Read-through for nonsense mutations in type XVII collagen deficient junctional epidermolysis bullosa

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Abbreviations:

C17, type XVII collagen; DEJ, dermal epidermal junction EB, epidermolysis bullosa; JEB, junctional epidermolysis bullosa; TRIDs, translational read-through inducing drugs.

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TO THE EDITOR

Junctional epidermolysis bullosa (JEB) is caused by mutations in genes encoding adhesion proteins, such as laminin 332, type XVII collagen (C17), integrin α6β4 or integrin α3. Absence of C17 leads to intermediate JEB that manifests with generalized skin blisters, chronic wounds, hair loss, nail loss or dystrophy, and enamel hypoplasia (Has et al., 2020). There is no cure, and no experimental therapy has been developed for JEB with C17 deficiency (Prodinger et al., 2020). About 20% of the *COL17A1* pathogenic variants are nonsense mutations leading to absence of C17. Studies of genotype-phenotype correlations showed that small amounts of partially functional C17 have biological relevance and translate into mild phenotypes (Condrat et al., 2018; Kroeger et al., 2019, 2017; Ruzzi et al., 2001). Here, we address the question whether translational read-through inducing drugs (TRIDs) have effect on *COL17A1* nonsense mutations and represent a therapeutic option to alleviate disease severity.

After written informed consent, keratinocytes from seven JEB patients with *COL17A1* nonsense mutations were studied (Supplementary Methods). *COL17A1* mRNA was expressed in all cells with various degrees of decay in cases 3, 5, 6 and 7. Four cell lines were completely devoid of C17 (cases 1, 2, 4 and 7), while three of them (cases 3, 5 and 6) expressed low levels of C17 as compared to normal keratinocytes (Figure 1a). This was due either to a second heterozygous splicing mutation (cases 3 and 6), or to natural alternative splicing of exon 33 containing the pathogenic variant p.R795* (case 5) (Ruzzi et al., 2001). We focused on the C17 negative keratinocytes carrying the homozygous mutations p.W464*, p.R688*, p.R1226*, and p.S140*, and treated them with the TRIDs gentamicin, paromomycin, G418, amlexanox or PTC124. While amlexanox and PTC124 had no effect on any of the mutant keratinocytes, a differential response to aminoglycosides was observed (Figure 1b). The p.W464* mutant keratinocytes demonstrated a robust and dose dependent response to gentamicin (125-1000 μg/ml), paromomycin (500-2000 μg/ml) and G418 (1-10 μg/ml) (Figure 1c). The amount of C17 expressed after treatment with 500 μg/ml gentamicin was much smaller than that of normal cells (Figure 2a). The effect of gentamicin, paromomycin and G418 on the p.R688* mutant keratinocytes was subtle, and C17 was clearly detectable when the highest TRIDs concentrations were used (Figure 1c). The other two mutations, p.R1226* and p.S140* did not respond to any TRIDs (Figure 1b and data not shown).

Next, we asked how long C17 was able to persist in JEB keratinocytes after a single treatment with gentamicin. Mutant p.W464* keratinocytes demonstrated the highest C17 level two days

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TO THE EDITOR

Junctional epidermolysis bullosa (JEB) is caused by mutations in genes encoding adhesion proteins, such as laminin 332, type XVII collagen (C17), integrin α6β4 or integrin α3. Absence of C17 leads to intermediate JEB that manifests with generalized skin blisters, chronic wounds, hair loss, nail loss or dystrophy, and enamel hypoplasia (Has et al., 2020). There is no cure, and no experimental therapy has been developed for JEB with C17 deficiency (Prodinger et al., 2020). About 20% of the *COL17A1* pathogenic variants are nonsense mutations leading to absence of C17. Studies of genotype-phenotype correlations showed that small amounts of partially functional C17 have biological relevance and translate into mild phenotypes (Condrat et al., 2018; Kroeger et al., 2019, 2017; Ruzzi et al., 2001). Here, we address the question whether translational read-through inducing drugs (TRIDs) have effect on *COL17A1* nonsense mutations and represent a therapeutic option to alleviate disease severity.

After written informed consent, keratinocytes from seven JEB patients with *COL17A1* nonsense mutations were studied (Supplementary Methods). *COL17A1* mRNA was expressed in all cells with various degrees of decay in cases 3, 5, 6 and 7. Four cell lines were completely devoid of C17 (cases 1, 2, 4 and 7), while three of them (cases 3, 5 and 6) expressed low levels of C17 as compared to normal keratinocytes (Figure 1a). This was due either to a second heterozygous splicing mutation (cases 3 and 6), or to natural alternative splicing of exon 33 containing the pathogenic variant p.R795* (case 5) (Ruzzi et al., 2001). We focused on the C17 negative keratinocytes carrying the homozygous mutations p.W464*, p.R688*, p.R1226*, and p.S140*, and treated them with the TRIDs gentamicin, paromomycin, G418, amlexanox or PTC124. While amlexanox and PTC124 had no effect on any of the mutant keratinocytes, a differential response to aminoglycosides was observed (Figure 1b). The p.W464* mutant keratinocytes demonstrated a robust and dose dependent response to gentamicin (125-1000 μg/ml), paromomycin (500-2000 μg/ml) and G418 (1-10 μg/ml) (Figure 1c). The amount of C17 expressed after treatment with 500 μg/ml gentamicin was much smaller than that of normal cells (Figure 2a). The effect of gentamicin, paromomycin and G418 on the p.R688* mutant keratinocytes was subtle, and C17 was clearly detectable when the highest TRIDs concentrations were used (Figure 1c). The other two mutations, p.R1226* and p.S140* did not respond to any TRIDs (Figure 1b and data not shown).

Next, we asked how long C17 was able to persist in JEB keratinocytes after a single treatment with gentamicin. Mutant p.W464* keratinocytes demonstrated the highest C17 level two days

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after addition of 500 µg/ml gentamicin. The C17 amount decreased progressively and was barely detectable six days after treatment (Figure 2b).

To investigate whether C17 expressed after read-through in JEB keratinocytes was functional and able to be deposited at the dermal-epidermal junction (DEJ), we analyzed its presence in cell culture media and in epidermal equivalents. The 120 kDa C17 shed ectodomain was found in conditioned media of p.W464* mutant keratinocytes after G418 treatment, suggesting that insertion of a cognate amino acid did not alter protein functionality, its transmembrane localization and its physiological cleavage (Figure 2c).

Epidermal equivalents containing p.W464* JEB keratinocytes were built and grafted subcutaneously in the back of immunodeficient mice as described (Benati et al., 2018). Engrafted mice (n=3) treated daily intraperitoneally for one week with 150 µg/ml gentamicin, but not with 75 µg/ml gentamicin or PBS, showed deposition of C17 at the DEJ (Figure 2d). However, C17 could not be visualized in gentamicin-treated epidermal equivalents constructed with p.R688* keratinocytes (Figure 2d), consistently with the very low efficacy achieved with gentamicin in vitro. Taken together, these results show that mutations p.W464* and p.R688* respond to aminoglycosides TRIDs, albeit differentially, and the newly formed C17 can be deposited at the DEJ.

Although gentamicin and paromomycin have been shown to be clinically effective in different settings, when applied topically (Lincoln et al., 2018; Ohguchi et al., 2018; Peled et al., 2020), their global effects on epidermal cells have not been characterized. We therefore assessed the impact of these drugs on keratinocytes through transcriptome analysis. Total RNA was extracted from p.W464* mutant keratinocytes that were either treated with 500 µg/ml gentamicin, 1000 µg/ml paromomycin, or not treated, and analyzed by RNASeq (Supplementary Methods and Supplementary Tables S1 and S2). The analysis revealed that gentamicin induces deeper transcriptional changes than those elicited by paromomycin. In fact, the expression of 234 genes was significantly upregulated and 884 genes were significantly downregulated after gentamicin treatment (FDR $\leq$0.05; fold change$\geq$2), whereas paromomycin only significantly upregulated 41 and downregulated 6 genes (Supplementary Tables S3 and S4). Among the common alterations due to both treatments, 44 genes were found to be similarly deregulated (Supplementary Figure S1a-b) and mainly associated to biological processes and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways including cell division, chromosome segregation, G2/M cell cycle, cell proliferation, and p53 signaling pathway (false discovery rate $\leq$0.05; Supplementary Table S5). The 20 most up- and down- regulated genes are shown in Supplementary Figure S1c. Whether these changes are associated with a common

effect of aminoglycoside TRIDs or with changes derived from the restoration of C17, remain to be determined. Interestingly, a significant reduction of mRNA levels for several adhesion proteins, such as *FERMT1*, *COL7A1*, *FN1* and *ITGB6* was noted after gentamicin, but not after paromomycin treatment (Supplementary Table S3, Supplementary Figure S1d). Genes involved in apoptosis were also de-regulated but did not reach statistical significance in this experiment, as shown for other cell types (Tao and Segil, 2015).

In summary, we show that aminoglycosides can induce read-through of *COL17A1* nonsense mutations, but the effects must be tested *in vitro* for each mutation before this treatment can be proposed to patients as a personalized therapy. Gentamicin has been already successfully used as a topical and systemic treatment in patients with different EB types (Hammersen et al., 2019; Li et al., 2020; Lincoln et al., 2018; Woodley et al., 2017). However, topical paromomycin may be an interesting alternative, as it seems to have a lower global effect on keratinocytes *in vitro*.

Data availability statement

Data related to this article are available under https://data.mendeley.com/datasets/9rbd5t9gy5/1.

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CRediT Contribution Statement:

Conceptualization: CH, MR, FLL; Data Curation: CH, MR, FLL; Formal Data Analysis: CH, SBS, SZ, IC, ECS, AY, MR, FLL; Funding Acquisition: CH, MR, FLL; Investigation: BSS, SZ, IC, AY; Methodology: CH, SBS, SZ, IC, ECS, AY, MR, FLL; Project Administration: CH, FLL; Resources: ; Software: CH, ECS; Supervision: CH, MR, FLL; Validation: BSS, SZ, ECS; Visualization: CH, SBS, SZ, IC, ECS; Writing – Original Draft Preparation: CH; Writing – Review and Editing: CH, SBS, SZ, IC, ECS, AY, MR, FLL.

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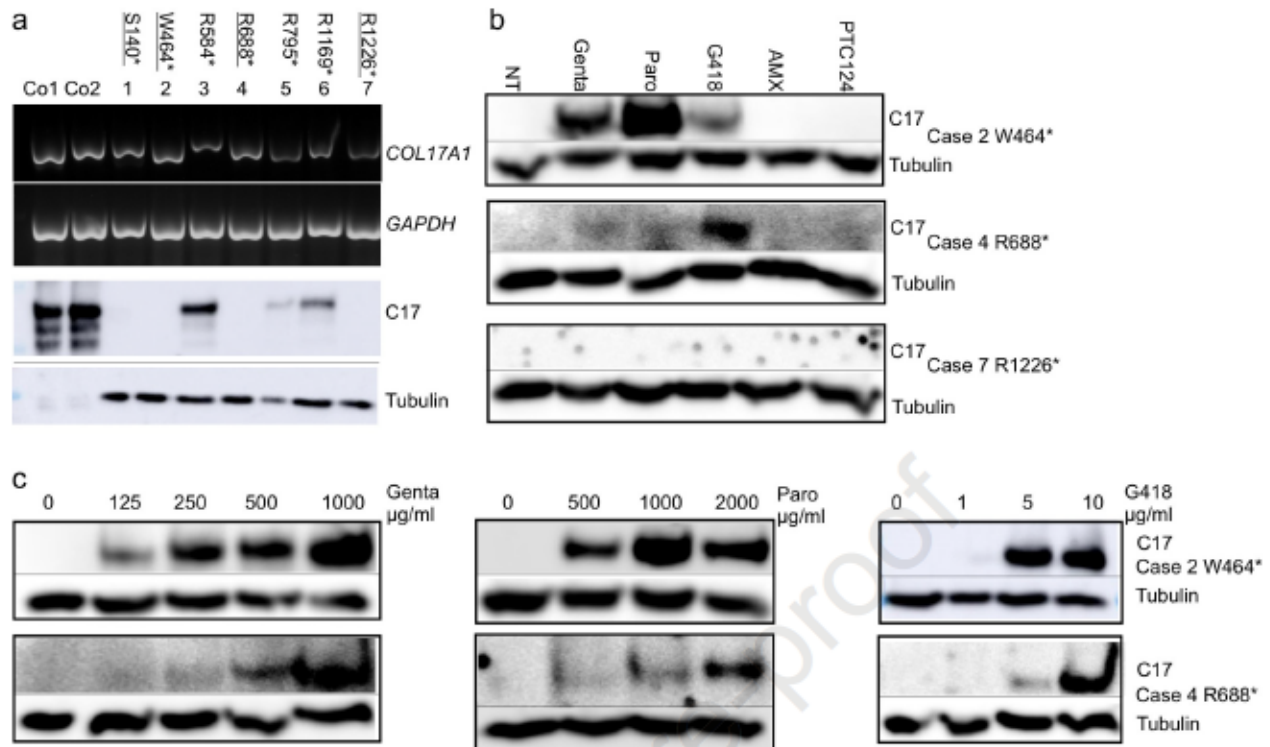
Conflicts of interest: 'none to declare'.
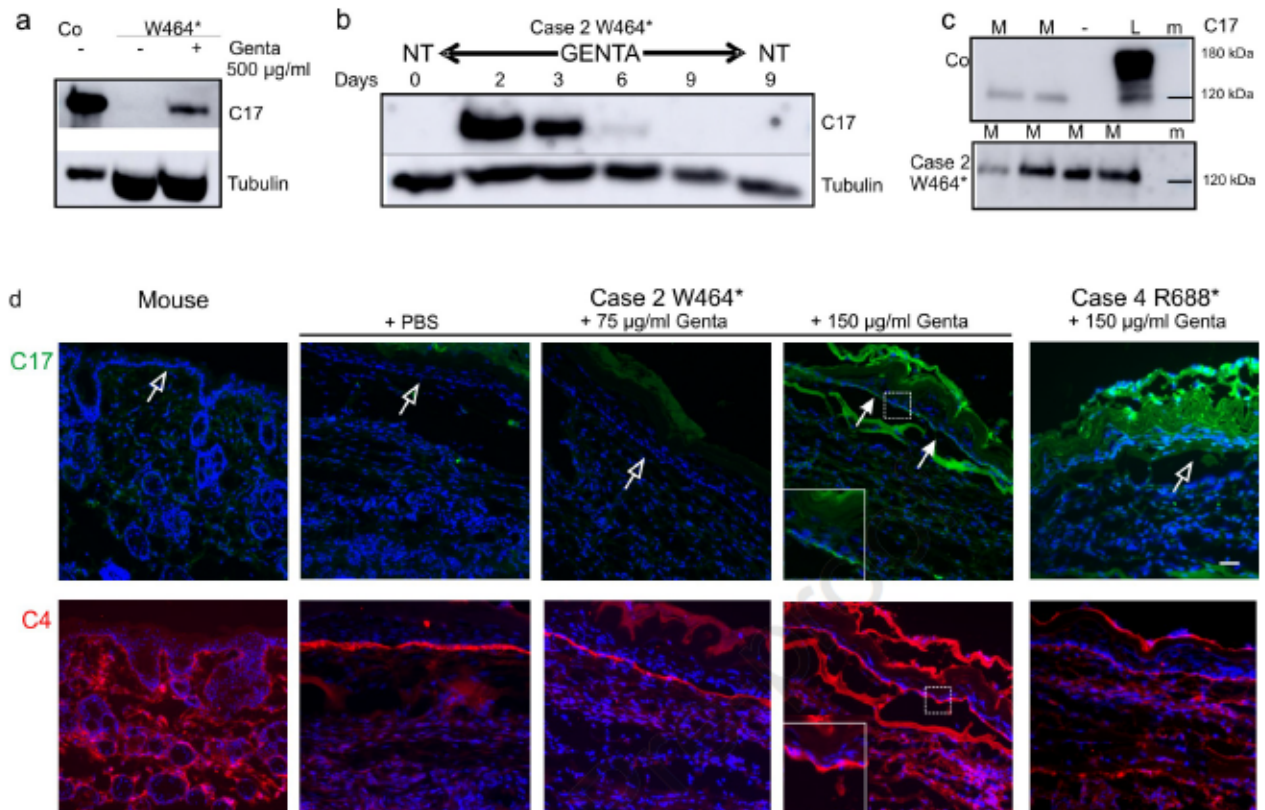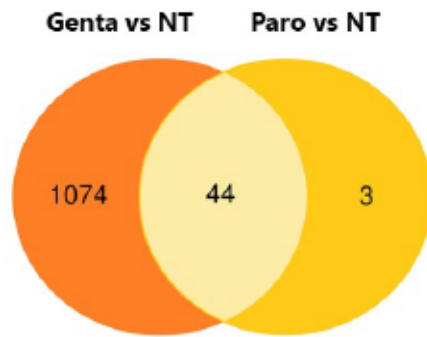
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Figure 1. JEB keratinocytes and their response to translational read-through inducing drugs. (a) Reverse transcriptase PCR (up) and immunoblots (down) show collagen XVII mRNA (*COL17A1*) and protein (C17) levels in keratinocytes in normal controls (Co1 and Co2) and cases 1-7 (nonsense mutations indicated on top). (b) Immunoblots show C17 in keratinocytes of cases 2, 4 and 7, without treatment (NT), and with single treatment of gentamicin (Genta, 1000 µg/ml), paromonycin (Paro, 2000 µg/ml), G418 (10µg/ml), amlexanox (AMX, 75 µg/ml) or PTC124 (20 µg/ml). Cells were incubated with indicated drugs for 48 hours. (c) Immunoblots show C17 in keratinocytes of cases 2 and 4 treated with the indicated concentrations of gentamicin (Genta), paromomycin (Paro) or G418 for 48 hours. GAPDH and tubulin, loading controls.

Figure 2. Characterization of read-through induced C17 in case 2 keratinocytes (W464*). (a) Immunoblot shows C17 and tubulin in control (Co) and in W464* keratinocytes. (b) W464* keratinocytes cultured without or with gentamicin (GENTA, 500 µg/ml) were lysed at the indicated time points after treatment and analyzed by immunoblot. (c) C17 was detected in the media (M) and lysate (L) of Co and in the media of W464* keratinocytes treated with G418 (10 µg/ml) (down). The 180 kDa full-length C17 is present only in the lysate and the 120 kDa ectodomain is present in both lysate and media. (d) C17 (white arrows) and collagen IV (C4) immunostaining in mouse skin and in grafts of epidermal equivalents. Open arrows, basement membrane; nuclei, blue; scale bar, 10 µm.
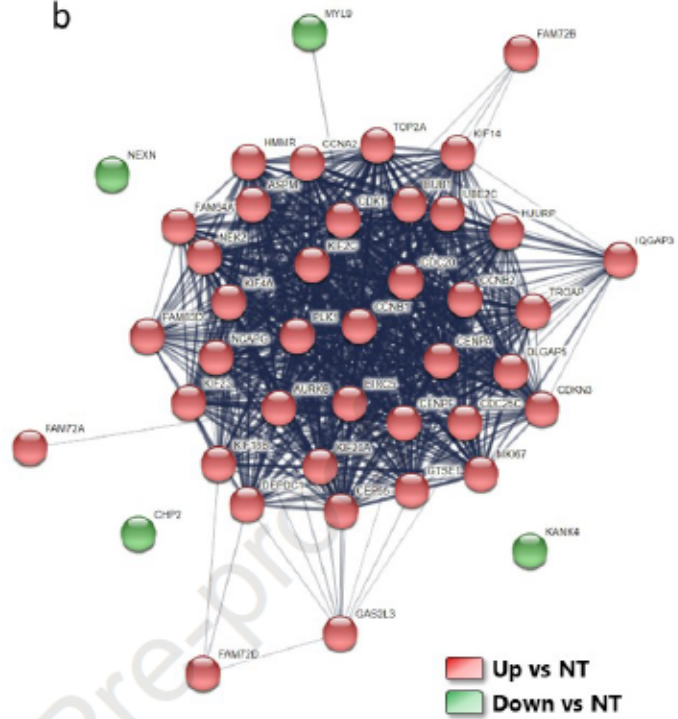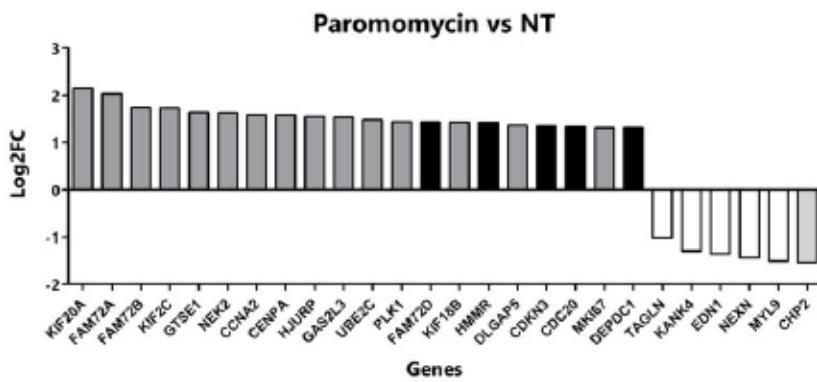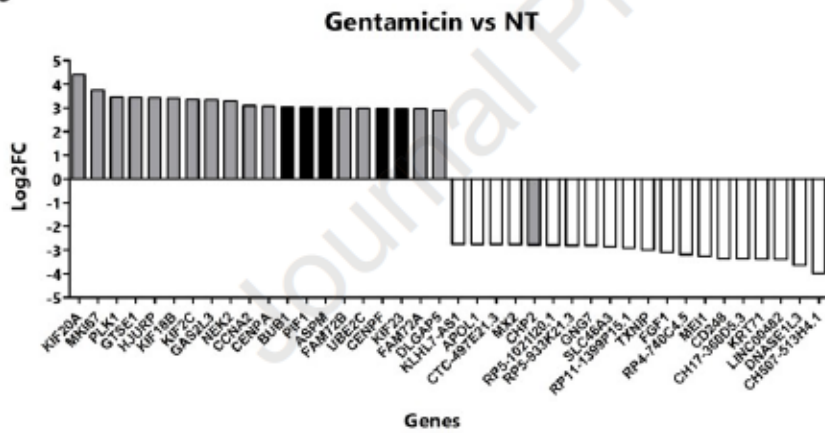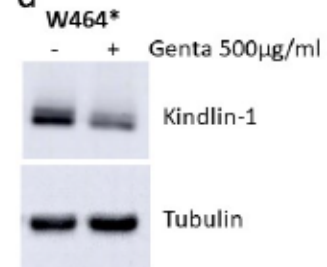
Supplementary Material

Supplementary Methods

Cell culture and treatments

After written informed consent, skin biopsies were obtained from seven JEB patients and healthy individuals who underwent surgery for therapeutic purposes, and used for isolation of keratinocytes with standard procedures (ethical approval Ethical Committee of the University of Freiburg EK-Freiburg 215/15). Cells were immortalized with a high titer lentivirus containing HPV-16 E6/E7 gene (BioCat), and cultured in defined keratinocyte growth medium supplemented with epidermal growth factor and bovine pituitary extract (Invitrogen, Karlsruhe, Germany)(He and Has 2019).

Cells were treated once with different concentrations of gentamicin (Sigma), paromomycin (Sigma), G418 (Gibco), amlexanox (AdipoGen) or PTC124 (ChemScene) and harvested in most experiments after 48 hours, or at the indicated time points (see Figure 2b).

Immunoblotting

For immunoblotting, cultured keratinocytes were homogenized either with a buffer containing 0.15 M NaCl, 50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1 mM Pefabloc and 5 mM EDTA as described previously (Tholen et al. 2016) or with RIPA buffer. Total protein content was determined using the micro Lowry assay (DC Protein Assay, Bio-Rad, Munich, Germany), and 40 μg of total protein from mutant samples and 10 μg total protein from healthy cells were used for SDS-PAGE. Proteins of the medium were concentrated with phenol/methanol precipitation. Normalized amounts of the proteins were subjected to SDS-PAGE, immunoblotted, and incubated with a monoclonal anti-C17 antibody (clone EPR18614, Abcam) or with an affinity purified antibody to kindlin-1 (Has et al. 2009). Antibodies to β-tubulin (Abcam, Cambridge, UK) were used as loading control. For semiquantitative quantification of C17, 0.5 μg total protein from normal keratinocytes and 35 μg total protein from W464* keratinocytes not treated and treated with 500 μg gentamicin was loaded on SDS gels. Quantification was performed in three independent experiments with ImageJ (https://imagej.nih.gov/ij/download.html).

RNA isolation, RT-PCR and RNA sequencing

Total RNA of keratinocytes was isolated using RNeasy Plus Mini kit (Qiagen, Hilden, Germany). One µg RNA complementary DNA was reverse transcribed using First Strand cDNA Synthesis kit (Thermo Fisher Scientific). RT-PCR was performed with the following primers F: TACCATGTACGTGTCAGGCC and R: TGATGCTGGACCACACATTG.

For RNA sequencing, for each condition (not treated - NT, treated with 500µg/ml gentamicin or with 1000 µg/ml paromomycin), RNA was extracted from three distinct culture flasks and sequenced independently.

RNA sequencing and bioinformatics analysis were performed by Novogene (en.novogene.com). In brief, mRNA was enriched using oligo(dT) beads. Library concentration was first quantified using a Qubit 2.0 fluorometer (Life Technologies). Insert size was checked on an Agilent 2100 and quantified using quantitative PCR (Q-PCR). Sequencing was performed with Illumina technology. Raw reads were filtered to remove reads containing adapters or reads of low quality, so that downstream analyses are based on clean reads. The filtering process was as follows: discard reads with adapter contamination, discard reads when uncertain nucleotides constitute more than 10 percent of either read (N > 10%), and discard reads when low quality nucleotides (base quality less than 20) constitute more than 50 percent of the read. Alignments were performed with HISAT2 to the reference, and quantification was performed with HTSeq. The differential gene expression analysis was performed with the software DESeq2. The Gene Ontology (GO, http://www.geneontology.org/) and KEGG (Kyoto Encyclopedia of Genes and Genomes) enrichment analyses of the differential expressed genes were performed to find out which biological functions or pathways are significantly associated with differential expressed genes. Graphs were performed with GraphPad Prism version 9.0.0 (GraphPad Software, La Jolla, CA).

Epidermal equivalents and immunofluorescence staining

Epidermal equivalents containing JEB keratinocytes were built and grafted on the back of immunodeficient mice as described previously (Benati et al. 2018). Mice were treated with intraperitoneal injections of gentamicin or with PBS for one week. Immunofluorescence staining of cryosections was performed by using a rabbit polyclonal antibody to type XVII collagen (Schäcke et al. 1998) and with DAPI for visualization of nuclei as described before (Has and He 2016).

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Supplementary Figure

Sup. Figure S1. Differentially expressed genes (DEGs) in JEB keratinocytes treated with gentamicin or paromomycin. (a) Venn Diagram of DEGs in gentamicin and paromomycin treated *vs* non-treated cells, shows 44 genes commonly deregulated in both treatments. **(b)** Protein-protein interaction network (STRING; string-db.org, version 11.0) of those 44 genes are shown. Line thickness represents the strength of data supporting the interaction. Nodes in red indicate up-regulation and green nodes indicate down-regulation. **(c)** The 20 most up- (black) and downregulated (white) genes after treatment with gentamicin or paromomycin are shown ($\log_2$fold change)   Genes represented in grey are those common in both datasets. **(d)** Immunoblot for kindlin-1 and tubulin with lysates of W464* keratinocytes without, and with 500 µg/ml gentamicin single treatment (cells were lysed 48 hours after treatment).

Supplementary Tables

Supplementary Table S1. Quality control data of RNA sequencing

Legend: JEB_Genta, JEB keratinocytes treated with gentamicin sample 1-3; JEB_NT, JEB keratinocytes not treated sample 1-3; JEB_Paro, JEB keratinocytes treated with paromomycin samples 1-3; G, Giga; raw_Reads: the original sequencing reads counts; clean_Reads: number of reads after filtering; raw_data: raw reads number multiply read length, saved in G unit; clean_data: clean reads number multiply read length, saved in G unit; error_rate: average sequencing error rate, which is calculated by Qphred=-10log10(e); Q20: percentages of bases whose correct base recognition rates are greater than 99% in total bases; Q30: percentages of bases whose correct base recognition rates are greater than 99.9% in total bases; GC_content: ercentages of G and C in total bases.

Supplementary Table S2. Overview of mapping status of RNA sequencing data

Legend: JEB_Genta, JEB keratinocytes treated with gentamicin sample 1-3; JEB_NT, JEB keratinocytes not treated sample 1-3; JEB_Paro, JEB keratinocytes treated with paromomycin samples 1-3; Total reads: Total number of filtered reads (Clean data); Total mapped: Total number of reads that can be mapped to the reference genome; Multiple mapped: Number of reads that can be mapped to multiple sites in the reference genome; Uniquely mapped: Number of reads that can be uniquely mapped to the reference genome; Read-1: Number of left read that can be mapped to the reference genome; Read-2: Number of right read that can be mapped to the reference genome; Reads map to '+': Number of reads that can be mapped to the positive strand (+); Reads map to '-': Number of reads that can be mapped to the minus strand (-); Non-splice reads: Number of reads that can be mapped entirely to a single exon; Splice reads: Number of reads that can be segmented and mapped to two exons (also named junction reads).

Supplementary Table S3. Differentially expressed genes in JEB keratinocytes treated with 500 μg/ml gentamicin *versus* not treated.

Supplementary Table S4. Differentially expressed genes in JEB keratinocytes treated with 1000 μg/ml paromomycin *versus* not treated.

Supplementary Table S5. Gene Ontology (GO) terms and KEGG pathways enriched in the list of 44 genes commonly deregulated in both treatments *vs* non-treated cells (FDR ≤0.05).

Supplementary Tables

Supplementary Table S1. Quality control data of RNA sequencing

Sample	raw_reads	clean_reads	raw_data(G)	clean_data(G)	error_rate(%)	Q20(%)	Q30(%)	GC_content(%)
JEB_Genta1	32632325	32044746	9.8	9.6	0.03	97.68	93.46	52.31
JEB_Genta2	19874924	19594293	6.0	5.9	0.03	97.49	92.91	51.95
JEB_Genta3	22772237	22387830	6.8	6.7	0.03	97.45	92.89	52.71
JEB_NT1	30577075	30043418	9.2	9.0	0.03	97.51	93.12	53.23
JEB_NT2	29389834	28857141	8.8	8.7	0.03	97.61	93.28	52.21
JEB_NT3	22028484	21763781	6.6	6.5	0.03	97.59	93.14	51.82
JEB_Paro1	25202673	24755668	7.6	7.4	0.03	97.72	93.53	51.95
JEB_Paro2	22596461	22284207	6.8	6.7	0.03	97.19	92.28	51.90
JEB_Paro3	26038147	25669945	7.8	7.7	0.03	97.74	93.59	51.61

Legend: JEB_Genta, JEB keratinocytes treated with gentamicin sample 1-3; JEB_NT, JEB keratinocytes not treated sample 1-3; JEB_Paro, JEB keratinocytes treated with paromomycin samples 1-3; G, Giga; raw_Reads: the original sequencing reads counts; clean_Reads: number of reads after filtering; raw_data: raw reads number multiply read length, saved in G unit; clean_data: clean reads number multiply read length, saved in G unit; error_rate: average sequencing error rate, which is calculated by Qphred=-10log10(e); Q20: percentages of bases whose correct base recognition rates are greater than 99% in total bases; Q30: percentages of bases whose correct base recognition rates are greater than 99.9% in total bases; GC_content: ercentages of G and C in total bases.

Supplementary Table S2. Overview of mapping status of RNA sequencing data

Sample name	JEB_Genta1	JEB_Genta2	JEB_Genta3	JEB_NT1	JEB_NT2	JEB_NT3	JEB_Paro1	JEB_Paro2	JEB_Paro3
Total reads	64089492	39188586	44775660	60086836	57714282	43527562	49511336	44568414	51339890
Total mapped	62400727 (97.36%)	38114614 (97.26%)	43537008 (97.23%)	58373349 (97.15%)	56208405 (97.39%)	42462358 (97.55%)	48289025 (97.53%)	43300757 (97.16%)	50088818 (97.56%)
Multiple mapped	4022683 (6.28%)	1711208 (4.37%)	3802354 (8.49%)	7235732 (12.04%)	1833745 (3.18%)	1492697 (3.43%)	1539619 (3.11%)	1810682 (4.06%)	1649654 (3.21%)
Uniquely mapped	58378044 (91.09%)	36403406 (92.89%)	39734654 (88.74%)	51137617 (85.11%)	54374660 (94.21%)	40969661 (94.12%)	46749406 (94.42%)	41490075 (93.09%)	48439164 (94.35%)
Read-1	29326266 (45.76%)	18312057 (46.73%)	19984670 (44.63%)	25694505 (42.76%)	27326987 (47.35%)	20596159 (47.32%)	23474407 (47.41%)	20919132 (46.94%)	24320396 (47.37%)
Read-2	29051778 (45.33%)	18091349 (46.16%)	19749984 (44.11%)	25443112 (42.34%)	27047673 (46.86%)	20373502 (46.81%)	23274999 (47.01%)	20570943 (46.16%)	24118768 (46.98%)
Reads map to '+'	29147339 (45.48%)	18177732 (46.39%)	19839554 (44.31%)	25528076 (42.49%)	27156463 (47.05%)	20462648 (47.01%)	23347505 (47.16%)	20720897 (46.49%)	24194772 (47.13%)
Reads map to '-'	29230705 (45.61%)	18225674 (46.51%)	19895100 (44.43%)	25609541 (42.62%)	27218197 (47.16%)	20507013 (47.11%)	23401901 (47.27%)	20769178 (46.60%)	24244392 (47.22%)
Non-splice reads	30246517 (47.19%)	19022344 (48.54%)	20769895 (46.39%)	26470046 (44.05%)	27758867 (48.10%)	20813557 (47.82%)	23799114 (48.07%)	21296907 (47.78%)	24498889 (47.72%)
Splice reads	28131527 (43.89%)	17381062 (44.35%)	18964759 (42.36%)	24667571 (41.05%)	26615793 (46.12%)	20156104 (46.31%)	22950292 (46.35%)	20193168 (45.31%)	23940275 (46.63%)

Legend: JEB_Genta, JEB keratinocytes treated with gentamicin sample 1-3; JEB_NT, JEB keratinocytes not treated sample 1-3; JEB_Paro, JEB keratinocytes treated with paromomycin samples 1-3; Total reads: Total number of filtered reads (Clean data); Total mapped: Total number of reads that can be mapped to the reference genome; Multiple mapped: Number of reads that can be mapped to multiple sites in the reference genome; Uniquely mapped: Number of reads that can be uniquely mapped to the reference genome; Read-1: Number of left read that can be mapped to the reference genome; Read-2: Number of right read that can be mapped to the reference genome; Reads map to '+': Number of reads that can be mapped to the positive strand (+); Reads map to '-': Number of reads that can be mapped to the minus strand (-); Non-splice reads: Number of reads that can be mapped entirely to a single exon; Splice reads: Number of reads that can be segmented and mapped to two exons (also named junction reads).

1

Supplementary Table S3. Differentially expressed genes in JEB keratinocytes treated with 500 µg/ml gentamicin *versus* not treated.

Supplementary Table S4. Differentially expressed genes in JEB keratinocytes treated with 1000 µg/ml paromomycin *versus* not treated.

Supplementary Table S5. Gene Ontology (GO) terms and KEGG pathways enriched in the list of 44 genes commonly deregulated in both treatments *vs* non-treated cells (FDR ≤ 0.05).

after addition of 500 µg/ml gentamicin. The C17 amount decreased progressively and was barely detectable six days after treatment (Figure 2b).

To investigate whether C17 expressed after read-through in JEB keratinocytes was functional and able to be deposited at the dermal-epidermal junction (DEJ), we analyzed its presence in cell culture media and in epidermal equivalents. The 120 kDa C17 shed ectodomain was found in conditioned media of p.W464* mutant keratinocytes after G418 treatment, suggesting that insertion of a cognate amino acid did not alter protein functionality, its transmembrane localization and its physiological cleavage (Figure 2c).

Epidermal equivalents containing p.W464* JEB keratinocytes were built and grafted subcutaneously in the back of immunodeficient mice as described (Benati et al., 2018). Engrafted mice (n=3) treated daily intraperitoneally for one week with 150 µg/ml gentamicin, but not with 75 µg/ml gentamicin or PBS, showed deposition of C17 at the DEJ (Figure 2d). However, C17 could not be visualized in gentamicin-treated epidermal equivalents constructed with p.R688* keratinocytes (Figure 2d), consistently with the very low efficacy achieved with gentamicin *in vitro*. Taken together, these results show that mutations p.W464* and p.R688* respond to aminoglycosides TRIDs, albeit differentially, and the newly formed C17 can be deposited at the DEJ.

Although gentamicin and paromomycin have been shown to be clinically effective in different settings, when applied topically (Lincoln et al., 2018; Ohguchi et al., 2018; Peled et al., 2020), their global effects on epidermal cells have not been characterized. We therefore assessed the impact of these drugs on keratinocytes through transcriptome analysis. Total RNA was extracted from p.W464* mutant keratinocytes that were either treated with 500 µg/ml gentamicin, 1000 µg/ml paromomycin, or not treated, and analyzed by RNASeq (Supplementary Methods and Supplementary Tables S1 and S2). The analysis revealed that gentamicin induces deeper transcriptional changes than those elicited by paromomycin. In fact, the expression of 234 genes was significantly upregulated and 884 genes were significantly downregulated after gentamicin treatment (FDR $\leq$0.05; fold change$\geq$2), whereas paromomycin only significantly upregulated 41 and downregulated 6 genes (Supplementary Tables S3 and S4). Among the common alterations due to both treatments, 44 genes were found to be similarly deregulated (Supplementary Figure S1a-b) and mainly associated to biological processes and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways including cell division, chromosome segregation, G2/M cell cycle, cell proliferation, and p53 signaling pathway (false discovery rate $\leq$0.05; Supplementary Table S5). The 20 most up- and down- regulated genes are shown in Supplementary Figure S1c. Whether these changes are associated with a common

effect of aminoglycoside TRIDs or with changes derived from the restoration of C17, remain to be determined. Interestingly, a significant reduction of mRNA levels for several adhesion proteins, such as *FERMT1*, *COL7A1*, *FN1* and *ITGB6* was noted after gentamicin, but not after paromomycin treatment (Supplementary Table S3, Supplementary Figure S1d). Genes involved in apoptosis were also de-regulated but did not reach statistical significance in this experiment, as shown for other cell types (Tao and Segil, 2015).

In summary, we show that aminoglycosides can induce read-through of *COL17A1* nonsense mutations, but the effects must be tested *in vitro* for each mutation before this treatment can be proposed to patients as a personalized therapy. Gentamicin has been already successfully used as a topical and systemic treatment in patients with different EB types (Hammersen et al., 2019; Li et al., 2020; Lincoln et al., 2018; Woodley et al., 2017). However, topical paromomycin may be an interesting alternative, as it seems to have a lower global effect on keratinocytes *in vitro*.

Data availability statement

Data related to this article are available under https://data.mendeley.com/datasets/9rbd5t9gy5/1.

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Conceptualization: CH, MR, FLL; Data Curation: CH, MR, FLL; Formal Data Analysis: CH, SBS, SZ, IC, ECS, AY, MR, FLL; Funding Acquisition: CH, MR, FLL; Investigation: BSS, SZ, IC, AY; Methodology: CH, SBS, SZ, IC, ECS, AY, MR, FLL; Project Administration: CH, FLL; Resources: ; Software: CH, ECS; Supervision: CH, MR, FLL; Validation: BSS, SZ, ECS; Visualization: CH, SBS, SZ, IC, ECS; Writing – Original Draft Preparation: CH; Writing – Review and Editing: CH, SBS, SZ, IC, ECS, AY, MR, FLL.

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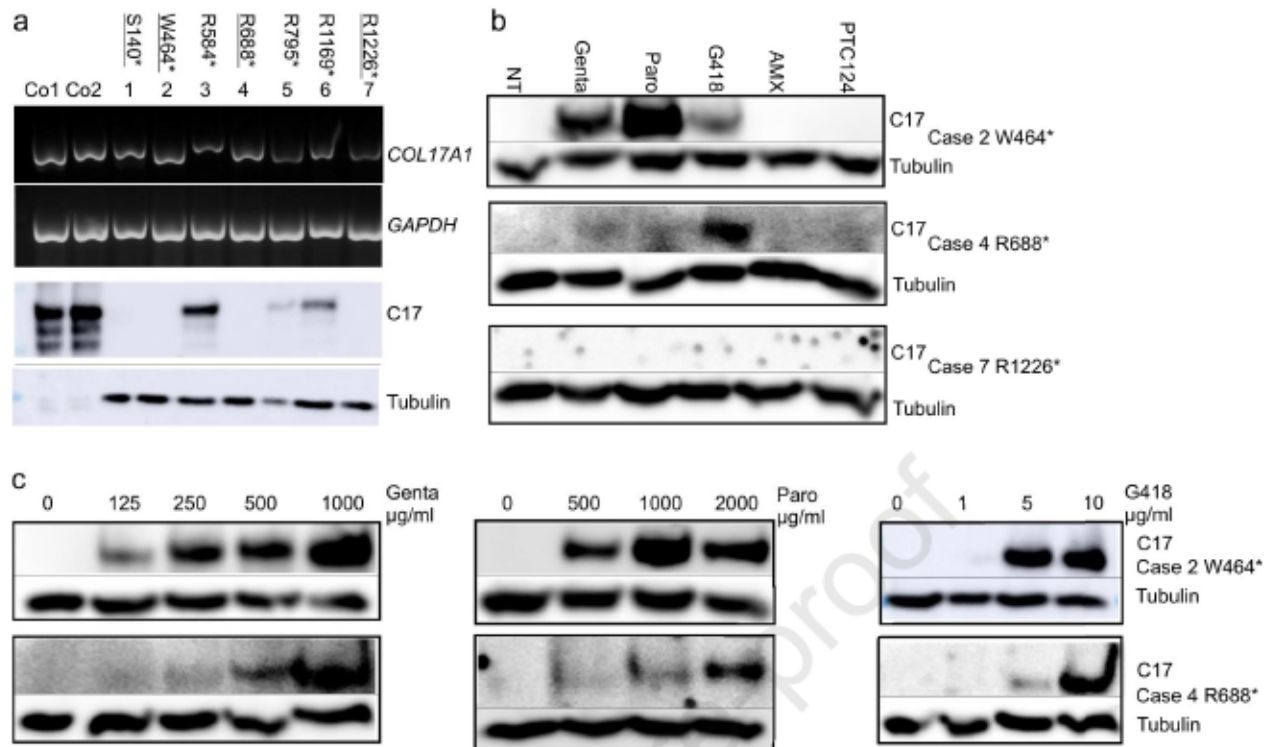
Conflicts of interest: 'none to declare'.
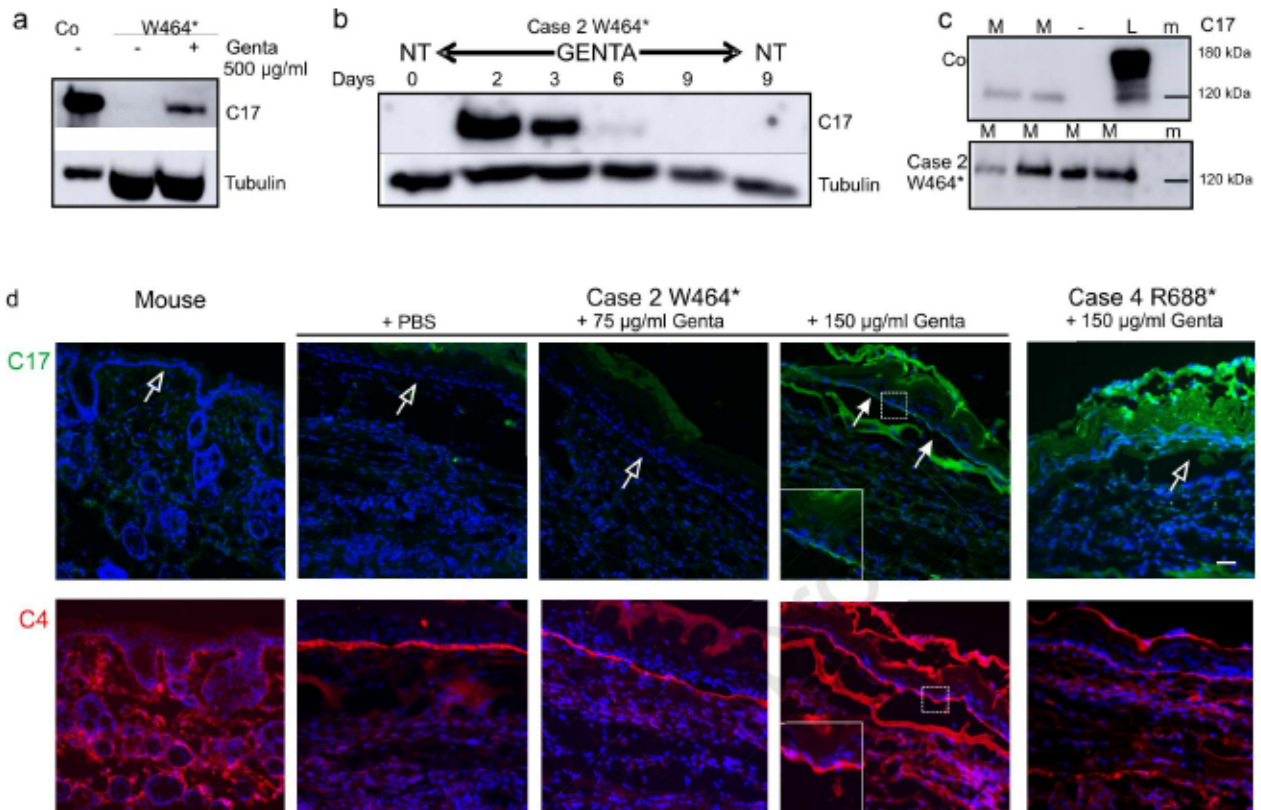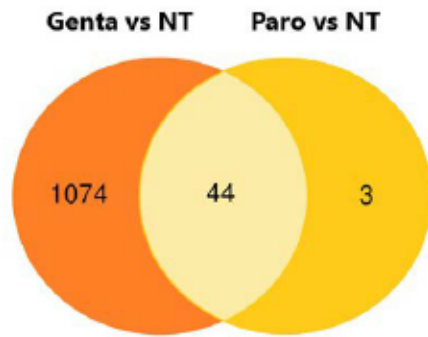
Figure legends

Figure 1. JEB keratinocytes and their response to translational read-through inducing drugs. (a) Reverse transcriptase PCR (up) and immunoblots (down) show collagen XVII mRNA (*COL17A1*) and protein (C17) levels in keratinocytes in normal controls (Co1 and Co2) and cases 1-7 (nonsense mutations indicated on top). (b) Immunoblots show C17 in keratinocytes of cases 2, 4 and 7, without treatment (NT), and with single treatment of gentamicin (Genta, 1000 µg/ml), paromonycin (Paro, 2000 µg/ml), G418 (10µg/ml), amlexanox (AMX, 75 µg/ml) or PTC124 (20 µg/ml). Cells were incubated with indicated drugs for 48 hours. (c) Immunoblots show C17 in keratinocytes of cases 2 and 4 treated with the indicated concentrations of gentamicin (Genta), paromomycin (Paro) or G418 for 48 hours. GAPDH and tubulin, loading controls.

Figure 2. Characterization of read-through induced C17 in case 2 keratinocytes (W464*). (a) Immunoblot shows C17 and tubulin in control (Co) and in W464* keratinocytes. (b) W464* keratinocytes cultured without or with gentamicin (GENTA, 500 µg/ml) were lysed at the indicated time points after treatment and analyzed by immunoblot. (c) C17 was detected in the media (M) and lysate (L) of Co and in the media of W464* keratinocytes treated with G418 (10 µg/ml) (down). The 180 kDa full-length C17 is present only in the lysate and the 120 kDa ectodomain is present in both lysate and media. (d) C17 (white arrows) and collagen IV (C4) immunostaining in mouse skin and in grafts of epidermal equivalents. Open arrows, basement membrane; nuclei, blue; scale bar, 10 µm.

a

S140*
W464*
R584*
R688*
R795*
R1169*
R1226*

Co1 Co2
1 2 3 4 5 6 7

COL17A1

GAPDH

C17

Tubulin

b

NT
Genta
Paro
G418
AMX
PTC124

C17
Case 2 W464*
Tubulin

C17
Case 4 R688*
Tubulin

C17
Case 7 R1226*
Tubulin

c

0 125 250 500 1000 Genta µg/ml

0 500 1000 2000 Paro µg/ml

0 1 5 10 G418 µg/ml

C17
Case 2 W464*
Tubulin

C17
Case 4 R688*
Tubulin

a

Co   W464*
-      -   +   Genta
              500 µg/ml

C17

Tubulin

b

Case 2 W464*
NT ◄───GENTA───► NT

Days   0   2   3   6   9   9

C17

Tubulin

c

M   M   -   L   m   C17

Co   180 kDa
                          120 kDa

M   M   M   M   m

Case 2
W464*   120 kDa

d   Mouse   + PBS   Case 2 W464*   + 150 µg/ml Genta   Case 4 R688*
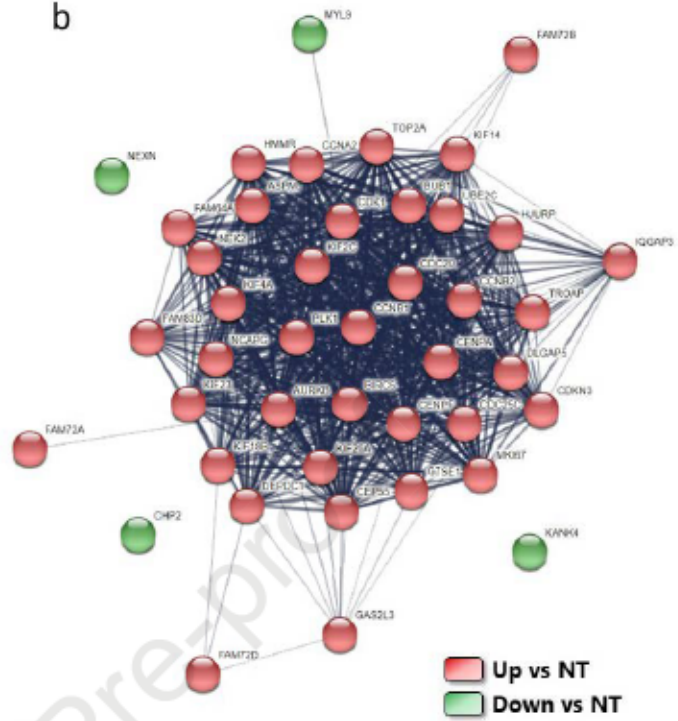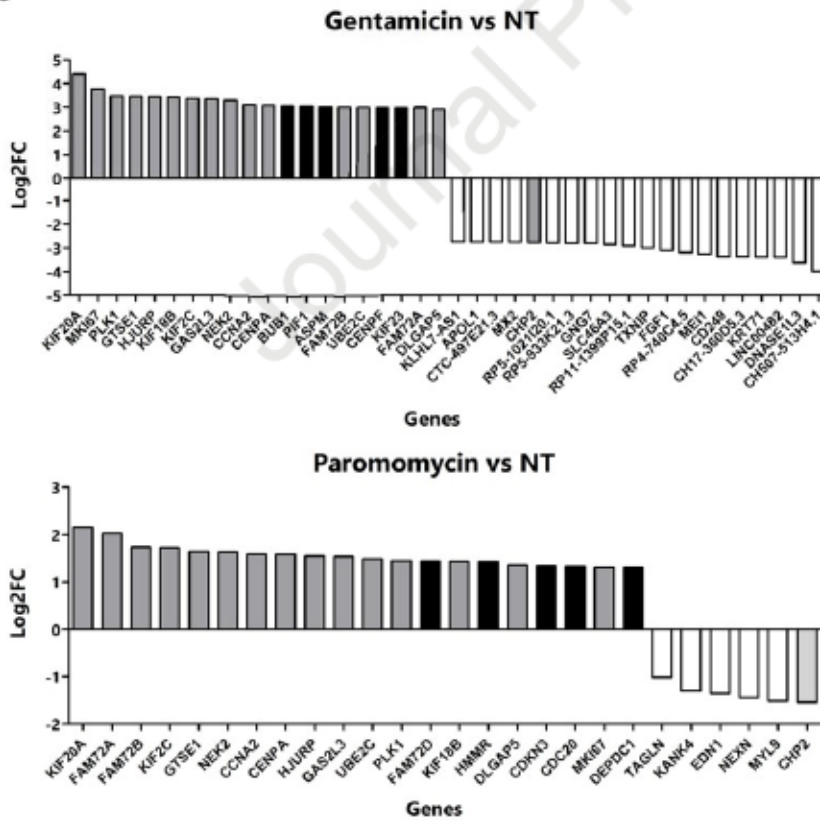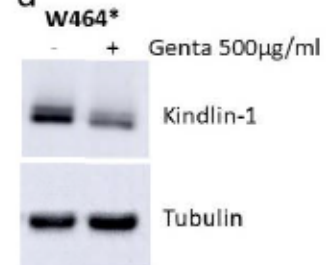   + 75 µg/ml Genta   + 150 µg/ml Genta

C17

C4

Supplementary Material

Supplementary Methods

Cell culture and treatments

After written informed consent, skin biopsies were obtained from seven JEB patients and healthy individuals who underwent surgery for therapeutic purposes, and used for isolation of keratinocytes with standard procedures (ethical approval Ethical Committee of the University of Freiburg EK-Freiburg 215/15). Cells were immortalized with a high titer lentivirus containing HPV-16 E6/E7 gene (BioCat), and cultured in defined keratinocyte growth medium supplemented with epidermal growth factor and bovine pituitary extract (Invitrogen, Karlsruhe, Germany)(He and Has 2019).

Cells were treated once with different concentrations of gentamicin (Sigma), paromomycin (Sigma), G418 (Gibco), amlexanox (AdipoGen) or PTC124 (ChemScene) and harvested in most experiments after 48 hours, or at the indicated time points (see Figure 2b).

Immunoblotting

For immunoblotting, cultured keratinocytes were homogenized either with a buffer containing 0.15 M NaCl, 50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1 mM Pefabloc and 5 mM EDTA as described previously (Tholen et al. 2016) or with RIPA buffer. Total protein content was determined using the micro Lowry assay (DC Protein Assay, Bio-Rad, Munich, Germany), and 40 μg of total protein from mutant samples and 10 μg total protein from healthy cells were used for SDS-PAGE. Proteins of the medium were concentrated with phenol/methanol precipitation. Normalized amounts of the proteins were subjected to SDS-PAGE, immunoblotted, and incubated with a monoclonal anti-C17 antibody (clone EPR18614, Abcam) or with an affinity purified antibody to kindlin-1 (Has et al. 2009). Antibodies to β-tubulin (Abcam, Cambridge, UK) were used as loading control. For semiquantitative quantification of C17, 0.5 μg total protein from normal keratinocytes and 35 μg total protein from W464* keratinocytes not treated and treated with 500 μg gentamicin was loaded on SDS gels. Quantification was performed in three independent experiments with ImageJ (https://imagej.nih.gov/ij/download.html).

RNA isolation, RT-PCR and RNA sequencing

Total RNA of keratinocytes was isolated using RNeasy Plus Mini kit (Qiagen, Hilden, Germany). One µg RNA complementary DNA was reverse transcribed using First Strand cDNA Synthesis kit (Thermo Fisher Scientific). RT-PCR was performed with the following primers F: TACCATGTACGTGTCAGGCC and R: TGATGCTGGACCACACATTG.

For RNA sequencing, for each condition (not treated - NT, treated with 500µg/ml gentamicin or with 1000 µg/ml paromomycin), RNA was extracted from three distinct culture flasks and sequenced independently.

RNA sequencing and bioinformatics analysis were performed by Novogene (en.novogene.com). In brief, mRNA was enriched using oligo(dT) beads. Library concentration was first quantified using a Qubit 2.0 fluorometer (Life Technologies). Insert size was checked on an Agilent 2100 and quantified using quantitative PCR (Q-PCR). Sequencing was performed with Illumina technology. Raw reads were filtered to remove reads containing adapters or reads of low quality, so that downstream analyses are based on clean reads. The filtering process was as follows: discard reads with adapter contamination, discard reads when uncertain nucleotides constitute more than 10 percent of either read (N > 10%), and discard reads when low quality nucleotides (base quality less than 20) constitute more than 50 percent of the read. Alignments were performed with HISAT2 to the reference, and quantification was performed with HTSeq. The differential gene expression analysis was performed with the software DESeq2. The Gene Ontology (GO, http://www.geneontology.org/) and KEGG (Kyoto Encyclopedia of Genes and Genomes) enrichment analyses of the differential expressed genes were performed to find out which biological functions or pathways are significantly associated with differential expressed genes. Graphs were performed with GraphPad Prism version 9.0.0 (GraphPad Software, La Jolla, CA).

Epidermal equivalents and immunofluorescence staining

Epidermal equivalents containing JEB keratinocytes were built and grafted on the back of immunodeficient mice as described previously (Benati et al. 2018). Mice were treated with intraperitoneal injections of gentamicin or with PBS for one week. Immunofluorescence staining of cryosections was performed by using a rabbit polyclonal antibody to type XVII collagen (Schäcke et al. 1998) and with DAPI for visualization of nuclei as described before (Has and He 2016).

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Supplementary Figure

Sup. Figure S1. Differentially expressed genes (DEGs) in JEB keratinocytes treated with gentamicin or paromomycin. (a) Venn Diagram of DEGs in gentamicin and paromomycin treated *vs* non-treated cells, shows 44 genes commonly deregulated in both treatments. **(b)** Protein-protein interaction network (STRING; string-db.org, version 11.0) of those 44 genes are shown. Line thickness represents the strength of data supporting the interaction. Nodes in red indicate up-regulation and green nodes indicate down-regulation. **(c)** The 20 most up- (black) and downregulated (white) genes after treatment with gentamicin or paromomycin are shown ($\log_2$fold change)   Genes represented in grey are those common in both datasets. **(d)** Immunoblot for kindlin-1 and tubulin with lysates of W464* keratinocytes without, and with 500 µg/ml gentamicin single treatment (cells were lysed 48 hours after treatment).

Supplementary Tables

Supplementary Table S1. Quality control data of RNA sequencing

Legend: JEB_Genta, JEB keratinocytes treated with gentamicin sample 1-3; JEB_NT, JEB keratinocytes not treated sample 1-3; JEB_Paro, JEB keratinocytes treated with paromomycin samples 1-3; G, Giga; raw_Reads: the original sequencing reads counts; clean_Reads: number of reads after filtering; raw_data: raw reads number multiply read length, saved in G unit; clean_data: clean reads number multiply read length, saved in G unit; error_rate: average sequencing error rate, which is calculated by Qphred=-10log10(e); Q20: percentages of bases whose correct base recognition rates are greater than 99% in total bases; Q30: percentages of bases whose correct base recognition rates are greater than 99.9% in total bases; GC_content: ercentages of G and C in total bases.

Supplementary Table S2. Overview of mapping status of RNA sequencing data

Legend: JEB_Genta, JEB keratinocytes treated with gentamicin sample 1-3; JEB_NT, JEB keratinocytes not treated sample 1-3; JEB_Paro, JEB keratinocytes treated with paromomycin samples 1-3; Total reads: Total number of filtered reads (Clean data); Total mapped: Total number of reads that can be mapped to the reference genome; Multiple mapped: Number of reads that can be mapped to multiple sites in the reference genome; Uniquely mapped: Number of reads that can be uniquely mapped to the reference genome; Read-1: Number of left read that can be mapped to the reference genome; Read-2: Number of right read that can be mapped to the reference genome; Reads map to '+': Number of reads that can be mapped to the positive strand (+); Reads map to '-': Number of reads that can be mapped to the minus strand (-); Non-splice reads: Number of reads that can be mapped entirely to a single exon; Splice reads: Number of reads that can be segmented and mapped to two exons (also named junction reads).

Supplementary Table S3. Differentially expressed genes in JEB keratinocytes treated with 500 µg/ml gentamicin *versus* not treated.

Supplementary Table S4. Differentially expressed genes in JEB keratinocytes treated with 1000 µg/ml paromomycin *versus* not treated.

Supplementary Table S5. Gene Ontology (GO) terms and KEGG pathways enriched in the list of 44 genes commonly deregulated in both treatments *vs* non-treated cells (FDR ≤0.05).

Supplementary Tables

Supplementary Table S1. Quality control data of RNA sequencing

Sample	raw_reads	clean_reads	raw_data(G)	clean_data(G)	error_rate(%)	Q20(%)	Q30(%)	GC_content(%)
JEB_Genta1	32632325	32044746	9.8	9.6	0.03	97.68	93.46	52.31
JEB_Genta2	19874924	19594293	6.0	5.9	0.03	97.49	92.91	51.95
JEB_Genta3	22772237	22387830	6.8	6.7	0.03	97.45	92.89	52.71
JEB_NT1	30577075	30043418	9.2	9.0	0.03	97.51	93.12	53.23
JEB_NT2	29389834	28857141	8.8	8.7	0.03	97.61	93.28	52.21
JEB_NT3	22028484	21763781	6.6	6.5	0.03	97.59	93.14	51.82
JEB_Paro1	25202673	24755668	7.6	7.4	0.03	97.72	93.53	51.95
JEB_Paro2	22596461	22284207	6.8	6.7	0.03	97.19	92.28	51.90
JEB_Paro3	26038147	25669945	7.8	7.7	0.03	97.74	93.59	51.61

Legend: JEB_Genta, JEB keratinocytes treated with gentamicin sample 1-3; JEB_NT, JEB keratinocytes not treated sample 1-3; JEB_Paro, JEB keratinocytes treated with paromomycin samples 1-3; G, Giga; raw_Reads: the original sequencing reads counts; clean_Reads: number of reads after filtering; raw_data: raw reads number multiply read length, saved in G unit; clean_data: clean reads number multiply read length, saved in G unit; error_rate: average sequencing error rate, which is calculated by Qphred=-10log10(e); Q20: percentages of bases whose correct base recognition rates are greater than 99% in total bases; Q30: percentages of bases whose correct base recognition rates are greater than 99.9% in total bases; GC_content: ercentages of G and C in total bases.

Supplementary Table S2. Overview of mapping status of RNA sequencing data

Sample name	JEB_Genta1	JEB_Genta2	JEB_Genta3	JEB_NT1	JEB_NT2	JEB_NT3	JEB_Paro1	JEB_Paro2	JEB_Paro3
Total reads	64089492	39188586	44775660	60086836	57714282	43527562	49511336	44568414	51339890
Total mapped	62400727 (97.36%)	38114614 (97.26%)	43537008 (97.23%)	58373349 (97.15%)	56208405 (97.39%)	42462358 (97.55%)	48289025 (97.53%)	43300757 (97.16%)	50088818 (97.56%)
Multiple mapped	4022683 (6.28%)	1711208 (4.37%)	3802354 (8.49%)	7235732 (12.04%)	1833745 (3.18%)	1492697 (3.43%)	1539619 (3.11%)	1810682 (4.06%)	1649654 (3.21%)
Uniquely mapped	58378044 (91.09%)	36403406 (92.89%)	39734654 (88.74%)	51137617 (85.11%)	54374660 (94.21%)	40969661 (94.12%)	46749406 (94.42%)	41490075 (93.09%)	48439164 (94.35%)
Read-1	29326266 (45.76%)	18312057 (46.73%)	19984670 (44.63%)	25694505 (42.76%)	27326987 (47.35%)	20596159 (47.32%)	23474407 (47.41%)	20919132 (46.94%)	24320396 (47.37%)
Read-2	29051778 (45.33%)	18091349 (46.16%)	19749984 (44.11%)	25443112 (42.34%)	27047673 (46.86%)	20373502 (46.81%)	23274999 (47.01%)	20570943 (46.16%)	24118768 (46.98%)
Reads map to '+'	29147339 (45.48%)	18177732 (46.39%)	19839554 (44.31%)	25528076 (42.49%)	27156463 (47.05%)	20462648 (47.01%)	23347505 (47.16%)	20720897 (46.49%)	24194772 (47.13%)
Reads map to '-'	29230705 (45.61%)	18225674 (46.51%)	19895100 (44.43%)	25609541 (42.62%)	27218197 (47.16%)	20507013 (47.11%)	23401901 (47.27%)	20769178 (46.60%)	24244392 (47.22%)
Non-splice reads	30246517 (47.19%)	19022344 (48.54%)	20769895 (46.39%)	26470046 (44.05%)	27758867 (48.10%)	20813557 (47.82%)	23799114 (48.07%)	21296907 (47.78%)	24498889 (47.72%)
Splice reads	28131527 (43.89%)	17381062 (44.35%)	18964759 (42.36%)	24667571 (41.05%)	26615793 (46.12%)	20156104 (46.31%)	22950292 (46.35%)	20193168 (45.31%)	23940275 (46.63%)

Legend: JEB_Genta, JEB keratinocytes treated with gentamicin sample 1-3; JEB_NT, JEB keratinocytes not treated sample 1-3; JEB_Paro, JEB keratinocytes treated with paromomycin samples 1-3; Total reads: Total number of filtered reads (Clean data); Total mapped: Total number of reads that can be mapped to the reference genome; Multiple mapped: Number of reads that can be mapped to multiple sites in the reference genome; Uniquely mapped: Number of reads that can be uniquely mapped to the reference genome; Read-1: Number of left read that can be mapped to the reference genome; Read-2: Number of right read that can be mapped to the reference genome; Reads map to '+': Number of reads that can be mapped to the positive strand (+); Reads map to '-': Number of reads that can be mapped to the minus strand (-); Non-splice reads: Number of reads that can be mapped entirely to a single exon; Splice reads: Number of reads that can be segmented and mapped to two exons (also named junction reads).

Supplementary Table S3. Differentially expressed genes in JEB keratinocytes treated with 500 µg/ml gentamicin *versus* not treated.

Supplementary Table S4. Differentially expressed genes in JEB keratinocytes treated with 1000 µg/ml paromomycin *versus* not treated.

Supplementary Table S5. Gene Ontology (GO) terms and KEGG pathways enriched in the list of 44 genes commonly deregulated in both treatments *vs* non-treated cells (FDR ≤0.05).