Energy Shortage in Human and Mouse Models of SLC4A11-Associated Corneal Endothelial Dystrophies

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Purpose. To elucidate the molecular events in solute carrier family 4 member 11 (SLC4A11)-deficient corneal endothelium that lead to the endothelial dysfunction that characterizes the dystrophies associated with SLC4A11 mutations, congenital hereditary endothelial dystrophy (CHED) and Fuchs endothelial corneal dystrophy 4.

Methods. Comparative transcriptomic analysis (CTA) was performed in primary human corneal endothelial cells (pHeCNc) and murine corneal endothelial cells (mHeCNc) with normal and reduced levels of SLC4A11 (SLC4A11 KD pHeCNc and Slc4a11 mice (Slc4a11−/− mHeCNc), respectively. Validation of differentially expressed genes was performed using immunofluorescence staining of CHED corneal endothelium, as well as western blot and quantitative PCR analysis of SLC4A11 KD pHeCNc and Slc4a11−/− mHeCNc. Functional analyses were performed to investigate potential functional changes associated with the observed transcriptomic alterations.

Results. CTA revealed inhibition of cell metabolism and ion transport function as well as mitochondrial dysfunction, leading to reduced adenosine triphosphate (ATP) production, in SLC4A11 KD pHeCNc and Slc4a11−/− mHeCNc. Co-localization of SNARE protein STX17 with mitochondria marker COX4 was observed in CHED corneal endothelium, as was activation of AMPK–p53/ULK1 in both SLC4A11 KD pHeCNc and Slc4a11−/− mHeCNc, providing additional evidence of mitochondrial dysfunction and mitophagy. Reduced Na+/H+ transport activity and altered NH4Cl-induced membrane potential changes were observed in Slc4a11−/− mHeCNc.

Conclusions. Reduced steady-state ATP levels and subsequent activation of the AMPK–p53 pathway provide a link between the metabolic functional deficit and transcriptome alterations, as well as evidence of insufficient ATP to sustain the Na+/K+ -ATPase corneal endothelial pump as the cause of the edema that characterizes SLC4A11-associated corneal endothelial dystrophies.

Keywords: SLC4A11, mitochondria dysfunction, AMPK, corneal endothelium, congenital hereditary endothelial dystrophy

Solvent carrier family 4 member 11 (SLC4A11) is one of the highly expressed differentiation markers for corneal endothelium.1–3 Mutations in SLC4A11 are associated with congenital hereditary endothelial dystrophy (CHED), Harboyan syndrome (CHED with perceptive deafness) and a subset of Fuchs endothelial corneal dystrophy (FECD4). Children with CHED often present with bilateral corneal edema at or shortly after birth with significant vision impairment. Corneal transplantation is the only means of restoring vision and is associated with a guarded prognosis in terms of graft survival and long-term recovery of vision.5 In addition, these children are at risk of developing perceptive deafness later in life (Harboyan syndrome).9 FECD affects as many as 5% of the US population over 40 years of age,10 and visually significant corneal edema secondary to FECD is the most common indication for keratoplasty in the United States and worldwide.11,12 Together, CHED and FECD constitute common indications for corneal transplantation in published series from around the world.13,14

SLC4A11 is functionally characterized as an NH3 and alkaline pH-stimulated H+ transporter, while permeability to Na+, OH− and water has also been reported.13,15 SLC4A11 is essential in facilitating energy-producing glutaminolysis, maintaining antioxidant signaling, and preventing apoptosis in corneal endothelial cells (CEnC).16,17 During development and in the event of oxidative DNA damage, SLC4A11 gene expression is upregulated by direct binding of phosphorylated (activated) p53.18 In SLC4A11-associated corneal endothelial dystrophies, the corneal edema that develops as a result of pathologic SLC4A11 mutations is evidence of CEnC dysfunction, either from direct cell loss/death or from disturbances in the CEnC “pump–leak” system.19,20 Corneal transparency is maintained by the CEnC “pump–leak” system through a dynamic balance between the passive leak of...
aqueous humor fluid from the anterior chamber into the corneal stroma and the active pumping of fluid from the corneal stroma into the anterior chamber. The fluid pump activity is driven by an ionic electrochemical gradient set up by the highly expressed Na+/K+-ATPase. As such, CEnC have the second highest density of mitochondria among any cell types in the body (second to photoreceptors) to generate sufficient adenosine triphosphate (ATP) to fuel the Na+/K+-ATPase-driven endothelial pump. As SLC4A11 plays a significant role in facilitating ATP-generating glutaminolysis in CEnC, it is not surprising that glutaminolysis inhibition, mitochondria membrane potential depolarization, enriched mitochondrial reactive oxidative species (ROS), and increased mitochondria turnover have been observed in the CEnC of the Slc4a11−/− mouse. Thus, the association between SLCA11 and CEnC mitochondrial function suggests that SLCA11 is involved not only in moving ions across the plasma membrane but also in the supply of energy to the endothelial pump.

Approximately 94 SLCA11 mutations have been identified in individuals with CHED. Although a large number of these mutations result in SLCA11 protein misfolding and failure to mature to the plasma membrane, some mutations affect SLCA11 transporter function without impacting membrane trafficking or cause aberrant SLCA11 pre-mRNA splicing and subsequent reduced SLCA11 expression. Collectively, these observations support the hypothesis that loss of SLCA11 function is the primary pathogenetic mechanism in CHED rather than mutant SLCA11 protein misfolding/mislocalization in the endoplasmic reticulum (ER). Therefore, we investigated the impact of reduced SLCA11 function on the CEnC transcriptome in primary human and murine immortalized CEnC, with validation in corneal endothelium from individuals with CHED, and elucidated the upstream molecular mechanism leading to the observed transcriptomic changes.

**Materials and Methods**

**Primary Human Corneal Endothelial Cell Culture and Knockdown of SLC4A11**

Primary cultures of human corneal endothelial cells (pHCEnC) were established from donor corneas as previously described. After achieving a confluent monolayer, passage 1 of pHCEnC were transfected with 10 nM anti-SLC4A11 siRNA (OriGene Technologies, Rockville, MD, USA) using Lipofectamine LTX (Life Technologies, Carlsbad, CA, USA). At 72 hours post-transfection, the cells were lysed for RNA isolation.

**Immortalized Mouse Corneal Endothelial Cell Culture**

Immortalized Slc4a11+/+ and Slc4a11−/− mice corneal endothelium and were cultured at 33°C in Opti-MEM I medium (Thermo Fisher Scientific, Waltham, MA, USA) with supplements as previously described. Passage 6 and 39 of Slc4a11+/+ and passages 7 and 40 of Slc4a11−/− MCEnc were lyzed for RNA isolation. Passages 10 and 44 of Slc4a11−/− and passages 11 and 45 of Slc4a11+/+ MCEnc were lyzed for protein and ATP isolation.

**Total RNA Isolation from pHCEnC and MCEnc**

Total RNA from cultured pHCEnC was isolated in TRI Reagent and purified with the RNeasy Clean-Up Kit (Qiagen, Hilden, Germany). Total RNA from cultured MCEnc was isolated and purified using the Qiagen RNeasy Plus Mini Kit.

**RNA Sequencing of Total RNA from pHCEnC and MCEnc**

Purified total RNA from pHCEnC was prepared for RNA sequencing libraries using the KAPA mRNA HyperPrep Kit (Roche Sequencing Solutions, Pleasanton, CA, USA). Libraries were sequenced on the HiSeq 4000 (Illumina, San Diego, CA, USA), and paired-end 150-bp reads were generated. Purified total RNA from the MCEnc was submitted to the UCLA Technology Center for Genomics & Bioinformatics for library preparation and sequencing. Single-end 50-bp reads were generated using the Illumina HiSeq 3000. The generated FASTQ files and quantitative results are available from the NCBI Gene Expression Omnibus database (accession numbers GSE142635 and GSE142636).

**RNA Sequencing Data Analyses**

Raw reads from pHCEnC and MCEnc samples were aligned to the human (GRCh38/hg38) and mouse (GRCm38/mm10) genomes, respectively, using HISAT2. Raw counts of aligned reads were converted to counts per million (CPM) mapped reads and normalized by the method of trimmed mean of M-values to adjust for library size differences. Linear models for microarray analysis coupled with variance modeling on the linear observation level were used for differential gene expression analysis. The CPM fold changes of gene transcripts were calculated by comparing each sample set to the appropriate control: (1) pHCEnC sample set, pHCEnC transfected with siRNA targeting Slc4a11 (Slc4a11 KD pHCEnC, n = 3) versus pHCEnC transfected with scrambled RNA (scRNA pHCEnC, n = 3); (2) MCEnc early sample set, Slc4a11+/− MCEnc passage 6 (n = 4) versus Slc4a11−/− MCEnc passage 7 (n = 4); and (3) MCEnc late sample set, Slc4a11−/− MCEnc passage 39 (n = 4) versus Slc4a11+/+ MCEnc passage 40 (n = 4). The following thresholds were applied to define genes with differential expression: CPM > 1, fold change > 1, and adjusted P < 0.05. A differential gene expression (DGE) list was created for each of the three sample sets, after which comparative transcriptome analysis was performed by comparing the three DGE lists to identify common differentially expressed genes (DEG) and enriched pathways.

**Ingenuity Pathway Analysis**

Qiagen Ingenuity Pathway Analysis (IPA) was used to perform comparative transcriptome analysis among the three DGE lists (pHCEnC, MCEnc early, and MCEnc late), including canonical/biological function pathway enrichment and upstream regulator prediction analyses. Enriched canonical and biological function pathways with a predicted activation z-score in each of the three DGE lists were sorted by the sign and value of the z-score to identify the most enriched pathways. Enriched pathways without an assigned z-score and predicted upstream regulators were ranked by mean of enrichment P values.
Quantitative PCR

Quantitative PCR (qPCR) was performed on separate batches of RNA samples isolated from passage 1 of SLC4A11 KD and scRNA pHcEnC, as well as Slc4a11−/− MCEnc passages 6 and 39 and of Scl4a11+/+ MCEnc passages 7 and 40. Total RNA of each sample was reverse-transcribed using the SuperScript III First-Strand Synthesis System (Sigma-Aldrich, St. Louis, MO, USA). Quantitative PCR was performed on the LightCycler 480 System (Roche, Basel, Switzerland) using Kapa SYBR Fast Universal Kit (Roche) and primers listed in Supplementary Table S1. Relative gene expression was calculated by the comparative Ct (2−ΔΔCt) method in comparison to the housekeeping gene PPIA/Ppia or ACTB/Actb.

Immunofluorescence

Five-micrometer sections of paraffin-embedded corneas from seven healthy donors and two individuals with CHED were de-paraffinized and rehydrated in a graded ethanol series (100%, 95%, 70% and 50%) for 5 minutes each and subject to antigen retrieval in 10 mM sodium citrate. Sections were incubated with primary antibodies overnight at 4°C and with secondary antibodies for 1 hour (Supplementary Table S2). Sections were mounted with Invitrogen ProLong Antifade Mountant (Thermo Fisher Scientific) and imaged with an Olympus FV-1000 inverted confocal fluorescence microscope (Olympus Corporation, Tokyo, Japan). Fluorescence intensity of the images was quantified using Olympus Fluoview 4.2 and ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Single Cell Patch-Clamp Recordings

Single cell recordings were made from a single adhered MCEnc after culturing of MCEnc on 25-mm glass coverslips to ~10% to 20% confluence. Membrane voltages were measured using whole-cell patch electrodes in current clamp mode (Ihold = 0) as previously described.55 Data were reported as mean (95% CI). Details of the experimental setup and solutions used are provided in the Supplementary Materials.

Intracellular pH Measurement

Intracellular pH (pH) measurements were performed by monitoring intracellular free H+ concentration using the pH-sensitive fluorescence dye BCECF-AM (B1170; Thermo Fisher Scientific) as described previously.19,20 Details of the experimental setup and solutions used are provided in the Supplementary Materials.

Western Blotting

Whole-cell lysates from pHcEnC and MCEnc were prepared with radioimmunoprecipitation assay buffer with protease and phosphatase inhibitors. Total protein was quantified with radioimmunoprecipitation assay buffer with proteinase and phosphatase inhibitors. Total protein was quantified with a bicinchoninic acid (BCA) assay, separated and detected using the Simple Western assay kit (ProteinSimple, San Jose, CA, USA). Quantification and data analysis were performed in Compass software, Simple Western software (ProteinSimple). Antibodies used are listed in Supplementary Table S2.

Intracellular ATP Assay

Slc4a11−/− MCEnc passages 10 and 44, Scl4a11+/+ MCEnc passages 11 and 45 and pHCEnC passage 1 were seeded at 1 × 105 cells/mL in 12-well (MCEnc) and 24-well (pHcEnC) plates and cultured to subconfluence. SLC4A11 was knocked down in confluent pHCEnC with siRNA as described above. ATP was extracted by a boiling water method56 and measured using a luciferin–luciferase ATP determination kit (Molecular Probes, Eugene, OR, USA).

Human Corneal Endothelium from Individuals with CHED

The authors followed the tenets of the Declaration of Helsinki in the treatment of the subjects reported herein. This study was approved by the Institutional Review Board at The University of California, Los Angeles (UCLA IRB #11-000020) and was performed after obtaining informed written consent from the parents of affected individuals with CHED who underwent penetrating keratoplasty.

Statistical Analysis

All P values used in the transcriptomic analysis to identify gene differential expression and pathway enrichment were false discovery rate adjusted P values. Statistical analysis, for data other than transcriptomes and patch-clamp recordings, was performed in Prism 7.0 (GraphPad, San Diego, CA, USA) with appropriate statistical tests based on the data structure. Specific statistical tests used for each comparison are indicated in figure legends. Data are presented as mean ± SEM. Statistical significance is denoted as follows in the figures: *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001.

RESULTS

CHED Corneal Specimens Suggest That SLC4A11 Mutations Are Not Associated with Decreased Mutant SLC4A11 Expression

Corneal specimens from two individuals with CHED were examined with light microscopy and immunohistochemistry. One of the two individuals (SLC4A11p.R158PfsX3/Mu/Mu) demonstrated compound heterozygous mutations in SLC4A11 (NM_032034) (c.473_480delGCTTCGCinsC; 2623G>T), p.R158PfsX3; Arg875* and the other (SLC4A11p.R875W/WT) demonstrated a single heterozygous SLC4A11 coding region mutation (c.2146C>T; p.Pro716Ala). Both corneal specimens demonstrated a significantly thickened Descemet membrane and an attenuated aberrant CEnC layer with cytoplasmic inclusions present in some cells (Fig. 1A). Immunofluorescence staining for SLC4A11 protein in the corneal endothelium was performed in conjunction with the use of an antibody against an ER marker, protein disulfide isomerase (PDI), to investigate whether these SLC4A11 mutations lead to protein misfolding and retention in the ER, as previously reported.5,6 No apparent difference in SLC4A11 protein staining intensity between the two specimens from the individuals with CHED, in which the staining localized to the cellular membrane and did not colocalize with PDI, and seven healthy controls (Fig. 1B).
SLC4A11/Slc4a11 Reduction Induces Corneal Endothelial Transcriptional Changes

Next, to mimic the loss of SLC4A11 function in CHED, we knocked down SLC4A11 in pHEnC (Fig. 1C) and utilized an immortalized MCEnc cell line from a Slc4a11−/− mouse. We then performed transcriptomic analysis of pHEnC and MCEnc with normal and reduced levels of SLC4A11 and Slc4a11, respectively. Based on the shared corneal endothelial phenotypes between individuals with CHED and the Slc4a11−/− mouse, we compared the transcriptomes from SLC4A11 KD pHEnC and early and late passage MCEnc derived from Slc4a11−/− mice.24 Both early and late MCEnc passages were included in the analysis so that transcriptional changes due to the loss of SLC4A11 could be differentiated from the transcriptional changes introduced by prolonged culture. A comparison of the DEG identified from each sample set revealed 3171 genes that were consensually differentially expressed across three sample sets (Fig. 1D), of which 1041 genes were differentially expressed in the same direction (Fig. 1E). Over a third of the 30 most highly DEG (Fig. 1F) have been previously demonstrated to play important roles in cellular functions, including SEMA3E,1 MSTR,57 RNF43,58 SLCA43,58 WNK4,59 SLC4A4,60 CYGB,61 SLC9A7,1,61 ZNF469,62-65 BNC3,66 and TTC22.67

Loss of SLC4A11 Leads to Generalized Inhibition of Cellular Metabolism

Comparison of enriched canonical pathways in Slc4a11 KD pHEnC, Slc4a11−/− MCEnc early (passage 6) and Slc4a11−/− MCEnc late (passage 39) sample sets identified a shared generalized inhibition (defined by negative activation z-score) of multiple metabolic pathways that were interconnected via intermediate metabolites (Figs. 2A, 2B). A generalized decrease in the expression of enzyme-encoding genes in these pathways was observed (Figs. 2C–2I), a finding that was confirmed for selected genes from each pathway using qPCR (Fig. 2J).

Altered Expression of Ion Channels and Transporters Impairs Transport Function of Corneal Endothelium

IPA biological function enrichment analysis identified “transport of molecule” as the top inhibited function (negative z-score) shared between the transcriptomes of the pHEnC, MCEnc early and MCEnc late samples sets (Fig. 3A). Because SLC4A11 is an electrogenic NH3:H+ co-transporter,15 we performed single cell recordings of the membrane potential of Slc4a11+/− and Slc4a11−/− MCEnc, in which the resting membrane potential is not statistically different (Fig. 3B), in response to extracellularly perfused 10 mM NH4Cl. Although exposure to NH4Cl induced a +12.7 mV (95% CI, −5 to +20 mV) depolarization in Slc4a11+/− MCEnc, likely due to the NH3:H+ permeability provided by Slc4a11, exposure to NH4Cl induced a −9.50 mV (95% CI, −19.3 to −0.5 mV) hyperpolarization in Slc4a11−/− MCEnc (Figs. 3C, 3D).

To investigate the nature of this depolarization to hyperpolarization shift resulting from loss of Slc4a11, we examined the list of 1041 genes differentially expressed in the same direction in the SLC4A11 KD pHEnC, Slc4a11−/− MCEnc early and MCEnc late sample sets to identify ion channels and transporters. We identified 2 genes encoding ion channels and 26 genes encoding transporters were either upregulated or downregulated, including SLC4A11 (Fig. 3E, Table). The electrogenic Na+-HCO3− co-transporter
(NBCe1, SLC4A4), which plays an essential role in the CEnC pump function, was downregulated fourfold in SLC4A11 KD pHeCEnC and >100-fold in Slc4a11−/− MCEnc early and late passages. The downregulation of SLC4A4/Slc4a4 was verified by qPCR in SLC4A11 KD pHeCEnC and Slc4a11−/− MCEnc (Fig. 3F), by western blot in SLC4A11 KD pHeCEnC (Fig. 3G) and by immunofluorescence staining for NBCe1 (SLC4A4) in the corneal endothelium of two individuals with CHED (Fig. 3H), which showed decreased NBCe1 expression compared to controls (Fig. 3I).

Next, functional measurement of Na+-dependent HCO3− transport in Slc4a11−/− MCEnc showed reduced Na+-.
Energy Shortage in SLC4A11 Deficient Corneal Endothelial Cells

**FIGURE 3.** SLC4A11 deficiency impacts corneal endothelial ion and solute transport function. (A) Heat map showing consensually enriched IPA biological function pathways from comparison of transcriptomes of SLC4A11 KD pHEnC and Slc4a11−/− MCEC early and late passages (sorted by mean activation z-score). (B) Scatterplot of resting membrane potential (V_m) in Slc4a11+/+ (n = 6) and Slc4a11−/− (n = 7) MCEC. Two-tailed paired-samples t-test, P = 0.408. (C) Representative trace of current-clamped single cell recording during 10 mM NH4Cl superfusion of Slc4a11+/+ and Slc4a11−/− MCEC. (D) Scatterplot of membrane potential changes (dV_m) in Slc4a11+/+ (n = 6) and Slc4a11−/− (n = 7) MCEC in response to NH4Cl superfusion. Monte Carlo resampling, two-tailed paired-samples t-test, difference between genotype dV_m = –22.2 (–34.8, –10) mV, P = 0.0069. (E) Heat map showing common differentially expressed genes encoding ion channel and transporter proteins in SLC4A11 KD pHEnC and early and late Slc4a11−/− MCEC. (F) The differential expression of Na+–HCO3− transporter (NBCe1, encoded by SLC4A4) mRNA was validated by qPCR in separate RNA isolations from SLC4A11 KD pHEnC and Slc4a11−/− MCEC. (G) Western blot for NBCe1 in SLC4A11 KD pHEnC and scRNA pHEnC control showing decreased NBCe1 protein level in SLC4A11 KD pHEnC. (H) Representative images of immunofluorescence staining for NBCe1 (green signal) and tight junction Zonula occludens-1 (ZO-1) (red signal) in corneal endothelium of two individuals with CHED and healthy control. Nuclei were stained with DAPI (gray signal). Scale bar: 5 μm. (I) Scatterplot of mean fluorescence intensity (MFI) ratio of NBCe1 over ZO-1 in corneal endothelium of two individuals with CHED and of seven healthy controls. Two-tailed unpaired t-test with Welch’s correction, P = 0.0031. (J) Representative trace of pH response in Slc4a11−/− and Slc4a11+/+ MCEC to the addition of extracellular Na+ in HCO3−-containing solution. (K) Bar graph of the rate of intracellular [H+] change (d[H+] / dt) in Slc4a11+/+ (n = 6) and Slc4a11−/− (n = 8) MCEC. Two-tailed unpaired t-test, P < 0.0001.

HCO3− co-transporter activity when compared to Slc4a11+/+ MCEC (Fig. 3J), consistent with reduced NBCe1 expression. In Figure 3J, pH1 was maintained at a low value when Slc4a11+/+ and Slc4a11−/− MCEC were perfused with a 28.3 mM bicarbonate (HCO3−) solution that was Na+ free. When switched to a bicarbonate solution containing Na+, pH1 increased as NBCe1 started to move the weak base HCO3− inward using the Na+ inward transmembrane electrochemical gradient. We determined the initial slope of this pH1 rise to serve as an indirect measure of Na+–HCO3− cotransport activity and observed a reduced Na+–dependent pH1 rise in Slc4a11−/− MCEC compared with Slc4a11+/+ MCEC (Figs. 3J, 3K).

**Mitochondria Dysfunction Leads to Reduced ATP Production**

Dilated mitochondria is a characteristic electron microscopy finding in CHED corneal endothelium, suggestive of mitochondrial involvement in the pathogenesis. Correspondingly, “mitochondria dysfunction” was the top enriched
canonical pathway in the pHEnC and MCEnc early and late sample sets (ranked by P value, Fig. 4A). Additionally, in the list of 1041 genes differentially expressed in the same direction in the pHEnC and MCEnc early and late sample sets, genes encoding proteins involved in the mitochondria electron transport chain, mediating mitochondrial ATP flux, import machinery and translation machinery showed a generalized decreased expression (Table 1).

Table 1. Differentially Expressed Genes Encoding Ion Channels and Transporters in Slc4a11 KD pHEnC, Slc4a11−/− MCEnc Early, and MCEnc Late Sample Sets

| Ion channels | Upregulated | Downregulated |
|--------------|-------------|---------------|
| Cl− channels | ClCN2, ANO5 | ClCN3         |
| Ca2+ channels | TRPV2, CACNB1, CACNB4 | TRPV4, TRPC1 |
| K+ channels | — | KCNA2, KCNA1 |
| Transporters | — | SLC4A11 |
| NH3:H+ transporter | — | SLC4A4 |
| Na+−HCO3− cotransporter | — | ATP2B1 |
| Ca2+−-ATPase | — | Glucose transporter SLC2A3, SLC2A12 |
| Glucose transporter SLC2A3, SLC2A12 | — | Glutamine transporter SLC38A1 |
| Cl−/HCO3− exchanger SLC4A3 | — | ATP-binding cassette transporters ABCG3 |
| ATP-binding cassette transporters ABCG3 | — | K+−Cl− cotransporter SLC12A7 |
| K+−Cl− cotransporter SLC12A7 | — | Na+−H+ exchanger regulator SLC9A3R2 |
| Na+−H+ exchanger regulator SLC9A3R2 | — | Na+−−HPO3− cotransporter SLC2O1 |
| Lactate transporter SLC16A4 | — | Lactate transporter SLC2A18 |
| Na+−carnitine cotransporter SLC2A18 | — | SO42−− transporter SLC26A2 |

To identify the upstream signaling pathway responsible for the observed transcriptomic changes in Slc4a11 KD pHEnC and Slc4a11−/− MCEnc, we performed upstream regulator prediction in IPA, which identified p53 (encoded by the TP53 gene) as the top candidate transcription factor (Fig. 5A). Western blot analysis in Slc4a11 KD pHEnC demonstrated increased Ser15 phosphorylation of p53 compared to scRNA pHEnC controls (Fig. 5B), indicative of post-translational activation of p53 transcriptional activity. Similarly, in Slc4a11−/− MCEnc, we observed increased Ser18 phosphorylation of p53 (corresponding to Ser15 of human p53) in Slc4a11−/− MCEnc late passage, although this was not observed in Slc4a11−/− MCEnc early passage (Fig. 5C). However, there was increased total p53 levels in both Slc4a11−/− MCEnc early and late passages (Figs. 5C, 5D), indicative of transcriptional activation of p53.

We then sought to identify the kinase responsible for the Ser15 (Ser18 in mouse) phosphorylation and transcriptional activation of p53 in Slc4a11−/− MCEnc. Given the observed ATP depletion in Slc4a11−/− MCEnc, as well as the reported capacity of the cellular ATP sensor AMP-activated protein kinase (AMPK) to mediate Ser15 (Ser18 in mouse) phosphorylation and transcriptional activation of p53, we investigated the potential role of AMPK. In the setting of a decreased ATP-to-adenosine diphosphate (ADP) or ATP-to-AMP ratio, the AMPK catalytic α subunit will be phosphorylated at Thr172, whereas phosphorylation of the regulatory β1 subunit at Ser182 is not dependent upon cellular ATP levels. In Slc4a11 KD pHEnC and Slc4a11−/− MCEnc, we observed increased Thr172 phosphorylation of AMPKα and no change in Ser182 phosphorylation of AMPKβ1 (Figs. 5B, 5C). Examination of another downstream substrate of AMPK, Unc-51 like autophagy activating kinase 1 (ULK1), showed increased phosphorylation (Ser555) in Slc4a11−/− MCEnc compared with scRNA pHEnC (Figs. 5E, 5F).
DISCUSSION

In this manuscript, we utilized primary human CEnC, a CEnC cell line from Slc4a11−/− mice23 and corneal specimens from individuals with CHED to investigate the causes of CEnC dysfunction that characterize each of the SLC4A11-associated corneal endothelial dystrophies (CHED, FECD, and Harboyan syndrome). Although several previous reports have elucidated possible roles for mitochondrial uncoupling, ER unfolded protein response, oxidative stress, and apoptosis in SLC4A11-deficient human and mouse cell lines,9,20,21,30,50,76 none utilized primary human CEnC, and only one report examined CHED patient corneal endothelium, in which increased oxidative stress was demonstrated.20 We identified ATP depletion in CEnC with reduced SLC4A11, in both transient (72 hours) SLC4A11 knockdown in pHCEnC and permanent Slc4a11−/− MCEnc. The reduced CEnC ATP levels provide a proposed pathogenesis for the CEnC dysfunction that characterizes the SLC4A11-associated corneal endothelial dystrophies. The fact that ATP depletion and ATP-sensor AMPKα activation were detected within 72 hours of SLC4A11 knockdown in pHCEnC and Slc4a11−/− MCEnc controls. One-tailed unpaired t-test, P = 0.034, n = 6 each) and late passage (P = 0.039, n = 6 each) Slc4a11+/+ and Slc4a11−/− MCEnc.
Although the prevailing hypothesis regarding the pathogenesis of CHED is that the majority of SLC4A11 mutations result in protein misfolding and retention in the ER, \cite{5,6,50,51} we provide preliminary evidence that mutant SLC4A11 protein is not retained in the ER of the corneal endothelium in CHED. In addition, immunostaining of corneal endothelium from two individuals with CHED did not show a reduced level of the SLC4A11 mutant protein, consistent with a previous report that CHED corneal endothelium does not demonstrate reduced SLC4A11 expression at the mRNA level. \cite{20} Instead, our data, which we recognize is derived from a very limited number of observations, support the alternative hypothesis that identified SLC4A11 mutations affect the ion transport function of SLC4A11 protein in the corneal endothelium. \cite{17,52,53}

We used a comparative transcriptomics approach based on the observation of phenotypic similarities between CHED and the S\textit{c}l\textit{e}a\textit{a}11\textsuperscript{−/−} mouse corneal phenotype. \cite{5,7,8} This approach is based on the high degree of gene orthology between mouse and human and the organ-dominated hierarchical clustering observed across mammals on real gene expression data. \cite{79} Such a comparative transcriptomic approach enables the differentiation of transcriptome alterations attributable to the loss of SLC4A11/S\textit{c}l\textit{e}a\textit{a}11 in ph\textit{c}En\textit{c} and m\textit{C}En\textit{c} from confounding biological or technical factors associated with each cell preparation, including differences in primary cell isolation and passaging, siRNA treatment, cell line immortalization, and cell culture media. \cite{80} With this approach, we identified generalized inhibition of multiple metabolic pathways, as well as mitochondrial dysfunction in both S\textit{LC}4\textit{A}11\textsuperscript{KD} p\textit{hcEnC} and S\textit{lc}4\textit{a}11\textsuperscript{−/−} m\textit{C}En\textit{C}. We also identified the reduced expression of several ion channels and transporters, including NBCe1, and a decrease in Na\textsuperscript{+}-dependent HCO\textsubscript{3}− transport activity, which was in contrast to a previous functional examination of HCO\textsubscript{3}− transport in S\textit{lc}e\textit{a}11\textsuperscript{−/−} m\textit{C}En\textit{C} that did not reveal any difference in comparison to S\textit{lc}e\textit{a}11\textsuperscript{+/+} m\textit{C}En\textit{C}. \cite{25} The discrepancy is likely because previous experiments were performed in a Na\textsuperscript{+}-rich, HCO\textsubscript{3}−-free solution with subsequent reintroduction of HCO\textsubscript{3}−, whereas we performed the experiment in a Na\textsuperscript{+}-free, HCO\textsubscript{3}−-rich solution with subsequent reintroduction of Na\textsuperscript{+}. While a previous report provided an estimate of HCO\textsubscript{3}− transport activities in S\textit{lc}e\textit{a}11\textsuperscript{−/−} m\textit{C}En\textit{C}, \cite{23} we provided an estimate of Na\textsuperscript{+}-dependent HCO\textsubscript{3}− transport activities. We attributed the observed transcriptomic changes in S\textit{LC}4\textit{A}11\textsuperscript{KD} p\textit{hcEnC} and S\textit{lc}4\textit{a}11\textsuperscript{−/−} m\textit{C}En\textit{C} to activation of the AMPK–p53 pathway. Although post-translational activation (phosphorylation) of p53 was observed with transient S\textit{LC}4\textit{A}11 knockout in ph\textit{cEnC}, transcriptional activation (upregulation) of p53 was observed with permanent S\textit{LC}4\textit{A}11 knockout in m\textit{C}En\textit{C}, both attributed to AMPK activation. \cite{3} The observation that ATP depletion and AMPK activation occur within 72 hours after S\textit{LC}4\textit{A}11 knockout suggests that they are likely to be among the initial cellular events in response to S\textit{LC}4\textit{A}11 deficiency.

In summary, we postulate that S\textit{LC}4\textit{A}11 deficiency leads to CEnC dysfunction primarily through decreased generation of ATP via glutaminolysis to fuel the Na\textsuperscript{+}/K\textsuperscript{+}-ATPase-driven endothelial pump. The decreased ATP levels also result in the activation of AMPK and its downstream substrates, p53 and ULK1, leading to transcriptional alterations and increased mitophagy, respectively. Similarly, given the role of S\textit{LC}4\textit{A}11 in preventing oxidative damage, the loss of
SLC4A11 leads to increased mitochondrial ROS production, subsequent mitochondrial dysfunction, and increased mitophagy (Fig. 5G). This proposed pathogenesis is shown to be efficacious in the Slec4a11−/− mice, in which the alterations resulting from Slc4a11 depletion mirror those observed in SLC4A11 KD pHCornea, as a model for the SLC4A11-associated corneal endothelial dystrophies and indicates a favorable translational potential for therapeutic approaches shown to be efficacious in the Slec4a11−/− mice.

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