		29 <b>31</b>	44 45	48	56	59	61	65	<b>68</b> 8	1 146 <b>149</b>	154	164
DE 6750	CID17 HUMAN	OWDUSAFUC	SNTTVEFPT	WEC			PLOC	F	SSI.I.AI.PPALETA			KDELCAA
P36730	CLDI7 HOMAN	OWDUSAFUC	SNTTVEEDI	WEC			DT.OC	KFV	SST.T.AT. PPAT.FTAL		DATHICC	KRELCAA
GSQEDS	GSQLDA GOKGO	OWDUSAFUC	SNTTVEEDT				DIOC					KDELCAA
HZQKW9	HZQKW9 PANTR	OWDUCAEVC	CNTTVEEDU		TWMNCTPC		DT OC	L FV	CCLUAT DDAT FTAL			KDELGAA
GISA44	GISA44 NOMLE	QWRVSAFVG	SNITVEED				RLQC					KDELGAA
G/PIL6	G/PIL6_MACFA	QUINDALING	CNITVEEDI				TT OC					KDELGAA
F60F88	F60F88 MACMU	QWRVSAFVG	SNILVFERL		LWMNCIR,		RLQC			C RDFIN	PAVHIG	KRELGAA
AUAU96N9XI	AUAU96N9XI_PAPAN	QWRVSAFVG	SNILVEERL				RLQC			C RDFIN	PAVITIC	KRELGAA
AUAUD9SB18	AUAUD9SB18 CHLSB	QWRVSAFVG	SNILVERL				RLQC			C RDFIN	PAVHLG	KRELGAA
E2RSC8	E2RSC8_CANFA	QWRVSAFVG	SNIIVFERL	WEG	LWMNCVR	2AK1	RLQC	KFI	SOLLALPPALEAA	C RDFIN		KRELGAA
L5KXP8	L5KXP8_PTEAL	QWRVSAFVG	SNILVFERL	WEG	LWMNCIR	ZVKV	TLQC	KFI	NDLLALPPELEAA	C RDFIN		KRELGAA
L9KDM8	L9KDM8_TUPCH	QWRVSAFVG	SNIIVFERL	WEG	TWMNCIK	ZAKV	RLQU	KII	SOLLALPPALEAA	C RDFIN	PATHVG	KRELGAA
F6VLZ2	F6VLZ2_HORSE	QWRVSAFIG	SNIIVFERL	WEG	LWMTCVRC	ZARV	RTŐC	KFI	SELLALPPALLAA	C RDEYN	PAINIG	KRELGAA
HOWN64	HOWN64_OTOGA	QWRVSAFVG	SNIIVFERL	WEG	LWMNCIRC	ĮAKV	RTŐC	KFI	SSLLALPPVLEAA	C RDEYD	PAVHVGÇ	OKRELGAA
HOUYE1	HOUYE1_CAVPO	QWRVSAFIG	SNIIIFERL	WEG	;LWMNCVRÇ	QARI	RLQC	KFI	TOMLALPPAVETA	R REFYS	PNVPIGÇ	2KRELGAA
M3WXT4	M3WXT4_FELCA	QWRVSAFVG	SNIIVFERL	WEG	LWMNCVRC	2AKV	RLQC	KFY	SSLLALPPVLEAA	R RDFYD	PTIHVGÇ	2KRELGAA
C3VMW3	C3VMW3_PIG	QWRVSAF1G	SNIIVFERI	WEG	JWMNCVRÇ	2AKA	RLQC	K FY	SSMLALSPALEAA	R RDFYN	PAVHVGÇ	OKRELGAA
G5AZU4	G5AZU4_HETGA	QWRVSAFIG	SNIIIFERL	WEG	;LWMNCVRÇ	2AKV	TLQC	K FJ	TSMLALPPVVEAA	REFYN	PAIPIGÇ	OKRELGAA
I3MA62	I3MA62_SPETR	QWRVSAFIG	SNIIVFERL	WEG	JWMNCIRC	2VKV	RLQC	K FY	NSLLALPPVLEAA	R RDFYD	PTVHFGÇ	<u>KRE</u> LGAA
Q8BXA6	CLD17_MOUSE	QWRVSAFIG	SNIIIFERI	WEG	FIMMNCIQC	2AMV	TLQC	K FY	NSILALPPVLEAA	R RDFYD	PTVHAGÇ	KRELGGA
G3U508	G3U508 LOXAF	QWRVSAFIG	SNIIVFERI	WEG	;LWMACV <mark>R</mark> Ç	2ATV	RLQC	K FY	SEMLELPPALEAA	R RDFYN	PAIHVGÇ	KRELGAA
G1TI80	G1TI80 RABIT	QWRVSAFIG	SNIIVFERI	WEG	;LWMNCV <mark>R</mark> Ç	2ARV	RFQC	K FY	SELLALPPVLEAA	R RDFYN	PTINAGÇ	KRELGAA
D4A6L7	D4A6L7 RAT	QWRVSAFIG	SNIIIFERI	WEG	JWMNCIQ	2AMV	TLQC	K FJ	NSILALPPVLEAA	R RDFYD	PTIHAGÇ	KRELGGA
W5NSG1	W5NSG1 SHEEP	QWRVSAFIG	SNIIVFERV	WEG	;LWMNCV <mark>R</mark> Ç	)AKI	KLQC	KAY	DSLLALPPALEAS	R RDFYN	PAVHVGÇ	KRELGAS
E1BMV0	E1BMV0 BOVIN	QWRVSAFIG	SNIIVFERV	WEG	;LWMNCV <mark>R</mark> Ç	)aki	KLQC	KVY	DSLLALPPALEAA	R RDFYN	PAV <mark>H</mark> VGÇ	KRELGAS
L8IB00	L8IB00 9CETA	QWRVSAFIG	SNIIVFERV	WEG	JWMNCVRC	)AKI	KLQC	KVY	DSLLALPPALEAA	R RDFYN	PAV <mark>H</mark> VGÇ	KRELGAS
G1PN91	G1PN91 MYOLU	QWRVSAFVG	SNIIVFERI	WEG	J.WMTCVRC	)ARA	TLQC	K L7	SELLALPPVLEAA	R RDFHN	PAV <mark>H</mark> VGÇ	KRELGAA
F6ZAA7	F6ZAA7 MONDO	QWRVSAFIG	SNIVIFERI	WEG	JUMMNCIQC	2VNV	RWQC	K YY	SSILALPPALEAA	RDFYN	PTIHVGQ	KRELGAA
G3WHJ3	G3WHJ3 SARHA	QWRVSAFIG	SNIVIFERI	WEG	JUMMNCIQ	2AR I	RWQC	K YY	NSLLALPHDLEAAH	R KDFYN	PAIHVGÇ	KRELGAA
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**Figure S1**. Multiple sequence alignment of ECL1 (left) and ECL2 (right) for Cldn17. 28 orthologous sequences available in UNIPROTKB were aligned using CLUSTAL O (McWilliam et al., Nucleic Acids Res. 2013 Jul; 41(Web Server issue: W597-600.)). Accession number and species is given on the left; residue number is given on top; green, positively charged; red negatively charged; cyan Ser/Thr; \*, identical; :, strongly similar properties;., weakly similar properties. The sequences are shown with positions 29 - 81 and 146-164 (ECL1 and ECL2 as defined by uniprot.org). Boxes mark the residues analyzed in this study. Blue boxes mark positions with identical or highly similar residues whereas red boxes mark positions with residues containing different properties (TIFF 2337 kb)



**Figure S2**. Exemplary western blots for other TJ proteins. The blots show exemplary clones of each mutation in comparison to wt Cldn17 and vector control (vec). Detected were Cldn1, Cldn4, occludin, the FLAG-tagged Cldn17 and  $\beta$ -actin as loading control. Expression changes occurred in some, but not all, clones of one mutant. However, these changes had no impact on functional results, as these were similar in all clones of one mutant (TIFF 221 kb)



**Figure S3**. Exemplary comparison of different clones (E44A). **a** The blots show exemplary clones of mutation E44A in comparison to wt Cldn17 and a vector control (vec). Detected were Cldn1, Cldn4, occludin, the FLAG-tagged Cldn17 and  $\beta$ -actin as loading control. **b** The densitometric analysis revealed expression differences which may influence the results of further experiments. However, only a very low mutant expression of 14 % (clone #1) compared to the wt, resulted in P<sub>Cl</sub>/P<sub>Na</sub> as observed for the vec, while ranges of expression above 43 % to 86 % were comparable to each other **c**. Also permeabilities for other ions **d** were only different from the other clones at very low expression levels. Additional expression variations in endogenous TJ protein levels had no effect in the ranges observed. However, to keep clonal variation as low as possible, several clones were analyzed for each mutant and outliers with extreme differences in expressions were excluded (TIFF 3315 kb)



**Figure S4**. Immunofluorescent staining of exemplary clones. Localization of the 3 × FLAG-tagged mutants of Cldn17 (red) was analyzed using occludin or ZO-1 (green) as TJ marker. All mutant Cldn17 constructs were colocalized (yellow) with the TJ marker, indicating correct insertion into the TJ. Bar = 10  $\mu$ m (TIFF 16234 kb)



**Figure S5.** Exemplary images of freeze-fracture electron microscopic samples. Mutation from anionto cation-selective Cldn17 (mutant K65E) as well as the other mutations of K65 that led to loss of charge selectivity had no effect on TJ ultrastructure. **a** Vector-transfected control. **b** wt Cldn17. **c** Cldn17 K65E. **d** Cldn17 K65A. **e** Cldn17 K65R. Bar = 200 nm (TIFF 4714 kb)



**Figure S6**. Live cell imaging of HEK293 cells transfected with three CFP-tagged constructs. Subcellular localization of **a** wtCldn17, **b** Cldn17 Y149A and **c** Cldn17 H154A (green). Trypan blue (red) marks the cell membrane. All three Cldn17 constructs were expressed and localized within the cell membrane. Enrichment in cell membranes of neighboring cells indicates the ability to *trans*-interact. Bar 5  $\mu$ m (TIFF 20632 kb)



**Figure S7**. Overlay of Cldn17 and Cldn19 homology models. Comparison of Cldn17 homology models based on templates for Cldn15 (PDB: 4P79, backbone as cartoon in green, residues as sticks in magenta) and Cldn19 (PDB: 3X29, backbone as cartoon in cyan, residues as sticks in violet). Both models indicate a similar fold of Cldn17 and similar positions of the residues (sticks) mutated in this study. However, the models differ in the C-terminal half of ECL2, the loop between  $\beta$ 1- and  $\beta$ 2-strand and the region between  $\beta$ 4-strand and TM2. The latter region was not resolved in the Cldn19 crystal, in which Cldn19 was in complex with the C-terminal domain of the *Clostridium perfringens* enterotoxin [20]. Disulfide bridges in ECL1 are shown as yellow sticks (TIFF 2927 kb)