Claudin17 deficient mice display leaky vasculature and organ injury accompanied by oxidative stress and inflammation

Mir Adil

University of Georgia

Varun Parvathagiri

University of Georgia

Arti Verma

University of Georgia

Fang Liu

Augusta University

Madhuri Rudraraju

University of Georgia

S P Narayanan

University of Georgia

Payaningal R Somanath (✉ sshenoy@augusta.edu)

University of Georgia <https://orcid.org/0000-0003-3017-0230>

Article

Keywords: CLDN17, organ injury, vascular permeability, reactive oxygen species, leukocytosis

Posted Date: January 24th, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1270153/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

The Multi-gene claudin (*CLDN*) family of tight junction proteins have isoform-specific roles in blood-tissue barrier regulation. Although *CLDN17*, a putative anion pore-forming *CLDN*, is assumed to regulate anion balance across the blood-tissue barriers, our knowledge about *CLDN17* is limited. The current study investigated how *Cldn17* deficiency in mice affects blood-tissue barrier integrity and vascular permeability. *Cldn17*^{-/-} mice revealed no breeding abnormalities, but the newborn pups exhibited delayed growth until they turned 8-weeks. Adult *Cldn17*^{-/-} mice displayed electrolyte imbalance, oxidative stress, increased vascular leakage, increased bone density, and organ injury. In addition, these mice demonstrated increased leukocytosis. A xenograft model to study pathological neovascularization showed higher vascular density in tumors developed in *Cldn17*^{-/-} mice compared to wild-type controls. RNA-sequencing revealed hyperactivation of signaling pathways associated with inflammation and reactive oxygen species generation, demonstrating the importance of *Cldn17* in blood-tissue barrier maintenance.

Introduction

Claudins (*CLDNs*) are critical tight junction (TJ) proteins involved in the transcellular and paracellular transport coupling^{1,2}, ion and electrolyte homeostasis, and blood-tissue-barrier maintenance^{3,4}. Besides ion selectivity, specific *CLDNs* are also responsible for cell-cell barrier formation and regulation of vascular permeability⁵. Blood-tissue barriers safeguard organs and control vital physiological processes through highly coordinated paracellular and transcellular transport⁶. Whereas injury to the alveolar-capillary unit causes pulmonary edema⁷, a similar condition in the kidneys causes renal arterial stenosis⁸. Modulations in *CLDN* expression in the blood-tissue barrier often result in inflammation⁹ and the generation of reactive oxygen species (ROS), and *vice versa*^{10,11}. As a prime example, *CLDN4* was upregulated *via* ROS-mediated pathway in MDCK cells in hyperosmolar conditions¹². Likewise, other pore-forming *CLDNs* could also play a role in ROS homeostasis.

A major pathway known to regulate the expression of *CLDNs* in vascular endothelial cells (ECs) is the Akt signaling pathway^{13,14,15,16,17}. The over-expression of VE-cadherin in the adherens junctions (AJs) resulted in increased *CLDN5* expression in ECs *via* the activation of Akt, in turn, blocking the suppressive effects of FoxO1/3a and β -catenin¹³. Similarly, treatment with angiopoietin-1 activated Akt to promote *CLDN5* expression in human microvascular ECs¹⁴. Although VEGF, a short-term vascular permeability inducer, promoted its effects *via* Src activation, it protected the EC-barrier in the long-term *via* activation of Akt and suppression of FoxO and β -catenin to promote *CLDN5* expression^{14,15,17}. Our past studies have revealed that Akt1-deficient ECs express significantly reduced levels of several *CLDNs* such as *CLDN3-7*, *CLDN9*, *CLDN17*, and *CLDN19*, with the highest decrease observed in *CLDN17*¹⁴, suggesting that these *CLDNs* may likely be primary mediators of EC-barrier regulation.

CLDN17 is widely expressed in the brain and the kidneys¹⁸ that forms a distinct anion-selective channel, which is structurally and functionally different from the paracellular cation channels¹⁹. Interestingly, it belongs to a class of 'intron-less genes' that constitutes ~3% of total human genes²⁰ that regulate the evolution of tissue-specific functions in mice and have diverse functions²¹. In the kidneys, *CLDN17* is predominantly expressed in the proximal segments of the nephron²². A recent study reported the downregulation of *CLDN17* associated with increased *CLDN3* and *CLDN8* in bronchial epithelial cells (EpCs) on dexamethasone-induced glucocorticoid activation²³. It has also been reported that *CLDN17* replaces *CLDN8* to recruit occludin (*OCLN*) in human bronchial EpCs²³, suggesting that *CLDN17* assumes the role of *CLDN8* in tissues where *CLDN8* is absent and *vice versa*. *E.g.*, *CLDN8* is abundant in the airways but not in the alveolar EpCs²³, suggesting that *CLDN17* may be a primary mediator of the blood-air barrier in the alveolar-capillary units.

As of today, the precise role of *CLDN17* in physiology and pathology is unknown. Literature on *CLDN17* is scarce, and the reagents and tools to study the role of *CLDN17* in cell-cell and blood-tissue-barrier regulation are limited. Although a few *CLDNs* have been studied *in vivo* employing knockout mouse models, no laboratory, to date, has studied the effects of *CLDN17* in a living organism. Hence, in the current study, we generated a *Cldn17*^{-/-} mouse model on a C57BL/6 background using the CRISPR/Cas9 technology and characterized its phenotype to reveal the significance of the gene with regards to tissue homeostasis, blood-tissue-barrier function, vascular permeability, redox signaling, and inflammation, etc. Our study revealed an important role for *Cldn17* in vascular and tissue homeostasis.

Results

***Cldn17*^{-/-} mice exhibit delayed growth.** *Cldn17*^{-/-} mice generated using the CRISPR/Cas9 technology were genotyped and further confirmed for gene loss by cell validation assay (Fig. 1a, b), Western, and mRNA analysis in the kidney and the lung (Fig. 1c-d). No breeding abnormalities were detected in the newly generated mice. To ascertain if *Cldn17* loss influences the growth of these mice, we recorded lengths and weights of the *Cldn17*^{-/-} mice along with age- and gender-matched wild-type (WT) controls at various stages. Remarkably, *Cldn17*^{-/-} mice appeared smaller than the WT controls on day 7 (Fig. 1e), and the length measurements revealed a significant size reduction in mice lacking *Cldn17* (Fig. 1g). We also assessed the levels of growth hormones in the WT and the *Cldn17*^{-/-} mice to determine if *Cldn17* has any control over this hormone. Liver lysates from both groups at early and late stages were subjected to ELISA to determine the levels of growth hormones. Although there was an expected reduction in the growth hormone level in the adult stage mice compared to the early-stage pups, no significant difference was observed between the WT and the *Cldn17*^{-/-} mice (Fig. 1h). Although the *Cldn17*^{-/-} mice demonstrated a significant reduction in size also when measured at weeks 2 to 4, there was a gradual decline in the difference, the *Cldn17*^{-/-} mice reached parity with the WT at a later stage (Fig. 1i), which was quantified by weights in the age range of 8 to 18 weeks involving both genders (Fig. 1f, j).

Collectively, these findings signify that *Cldn17* plays a role in the overall growth of mice, at least in early life.

Impact of *Cldn17* loss on serum and urine parameters. Since *Cldn17* is believed to be an anionic pore-forming *CLDN* with a plausible role in ion homeostasis, we decided to analyze the levels of various electrolytes in serum samples obtained from WT and *Cldn17*^{-/-} mice. Blood was collected through retro-orbital puncture before it was processed into serum and analyzed at the University of Georgia veterinary diagnostic laboratory. As anticipated, serum electrolytes were found to be altered in the *Cldn17*^{-/-} mice with significantly elevated negatively charged chloride and bicarbonates (Fig. 2a-b) and reduced levels of positively charged ions in the *Cldn17*^{-/-} mice compared to WT (Fig. 2c-d). A reduction in the anion gap was also observed in *Cldn17*^{-/-} mice though the difference was not statistically significant (Fig. 2e). In addition to the electrolytes, modulation in serum proteins was assessed in the absence of *Cldn17*. While total protein and albumin levels were unaltered in *Cldn17*^{-/-} mice, total globulin and the albumin to globulin ratio were significantly reduced (Fig. 2f). These results signify the vital role of *Cldn17* in ion and protein homeostasis in the blood.

Anticipating similar alterations in the urine, we collected urine from WT and *Cldn17*^{-/-} mice using metabolic chambers for analysis. Interestingly, rare WBCs (white blood cells) presence in WT mice, considered normal, was significantly reduced in the *Cldn17*^{-/-} mouse urine. Surprisingly, all other urine parameters such as the pH, specific gravity, color, bilirubin, bacteria, turbidity, and crystals showed no significant difference between WT and *Cldn17*^{-/-} mice (Supplementary Fig. 1).

***Cldn17*^{-/-} mice display increased bone density.** The observed alterations in various ions in the *Cldn17*^{-/-} mouse serum prompted us to determine the effect of *Cldn17* loss on bone density. We performed μ CT analysis of femurs and tibiae from WT and *Cldn17*^{-/-} mice. Interestingly, femurs from *Cldn17*^{-/-} mice revealed significantly higher bone density compared to the WT group (Fig. 3a). Quantification of these bones revealed significant changes in both trabecular (Fig. 3b) and cortical (Fig. 3c) bones. While volume, surface, trabecular thickness, and trabecular number were increased in the *Cldn17*^{-/-} group, open porosity was decreased in these mice. These alterations in bone formation in the mice deficient in *Cldn17* indicate a critical involvement of this gene in bone remodeling.

Lack of *Cldn17* promotes vascular permeability in vivo. Based on the critical function of *CLDNs* in the maintenance of blood-tissue barrier integrity, we assessed vascular permeability *in vivo* in *Cldn17*^{-/-} and WT mice using the Miles (Evan's blue dye leakage) assay, which was performed through intraperitoneal as well as intravenous (tail-vein) administration of Evan's blue dye. Organs isolated from the *Cldn17*^{-/-} mice revealed a higher content of Evan's blue dye compared to the control group upon absorbance quantification. While the intravenously administered dye showed a significantly higher permeability in the ears (Fig. 4a), intraperitoneally administered dye resulted in a higher leakage in ears and lungs in the *Cldn17*^{-/-} groups compared to the WT control group (Fig. 4b).

Alternately, a Matrigel plug assay was performed to confirm the significance of *Cldn17* in vascular integrity. Subcutaneously injected Matrigel was isolated from the WT and the *Cldn17*^{-/-} mice in the form of plugs, which were then immersed in Drabkin's reagent before assessing the hemoglobin content of the plugs by measuring the absorbance at a wavelength of 540 nm. The isolated plugs from *Cldn17*^{-/-} mice exhibited a brighter red color against the plugs from WT mice that appeared pale (Fig. 4c). Consistent with the physical appearance, quantification of these plugs revealed a significant increase in hemoglobin content in the plugs implanted in *Cldn17*^{-/-} mice compared to the WT group (Fig. 4d), signifying increased leakiness in the mice deficient of the *Cldn17* gene.

***Cldn17*^{-/-} mice exhibit hypophagia and polydipsia.** Given the retarded early growth in the absence of the *Cldn17* gene, we anticipated alterations in the food consumption in the genetically modified mice. We observed a significant reduction in the food consumption in *Cldn17*^{-/-} mice against the control (Fig. 4e). Since *Cldn17* is a TJ protein expressed in the proximal tubule responsible for water reabsorption in the kidneys, we also determined water intake, which revealed significantly increased water consumption by *Cldn17*^{-/-} mice compared to the WT (Fig. 4f). Analysis of fecal pellets revealed a significant reduction in the number of pellets excreted over 24 hours by *Cldn17*^{-/-} mice compared to the WT group (Fig. 4g). Wet/dry ratio analysis of these fecal pellets was also performed, which revealed a significantly lower moisture content in the fecal pellets collected from mice lacking *Cldn17* (Fig. 4h), signifying constipation-like effects in *Cldn17*^{-/-} mice.

***Cldn17*^{-/-} mice display lung and kidney injury.** Age, gender, and weight-matched WT, *Cldn17*^{+/-} and *Cldn17*^{-/-} mice were euthanized, and their organs including lungs, kidneys, liver, and skin were collected, sectioned, and subjected to H&E or Masson's trichrome staining. We observed alveolar flooding, inflammatory cell infiltration, and lung injury obtained from *Cldn17*^{+/-} and *Cldn17*^{-/-} mice compared to WT controls (Fig. 5a). Quantification was performed by blinded injury scoring, which revealed a higher injury in the mice lacking *Cldn17* (Fig. 5b), corroborated with pulmonary edema as analyzed by the wet/dry ratio by recording organ weights at two different time points separated by incubation in an oven for 72 hours. Higher edema was clear in both *Cldn17*^{+/-} and *Cldn17*^{-/-} groups compared to the WT controls (Fig. 5c).

Since *Cldn17* is expressed in the kidneys, a potential role of *Cldn17* in kidney function is expected. As anticipated, there was an increased injury in the *Cldn17*^{+/-} and *Cldn17*^{-/-} mouse kidneys compared to WT, characterized by vacuolar degeneration, mineralization, and increased Bowman's capsule spacing (Fig. 5a). Like the lungs, injury scoring by blinded reviewers on a scale of 1-5 revealed a higher injury in the *Cldn17*^{-/-} mice compared to WT (Fig. 5d). Wet/dry ratio analysis assessed renal edema, which showed no significant difference between the two groups (Fig. 5e). Further, ImageJ software quantification of the area of Bowman's capsule revealed significantly enlarged capsule size in *Cldn17*^{-/-} mice compared to WT (Fig. 5f). In addition, analysis of skin and liver tissues from WT, *Cldn17*^{+/-}, and *Cldn17*^{-/-} mice revealed no significant difference between the three groups (Supplementary Fig. 2).

RNA-seq analysis of kidneys reveals gene modulations regulating inflammation, angiogenesis, ROS generation, and myeloid cell differentiation. A series of intriguing observations from the *Cldn17*^{-/-} mice prompted us to perform RNA-seq analysis of their kidneys. Analysis of 12,031 genes in WT and *Cldn17*^{-/-} mouse kidneys revealed significant changes in 424 genes, with 295 upregulations and 119 downregulations in gene expression in the *Cldn17*^{-/-} group compared to WT (Fig. 6a and Supplementary Fig. 3). Interestingly, *Cldn17*^{-/-} kidneys had *de novo* expression of ten genes that are not expressed in the WT kidneys.

Next, we identified the most deregulated signaling pathways using the Panther database (Fig. 6b). The top three downregulated pathways were associated with coagulation, nicotine degradation, and androgen/estrogen/progesterone biosynthesis-related signaling. In contrast, upregulated genes were linked to inflammation, integrins (cell adhesion and migration), angiogenesis, lipid metabolism, and gonadotropin hormone receptor signaling. In the next step, RNA-seq data were subjected to gene ontology (GO) and gene set enrichment analysis (GSEA) analyses based on genes modulated more than two-fold. From the highly influenced signaling pathways, we identified some pathways of interest based on our study objectives. Interestingly, inflammatory pathways, including TNF α and IL-related signaling, were enhanced in the *Cldn17*^{-/-} mice compared to WT (Fig. 6c-d). Furthermore, we observed increased expression of genes regulating myeloid differentiation, epithelial-to-mesenchymal transition, and p53 pathway (Supplementary Fig. 5), associated with altered expression of CytP450 enzymes, calgranulins, CD receptors, histone proteins, and collagens (Supplementary Tables 1-5), all of which are associated with inflammation, organ injury, and fibrogenesis. Apart from the aforesaid signaling pathways, genes related to KRAS signaling, xenobiotic metabolism, cell cycle regulation, and symporter activity were also modulated in the *Cldn17*^{-/-} mice (Supplementary Fig. 6-7).

***Cldn17*^{-/-} mice exhibit leukocytosis.** Since we observed inflammation in the lung lysates and the RNA-seq analysis, we performed a complete analysis of blood collected *via* retro-orbital puncture from WT and *Cldn17*^{-/-} mice. Interestingly, WBCs were significantly higher in the *Cldn17*^{-/-} mice by more than two folds (Fig. 7a). A differential count analysis showed considerably higher neutrophils, lymphocytes, eosinophils, and basophils in the *Cldn17*^{-/-} group against the WT (Fig. 7b). Apart from the leukocytes, the *Cldn17*^{-/-} mice displayed significantly reduced platelet count compared to the WT group (Fig. 7c). Although not significant, we observed enlarged spleen in *Cldn17*^{-/-} mice compared to WT (Fig. 7d). To confirm the elevated leukocyte count, we performed FACS analysis on fresh samples obtained from both groups (Fig. 7e). We observed a significant increase in granulocyte and lymphocyte counts (Fig. 7f). These findings reveal that *Cldn17* deficiency results in leukocytosis and thrombocytopenia.

***Cldn17* loss promotes pathological angiogenesis.** RNA-seq analysis suggested an important role for *Cldn17* in vasculature development. Signaling pathways related to vascular barrier were found to be altered with increased involvement of apical junctions and vasculature regulation in mice lacking *Cldn17* (Fig. 8a). To confirm the vital participation of *Cldn17* in the vascular barrier integrity, we performed *in vitro* studies on human dermal microvascular ECs (HMECs) and bronchial EpCs with the *CLDN17* gene

knocked by lentiviral particles expressing shRNAs for human *CLDN17* (Fig. 8b). Both HMECs and EpCs with or without *CLDN17* were subjected to electric cell-substrate impedance sensing (ECIS) to determine trans-endothelial or -epithelial electrical resistance (TEER). In the absence of *CLDN17*, both HMECs and EpCs displayed reduced TEER compared to their respective controls, as represented in Fig. 8c-d.

To confirm the effect of *Cldn17* loss on pathological angiogenesis *in vivo*, we developed murine RM1 metastatic prostate cancer cell tumor xenografts in mice and collected them on day 12. Laminin-staining of tumor sections revealed increased blood vessel count in the *Cldn17*^{-/-} mice against the control group (Fig. 8e). Quantification of these images by ImageJ software revealed a significant increase in the vascular density in *Cldn17*^{-/-} mice against the WT group (Fig. 8f). Surprisingly, there was no significant difference in the tumor size between the groups (Fig. 8g). These results collectively indicate that *Cldn17* loss promotes pathological angiogenesis *in vivo*.

Cldn17 deficiency promotes ROS generation. Since ROS are charged molecules, *Cldn17* may be potentially involved in their clearance from tissues. The RNA-seq data revealed alterations in signaling pathways pertaining to ROS and hydrogen peroxide responses in addition to the hypoxia pathway as analyzed by GO and GSEA analysis (Fig. 9a). Ingenuity pathway analysis (IPA) suggested a potential link between *CLDN17* and *SOD1* (Supplementary Fig. 4), which was confirmed by Western blot analysis of kidney lysates (Fig. 9b), revealing significantly lower *Sod1* expression in *Cldn17*^{-/-} kidneys (Fig. 9c). In addition, dihydroethidium (DHE) staining of frozen kidney sections indicated increased ROS levels in *Cldn17*^{-/-} kidneys compared to WT (Fig. 9d).

Discussion

Defects in endothelial and epithelial cell-barrier TJs cause perturbations in vascular homeostasis leading to blood-tissue-barrier damage, inflammation, and injury to organs^{24,25}. Among the various TJ proteins, the 27-gene member family of *CLDNs* plays a most significant role in blood-tissue barrier regulation and tissue integrity^{17,26}. Whereas several *CLDNs* have been studied in epithelial cells (EpC)^{1,17}, *CLDN5* is the only well-characterized member in ECs^{13,14}. Intriguingly, the blood-tissue barrier is intact in *Cldn5*^{-/-} mice except for a modest and selective leakage of very low molecular weight proteins in the brain²⁷. There is a significant gap in our knowledge of the expression, mechanisms, and role of various *CLDNs* in ECs and blood-tissue barriers. Akt1-FoxO-βCatenin pathway has been demonstrated to regulate vascular permeability by modulating the expression of TJ *CLDNs*^{14,15,28,29}. Although its significance is not clear, we observed a correlation between loss of *CLDN17*, a putative anion pore-forming *CLDN*^{19,30}, with aberrant EC-barrier disruption¹⁴. Until today, the role of *Cldn17* in physiology and pathology *in vivo* has not been investigated. In the current study, our results demonstrate a vital role for *Cldn17* in early development, vascular and blood-tissue-barrier homeostasis, bone and organ health, and control of ROS generation and inflammation. Despite the early delay in their growth, *Cldn17*^{-/-} mice reached parity with WT mice in their weight and length and exhibited no changes in growth hormone levels, breeding abnormalities, or mortality until 18 months of observation. While the exact reasons for this growth lag are

not clear, the literature indicates a similar phenotype in mice lacking the *Ocln* gene, where a size reduction was observed that subsided by 8 weeks³¹. Interestingly, both *Cldn17*^{-/-} mice and the *Ocln*^{-/-} mice exhibited analogous observations of increased bone density, suggesting that TJs, in general, may either be directly or indirectly linked to bone density and bone health. However, the newly generated *Cldn17*^{-/-} mice displayed myeloid cell differentiation and leukocytosis.

Cldn17 is an intron-less gene reported to be primarily expressed in the kidneys and brain^{17, 22, 30}. *Cldn17* is a paracellular anion channel that is permeable for halides, HCO₃⁻, NO₃⁻ and organic anions^{19, 30} suggesting a potential role in buffering, electrolyte, and fluid balance across the cell barrier. An increase in serum chloride and bicarbonate levels accompanied by a decrease in sodium and potassium levels and a reduced anion gap in *Cldn17*^{-/-} mice compared to WT is a first-time report signifying the importance of *Cldn17*^{-/-} in ion, electrolyte, and pH homeostasis. It is a confirmation of its anticipated role based on the structural evidence. An increase in serum globulin levels and a decreased albumin to globulin ratio in *Cldn17*^{-/-} mice vs. WT further confirms this conclusion. Furthermore, our observations on the anion-channel property of *Cldn17* were corroborated with reduced symporter activity in an RNA-Seq analysis in *Cldn17*^{-/-} mice. Surprisingly, no significant proteinuria was observed in *Cldn17*^{-/-} mice. Deregulated symporter activity has been linked to an imbalance in ions and electrolytes such as calcium, which plays an integral role in bone formation³². Supporting this view, *Cldn17*^{-/-} animals exhibited increased bone density in the femur compared to the WT controls. Literature indicates that osteoclast activity and bone homeostasis are also dependent on chloride ions levels^{30, 32, 33}, and inhibition of chloride channels cause osteopetrosis³³ characterized by abnormally higher bone density resulting from defective osteoclasts³⁴. A similar observation in *Cldn17*^{-/-} mice femur strongly suggests a key role for *CLDN17* in regulating chloride balance and bone health. Since chloride channel activation regulates fluid balance in the intestines helping with smooth passage of stools³⁵, reduced number of fecal pellets in the excreta and reduced fecal water content in *Cldn17*^{-/-} mice compared to WT controls could also be a result of chloride imbalance in *Cldn17*^{-/-} mice.

Being an EC and EpC TJ protein, loss of *CLDN17* is expected to affect the cell-cell monolayer integrity. Confirming this, shRNA-mediated reduction in *CLDN17* expression in HMECs and human lung EpCs resulted in impaired monolayer resistance, indicating the importance of *CLDN17* in EC and EpC barrier regulation. This was corroborated *in vivo* by the leakage of Evans blue dye extravasation in a Miles's assay. Furthermore, vascular hyperpermeability has been indicated to promote pathological angiogenesis³⁶. Supporting this, murine RM1 prostate cancer tumor xenografts grown in *Cldn17*^{-/-} mice displayed increased laminin-positive vascular area compared to control tumors in WT mice. Increased hemoglobin content in Matrigel plugs implanted in *Cldn17*^{-/-} mice compared to WT mice in our study is also an attestation of increased angiogenesis and vascular leakage. Interestingly, a pro-angiogenesis phenotype was supported by RNA-seq data illustrating enhanced expression of pro-angiogenic genes in *Cldn17*^{-/-} mice compared to WT.

Apart from pathological angiogenesis, vascular hyperpermeability is associated with inflammation and injury to organs such as the lungs and kidneys³⁷. In agreement, *Cldn17*^{-/-} mice displayed damage in the lungs and the kidneys associated with pulmonary edema. The lung injury characteristics of *Cldn17*^{-/-} mice mimics immunologic lung injury that causes ARDS³⁸. Modulation in calgranulins in *Cldn17*^{-/-} mice, known to be dysregulated in lung injury³⁹, signifies a crucial role for *CLDN17* in lung physiology. Persistent lung injury in *Cldn17*^{-/-} animals may likely be due to the elevated S100A9 in *Cldn17*^{-/-} mice, a calgranulin known to be associated with chronic lung disease⁴⁰. Similarly, *Cldn17*^{-/-} mouse phenotype can be compared to human vascular leak syndrome (VLS), also related to vascular permeability, interstitial edema, organ failure, increased WBCs and globulin, and reduced albumin^{41,42}. VLS is characterized by splenomegaly⁴¹, ARDS, and AKI⁴³, all of which are evident also in the *Cldn17*^{-/-} mice.

The findings from RNA-seq analysis further reinforced our notion that systemic inflammation accompanies *Cldn17* deficiency. These observations coincide with a clinical study linking altered *CLDN17* expression on adhesion and diapedesis of agranulocytes and granulocytes, leading to bronchopulmonary dysplasia, a chronic lung disease⁴⁴. Another clinical study reported a significant upregulation of *CLDN17* in the biopsy samples from oral lichen planus, a chronic, T cell-mediated inflammatory, autoimmune mucosal disease⁴⁵, thus suggesting a potential role for *CLDN17* in inflammation. Interestingly, thrombocytopenia observed in *Cldn17*^{-/-} mice is another possible factor indirectly promoting inflammation as platelets are reported to be involved in the maintenance of vascular barriers⁴⁶, and their loss is linked to vascular leakage⁴⁷. This is further supported by RNA-seq data indicating an increased level of platelet factor 4 (PF4), which has been linked to thrombocytopenia⁴⁸. Furthermore, IPA analysis and the RNA-seq data revealed potential involvement of ROS in inflammation and organ injuries observed in *Cldn17*^{-/-} mice. These analyses established a robust relationship between *CLDN17*, and pathways linked to oxygen homeostasis. IPA data predicated a potential link between *CLDN17* and *SOD1*, a potent regulator of ROS levels in tissues⁴⁹. As predicted, *Sod1* levels were reduced in *Cldn17*^{-/-} kidneys accompanied by increased ROS levels in *Cldn17*^{-/-} kidneys, evidenced by DHE staining. Moreover, RNA-seq revealed increased expression of genes regulating oxidative stress and cytochrome P450 enzymes, which have been indicated in ROS generation⁵⁰. Altogether, the observations from the current study, including the data from gene expression profiling, collectively suggest a regulatory role of *CLDN17* in many physiological mechanisms that include maintenance of vascular barrier integrity and ion, electrolyte, and ROS homeostasis. Further, deficiency of *Cldn17* in mice leads to negative effects, including but not limited to vascular leakage, inflammation, and organ damage.

Methods

Generation and maintenance of *Cldn17*^{-/-} mice. All the mouse-related experiments were performed with the approval of Charlie Norwood Veterans Affairs Medical Center Institutional Animal Care and Use Committee (approval references #19-04-114 and #20-01-118) and were in accordance with the ARRIVE guidelines for reporting experiments involving mice. Ketamine and xylazine were administered

intraperitoneally in combination for inducing anesthesia. Carbon dioxide asphyxiation followed by cervical dislocation was used for euthanizing undesired mice. To generate knockout mice (Horizon Discovery, Lafayette, LA), ten suitable sgRNAs for CRISPR/Cas9 knockout in C57BL/6 mice zygotes were performed, and the most potent sgRNA with minimal off-target potential was assembled into the ribonucleoprotein complex with Cas9 endonuclease (gRNA Binding/PAM Site 5' $\diamond$ 3': tcggtttggttgggacgattGGG; Forward Primer 5' $\diamond$ 3': agaagaccaggcactcctct and Reverse Primer 5' $\diamond$ 3': tcatgccgcagatgccaata for genotyping). These were delivered into zygotes from C57BL/6 mice followed by embryo transfer into pseudo-pregnant females. Genomic PCR and DNA sequencing analyzed viable progenies for the presence of the desired mutation. Each small guide RNA were validated by transfection into cultured cells, and assessed cutting efficiency by the Surveyor Cel 1 Mutation Detection assay. The two smaller cleavage bands added up to the size of the parental band. The ratio of the intensity of the cleavage bands relative to the parental band was used to assess cutting efficiency. Up to two selected transgenic founder mice were bred with C57BL/6 mice to produce F1 progeny heterozygous for the *Cldn17*^{-/-}, as confirmed by the PCR mediated genotyping and DNA sequencing analysis. Off-target analysis and on-target sequence confirmation in F1 mice were also performed, which consisted of PCR and sequencing of the top 10 predicted off-target sites. *Cldn17*^{+/-} mice were eventually bred to obtain homozygous *Cldn17*^{-/-} mice used in the study. A detailed genotyping protocol is provided in (Supplementary Figure 8).

RNA isolation, cDNA preparation, and qRT-PCR. Mice lungs were collected, and RNA was extracted using an RNA isolation kit (RNeasy Plus, Qiagen, Valencia, CA), and RNA quality was confirmed using Nanodrop 2000 spectrophotometer (Thermo Scientific). Complementary DNA (cDNA) was synthesized from 700 ng of RNA using RT2 First Strand kit (Qiagen) using a StepOne Plus thermal cycler and detection software (Applied Biosystems, Foster City, CA), and quantitative real-time PCR (qRT-PCR) was performed using the RT2 SYBR Green ROX qPCR Mastermix (Qiagen) in real-time PCR equipment (Applied Biosystems). Sample cDNA was amplified and quantified over many shorter cycles under the following conditions: an initial 10 min 95°C period followed by 45 cycles of 95°C for 15 s, 60°C for 1 min and 72°C for 15 s. The threshold cycle (Ct) was determined using the exponential growth phase and the baseline signal from fluorescence vs. cycle number plots. The mouse primers used for the messenger RNA (mRNA) analysis of *Cldn17* were 5'-tctccctccggtactgga ag-3' (forward) and 5'-gctcctccaagtctcgc tt-3' (reverse) and the primers for β -actin, used as a house-keeping control gene, were 5'-tttgagaccttcaacacccc -3' (forward) and 5'-atagctcttctccaggagg -3' (reverse).

Western blot analysis. It was performed as described in previously published studies^{51, 52}. Western analysis was performed on the cell lysates prepared using 1X RIPA lysis buffer (Millipore, Temecula, CA) supplemented with protease and phosphatase inhibitor tablets (Roche Applied Science, Indianapolis, IN). The DC protein assay reagent was used for protein estimation in the lysates (Bio-Rad Laboratories, Hercules, CA). Approximately 30–40 μ g of heat-denatured cell lysates prepared in Laemmli buffer were loaded onto the SDS-PAGE gels. Densitometry was performed using the NIH ImageJ software. Antibodies

used include *Cldn17* from Abcam (ab23333) and LSBio (LS-C3063), SOD1 (2770S, Cell Signaling Technology, and β -actin (A5441, Sigma).

Analysis of serum electrolytes, blood, and urine parameters. WT and *Cldn17*^{-/-} mice were subjected to retro-orbital puncture to collect around 800 to 1000 μ L of blood. A part of the collected volume was used for a complete blood count. The rest was processed for the analysis of serum electrolytes and proteins levels. While ion-specific electrode (ISE) method was used to assess serum sodium, potassium, and chloride levels, Siemens Advia 2120i hematology analyzer was utilized for complete blood count, which is a flow cytometry-based system that uses light scattering, differential WBC) lysis, and myeloperoxidase and oxazine 750 staining to provide a total blood cell count, a WBC differential, and a reticulocyte count. A cyanide-free method was used to measure hemoglobin calorimetrically. Urine was collected from WT and *Cldn17*^{-/-} mice using metabolic cages to determine various parameters, including pH, EpCs, blood cells, and others.

Bone analysis using micro-computed tomography (μ CT). Three-dimensional analyses of femurs and tibiae were performed using a μ CT scanner (Skyscan 1272, Skyscan, Kontich, Belgium). The X-ray tube was set to a voltage of 80 kV, and a 1 mm aluminum filter was used to reduce beam hardening artifacts. Samples were scanned in 70% ethanol. For each sample, 265 section images were reconstructed with NRecon software (version 1.7.3.1, Skyscan). Three-dimensional modeling and analysis of BV (Bone Volume)/TV (Total Volume) ratio (percentage of bone tissue), trabecular number and thickness, and Bone marrow density were obtained with the CTAn (version 1.17.7.2, Skyscan) and CTVol (Realistic 3D visualization version 2.3.2.0, Skyscan) software. The Dataviewer (version 1.5.6.2, Skyscan) software was used to generate 3D cross-sectional imaging of bones. The dissected bones were then processed for histological and histomorphometry analysis.

Mile's assay. Vascular permeability *in vivo* was assessed using the Miles assay as described previously⁵³. WT and *Cldn17*^{-/-} mice were injected with Evan's blue dye at a concentration of 1% intravenously or 2% intraperitoneally followed by a 30 minute or three-hour interval, respectively, before the mice were euthanized using CO₂. The mice were then perfused with 1X PBS to wash out the excess dye, and finally, the organs (lung, liver, kidney, ear, eye, and brain) were collected and stored in formamide for the next 24 hours at 42°C. The absorbance of formamide was then assessed at a wavelength of 610 nm using a calorimetric device which was normalized to the weight of the organ used to determine the vascular leakage.

Matrigel plug assay. Matrigel plug assay was performed in accordance with a procedure described in a previous study²⁸. On day 7, mice were euthanized to collect Matrigel plugs which were then digested using 5 ml Drabkin's reagent (Sigma D5941) and quantified for neo-vessel formation using a hemoglobin assay according to the manufacturer's protocol.

Organ injury and wet/dry ratio analysis. Age- and gender-matched WT and *Cldn17*^{-/-} mice were euthanized to collect their organs, then processed for histological examination and determination of

tissue edema. One of the liver lobes, the right superior lobe of the lung, and one of the kidneys were fixed in 4% paraformaldehyde, and 10 µm thick sections were processed for H&E staining. Histological assessment of these slides was performed by examination of tissue fields by blinded reviewers based on scoring from 1 to 5, with one being the best and five being the worst (severe most injury), and averages of these scores were considered for statistical analysis. While interstitial congestion, inflammatory cell infiltration, and consolidation were considered for lung analysis, mineralization, vacuolar degeneration, and Bowman's capsule spacing were taken into consideration for kidney scoring. The left lobe of the lung, one of the kidneys, and fecal pellets was weighed when fresh, followed by incubation in an oven for 72 hours on sterile papers at 80°C to remove all moisture content. The dry weights were then recorded to calculate the wet/dry ratios, which determine edema or fluid content.

RNA-Seq and bioinformatics analysis. Kidneys were isolated from WT and *Cldn17*^{-/-} mice and stored in Trizol® before freeze-drying. Samples were then subjected to RNA isolation, and total RNA purity and concentration were evaluated by spectrophotometry using NanoDrop ND-1000 (ThermoFisher). Total RNA quality was assessed by the Agilent 2100 bioanalyzer (Agilent Technologies) and assured of an RNA Integrity Number (RIN) greater than 5. Total RNA samples were processed for cDNA library preparations using TruSeq Stranded Total RNA kit (Illumina), which depletes cytoplasmic ribosomal RNA. Briefly, 800 ng of total RNA was treated with rRNA Removal Mix to deplete rRNA. Following purification, the RNA was fragmented into small segments, 200-300 bp in size, and converted to cDNA fragments. These cDNA fragments then had the addition of a single 'A' base and subsequent ligation of the adapter. The products were purified and enriched with PCR to create the final cDNA library. The prepared library was examined by bioanalyzer and Qubit (Thermofisher) to test library quality and quantity, respectively. The libraries were pooled and ran on the NextSeq500 sequencing system using a 75-cycle paired-end protocol. BCL files generated by the NextSeq500 were converted to FASTQ files for the downstream analysis. Reads passed the quality control were aligned to the reference genome, starting with the STAR aligner. The generated BAM files in a comparative setup were imported to the following Cufflinks and Cuffdiff tools from Tuxedo Suite for outputting differentially expressed genes with log2 fold changes and q values having replicates. Genes with p-values of less than 0.05 were taken into consideration for bioinformatics analysis which was performed using gene set enrichment analysis (GSEA) and gene ontology (GO). R programming was utilized to generate graphs, including the volcano plot for the modulated genes, and the Panther database was used to identify signaling pathways based on significantly altered genes.

Flow cytometry analysis. Blood was collected through retro-orbital technique in EDTA-treated collection tubes. Around 100 µL of blood was mixed with 1 mL of ACK lysing buffer and allowed to incubate at room temperature for 3 to 5 minutes for red cells lysis. White blood cells were then collected as a pellet after centrifugation at 300g for 5 minutes at room temperature and aspiration of supernatant. Pellet was washed with 0.5 mL ice-cold PBS followed by centrifugation at 300g for 5 minutes at 2-8°C before resuspending the cells in PBS for flow cytometry. Forward vs. side scatter plotting was performed on these samples for a cell count of 10,000 in Cytoflex LX from Beckman Coulter.

Generation of stable CLDN17 deficient cell lines. Telomerase-immortalized HMECs (CRL-4025; ATCC, Manassas, VA, USA) were maintained in Endothelial Cell Basal Medium-2 with a Growth Medium-2 Bullet Kit (Lonza; Walkersville, MD, USA), and human lung EpCs (H441; HTB-174; ATCC) were maintained in RPMI-1640 medium (ATCC 30-2001). All cultures were maintained in a humidified 5% CO₂ incubator at 37°C and routinely passaged when 80–90% confluent. Stable *shControl* and *shCLDN17* HMECs were generated using 2.5 µL/ml lentivirus particles (Dharmacon™, Lafayette, CO). The 3 validated lentiviral shRNA sequences include tgctgccaacaaaagctga (Sequence 1; Cat # V3SH7591-225585343), ggatggctgggttagaa (Sequence 2; Cat # V3SH7591-225642336), and gcactggacctgcttcag (Sequence 2; Cat # V3SH7591-225656194). Whereas all three ShRNAs reduced *CLDN17* expression in ECs and EpCs, the best results were obtained with sequence 2, which was used in all the in vitro studies. Cells were treated with 2 µL/ml polybrene (10mg/ml, American bioanalytical, Natick, MA) followed by transfection with the lentiviral particles. Three days later, transfection efficiency was examined through Turbo-GFP expression and subjected to 2 µg/ml puromycin (Life Technologies, Grand Island, NY) selection until all cells expressed GFP.

Measurement of EC and EpC monolayer barrier resistance in vitro. Endothelial and epithelial cell monolayer-barrier integrity (measured as electrical resistance of the endothelial monolayer) was determined using electric cell-substrate impedance sensing (ECIS) equipment (Applied Biophysics, Troy, NY, USA) as described previously⁵⁴. Cell monolayer-barrier resistance was measured at multiple frequency modes, but the final analysis was performed using a frequency of 4000 Hz.

In vivo mouse tumor xenograft model. Murine RM1 metastatic prostate cancer cells were grown to confluency in 75-ml flasks, resuspended in PBS, and counted. An equal number of cells (1×10^6) was injected subcutaneously in 7- to 8-week-old WT and *Cldn17*^{-/-} mice as described in a previous study²⁹. Mice were euthanized on day 12, and tumors were isolated, weighed, and fixed in 4% PFA for immunohistochemistry analysis.

Determination of vascular length density. Tumors isolated from mice were sectioned and stained with laminin to determine the vascular length density using Image J software.

Ingenuity pathway analysis (IPA). IPA database (Qiagen Bioinformatics) transforms a list of genes into a set of relevant networks associated with pathology based on extensive records maintained in the Ingenuity Pathways Knowledge Base⁵⁵. Highly interconnected networks are predicted to represent a significant biological function. Only those genes that were directly affected by the pathway of interest and *Cldn17* are shown.

ROS analysis. Production of ROS was estimated in WT and *Cldn17*^{-/-} mice with Dihydroethidium (DHE) staining on kidney sections frozen using optimal cutting temperature compound as described in a previous study⁵⁶. Stained images were then captured on confocal microscopy and subjected to fluorescence quantification using ImageJ software.

Analysis of growth hormone expression. Growth hormone level was determined in the WT and *Cldn17*^{-/-} mice using an ELISA kit from Novus Biologicals (NBP3-08143) according to the instructions provided by the manufacturer. Liver samples in PBS were used for the detection of growth hormone from pups and adult mice.

Statistical analysis. Data are presented as mean $\pm$ s.d. or mean $\pm$ s.e.m. The 'n' value for each figure implies the number of samples in each group which was determined by power analysis. All the data were analyzed by parametric testing using the student's unpaired t-test or one-way ANOVA using the GraphPad Prism 6.01 software. The Chi-square test was also used wherever applicable. Data with $p < 0.05$ were considered significant.

Declarations

Data availability. The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request. Source data are provided with this paper.

Acknowledgments: Funds were provided by the NCATS grant UL1TR002378 to PRS and NEI grant R01EY028569 to SPN. Technical support by Sam Chang and his team at the Integrated Genomics Facility, Georgia Cancer Center, Augusta University for RNA-seq analysis is acknowledged.

Competing interests: Authors declare that there are no financial or other conflicts of interest exist.

Author contributions: M.S.A., S.P.N., and P.R.S. all contributed to the intellectual conceptualization and design of the overall study. M.S.A., and V.P. designed, performed, and analyzed data for the animal phenotyping, vascular permeability, organ injury and edema, RNA-seq analysis, and flow cytometry analysis. A.V. designed, performed, and analyzed bone imaging. F.L. performed and analyzed ROS analysis. M.R. performed and analyzed kidney pathologies. M.S.A., S.P.N., and P.R.S. wrote the manuscript and made the figures.

Correspondence and requests for materials should be addressed to Payaningal R. Somanath.

References

1. Günzel D. Claudins: vital partners in transcellular and paracellular transport coupling. *Pflügers Arch* **469**, 35–44 (2017).
2. Elkouby-Naor L, Ben-Yosef T. Functions of claudin tight junction proteins and their complex interactions in various physiological systems. *Int Rev Cell Mol Biol* **279**, 1–32 (2010).

3. Shekarabi M, Zhang J, Khanna AR, Ellison DH, Delpire E, Kahle KT. WNK Kinase Signaling in Ion Homeostasis and Human Disease. *Cell Metab* **25**, 285–299 (2017).
4. Kawedia JD, *et al.* Interaction between transcellular and paracellular water transport pathways through Aquaporin 5 and the tight junction complex. *Proc Natl Acad Sci U S A* **104**, 3621–3626 (2007).
5. Park-Windhol C, D'Amore PA. Disorders of Vascular Permeability. *Annu Rev Pathol* **11**, 251–281 (2016).
6. Wen Q, *et al.* Signaling pathways regulating blood-tissue barriers - Lesson from the testis. *Biochim Biophys Acta Biomembr* **1860**, 141–153 (2018).
7. Frank JA. Claudins and alveolar epithelial barrier function in the lung. *Ann N Y Acad Sci* **1257**, 175–183 (2012).
8. Bhattad PB, Jain V. Renal Artery Stenosis As Etiology of Recurrent Flash Pulmonary Edema and Role of Imaging in Timely Diagnosis and Management. *Cureus* **12**, e7609-e7609 (2020).
9. Owen-Woods C, *et al.* Local microvascular leakage promotes trafficking of activated neutrophils to remote organs. *J Clin Invest* **130**, 2301–2318 (2020).
10. Lee KS, *et al.* Hydrogen peroxide induces vascular permeability via regulation of vascular endothelial growth factor. *Am J Respir Cell Mol Biol* **35**, 190–197 (2006).
11. Incalza MA, D'Oria R, Natalicchio A, Perrini S, Laviola L, Giorgino F. Oxidative stress and reactive oxygen species in endothelial dysfunction associated with cardiovascular and metabolic diseases. *Vascul Pharmacol* **100**, 1–19 (2018).
12. Ikari A, *et al.* Hyperosmolarity-induced up-regulation of claudin-4 mediated by NADPH oxidase-dependent H₂O₂ production and Sp1/c-Jun cooperation. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* **1833**, 2617–2627 (2013).
13. Taddei A, *et al.* Endothelial adherens junctions control tight junctions by VE-cadherin-mediated upregulation of claudin-5. *Nat Cell Biol* **10**, 923–934 (2008).
14. Gao F, *et al.* Akt1 promotes stimuli-induced endothelial-barrier protection through FoxO-mediated tight-junction protein turnover. *Cell Mol Life Sci* **73**, 3917–3933 (2016).
15. Gao F, Sabbineni H, Artham S, Somanath PR. Modulation of long-term endothelial-barrier integrity is conditional to the cross-talk between Akt and Src signaling. *J Cell Physiol* **232**, 2599–2609 (2017).
16. Alwhaibi A, Verma A, Adil MS, Somanath PR. The unconventional role of Akt1 in the advanced cancers and in diabetes-promoted carcinogenesis. *Pharmacol Res* **145**, 104270 (2019).
17. Adil MS, Narayanan SP, Somanath PR. Cell-cell junctions: structure and regulation in physiology and pathology. *Tissue Barriers*, 1848212 (2020).
18. Rosenthal R, Günzel D, Theune D, Czichos C, Schulzke JD, Fromm M. Water channels and barriers formed by claudins. *Ann N Y Acad Sci* **1397**, 100–109 (2017).
19. Conrad MP, Piontek J, Günzel D, Fromm M, Krug SM. Molecular basis of claudin-17 anion selectivity. *Cell Mol Life Sci* **73**, 185–200 (2016).

20. Marcelino Cereijido JMA. Tight Junctions. *CRC Press 2nd Edition*, (2001).
21. Shabalina SA, Ogurtsov AY, Spiridonov AN, Novichkov PS, Spiridonov NA, Koonin EV. Distinct patterns of expression and evolution of intronless and intron-containing mammalian genes. *Mol Biol Evol* **27**, 1745–1749 (2010).
22. Günzel D, Yu AS. Claudins and the modulation of tight junction permeability. *Physiol Rev* **93**, 525–569 (2013).
23. Kielgast F, *et al.* Glucocorticoids Regulate Tight Junction Permeability of Lung Epithelia by Modulating Claudin 8. *Am J Respir Cell Mol Biol* **54**, 707–717 (2016).
24. Park-Windhol C, D'Amore PA. Disorders of Vascular Permeability. *Annu Rev Pathol* **11**, 251–281 (2016).
25. Cong X, Kong W. Endothelial tight junctions and their regulatory signaling pathways in vascular homeostasis and disease. *Cellular Signalling* **66**, 109485 (2020).
26. Rudraraju M, Narayanan SP, Somanath PR. Regulation of blood-retinal barrier cell-junctions in diabetic retinopathy. *Pharmacol Res* **161**, 105115 (2020).
27. Nitta T, *et al.* Size-selective loosening of the blood-brain barrier in claudin-5-deficient mice. *J Cell Biol* **161**, 653–660 (2003).
28. Chen J, *et al.* Akt1 regulates pathological angiogenesis, vascular maturation and permeability in vivo. *Nat Med* **11**, 1188–1196 (2005).
29. Gao F, Alwhaibi A, Artham S, Verma A, Somanath PR. Endothelial Akt1 loss promotes prostate cancer metastasis via beta-catenin-regulated tight-junction protein turnover. *Br J Cancer*, (2018).
30. Krug SM, *et al.* Claudin-17 forms tight junction channels with distinct anion selectivity. *Cell Mol Life Sci* **69**, 2765–2778 (2012).
31. Runkle EA, Mu D. Tight junction proteins: from barrier to tumorigenesis. *Cancer Lett* **337**, 41–48 (2013).
32. Jiao D, Guo S, Chen Y, Zhan Y, An H. Ion Channels and Bone Homeostasis Imbalance. *Biomedical Journal of Scientific & Technical Research* **16**, 1–6 (2019).
33. Schaller S, Henriksen K, Sørensen MG, Karsdal MA. The role of chloride channels in osteoclasts: ClC-7 as a target for osteoporosis treatment. *Drug News Perspect* **18**, 489–495 (2005).
34. Sobacchi C, Schulz A, Coxon FP, Villa A, Helfrich MH. Osteopetrosis: genetics, treatment and new insights into osteoclast function. *Nat Rev Endocrinol* **9**, 522–536 (2013).
35. Portalatin M, Winstead N. Medical management of constipation. *Clin Colon Rectal Surg* **25**, 12–19 (2012).
36. McDonald DM, Baluk P. Significance of blood vessel leakiness in cancer. *Cancer Res* **62**, 5381–5385 (2002).
37. Koning NJ, *et al.* Reduction of vascular leakage by imatinib is associated with preserved microcirculatory perfusion and reduced renal injury markers in a rat model of cardiopulmonary bypass. *British Journal of Anaesthesia* **120**, 1165–1175 (2018).

38. Li H, Groshong SD, Lynch D, Brown KK, Frankel SK. Eosinophilic Lung Disease. *Clinical Pulmonary Medicine* **17**, (2010).
39. Kang JH, Hwang SM, Chung IY. S100A8, S100A9 and S100A12 activate airway epithelial cells to produce MUC5AC via extracellular signal-regulated kinase and nuclear factor- κ B pathways. *Immunology* **144**, 79–90 (2015).
40. Bargagli E, *et al.* Calgranulin B (S100A9) levels in bronchoalveolar lavage fluid of patients with interstitial lung diseases. *Inflammation* **31**, 351–354 (2008).
41. Kapoor P, *et al.* Idiopathic systemic capillary leak syndrome (Clarkson's disease): the Mayo clinic experience. *Mayo Clin Proc* **85**, 905–912 (2010).
42. Baluna R, Vitetta ES. Vascular leak syndrome: a side effect of immunotherapy. *Immunopharmacology* **37**, 117–132 (1997).
43. Siddall E, Khatrri M, Radhakrishnan J. Capillary leak syndrome: etiologies, pathophysiology, and management. *Kidney Int* **92**, 37–46 (2017).
44. Torgerson DG, *et al.* Ancestry and genetic associations with bronchopulmonary dysplasia in preterm infants. *Am J Physiol Lung Cell Mol Physiol* **315**, L858–L869 (2018).
45. Chen J, Du G, Wang Y, Shi L, Mi J, Tang G. Integrative analysis of mRNA and miRNA expression profiles in oral lichen planus: preliminary results. *Oral Surgery, Oral Medicine, Oral Pathology and Oral Radiology* **124**, 390–402.e317 (2017).
46. Cloutier N, *et al.* Platelets can enhance vascular permeability. *Blood* **120**, 1334–1343 (2012).
47. Ho-Tin-Noé B, Demers M, Wagner DD. How platelets safeguard vascular integrity. *J Thromb Haemost* **9 Suppl 1**, 56–65 (2011).
48. Nevzorova TA, *et al.* Platelet factor 4-containing immune complexes induce platelet activation followed by calpain-dependent platelet death. *Cell Death Discovery* **5**, 106 (2019).
49. Bunton-Stasyshyn RK, Saccon RA, Fratta P, Fisher EM. SOD1 Function and Its Implications for Amyotrophic Lateral Sclerosis Pathology: New and Renascent Themes. *Neuroscientist* **21**, 519–529 (2015).
50. Veith A, Moorthy B. ROLE OF CYTOCHROME P450S IN THE GENERATION AND METABOLISM OF REACTIVE OXYGEN SPECIES. *Curr Opin Toxicol* **7**, 44–51 (2018).
51. Alharthi A, Verma A, Sabbineni H, Adil MS, Somanath PR. Distinct effects of pharmacological inhibition of stromelysin1 on endothelial-to-mesenchymal transition and myofibroblast differentiation. *J Cell Physiol* **236**, 5147–5161 (2021).
52. Gah A, Adil MS, Sabbineni H, Verma A, Somanath PR. Differential regulation of TGF β type-I receptor expressions in TGF β 1-induced myofibroblast differentiation. *Can J Physiol Pharmacol* **98**, 841–848 (2020).
53. Adil MS, Somanath PR. Vascular Permeability Assays In Vivo. *Methods Mol Biol* **2367**, 165–175 (2021).

54. Adil MS, Somanath PR. Endothelial Permeability Assays In Vitro. *Methods Mol Biol* **2367**, 177–191 (2021).
55. Krämer A, Green J, Pollard J, Jr., Tugendreich S. Causal analysis approaches in Ingenuity Pathway Analysis. *Bioinformatics* **30**, 523–530 (2014).
56. Wang Q, Zou MH. Measurement of Reactive Oxygen Species (ROS) and Mitochondrial ROS in AMPK Knockout Mice Blood Vessels. *Methods Mol Biol* **1732**, 507–517 (2018).

Figures

Figure 1

***Cldn17* deficiency delays growth in young mice.** **a-b** Cel-1 assay validation gel representing *Cldn17* gene knockout and gRNA base-pair details for WT and cleavage bands. **c** Western blot images validating gene loss at the protein level in kidneys. **d** Bar graph showing mRNA level in lungs (n=5). **e** Images (dorsal view) of WT and *Cldn17*^{-/-} mice on day 7 and **f** Week 14. **g** Lengths of WT and *Cldn17*^{-/-} mice on day 7 (n=5). **h** Growth hormone levels in pups and adult mice from WT and *Cldn17*^{-/-} liver samples. **i** Weights

of mice recorded at weeks 1 to 4 (n=5) and j weeks 8 to 18 (n=5-12). Data presented as mean $\pm$ s.e.m.; +/+, WT; -/-, *Cldn17* knockout; gRNA, guide RNA; bp, base pair; g, gram; cm, centimeter; *p<0.05; **p<0.0001. **c** Weights of spleen in WT and *Cldn17*^{-/-} mice.

Figure 2

Loss of *Cldn17* affects serum parameters and proteins. Bar graphs showing variations in serum levels for **a** Chloride (n=17), **b** Bicarbonates (n=8), **c** Sodium (n=17), **d** Potassium (n=7), **e** anion gap (n=6), and **f** Proteins (total, albumin, globulin, and albumin/ globulin ratio) in WT and *Cldn17*^{-/-} mice. Data presented as mean ± s.e.m.; *p<0.05.

Figure 3

Remodeling of femur in *Cldn17*^{-/-} mice against WT. **a** Femur images from 8- to 12-week-old WT and *Cldn17*^{-/-} mice acquired from micro-computed tomography at various angles (exterior, coronal, and trans-axial planes). **b** Trabecular and **c** Cortical bone quantification obtained from Skyscan demonstrating various bone properties in WT and *Cldn17*^{-/-} mice. Data presented as mean $\pm$ s.e.m.; +/+ , WT; -/- , *Cldn17* knockout; Cl⁻, chloride; HCO₃⁻, bicarbonate; Na⁺, sodium; K⁺, potassium; n=6; *p<0.05

Figure 4

***Cldn17*^{-/-} mice reveal vascular leakage in Miles and the Matrigel plug assays and demonstrate altered consumption of food and water.** **a** Intravenous (n=6-8) and **b** Intraperitoneal (n=5-6) extravasation of Evan's blue dye into various organs determined at a wavelength of 610 nm in WT and *Cldn17*^{-/-} mice. **c** Representative images of Matrigel plugs isolated from WT and *Cldn17*^{-/-} mice and **d** Bar graph illustrating absorbance of hemoglobin present in the Matrigel plug lysates obtained from WT and *Cldn17*^{-/-} mice (n=6-7) using Drabkin's reagent at an absorbance of 540 nm. **e** Fold-change food (n=8) and **f** Fold-change water (n=9) consumption in WT and *Cldn17*^{-/-} mice. **g** Fecal pellets excreted over 24 hours (n=4 cages, 2-4 mice per cage) from WT and *Cldn17*^{-/-} mice. **h** Wet/dry ratio of fecal pellets demonstrating the moisture content (n=7-9). Data presented as mean ± s.e.m.; *p≤0.05; **p<0.01.

Figure 5

***Cldn17^{-/-}* mice display organ injury.** **a** Hematoxylin and eosin-stained images of lungs and kidneys with magnified Bowman's capsule area from WT, *Cldn17^{+/-}* and *Cldn17^{-/-}* mice. **b-e** Scoring of lung and kidney injury by blinded reviewers and quantification of pulmonary and renal edema by wet/dry ratio. **f** Quantification of Bowman's capsule area by Image J. Data presented as mean ± s.e.m.; pg/ml, picograms per milliliter; n=3-7; *p<0.05; **p<0.01.

Figure 6

RNA-Seq analysis reveals altered gene expression in *Cldn17*^{-/-} mouse kidneys. **a** Alteration in signaling pathways (involving at least 2 genes) in the *Cldn17*^{-/-} mice against the WT group based on the Panther database. **b** Distribution of genes between WT and *Cldn17*^{-/-} mice on a volcano plot. **c** Gene enrichment plots (GO) and **d** Heat maps (GSEA) for altered genes involved in the regulation of inflammatory. Data represented for genes with foldchange >2; n=3; GO, gene ontology; GSEA, gene set enrichment analysis;

NS, not significant; FC, fold-change; IL, interleukin; TNF, tumor necrosis factor; +/+, WT; -/-, *Cldn17* knockout; p<0.05.

Figure 7

Loss of *Cldn17* causes leukocytosis. **a** WBCs (n=7), **b** Platelets (n=8), and **c** Differential leukocyte count (n=7) in the 8- to 12-week-old WT and *Cldn17*^{-/-} mice. **d** Bar graph showing the difference in spleen size

between WT and *Cldn17*^{-/-} mice. **e** Forward vs. side scatter plots to confirm leukocytosis and **f** its densitometric graphs (n=5). Data presented as mean ± s.e.m.; WBC, white blood cells; µL, microliter; *p<0.05; **p<0.01.

Figure 8

***Cldn17*^{-/-} mice demonstrate modulation of cell junctions and enhanced pathological angiogenesis.** **a** Gene enrichment plots (GO) and heat maps (GSEA) from the RNA-seq analysis illustrating changes in genes related to the apical junction and vasculature development. Data represented for genes with foldchange >2; n=3. **b** Western blot images confirming *CLDN17* knockdown in human lung endothelial (left, n=4) and epithelial (right, n=3) cells. **c** ECIS graph representing changes in electrical resistance in shCtrl and shCLDN17 cell lines generated from human lung endothelial (top, n=6) and epithelial (bottom, n=3) cells. Data presented as mean ± s.d. **d** Laminin-stained images of tumor plugs isolated from WT and *Cldn17*^{-/-} mice showing blood vessels. **e** Bar graphs showing quantification of vascular length density (top, n=5-6) and tumor weights (bottom, n=5-6). Data presented as mean ± s.e.m.; GO, gene ontology; GSEA, gene set enrichment analysis; *p<0.05; #p<0.01.

Figure 9

Influence of *Cldn17* loss on oxidative stress. **a** Gene enrichment plots (GO) and heat maps (GSEA) depicting modulation of genes related to ROS, H₂O₂, and hypoxia in kidney samples from *Cldn17*^{-/-} mice against the WT group on RNA sequencing. Data represented for genes with foldchange>2; n=3. **b** Ingenuity pathway analysis predicting a potential link between *CLDN17* and SOD1. **c** Representative images of immunoblots and densitometric analysis of SOD1 expression in kidney lysates obtained from

WT and *Cldn17*^{-/-} mice. **d** DHE-stained images and fluorescence quantification of ROS on frozen kidney sections obtained from both groups of mice. Data presented as mean ± s.e.m.; GO, gene ontology; GSEA, gene set enrichment analysis; ROS, reactive oxygen species; H₂O₂, hydrogen peroxide; SOD1, superoxide dismutase1; DHE, dihydroethidium.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [CLDN17ManuscriptSuppl.Figures.pdf](#)
- [SourceData.zip](#)