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A heterozygous mutation in GJB2 (Cx26F142L) associated with deafness and recurrent skin rashes results in connexin assembly deficiencies

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Abstract
Mutations in GJB2 encoding Connexin 26 (CX26) are associated with hearing loss and hyperproliferative skin disorders of differing severity including keratitis-ichthyosis-deafness (KID) and Vohwinkel syndrome. A 6-year-old Caucasian girl who presented with recurrent skin rashes and sensorineural hearing loss harboured a heterozygous point mutation in GJB2 (c.424T > C; p.F142L). To characterize the impact of CX26F142L on cellular events. Plasmids CX26WT, CX26F142L, CX26G12R (KID) or CX26D66H (Vohwinkel) were transfected into HeLa cells expressing Cx26 or Cx43 or into HaCaT cells, a model keratinocyte cell line. Confocal microscopy determined protein localization. MTT assays assessed cell viability in the presence or absence of carbenoxolone, a connexin-channel blocker. Co-immunoprecipitation/Western blot analysis determined Cx43:Cx26 interactions. Quantitative real-time polymerase chain reaction assessed changes in gene expression of ER stress markers. Dye uptake assays determined Connexin-channel functionality. F142L and G12R were restricted to perinuclear areas. Collapse of the microtubule network, rescued by co-treatment with paclitaxel, occurred. ER stress was not involved. Cell viability was reduced in cells expressing F142L and G12R but not D66H. Unlike G12R that forms “leaky” hemichannels, F142L had restricted permeability. Cell viability of F142L and G12R transfected cells was greater in HeLa cells expressing Cx43 than in native Cx-free HeLa cells. Co-immunoprecipitation suggested a possible interaction between Cx43 and the three mutations. Expression of CX26F142L and G12R results in microtubule collapse, rescued by interaction with Cx43. The GJB2 mutations interacted with Cx43

Abbreviations: CBF, carboxyfluorescein; CBX, carbenoxolone; cDMEM, Dulbecco’s modified Eagle’s medium; Cx, generic connexin mouse, human or rat; CX, human Connexin; EKV, erythrokeratodermia variabilis; ERGIC, endoplasmic reticulum/Golgi; GFP, green fluorescent protein; GJ, gap junction; HC, hemichannel; IP, immunoprecipitation; KID, keratitis-ichthyosis-deafness; PBS, phosphate-buffered saline; PI, propidium iodide; SCC, squamous cell carcinoma; SEM, standard error of the mean; SFM, serum-free medium; SNHL, sensorineural hearing loss; TM3, transmembrane domain 3; WT, wild type.

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suggesting that unique Cx43:Cx26 channels are central to the diverse phenotype of CX26 skin-related channelopathies.

**KEYWORDS**
Connexin channelopathy, GJB2 mutation, hyperproliferative skin disorder, keratitis-ichthyosis-deafness

1 | INTRODUCTION

A spectrum of dominant mutations associated with deafness and syndromic hyperproliferative skin disorders is found in the coding region of GJB2, located on chromosome 13, encoding the gap junction protein Connexin 26 (CX26) (NCBI reference sequence NM_004004.6). Connexins, a family of four-transmembrane-spanning proteins, are classified according to their phylogenetic origins with up to 10 members of the alpha (eg GJA1 encoding CX43) and beta (encoded by genes GJB1-6) groups expressed throughout the epidermis. CX43 predominates, and CX26 is expressed in low levels in the spinous and granular layers of normal human epidermis but is up-regulated in hyperproliferative states, for example psoriasis. Six connexin subunits oligomerize forming a connexon or hemichannel (HC) that under normal conditions remain closed, although triggers such as pathogen challenge, changes in temperature, pH or oxygen levels can open them. Hemichannels align and dock at the plasma membrane with a HC from a neighbouring cell to form a dodecameric gap junction permitting the direct exchange of small metabolites (<1 kDa in size). CX43 and CX26 do not normally form heteromeric partners; however, many CX26 mutations induce transdominant effects on CX43 with pathological implications.

Mutations in CX26 are all associated with deafness, and dominant mutations also link with skin diseases that fall into two main groups: Class 1: loss of channel function and trafficking deficiencies associated with non-inflammatory skin conditions. For example, the mutation D66H linked with Vohwinkel syndrome where patients present with a starfish or honeycomb-like appearance around areas such as the knuckles and wrists and constriction bands encircle the digits ultimately resulting in autoamputation of the digits (pseudoainhum). Secondly, Class 2: "gain of channel function" mutations result in trafficking abnormalities and are associated with inflammatory skin conditions, for example the mutation G12R associated with keratitis-ichthyosis-deafness (KID) syndrome. KID syndrome is the severest of connexin-related skin disorder with a wide range of pathologies that are associated with specific mutations where patients develop hyperkeratosis on the extremities and suffer corneal vascularization ultimately resulting in blindness. Patients are particular sensitive to severe bacterial and fungal skin infections and have a predisposition to development of squamous cell carcinoma (reviewed by [1]).

In the present work, we present a case history of a child who presented with deafness and recurrent skin rashes caused by a heterozygous point mutation in GJB2 (c.424T > C; p.F142L referred to as F142L throughout). There are three other clinical reports of the F142L mutation (Table S1). [12-14] In the present work, we compare the functional effects of three CX26 mutations: D66H, G12R and F142L with wild-type (WT) CX26 in cells expressing connexins representative of the epidermis. The study is the first to provide empirical data on the pathophysiological consequences of the F142L mutation and suggests that F142L and G12R result in collapse of the microtubule network that can be partially rescued by interaction with CX43.

2 | MATERIALS AND METHODS

2.1 | Histology

Four mm² punch biopsies of clinically affected skin of the groin were fixed in formalin and processed for haematoxylin and eosin staining following standard procedures prior to routine clinical histological analysis.

2.2 | Sequencing

Mutation screening of the GJB2 gene was performed via East of Scotland Regional Genetics Service, NHS Laboratory, Ninewells Hospital, Dundee, UK

2.3 | Wild type and mutant CX26 expression plasmids

Human CX26WT cDNA fused in frame to pmCherry-N1 or pEGFP-N1 (BD Biosciences Clontech PT3975-5 and PT3027-5, respectively) yielding constructs CX26WT-mCherry or CX26WT-GFP, respectively, were used as previously described. The point mutation c.424T > C (encoding p.F142L) was introduced to CX26WT-GFP and CX26WT-mCherry as previously described for plasmids CX26D66H and G12R. All constructs were fully verified by sequencing (GATC Biotech (Germany)) and analysed by CLUSTALV sequence alignment.

2.4 | Cell Culture and transfection

Cell lines HeLa Ohio (ATCC, Manassas, VA, USA) and HaCaT (CLS, Eppelheim, Germany) were cultured at 37°C, 5% CO₂ in Dulbecco’s modified Eagle’s medium (DMEM, Lonza, Slough, UK) supplemented
with 10% (v/v) foetal calf serum and L-glutamine (2 mmol/L) and 100 µg/mL penicillin/streptomycin (cDMEM) (Lonza, Slough, UK). HeLa26 and HeLa43 (HeLa cells stably transfected to express mouse (m) Cx26 or mCx43, respectively) were maintained in cDMEM supplemented with puromycin (0.5 µmol/L). Cells were transfected using Lipofectamine 3000 kit for 18-24 hours with 0.5 µg of the appropriate plasmid cDNA (Thermo Fisher Scientific, UK). Transfection efficiency was determined by virtue of eGFP or mCherry autofluorescence.

2.5 | Drug treatments

Cells were treated with the Cx channel blocker carbenoxolone (CBX) (100 µmol/L), paclitaxel (stabilizer of the microtubular network) (0.25 µmol/L, Sigma) or thapsigargin (depletes ER calcium stores and induces ER stress) (1 µmol/L, Sigma) as required.

2.6 | Immunofluorescence

Cells were fixed in ice-cold methanol and processed for immunocytochemical analysis as previously described. Connexin localization was determined by either GFP or mCherry autofluorescence. CX26WT was detected using a mouse anti-Cx26 antibody (1:200 dilution 13-8100; Invitrogen, UK) and Cx43WT with a Rabbit anti-Cx43 antibody (1:500 dilution, kindly gifted by E. Leithe[20], and β-tubulin was identified using a β-tubulin monoclonal antibody (1:100 dilution, T4026; Sigma). Secondary antibodies were goat anti-rabbit or mouse conjugated to Alexa 488 or Alexa 594 (1:100 dilution, T4026; Sigma). Nuclei were stained with DAPI (5 µg/mL) (Sigma-Aldrich). Imaging was carried out on a Zeiss Axiovert microscope linked up to a 800 Airyscan confocal system under appropriate conditions and images processed using Zen Lite software.

2.7 | RNA isolation and quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR)

Total RNA was isolated from transfected HeLa Ohio cells using ISOLATE II RNA Mini Kit according to manufacturers' instructions (Bioline) and cDNA synthesized with the M-MLV reverse transcription kit (Promega, UK). Primers against CHOP, GADD34 and the reference gene GAPDH were used in TaqMan PCR reactions (Primer design) as previously described.

2.8 | Connexin Functionality assays

Eighteen hours post-transfection, the ability of cells to uptake propidium iodide (PI, MW 668) [10 µmol/L] or carboxyfluorescein (CBF, MW 473) [100 µmol/L] assessed hemichannel function, where appropriate cells were preblocked with CBX for 1 hour, which was maintained on the cells throughout the experiment. Cells were exposed to the relevant dye for 10 minutes at 37°C in serum-free medium (SFM) in the presence or absence of CBX. The dye was removed, and the wells washed twice with PBS containing CBX. Wells were filled with PBS (+Ca²⁺) to shut hemichannels and viewed on an EVOS fluorescent microscope equipped with filter sets permitting excitation at 498 nm and emission 520 nm.

Parachute assays determined the ability of F142L to form channels with CX26WT. HeLa Ohio cells were transfected with CX26WT or CX26F142L-mCherry tagged constructs. Twenty-four hours post-transfection, these “acceptor” cells were overlaid with a cell suspension of calcein AM-labelled HeLa26 “donor” cells. Cells were co-incubated for 6 hours at 37°C and the transfer of calcein AM to underlying transfected HeLa Ohio monolayers calculated as previously described.

2.9 | Cell viability assays

Cell viability was determined by MTT (3-(4,5-diethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma) assays. HeLa Ohio and HeLa43 cells seeded onto 96-well plates (5 × 10³ cells per well) were transfected with relevant GFP-tagged plasmids. Six hours post-transfection, CBX was added to selected wells. Twenty-four hours post-transfection, the cells were incubated in 50 µL SFM ± CBX and 50 µL MTT [5 mg/mL] at 37°C for 1 hour, followed by replacing the media with 100 µL DMSO, incubation at room temperature for 30 minutes and reading the absorbance at 570 nm. Results were expressed as the % viability compared to Cx26WT-GFP transfected cells ± SEM.

2.10 | Immunoprecipitation and Western Blotting

HeLa43 cells transfected with relevant GFP plasmids were harvested in RIPA buffer (125 mmol/L NaCl, 5 mmol/L EDTA, 1% (w/v) sodium deoxycholate and 0.5% (v/v) Triton X-100) 24 hours post-transfection. To 25 µL sheep anti-rabbit IgG dynabeads (Thermo Fisher Scientific, UK) previously incubated with anti-GFP antibody (1:100; ab290, Abcam, UK), 500 µg total protein was added and incubated at 4°C overnight followed by elution of bound protein in 5x Laemmli buffer. Protein was subjected to SDS-PAGE and Western blot analysis and probed with primary antibodies targeted to GFP (1:1000 dilution), Cx43 (1:500 dilution, Rivedal [20]), GRP78 BiP (1:1000 dilution, ab290, Abcam, UK) or GAPDH (1:5000 dilution, sc-32233, Santa Cruz, Heidelberg, Germany), at 4°C overnight. Fluorescent secondary goat anti-mouse IRDye 680RD or goat anti-rabbit IRDye 800 (Li-COR, Lincoln, Nebraska) was used, and the bands were detected by the Odyssey® FC Dual Mode Li-COR Imaging System.
2.11 | Statistical analysis

Experiments were performed in triplicate and repeated on at least three separate occasions (n = 3). Data were compiled using GraphPad Prism 8 software. Results are expressed as mean ± SEM, and statistical analysis was performed using ANOVA followed by unpaired Student’s t test or Dunnett’s multiple comparison test as appropriate. *P < .05; **P < .01; ***P < .001.

3 | RESULTS

3.1 | Clinical case study

A 6-year-old Caucasian girl with profound bilateral congenital sensory neuronal hearing loss was presented with recurrent skin rashes (Figure 1A–C). Hearing loss was identified at newborn screening, and bilateral cochlear implants were inserted at 2 years of age. At age 6 weeks, she developed well-defined erythematous hyperkeratotic plaques primarily affecting the perineal area and to a lesser extent affecting the face and trunk. Her nipples were hyperkeratotic, but palms, soles and nails appeared normal. There was no blistering. A diagnosis of acrodermatitis enteropathica was initially considered, but zinc levels were normal. The rash fluctuated in severity, with flares lasting about a week before subsiding to leave yellow-brown verrucous plaques. Her skin was empirically treated with topical emollients, barrier preparations and combined topical corticosteroid/antifungal creams, with some improvement.

She suffered recurrent abscesses in the left pre-auricular area, related to a complex left branchial cleft anomaly. At 6 years of age, the branchial cleft anomaly was excised, but wound healing was complicated by infection and the development of prolonged erythema and hyperkeratosis requiring topical emollients, keratolytics and steroid treatment. At this age, her height was tracking the 50th centile, but weight gain was faltering on the 0.4th centile. Her development was otherwise age appropriate. Serum mineral and vitamin levels were within acceptable limits, and there was no evidence of coeliac disease.

The child had flexion contractures of the hip, knee and ankle joints, causing a mixed crouch/taiptoe gait. This improved following surgery to lengthen the hamstrings and Achilles tendons, but again was complicated by hyperkeratotic erythema at the surgical scars.

Other noted features were mild angular cheilitis and poor primary dentition requiring dental extractions (Figure 1C). There was no other mucosal involvement and no eye involvement. At the age of 6 years, she appeared to have a normal head of hair, but her parents reported that her hair had been fine and sparse during her first few
years. MRI scanning revealed bilateral duplication of the internal auditory meatus and a probable maxillary dentigerous cyst. Her family history was unremarkable.

A skin biopsy from the groin demonstrated hyperkeratosis with a prominent granular layer, basket-weave keratin and no sign of parakeratosis, unlike that observed in psoriasis (Figure 1D). Genetic screening identified a single heterozygous point mutation (c.424T > C) in GJB2 (CX26), which substitutes phenylalanine with leucine at codon 142 (F142L; Figure 1E). The mutation was absent in the proband’s unaffected parents and younger sibling, suggesting it arose as a spontaneous mutation. Screening for two common deletions in the GJB6 gene encoding CX30 (del(GJB6-DB51830) and del(GJB6-D13518540)) was negative.[23,24]

### 3.2 | Comparison of the spatial localization of CX26 F142L with other CX26 mutations and CX26WT in keratinocytes and HeLa cells

The subcellular spatial localization of F142L was compared with CX26G12R and CX26D66H. In HaCaT cells, CX26WT-GFP formed large plaques at areas of cell-to-cell contact in similar regions to endogenously expressed CX43 (Figure 2Ai). All three mutations were distributed throughout the cytoplasm and at perinuclear areas with limited evidence of gap junction plaque formation; intracellular co-localization of endogenous CX43 was also evident (Figure 2Aii-iv).

Similar localizations were observed in HeLa43-transfected cells with evidence of intracellular co-localization with Cx43WT in cells co-expressing the CX26 mutations tagged with GFP (green) (Figure 2Bi-iv). In HeLa26 cells, transfected to express CX26WT-mCherry the two wild-type CX26 proteins also trafficked to the plasma membrane where gap junction plaques were evident (yellow staining at cell periphery) (Figure 2Cii). By contrast, in HeLa26 cells co-expressing F142L-mCherry the mutated protein was restricted to perinuclear areas, as was observed in cells expressing F142L-GFP (Figure 2Biii, Ciii). In HeLa26 cells co-expressing G12R-GFP, the mutant protein was predominantly located intracellularly with limited plasma membrane targeting observed (Figure 2Cii). In D66H-GFP expressing HeLa26 cells, the mutant protein was restricted to the endoplasmic reticulum Golgi intermediate compartment (ERGIC) region (Figure 2Civ).

### 3.3 | F142L and the KID mutation G12R disrupt the β-tubulin network

The microtubule network plays an important role in the trafficking of connexins to the plasma membrane. Co-staining of the cells with an antibody targeting β-tubulin revealed a disruption of the microtubule network in HeLa26 cells expressing F142L-mCherry (Figure 2Dii) and D66H-GFP (Figure 2Diii) in comparison with non-transfected cells and those expressing Cx26WT or D66H-GFP where the microtubular network remained intact (Figure 2Di and Div). To determine whether stabilization of the microtubule network could rescue the trafficking deficiency, cells were treated with paclitaxel for 20 hours post-transfection (Figure 2Ei-iv). The integrity of the microtubule network recovered in cells expressing both G12R and F142L; however, the proteins were still largely restricted to perinuclear areas (Figure 2Eii, iii). In cells expressing CX26WT or D66H, paclitaxel treatment did not influence the spatial localization of the connexin proteins (Figure 2Ei, iv).

### 3.4 | The trafficking deficiency of F142L mutation is not associated with ER stress

No significant change in ER stress, assessed via RT-PCR and Western blot analysis of GRP78 and GADD34 expression, was detected in HeLa Ohio cells (that do not express endogenous connexins) transfected with any of the Cx-expressing plasmids (supplementary data Figure 1A-C).

### 3.5 | Functional characteristics of Cx26F142L

Previously, we determined that in transfected HeLa Ohio cells, D66H does not form functional channels.[16,17,25] By contrast, ATP release and dye transfer assays determined that the KID mutation G12R forms leaky connexin channels.[16] In addition, HeLa Ohio cells expressing D66H were unable to take up propidium iodide and the level of PI uptake in G12R transfected HeLa Ohio cells was greater than those expressing Cx26WT, which was inhibited by co-incubation with CBX (Figure 3A). Following exposure of HeLa Ohio cells transfected to express CX26WT-mCherry to the fluorescent dye CBF (λex 494 nm; λem 518 nm), ~60% of transfected cells took up the fluorescent dye, inhibited by co-incubation with the Cx channel blocker CBX (Figure 3B). By contrast in cells expressing F142L-mCherry, less than 10% of cells took up CBF, which was again inhibited by co-incubation with CBX (Figure 3B). Parachute assays further determined the ability of CX26-F142L to form gap junction channels with CX26WT. HeLa Ohio cells transfected to express CX26WT-mCherry or F142L-mCherry were overlaid with a suspension of HeLa26 cells loaded with calcein AM. CX26WT transfected cells efficiently accepted the dye from the HeLa26 donor cells, while the % of F142L expressing cells accepting the dye was less than 10%, significantly lower than CX26WT (Figure 3C).

### 3.6 | The influence of CX26 mutations on cell viability

In HeLa Ohio cells expressing D66H, no significant difference in cell viability levels in the presence or absence of CBX was
FIGURE 2  Localization of CX26 mutations and the impact on CX43 and the microtubule network. HaCaT cells (A) and HeLa43 (B) were transfected with CX26WT-GFP (i), CX26F142L-GFP (ii), CX26G12R-GFP (iii) or CX26D66H-GFP (iv) (Cx - green) and co-stained for CX43 (red). HeLa26 cells (C) were transfected with CX26WT-mCherry (red) (i), CX26F142L-mCherry (red) (ii), CX26G12R-GFP (green) (iii) or CX26D66H-GFP (green) (iv) and co-stained CX26 (stained green in Ci, Cii and red in Ciii and Civ). Areas of co-localization stained yellow. White arrow indicates trapped F142L, and yellow arrow indicates CX43 Gap Junction staining. HeLa26 cells (D) were transfected with CX26WT-GFP (green) (i), CX26F142L-mCherry (red) (ii), CX26G12R-GFP (green) (iii) or CX26D66H-GFP (green) (iv) and were co-stained for β-tubulin (stained red Di, Dii, Div and green in Diii). HeLa26 cells (E) were transfected with CX26WT-GFP(i), CX26F142L-mCherry (ii), CX26G12R-GFP (iii) or CX26D66H-GFP (iv) and treated with paclitaxel throughout the transfection period followed by co-staining with β-tubulin (stained red Ei, Eii, Eiv and green in Eiii). Nuclei were stained with DAPI (blue). Scale bar = 10 µm
observed in comparison with cells expressing Cx26WT. Cell viability was reduced by ~50% for cells expressing F142L and G12R. In both cases, co-incubation with CBX throughout the transfection period increased cell viability (Figure 3D). By contrast when F142L and G12R mutations were expressed in HeLa43 cells, a significant improvement in cell viability occurred compared to expression in the HeLa Ohio cells (Figure 3E). Furthermore, no significant difference in cell viability was observed between these mutations with Cx26WT and D66H transfected cells (Figure 3E).

3.7 | CX26 mutations interact with CX43

To determine whether the mutant proteins alter CX43:CX26 compatibility, co-immunoprecipitation (IP) reactions were carried out in HeLa43 cells transfected to express eGFP, Cx26WT, G12R, D66H or F142L fused GFP plasmids. HeLa Ohio cells transfected with eGFP were used as a negative control. Western blot of the IP samples determined that anti-GFP-coated beads succeeded in pulling down all the GFP-tagged constructs. The eGFP expressed in either HeLa Ohio or HeLa43 cells showed bands corresponding to eGFP (~28kDA). The chimeric proteins (Cx26WT, G12R, F142L and D66H) were detected at the size of ~54kDA (Figure 4 IP:GFP:GFP blot). No bands were detected in the HeLa Ohio negative control membrane probed with a Cx43 antibody. CX26WT-GFP was not detected in the IP:GFP:Cx43 blot, indicating that there was no interaction between these two proteins. By contrast, all the mutants co-immunoprecipitated with Cx43. In addition, the eGFP control, that was transfected in either HeLa Ohio or HeLa 43 cells, showed no interaction with the Cx43 antibody (Figure 4 IP:GFP:Cx43). Thus, the
data suggest that interaction with Cx43 occurs and that this is a way in which aberrant CX26 mutations in the skin are rescued.

Table 1 summarizes the results obtained.

4 | DISCUSSION

In the present study, we provide a detailed clinical case study of a patient harbouring a heterozygous CX26 mutation resulting in amino acid change F142L in the third transmembrane domain of the protein. Using a range of cell-based assays, we determined that this mutation alters the ability of CX26 to assemble into gap junctions, affects the microtubule network and is able to form heteromeric channels with Cx43. There are three other clinical reports of the F142L mutation, and all cases demonstrate significant clinical overlap, particularly psoriasiform dermatitis, sensorineural deafness and recurrent infections. The genotype-phenotype has been described as “mucositis-deafness syndrome,” as marked oropharyngeal and oesophageal mucositis were present in the first four cases. However, apart from mild angular cheilitis, mucosal involvement was not evident in the present patient. A further case developed metastatic squamous cell carcinoma (SCC) of the hard palate. Musculoskeletal impairment with lower limb joint and muscle contractures was also evident in the present case (Table 1).

The chronic skin inflammation associated with the F142L mutation suggested similarities to KID syndrome where patients, including those with the G12R mutation, are also susceptible to severe bacterial and fungal infections. “Leaky” hemichannels, with altered gating characteristics, are proposed to be a causative event in the progression of KID syndrome. Each KID mutation studied to date has unique characteristics rendering the channels more sensitive to changes in calcium, zinc and other ions and are influenced by pro-inflammatory mediators such as peptidoglycan a key component of gram-positive bacterial cell wall, with several mutations resulting in lethal phenotypes. Overexpression of CX26 (for example in psoriasis) or these leaky KID hemichannels release ATP from the cells feeding into purinergic signalling pathways and associated pro-inflammatory signalling cascades leading to hyperproliferation loss of epidermal integrity and inflammation. CX channel blockers or inhibition of the overexpression of CX26 can reduce these events and may offer potential therapeutic strategies. However, the F142L mutation produced channels with

| TABLE 1 | Summary of clinical and cellular events of CX26 mutations |
|----------|----------------------------------|
| WTCX26 | G12R | D66H | F142L |
| Mutation domain | N/A | Amino terminus | EXL1 | TM3 |
| Clinical case | Normal | KID syndrome | Vohwinkel disease | Hyperkeratosis, hyperkeratotic plaques (supplementary table 1) |
| Associated with bacterial infection | Normal | Yes | No | Yes |
| Cellular Localization | PM | Perinuclear | Evidence microtubule instability | ERGIC/diffuse Intact cytoskeleton | Perinuclear Evidence microtubule instability |
| Cx Channel function | Normal | Increased HC activity | Loss of function | Decreased permeability |
| Impact on cell viability | No | Yes Rescued CBX | No | Yes Rescued CBX |
| Interaction with Cx43 evident | No | Yes Viability rescue Co-IP | Yes Co-IP | Yes Viability rescue Co-IP |

FIGURE 4 CX26 mutations co-immunoprecipitate with Cx43: HeLa43 cells were transfected to express eGFP, CX26WT, F142L, G12R or D66H-GFP and harvested for co-immunoprecipitation analysis. As a control, HeLa Ohio cells were transfected with eGFP. Western blots were probed with antibodies targeted to GFP (IP); Cx43 (IP) and the input lysates probed for Cx43 and GAPDH. n = 3 representative blot.
restricted permeability, that was further reduced by exposure to the channel inhibitor CBX. This suggests that metabolites exchanged via these restricted channels may influence cell viability pathways. The amino acid phenylalanine at position 142 in TM3 is highly conserved across Cx subtypes and species. Phenylalanine and leucine are both hydrophobic amino acids although leucine has an aliphatic side chain that likely alters the pore structure. The amino acid change interferes with channel activity but does not totally block channel function as is seen in CX26 mutations associated with non-inflammatory skin conditions (eg D66H, H73R), where the mutant proteins are trapped intracellularly, form non-functional channels and dominantly inhibit the function of wild-type connexins with no evidence of cell death observed.

The trapping of mutated Cx proteins in the ER and induction of an unfolded protein response (UPR) has been widely reported as a pathological consequence for connexin channelopathies. This includes some mutations in CX31 linked to EKV yet in other mutations associated with a lethal EKV phenotype no ER stress was evoked. A variety of CX26 mutations associated with hearing loss that were retained in the ER region did not evoke an ER stress response. No ER stress was observed in cells expressing CX26F142L, D66H or G12R suggesting that these CX26 mutations other events induce the pathogenic effects.

Connexins predominantly rely on an intact microtubule network and associated motor proteins such as consortin for efficient delivery to the plasma membrane. In cells expressing CX26F142L and the KID mutation G12R, a distinct loss in β-tubulin integrity occurred; however, both mutations were able to form heteromeric channels with CX26WT and CX43WT where reduced delivery of the WT protein to the plasma membrane occurred. Previously, we and others have shown that CX26 can adopt a microtubule-independent route to the plasma membrane and this may be a way in which the mutant proteins can be delivered. The β-tubulin loss was rescued by co-incubation with paclitaxol and suggests the mutations introduce alteration in cell cycle-mediated events, which was also evidenced by the loss of cell viability in cells expressing F142L and G12R. Similar events were recently reported for the GJB3 mutation CX31G45E associated with EKV. Indeed, the clinical features of the CX26F142L bare resemblance to some patterns of the EKV phenotype. Despite the diverse impact on channel gating observed in homomeric configurations a common feature of all of these β-connexin skin mutations is the ability to aberrantly interact with CX43 with a rescue of the loss of cell viability for G12R and F142L occurring. Thus, inhibition of F142L channel behaviour and interaction with CX43 influence cell viability. Recently, the importance of CX43 in polarity mediated cell cycle events and mitotic spindle orientation has been reported in luminal epithelial breast cancer cells. It is thus possible that altered CX43: CX26 heteromeric channels influence asymmetric cell division required for stratification of the epidermis and contribute to hyperproliferative status of the skin.

In conclusion, the molecular mechanisms underlying the CX26F142L mutation are aberrant intercellular communication via defective heterotypic CX26F142L:CX43 channels that trigger signalling pathways resulting in epidermal hyperproliferation (hyperkeratosis), inflammation and delays in wound healing. Interestingly, delays or complications in wound healing in reports of KID syndrome have been reported, especially in patients undergoing cochlear implant procedures. As peptides and siRNA therapies targeting connexins show success in preclinical wound healing trials, such agents could be considered to improve targeted wound closure in KID patients in the future. Further studies are now required to detail the signalling pathways affected and strategies to alleviate these conditions.

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CONFLICT OF INTEREST
The authors declare no conflicting interests.

AUTHORS CONTRIBUTION
ML, AW and MZ identified the patient and provided clinical follow up. MZ organized genetic screening and histological investigations. PEM, AA and MZ devised the study and AA performed the laboratory work with inputs from AS and YY. AA and ML wrote the first draft of the manuscript with inputs from AW, MZ and PEM. PEM collated the final version of the manuscript.

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Table S1. Summary of reported clinical features with the mutation comparison test compared to control ***

Additional supporting information may be found online in the Supporting Information section.

Supporting Information

Figure S1. Impact of Cx26 mutations on ER Stress: Twenty-four hours post transfection of HeLaOhio cells with eGFP, CX26WT, G12R, D66H or F142L cells were harvested, protein or mRNA extracted and ER stress levels determined. Relative gene expression of GADD34 compared to GAPDH by qPCR (\(n=3\)) (A); Representative western blot probed for expression of GRP78/BiP and GAPDH (B); Densitometric analysis of \(n=3\) western blots where protein expression of GRP78/BiP is expressed relative to GAPDH (C). Cells were exposed to Thapsigargin for 8 hours as a positive control. Statistical analysis performed by ANVOA followed by Dunnett’s multiple comparison test compared to control *** < .005.

Table S1. Summary of reported clinical features with the mutation CX26F142L.

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