaques (Fig. 2B). Likewise, HeLa cells engineered to express untagged Cx26 mutants displayed cellular localizations similar to HeLa cells expressing GFP-tagged mutants (data not shown). The ability of the disease-linked mutants to form functional gap junction channels was assessed by quantifying intercellular transfer of microinjected Alexa Fluor 350 in HeLa cells expressing Cx26 or the various mutants. Cx26 had nearly 100% incidence of dye transfer (Fig. 2D), whereas cells expressing the M163V mutant passed dye ~40% of the time. Cells expressing either the N54K or S183F mutants were essentially unable to establish gap junction channels capable of dye transfer (Fig. 2, D and E).

\textbf{Cx26 mutants exhibit dominant-negative effects on Cx26 function}

To assess the distribution and function of Cx26 mutants in conditions where both wild-type and mutant Cx26 are
expressed in the same cell, we engineered HeLa cells to express RFP-tagged Cx26 and GFP-tagged Cx26 mutants at approximately a 1:1 ratio. Cx26 and the M163V mutant formed numerous gap junction plaques at cell-cell interfaces (arrows). N54K and S183F mutants were mostly located within the cell, whereas the S183F mutant formed few gap junction plaques. B, the N54K and S183F mutants, but not the M163V mutant, formed fewer gap junction plaques compared with Cx26. One-way ANOVA; **, p < 0.01; n = 13. C, the N54K and S183F mutants, but not the M163V mutants, also displayed increased intracellular fluorescence. One-way ANOVA; **, p < 0.01; n = 6. D, Alexa Fluor 350 dye transfer in pairs or clusters of HeLa cells expressing N54K and S183F mutants was negligible, whereas ~40% of the cells expressing the M163V mutant transferred dye. E, example images of dye-transfer experiments showing successful (Cx26) and unsuccessful dye transfer (N54K). One-way ANOVA; ****, p < 0.0001; **, p < 0.01; n = 3. The number of cells that were microinjected to test for dye transfer in each case is noted in D. Ctrl, untransfected HeLa cells. Scale bar (A and E), 20 μm. Error bars, S.D.
Cx26 mutants predict disease severity

actions. Cx26 as well as the M163V and S183F mutants were able to form numerous gap junction plaques at the cell surface and often co-localized with Cx30 (Fig. 4, A and B). The N54K mutant formed very few plaques and remained in an endoplasmic reticulum-like distribution pattern but also appeared to impair the ability of Cx30 to form abundant gap junction plaques (Fig. 4, A and B). Whereas the N54K and S183F mutants were typically found in intracellular compartments, only the N54K mutant was able to significantly retain co-expressed Cx30 in intracellular compartments (Fig. 4C). Together these findings suggest the N54K mutant exhibits a transdominant effect on Cx30 trafficking. Next we determined whether the Cx26 mutants exhibited transdominant effects on Cx30 channel function using Alexa Fluor 350 dye-transfer experiments (Fig. 4D). Microinjected HeLa cells expressing Cx30 alone or co-expressing Cx30 and Cx26 had a nearly 100% incidence of dye transfer. However, cells co-expressing Cx26 and either the N54K or S183F mutants had a significantly reduced ability to pass dye, indicating that these mutants had a transdominant effect on Cx30 channel function. Interestingly, the M163V mutant did not significantly reduce the ability of Cx30-positive cells to pass dye.

Figure 3. Cx26 mutants exhibit dominant-negative properties on Cx26 in HeLa cells. A, HeLa cells expressing GFP-tagged Cx26 or N54K, M163V, and S183F mutants (green) together with Cx26-RFP (red) were stained with Hoechst (blue). All mutants formed gap junction plaques at cell-cell interfaces (arrows) and colocalized with Cx26-RFP. B, only the N54K mutant formed fewer gap junction plaques than Cx26, and none of the mutants significantly impaired the formation of wild-type Cx26 gap junction plaques. One-way ANOVA; *, p < 0.05; n = 13. C, all mutants displayed increased intracellular fluorescence, however, only the N54K mutant was able to significantly increase the intracellular fluorescence of wild-type Cx26. One-way ANOVA; ****, p < 0.0001; *, p < 0.05; n = 6. D, untransfected control cells (Ctrl) or cells expressing the N54K mutant together with Cx26 failed to pass microinjected Alexa Fluor 350 dye. Cells expressing S183F and M163F mutants together with Cx26-RFP had reduced incidences of dye transfer compared with cells expressing Cx26-GFP. One-way ANOVA; ****, p < 0.0001; n = 3. The number of cells that were microinjected in each case is noted in D. Scale bar, 20 μm. Error bars, S.D.
N54K displays mild transdominant effect on endogenous Cx43

It has been demonstrated that some Cx26 mutants have the abnormal ability to interact with Cx43 (12). Therefore, we engineered Cx43-positive rat epidermal keratinocytes (REKs) to express Cx26 mutants to determine their potential for transdominant effects on endogenous Cx43 in a tissue-relevant cell. Only the N54K mutant formed fewer gap junction plaques than Cx26, but none of the mutants impaired the formation of Cx43 plaques (Fig. 5, A and B). The S183F mutant extensively colocalized with Cx43, consistent with its reported interaction with Cx43 (26). The N54K and S183F mutants were frequently found in intracellular compartments, and interestingly, these mutants also increased the intracellular localization of co-expressed Cx43 (Fig. 5C), suggesting a possible transdominant interaction with endogenous Cx43. REKs engineered to express Cx26 mutants were microinjected with Alexa Fluor 350, which readily passes through both Cx26 and Cx43 gap junction channels. Only cells expressing the N54K mutant exhibited a significant decrease in dye transfer abilities similar to cells expressing Cx26 and Cx30, or Cx30 alone, whereas the incidences of dye transfer in cells expressing the N54K and S183F mutants together with Cx30 were markedly reduced. One-way ANOVA; ****, p < 0.0001; n = 3. The number of cells that were microinjected in each case is noted in D. Scale bar, 20 μm. Error bars, S.D.
A significant decrease in detectable dye transfer, suggesting that the N54K mutant had a modest transdominant-negative effect on endogenous Cx43 (Fig. 5D).

**N54K and S183F mutants display reduced hemichannel function**

Aside from GJIC and trafficking defects, mutant connexins may further disrupt hemichannel properties (18). We therefore used a propidium iodide (PI) dye uptake assay to assess the function of mutant hemichannels in normal, calcium-containing extracellular solution (ECS + Ca²⁺) and divalent-cation-free extracellular solution (ECS − Ca²⁺), which stimulates hemichannels to open (19). All mutant-expressing HeLa cells displayed minimal PI uptake in ECS + Ca²⁺ conditions, and similar to Cx26, the M163V mutant exhibited a nearly 100% incidence of PI uptake in ECS − Ca²⁺, indicating fully functional hemichannel activity (Fig. 6B). Interestingly, in ECS − Ca²⁺, cells expressing the N54K mutant demonstrated a highly variable incidence of PI uptake, whereas cells expressing the S183F mutant displayed no increase in PI uptake.

---

**Figure 5. The N54K mutant exhibits transdominant inhibition of endogenous Cx43 in REKs.**

A. REKs expressing GFP-tagged Cx26, N54K, M163V, and S183F mutants (green) with endogenous Cx43 immunolabeled in red were stained with Hoechst (blue). The N54K and S183F mutants were often found intracellularly but also formed gap junction plaques at the cell surface, whereas the M163V mutant exhibited a localization pattern similar to Cx26. B. the N54K mutant formed fewer gap junction plaques compared with Cx26; however, endogenous Cx43 gap junction formation was not impaired by any of the mutants. One-way ANOVA: **, p < 0.01; n = 24. C. the N54K and S183F mutants exhibited increased intracellular fluorescence and also increased the amount of intracellular fluorescence from wild-type Cx43. One-way ANOVA: ****, p < 0.0001; ***, p < 0.001; *, p < 0.05; n = 6. D. REKs expressing the N54K mutant had reduced dye transfer capabilities compared with untransfected REKs (Ctrl) and REKs expressing Cx26. One-way ANOVA: **, p < 0.01; n = 3. The number of cells microinjected is noted in D. Scale bar, 20 µm. Error bars, S.D.
indicating non-functional hemichannels (Fig. 6B). It is important to note that divalent-cation-free solution was used only to stimulate hemichannel opening. As a result, some cells exhibited a rounded morphology, which may indicate that the cells are beginning to become unhealthy (Fig. 6A).
non-traditional binding partner, such as Cx43, should be considered a gain-of-function property (11). Therefore, we also explored possible transdominant effects on Cx30, which is co-expressed with Cx26 in the skin and cochlea, as well as Cx43, which is co-expressed with Cx26 in the skin and also found in the cochlea. Furthermore, at least six additional syndromic mutants have been identified in patients that still require functional analysis (21). This report describes the M163V and S183F mutants (25, 26) and the N54K mutant, which was reported once in a patient with Bart-Pumphrey syndrome (15) and remains otherwise unexamined.

N14K and D50N mutants are linked to KIDS, which is one of the most severe Cx26-linked skin disorders. Patients present with erythrokeratoderma, PPK, and frequent cutaneous infections, which can lead to fatal septicemia early in life (27). We found that these mutants strongly induced cell death such that meaningful channel function information was unattainable. However, reports suggest they form “leaky” hemichannels when expressed in Xenopus oocytes, where they induce blebbing and substantially reduce cell viability (23, 28, 29). Cx26 hemichannels are sensitive to hyperpolarization by extracellular Ca	extsuperscript{2+}, such that they remain closed under physiological conditions (19). Interestingly, some studies have shown that high extracellular Ca	extsuperscript{2+} conditions can improve the viability of cells expressing such mutants, suggesting that elevated hemichannel activity is tightly linked to cell death in vitro (23, 30, 31). Our
 observations further demonstrate that Ca$^{2+}$-free conditions can elevate the activity of Cx26 and mutant hemichannels, which may lead to cytotoxic effects. Nevertheless, reduced Ca$^{2+}$ sensitivity by N14K, D50N, and several other KIDS mutants promotes hemichannel opening that can diminish transmembrane ion gradients and release molecules, including ATP, that affect cell viability (32). Excessive ATP release is also known to stimulate purinergic signaling capable of mobilizing intracellular Ca$^{2+}$ and releasing pro-inflammatory cytokines (33). In the epidermis, these mutants may also be able to disrupt the normal epidermal Ca$^{2+}$ gradient and lipid processing (34), which can result in barrier defects and a compensatory hyperproliferative response that drives hyperkeratosis in KIDS (35). Interestingly, Asn-14 and Asp-50 are pore-lining residues (35), suggesting that changes to the structure of the Cx26 channel pore can produce the aberrant hemichannel properties that stand at the forefront of KIDS skin pathogenesis.

The N54K mutant is linked to moderately severe Bart-Pumphrey syndrome, featuring PPK, knuckle pads, leukonychia, and deafness; the S183F mutant is linked to PPK with deafness. Although no well-defined pathogenic mechanisms for PPK have been established, our findings suggest that the high intracellular retention of N54K and S183F mutants is a common characteristic of Cx26 mutants linked to PPK. Indeed, this trafficking defect has been indicated in previous reports (21, 36–38). A patient with Bart-Pumphrey syndrome harboring the N54K Cx26 mutant (15) was reported to have a compensatory increase in epidermal Cx30 expression. Asn-54 is an invariantly conserved residue in the connexin family among numerous species (15) and forms hydrogen bonds with Leu-56 from the opposing hemichannel (10). Interestingly, we found that the N54K mutant impaired Cx30 trafficking and dye transfer, suggesting that epidermal Cx30 compensation may act to overcome a transdominant hindrance of Cx26:Cx30 heteromeric gap junction formation between keratinocytes. This study and only a handful of others (31, 39–42) investigated Cx26 mutants in a relevant keratinocyte model in addition to connexin-deficient reference cells. We found that only REKs expressing the N54K mutant had reduced dye transfer. This suggests that N54K Cx26 may also exert transdominant effects on endogenous Cx43, which may be an important etiological factor in Bart-Pumphrey syndrome skin. Recently, Shuja et al. (26) demonstrated that S183F Cx26 does not form gap junctions or hemichannels in Xenopus oocytes, and junctional conductance was reduced in cells co-expressing Cx43. We also found that the S183F mutant inhibited dye transfer in cells co-expressing Cx30 and promoted partial intracellular retention of co-expressed Cx43, pointing toward transdominant interactions of co-expressed connexins as a mechanism of disease. We suspect that we may not have observed the reported S183F mutant functional inhibition of Cx43 seen in Xenopus oocytes (26) in keratinocytes due to the limited sensitivity of the dye transfer assay or to the dosage of the expressed S183F mutant. Because Ser-183 is a highly conserved residue in the connexin family and among many species (16), we suggest that the Ser-183 residue may have an indirect role in interprotomer binding. Additionally, moderately leaky heteromeric hemichannels formed by S183F Cx26 and Cx43 (26), similar to those composed of S17F Cx26 and Cx43, may also contribute to PPK. Nevertheless, our findings provide additional evidence for the impact of transdominant interactions between connexins in skin disease, where disease severity may be linked to the extent of transdominant influence, particularly in skin regions exposed to greater mechanical stress.

In this study, only the M163V mutant is linked to hearing loss without added skin disease. Because Cx26 is proposed to play an important role in potassium buffering and/or recycling in the inner ear (4) and dozens of hearing loss Cx26 mutants display reduced or no gap junction function (22), it is not surprising that we found that the M163V mutant had a dominant-negative effect on Cx26. However, Cx30 is also highly expressed with Cx26 in the inner ear (4), such that compensation might be able to rescue hearing. Interestingly, several studies have shown that Cx30 does not functionally compensate for Cx26 in the inner ear (43–45). This may explain why the M163V mutant leads to hearing loss despite the finding that HeLa cells co-expressing the M163V mutant and Cx30 passed dye at levels not unlike those expressing Cx30 and/or Cx26. Including our findings from syndromic mutants, we suggest that mutants with trafficking defects and/or transdominant properties, and mutants that induce cell death lead to skin phenotypes, whereas mutants that have reduced gap junction function but do not interfere with other co-expressed connexins produce non-syndromic hearing loss.

Human epidermis expresses seven different connexins in overlapping populations of keratinocytes (6, 46), making the epidermis a more robust system from the prospective of connexin expression compared with the cochlea. Fortunately, this high degree of redundant intercellular communication affords the epidermis resiliency in response to GJB2 mutations that have minor effects on protein function. Because fewer connexin types are expressed within the cochlea and compensation may be limited, smaller perturbations in connexin function are capable of disrupting normal hearing. This may speak to the reason why GJB2 mutations almost never produce skin disease alone but Cx26-linked syndromic and non-syndromic deafness is common. Although the majority of autosomal dominant GJB2 mutations produce hearing loss in addition to skin disease, we posit that the strongest predictors of syndromic disease severity actually stem from the transdominant status and gain-of-function properties of Cx26 mutants. Finally, this study provides evidence in support of genetic screening when faced with complex syndromic diseases.

**Experimental procedures**

**Cell culture**

Connexin-deficient cervical cancer cells (HeLa) were purchased from ATCC, and REKs originally characterized in (47) were generously provided by Dr. Vincent Hascall. All cells were grown in DMEM (Life Technologies catalog no. 11965-092) supplemented with 10% fetal bovine serum, 2 mM L-glutamine (Life Technologies catalog no. 25030-081), 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen) (48).
Cx26 mutants predict disease severity

cDNA constructs and transfections

cDNA encoding human Cx26 was provided by Dr. C. C. Naus (University of British Columbia, Vancouver, Canada). PCR was used to add XhoI and EcoRI restriction sites to the 5’ and 3’ ends of Cx26, and the resulting cDNAs were cloned into the pEGFP-N1 vector (Clontech) and sequenced for verification with a 17-amino acid linker sequence separating the Cx26 and GFP moieties. Constructs encoding human N14K, D50N, N54K, M163V, and S183F Cx26-GFP were further obtained from NorClone by using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) in accordance with the manufacturer’s instructions. RFP-tagged Cx30 and Cx26 constructs were described previously (18), and all constructs were validated by sequencing. GFP and RFP tags were shown not to dramatically affect connexin trafficking or protein function, as shown previously (38). Cells at ~60% confluence were transiently transfected using Lipofectamine 2000. Transfection mixtures contained 200 μl of Opti-MEM reduced serum medium (Life Technologies catalog no. 31985-070); 1 μl of Lipofectamine 2000 transfection reagent (Invitrogen catalog no. 11668019); and 1 μg of GFP-tagged Cx26, N14K, D50N, N54K, M163V, or S183F cDNA constructs. For co-expression experiments, transfection mixtures differed in that 0.5 μg of GFP-tagged constructs plus 0.5 μg of RFP-tagged Cx26 or Cx30 were added to produce roughly equal expression of mutant to wild-type connexins. The mixture was gently swirled, incubated at room temperature for 10 min, and added dropwise to cells growing in DMEM. All cells were used for experiments between 24 and 48 h following transfection.

Immunofluorescent labeling

HeLa cells or REKs grown to ~80% confluence on sterile glass coverslips were washed with phosphate-buffered saline (PBS) and fixed in an ice-cold solution of 80% methanol and 20% acetone for 10 min at 4 °C. Coverslips were then washed in PBS, blocked in a 2% bovine serum albumin (BSA) solution (diluted in PBS) for 30 min, and then incubated at room temperature for 1 h with the following primary antibodies diluted in BSA solution: 1:500 mouse anti-PDI (Assay Designs catalog no. SPA-891), 1:500 rabbit anti-Cx43 (Sigma-Aldrich catalog no. C6219), or 1:200 rabbit anti-cleaved caspase-3 (Sigma-Aldrich catalog no. C8487). Secondary antibodies 1:500 Alexa Fluor 488-conjugated anti-mouse (Invitrogen catalog no. A11017) and 1:500 Alexa Fluor 555-conjugated anti-rabbit (Invitrogen catalog no. A21429) were used to detect primary antibodies. Cells were then incubated for 10 min at room temperature with Hoechst 33342 (1:1000 diluted in distilled water) (Molecular Probes catalog no. H3570), mounted, and imaged with a Zeiss LSM 800 confocal Airyscan microscope equipped with ZenWorks software. Images were captured with a ×63 oil immersion objective at room temperature. Gap junction plaques between cells were quantified in a blinded fashion by counting the number of green and red punctae at individual cell–cell interfaces. A minimum of 13 separate images were captured for each mutant, and a one-way ANOVA was performed on the means of three biological replicates. For each mutant, six images were used to quantify the fluorescent signal within intracellular compartments. Briefly, monochromatic fluorescence was measured using ImageJ by carefully tracing the cell–cell interface and the intracellular regions of each cell. Values indicate the percentage of total cellular fluorescence located within intracellular compartments.

Dye-transfer studies

HeLa cells or REKs grown to ~60% confluence and engineered to express GFP-tagged Cx26 or Cx26 mutants (and RFP-tagged connexins for co-expression experiments) as described above were microinjected with Alexa Fluor 350 (410 Da; Molecular Probes catalog no. A10439) to assess gap junction dye transfer as described previously (49). Briefly, cells were microinjected using a fine glass needle attached to an Eppendorf FemtoJet automated microinjector. Cells were imaged using a Leica DM IRE2 epifluorescent microscope to visualize GFP and RFP, and then 1 min following microinjection, they were imaged again to visualize the spread of Alexa Fluor 350. All images were captured with a ×20 objective at room temperature. The incidence of dye transfer within each trial was quantified as the percentage of microinjected cells that passed dye to neighboring cells, and a one-way ANOVA was performed on the means of three biological replicates. Tukey’s post hoc test compared the means of each condition with the condition in which cells expressed GFP-tagged Cx26. Untransfected HeLa cells were used as negative controls, and HeLa cells expressing Cx26-GFP only or Cx26-GFP in addition to Cx26-RFP or Cx30-RFP served as positive controls for all dye transfer experiments in HeLa cells. Last, for dye transfer experiments in REKs, untransfected REKs or REKs expressing Cx26-GFP or mutants were used.

Hemichannel assay

HeLa cells were seeded at low density to isolate cells from one another and then were engineered to express Cx26 or Cx26 mutants as described above. PI dye uptake assays to evaluate hemichannel activity were performed as noted previously (18). Briefly, cells were washed in ECS containing 142 mM NaCl, 5.4 mM KCl, 1.4 mM MgCl2, 2 mM CaCl2, 10 mM HEPES, 25 mM d-glucose, osmolality 298 mOsm, pH-adjusted to 7.35 using NaOH) and then twice in either ECS + Ca2+ or divalent-cation-free solution (ECS − Ca2+) (same as ECS + Ca2+ but with Ca2+ and Mg2+ substituted with 2 mM EGTA). ECS + Ca2+ or ECS − Ca2+ containing 1 mg/ml PI (668.4 Da; Invitrogen) was added to the cells and incubated at 37 °C for 15 min. Cells were washed three times with ECS + Ca2+, and then isolated cells were imaged to visualize GFP and PI using the Leica microscope and OpenLab software. For each replicate, the number of cells containing PI was recorded as a percentage of the total number of GFP-positive cells, and a two-way ANOVA was performed on the means of three biological replicates. Sidak’s post hoc test compared the means of ECS + Ca2+ and ECS − Ca2+ conditions.

Statistical analysis

GraphPad Prism version 6 was used for all statistical analysis, and statistical significance was noted when p was < 0.05. All plots display individual points and the mean ± S.D.
Acknowledgments—We thank Dr. Christian C. C. Naus for the Cx26 cDNA construct and Dr. Vincent C. Hascall for the REKs. We also thank Dr. Jessica Esseltine for assistance with some co-expression studies.
Cx26 mutants predict disease severity

epidermal lipid processing and calcium distribution in the KID syndrome mouse model Cx26S17F. FEBS Lett. 589, 1904–1910
35. García, I. E., Bosen, F., Mujica, P., Pupo, A., Flores-Muñoz, C., Jara, O., González, C., Willecke, K., and Martínez, A. D. (2016) From hyperactive Connexin26 hemichannels to impairments in epidermal calcium gradient and permeability barrier in the keratitis-ichthyosis-deafness syndrome. J. Invest. Dermatol. 136, 574–583
36. de Zwart-Storm, E. A., Hamm, H., Stoevesandt, J., Steijlen, P. M., Martin, P. E., van Geel, M., and van Steensel, M. A. (2008) A novel missense mutation in GJB2 disturbs gap junction protein transport and causes focal palmoplantar keratoderma with deafness. J. Med. Genet. 45, 161–166
37. Marziano, N. K., Casalotti, S. O., Portelli, A. E., Becker, D. L., and Forge, A. (2003) Mutations in the gene for connexin 26 (GJB2) that cause hearing loss have a dominant negative effect on connexin 30. Hum. Mol. Genet. 12, 805–812
38. Thomas, T., Jordan, K., Simek, J., Shao, Q., Jeddesko, C., Walton, P., and Laird, D. W. (2005) Mechanisms of Cx43 and Cx26 transport to the plasma membrane and gap junction regeneration. J. Cell Sci. 118, 4451–4462
39. Common, J. E., Di, W. L., Davies, D., Galvin, H., Leigh, I. M., O’Toole, E. A., and Kelsell, D. P. (2003) Cellular mechanisms of mutant connexins in skin disease and hearing loss. Cell Commun. Adhes. 10, 347–351
40. Di, W. L., Yu, C., Common, J. E., Aasen, T., O’Toole, E. A., Kelsell, D. P., and Zicha, D. (2005) Connexin interaction patterns in keratinocytes revealed morphologically and by FRET analysis. J. Cell Sci. 118, 1505–1514
41. Donnelly, S., English, G., de Zwart-Storm, E. A., Lang, S., van Steensel, M. A., and Martin, P. E. (2012) Differential susceptibility of Cx26 mutations associated with epidermal dysplasias to peptidoglycan derived from Staphylococcus aureus and Staphylococcus epidermidis. Exp. Dermatol. 21, 592–598
42. Man, Y. K. S., Trolle, C., Tattersall, D., Thomas, A. C., Papakonstantinopoulou, A., Patel, D., Scott, C., Chong, J., Jagger, D. J., O’Toole, E. A., Navsaria, H., Curtis, M. A., and Kelsell, D. P. (2007) A deafness-associated mutant human connexin 26 improves the epithelial barrier in vitro. J. Membr. Biol. 218, 29–37
43. Cohen-Salmon, M., Ott, T., Michel, V., Hardelin, J. P., Perfettini, L., Eybalin, M., Wu, T., Marcus, D. C., Wangemann, P., Willecke, K., and Petit, C. (2002) Targeted ablation of connexin26 in the inner ear epithelial gap junction network causes hearing impairment and cell death. Curr. Biol. 12, 1106–1111
44. Qu, Y., Tang, W., Zhou, B., Ahmad, S., Chang, Q., Li, X., and Lin, X. (2012) Early developmental expression of connexin26 in the cochlea contributes to its dominant functional role in the cochlear gap junctions. Biochem. Biophys. Res. Commun. 417, 245–250
45. Teubner, B., Michel, V., Pesch, J., Lautermann, J., Cohen-Salmon, M., Söhl, G., Jahnke, K., Winterhager, E., Herberhold, C., Hardelin, J. P., Petit, C., and Willecke, K. (2003) Connexin30 (Gjb6)-deficiency causes severe hearing impairment and lack of endocochlear potential. Hum. Mol. Genet. 12, 13–21
46. Di, W. L., Rugg, E. L., Leigh, I. M., and Kelsell, D. P. (2001) Multiple epidermal connexins are expressed in different keratinocyte subpopulations including connexin 31. J. Invest. Dermatol. 117, 958–964
47. Baden, H. P., and Kubilus, J. (1983) The growth and differentiation of cultured newborn rat keratinocytes. J. Invest. Dermatol. 80, 124–130
48. Penuela, S., Bhalla, R., Gong, X. Q., Cowan, K. N., Celetti, S. I., Cowan, B. J., Bai, D., Shao, Q., and Laird, D. W. (2007) Pannexin 1 and pannexin 3 are glycoproteins that exhibit many distinct characteristics from the connexin family of gap junction proteins. J. Cell Sci. 120, 3772–3783
49. Huang, T., Shao, Q., MacDonald, A., Xin, L., Lorentz, R., Bai, D., and Laird, D. W. (2013) Autosomal recessive GJA1 (Cx43) gene mutations cause ocuolodentodigial dysplasia by distinct mechanisms. J. Cell Sci. 126, 2857–2866