Ectopic GRHL2 Expression Due to Non-coding Mutations Promotes Cell State Transition and Causes Posterior Polymorphous Corneal Dystrophy 4

Petra Lisková, Lubica Dudakova, Cerys J. Evans, Karla E. Rojas Lopez, Nikolaos Pontikos, Dimitra Athanasiou, Hodan Jama, Josef Sach, Pavlina Skalická, Viktor Stranecký, Stanislav Kmoch, Caroline Thaung, Martin Filipček, Michael E. Cheetham, Alice E. Davidson, Stephen J. Tuft, and Alison J. Hardcastle

In a large family of Czech origin, we mapped a locus for an autosomal-dominant corneal endothelial dystrophy, posterior polymorphous corneal dystrophy 4 (PPCD4), to 8q22.3–q24.12. Whole-genome sequencing identified a unique variant (c.20+544G>T) in this locus, within an intronic regulatory region of GRHL2. Targeted sequencing identified the same variant in three additional previously unsolved PPCD-affected families, including a de novo occurrence that suggests this is a recurrent mutation. Two further unique variants were identified in intron 1 of GRHL2 (c.20+257delT and c.20+133delA) in unrelated PPCD-affected families. GRHL2 is a transcription factor that suppresses epithelial-to-mesenchymal transition (EMT) and is a direct transcriptional repressor of ZEB1. ZEB1 mutations leading to haploinsufficiency cause PPCD3. We previously identified promoter mutations in OVOL2, a gene not normally expressed in the corneal endothelium, as the cause of PPCD1. OVOL2 drives mesenchymal-to-epithelial transition (MET) by directly inhibiting EMT-inducing transcription factors, such as ZEB1. Here, we demonstrate that the GRHL2 regulatory variants identified in PPCD4-affected individuals induce increased transcriptional activity in vitro. Furthermore, although GRHL2 is not expressed in corneal endothelial cells in control tissue, we detected GRHL2 in the corneal “endothelium” in PPCD4 tissue. These cells were also positive for epithelial markers E-Cadherin and Cytokeratin 7, indicating they have transitioned to an epithelial-like cell type. We suggest that mutations inducing MET within the corneal endothelium are a convergent pathogenic mechanism leading to dysfunction of the endothelial barrier and disease.

Introduction

Posterior polymorphous corneal dystrophy (PPCD) is a rare autosomal-dominant disorder, primarily affecting the corneal endothelium and Descemet membrane. The severity and phenotype of PPCD is variable. Mild manifestations of the disease include asymptomatic corneal endothelial changes such as vesicular, band-like, and geographic lesions. In severe cases, corneal endothelial failure may occur and corneal transplantation is required to restore vision. Aberrant corneal endothelial cells have been shown to proliferate and migrate onto the trabecular meshwork and iris acquiring an epithelial-like morphology. Secondary complications are common and include corneal edema, glaucoma, iris adherence to the cornea, and corectopia.

PPCD is a genetically heterogeneous condition, with approximately a third of cases attributed to heterozygous mutations in the transcription factor encoding gene ZEB1 (MIM: 189909) (PPCD3 [MIM: 609141]). Recently, we and others have established that heterozygous regulatory mutations in the promoter of OVOL2 (MIM: 616441) cause PPCD1 (MIM: 122000). ZEB1 and OVOL2 control cell state, through regulation of epithelial-to-mesenchymal transition (EMT) and the converse process of mesenchymal-to-epithelial transition (MET), through a mutually inhibitory pathway. EMT and MET are central processes in development, and these finely tuned and reversible cell state transition pathways also support the maintenance of cellular identity and function. Aberrant regulation of MET and EMT underpins tumor progression and malignant transformation processes, as well as playing an important role in other disease conditions including fibrosis, wound repair, and inflammation.

Corneal endothelial cells are embryonically derived from the neural crest and form a monolayer of post-mitotic hexagonal cells on the inner surface of the cornea. They are specialized cells that have a barrier-pump function, governing fluid and solute transport across the posterior surface of the cornea and maintaining the cornea in a relatively dehydrated state that is essential for optical transparency. Haploinsufficiency and subsequent reduced expression of ZEB1 in the corneal endothelium is thought to underlie the pathology of PPCD3, whereas inappropriate ectopic expression of OVOL2 in corneal endothelial cells is the proposed mechanism for PPCD1. The

The American Journal of Human Genetics 102, 447–459, March 1, 2018 447

© 2018 The Author(s). This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).
disrupted balance of cell state transition regulators OVOL2 and ZEB1 within the diseased corneal endothelial cells could result in cellular trans-differentiation of the corneal endothelial cells into an epithelial-like state through the MET pathway.2,10,14,21,22 This hypothesis is supported by multiple studies demonstrating the epithelial-like phenotype of endothelial cells in PPCD, including histopathological and transcriptomic studies,1,5,6,21,22 and is likely a consequence of gene mutations specifically affecting corneal endothelial cells. Despite these recent advances in our understanding of the molecular basis of PPCD, there is evidence for further genetic heterogeneity of PPCD.12,21,23 Here, we describe an additional PPCD locus, PPCD4, which was mapped to 8q22.3–q24.12, and the subsequent identification of causative non-coding variants in GRHL2 with further evidence for the importance of MET in PPCD.

Material and Methods

Study Subjects and Clinical Examination

All participants signed informed consent approved by the ethics committee of the General University Hospital in Prague (reference no. 151/11 S-IV) or Moorfields Eye Hospital (REC references 13/LO/1084 and 09/H0724/25) before inclusion in the study. Ophthalmic examination included best corrected distance Snellen visual acuity (BCVA) converted to decimal values, intraocular pressure, slit-lamp biomicroscopy and specular microscopy (Noncon ROBO Pachy SP-9000; Konan Medical Inc.) and spectral-domain optical coherence tomography (SD-OCT) (Spectralis; Heidelberg Engineering GmbH). Genomic DNA was extracted from venous blood samples using a Gentra Puregene blood kit (QIAGEN) or from saliva using a Oragene kit (Oragene OG-300, DNA Genotek).

Linkage Analysis

Linkage analysis was performed using selected individuals from family C15 (Figure 1A). Nine affected (VI:2, VI:4, VII:1, VIII:1, VII:3, VII:7, IX:1, IX:3, IX:6) and seven unaffected samples (VII:2, VII:3, VIII:2, VIII:4, IX:2, IX:4, IX:5) were genotyped using an Illumina Omni2.5 Exome-8 array. Parametric linkage analysis, assuming dominant inheritance of a fully penetrant rare allele (disease allele frequency 0.00001) was performed using MERLIN.24 The following criteria were applied to select markers for linkage: only polymorphic SNPs with annotated rs numbers were analyzed, Mendelian inconsistent SNPs or SNPs with missing alleles were discarded, a SNP density of 0.1 cM was maintained.
Whole-Exome Sequencing (WES)
WES was performed using a TruSeq exome enrichment kit (Illumina) and Illumina HiSeq2000 sequence platform on DNA samples of affected individuals VIII:1, VIII:2, VII:6, and VII:10 from family C15, and using a SureSelect Human All Exon 50Mb Kit (Agilent) and Illumina HiSeq2000 sequence platform for individuals V:18, V:13, V:14, V:15, VIII:7, VIII:9, and VIII:5. Reads were aligned to the GRCh38/hg38 human reference sequence with Novoalign v.2.05 (Novocraft). The WES data were analyzed using the Phenopolis platform. ExomeDepth was used to identify copy-number variants (CNVs). Aligned data were visualized with the Integrated Genomics Viewer (IGV, Broad Institute). On the basis that PPCD is a rare dominant disease, WES data were filtered for rare variants with a minor allele frequency (MAF) \( \leq 0.005 \) according to ExAC in family C15 and a control WES dataset generated from 20 unrelated individuals of Czech origin (National Centre for Medical Genomics).

Whole-Genome Sequencing (WGS)
Four distantly related affected individuals (VI:7, VII:9, VII:8, and VIII:1) from C15 were analyzed by WGS using a TruSeq Nano DNA library preparation kit and a HiSeq X Ten sequencer (Illumina). Reads were aligned to the GRCh38/hg38 human reference sequence with Novoalign v.2.05 (Novocraft). Variant calling was performed with GATK HaplotypeCaller and annotated using the Variant Effect Predictor (VEP) which provides allele frequency annotation in various control datasets, predicts the effects of variants on nearby transcripts, and reports the potential regulatory role for non-coding regions.

Sanger Sequencing of Potential Regulatory Regions of GRHL2
For unsolved PPCD-affected case subjects, a region of 2,728 bp (chr8:hg38:101,491,361–101,494,128) encompassing potential regulatory regions of GRHL2, including the \( S \) untranslated region (UTR), exon 1, and partial intron 1 (Figure S1), was amplified by PCR (GoTaqGreen, Promega, primers and conditions available on request) and Sanger sequenced using BigDye terminator sequencing on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). GRHL2 variants associated with disease have been submitted to ClinVar.

In Silico Analysis of Variants
In addition to the annotation data provided by the VEP, variants of interest were also analyzed by splice site prediction tools Human Splicing Finder, NNSPLICE, MaxEntScan, and NetGene2. ENCODE (Encyclopedia of DNA elements) data were manually interrogated using IGV for transcription factor binding in the genomic region of interest containing candidate variants. The effect of variant on transcription factor binding was predicted by Alibaba 2.1 and MatInspector. Alibaba 2.1 predicts transcription factor binding sites in an input nucleotide sequence using binding sites collected from TRANSFAC. MatInspector predicts transcription factor binding sites using a library of weight matrices.

Cell Culture, RNA Extraction, and RT-PCR
RNA was extracted from whole corneal buttons donated after enucleation surgery for posterior segment melanoma and cell cultures, using an RNeasy Extraction Kit (QIAGEN). Primary endothelial cells were expanded and cultured as previously described. An immortalized human corneal endothelial cell line, B4G12, was cultured according to published protocols. Normal donor corneal rings stored in Optisol (Chiron Ophthalmics) were obtained from Moorfields Lions Eye Bank, and limbal epithelial stem cells (HLEC/HLE-S) and stromal fibroblasts (SF) were isolated and cultured as previously described. HEK293 cells were cultured with standard reagents and conditions, cDNA was reverse transcribed using oligo(dT) priming with a Tetro cDNA synthesis kit (Bioline). GRHL2 was amplified with intron-spanning primers from exon 4 to exon 8 forward 5\'-GGCCCTATCTCAAGAGAGCACT-3' and reverse 5\'-GTTCCAGAGTAAAGGA AACA-3' and beta actin was amplified using primers forward 5\'-CTGGGACGACATGGGAAGAAA-3' and reverse 5\'-AAGGAAG GCTGGAAGAGTGC-3'.

Histology and Immunostaining
Cornea tissue from individual III:1 (age 8.5 years) from family C23 removed during penetrating keratoplasty was fixed in 10% neutral-buffered formalin. The sample was then processed into paraffin wax and 5-\( \mu \)m sections were cut. Sections were stained with tinctorial haematoxylin and eosin (H&E) using conventional methods.

A second cornea removed from individual II:1 (age 41 years) from family C23, also during penetrating keratoplasty, and a control cornea (Miracles In Sight Inc.) were embedded in optimal cutting temperature compound and snap frozen. Tissue sections were then cut to 4-\( \mu \)m thickness using a cryostat, thaw-mounted onto histological slides, and air-dried for 30 min. Immunostaining was performed manually using the Bond Polymer Refine Detection kit (DS9800, Leica). Sections were fixed for 10 min in acetone followed by 10 min in methanol. After washing with distilled water, a peroxidase block was used for 30 min to quench any endogenous peroxidase activity, followed by three washes with Tris-based saline 0.1% (v/v) Tween (TBS-T). Immunodetection of proteins of interest was carried out with the following primary antibodies: rabbit anti-GRHL2 (1:100, HPA004820, Sigma Aldrich), rabbit anti-N-cadherin (1:300, ab18203, Abcam), mouse anti-E-Cadherin (1:200, M3612, Dako), and human anti-Cytokeratin 7 (CK7, 1:2,000, M7018, Dako) for 1 hr at 37°C. Subsequently tissue sections were washed three times with TBS-T and incubated with post-primary linker IgG for 15 min for localization of mouse antibodies followed by three washes with TBS-T and incubation with poly-HRP IgG for 30 min for localization of rabbit antibodies. After three washes with TBS-T and distilled water, staining was visualized with 3,3'-diaminobenzidine tetrahydrochloride hydrate (DAB), washed, and counterstained with Mayer's Haematoxylin to allow the visualization of nuclei. Tissue sections were then dehydrated in graded ethanol and in xylene prior to mounting with DPX mounting medium.

H&E staining was performed using a Leica Autostainer XL with integrated coverlipper (CV5030). Staining was visualized using a Nikon Eclipse 80i microscope equipped with a DVM2000C digital camera. Images of corneal sections were taken using the same magnification between the control and diseased tissue.

Luciferase Assay
Primers were designed to amplify a genomic region that encompasses potential GRHL2 regulatory regions, spanning all variants of interest. A 2,728-bp product (chr8:101,491,361–101,494,128) containing upstream sequence, exon 1, and partial intron 1 of...
the GRHL2 gene (Figure S1) was amplified from control genomic DNA, cloned into pGEM-TEasy (Promega) and sub-cloned into the promoter-less firefly luciferase reporter vector pGLO-Basic (Promega). Primers used for cloning incorporated KpnI and NheI restriction site to facilitate subcloning (forward 5’-GGTACGACGTTTACACGTCCTCC-3’ and reverse 5’-GCAGGAAAAACGGGGG-3’). Variants identified in PPCD4-affected individuals were introduced by site-directed mutagenesis using a QS Site-Directed Mutagenesis Kit (New England Biolabs) and all constructs were verified by Sanger sequencing. Wild-type or mutant GRHL2 promoter pGLO3-Basic plasmids (90 ng) were used to co-transfect HEK293 cells with 10 ng of pRL-CMV (CMV-promoter driven Renilla luciferase reporter, Promega) using TransIT-LT1 transfection reagent (Mirus). At 24 hr post-transfection, luciferase activity was measured using an Orion I. Microplate Luminometer (Titerk Biothl) and a dual-luciferase reporter assay system (Dual-Glo Luciferase Assay System, Promega).

Results

Defining a New Locus for Autosomal-Dominant Posterior Polymorphous Corneal Dystrophy (PPCD4)

In a large autosomal-dominant PPCD-affected family of Czech origin (C15, Figure 1A), targeted Sanger sequencing did not identify any likely disease-associated variants within established PPCD-associated genes, including the OVOL2 promoter region.11,23 Furthermore, quantitative real-time PCR and Illumina HumanOmniExpress BeadChip SNP array analysis did not detect CNVs encompassing known PPCD-associated genes.10 Therefore, we performed WES using DNA samples from affected (VII:7, VI:9, VII:8, VIII:1) and unaffected (VI:14, VII:9, VI:15) individuals from family C15. WES data were filtered for rare variants in affected individuals that were absent from unaffected individuals; no potential mutations were identified. We therefore considered that an additional PPCD locus and/or a variant not captured by WES might be causative in this family.

We therefore defined the locus segregating with disease in family C15 through linkage analysis by genotyping nine affected and seven unaffected individuals from a large branch of family C15 (Figure 1A). A single locus was identified, chr8: hg38:100,821,039–119,725,923, spanning chromosome 8q22.3-q24.12, with a maximum LOD score of 4.38 (Figure 1B), thereby delineating a locus for PPCD (PPCD4).

Identification of a Rare Non-coding GRHL2 Variant in the Index PPCD4-Affected Family

Next, we performed WGS in four distantly related affected individuals from family C15 (Figure 1A) and filtered for variants located within the PPCD4 locus (chr8:hg38:100,821,039–119,725,923) that were shared between all four affected individuals. We filtered our WGS datasets to exclude all variants that have a MAF ≥ 0.005 in the gnomAD, Kaviar, 1000G, GoNL, and UK10X datasets. Using this approach, three unique variants were identified in the linkage region that were confirmed by Sanger sequencing (Table 1). Two variants were intergenic, and one variant occurred within intron 1 of GRHL2. We found no bioinformatic evidence to implicate the intergenic variants in regions of active promoters or enhancers. In contrast, c.20+544G>T (chr8:hg38:101,493,333G>T), located in intron 1 of GRHL2, maps to a promoter region for this gene (ENSR00000228091), reflected in the CADD score (Figures 1C and 1D; Table 1).

To further delineate the PPCD4 locus in family C15, rare variants filtered from WGS data, including the c.20+544G>T variant in intron 1 of GRHL2, were genotyped and assessed for segregation in the extended pedigree by Sanger sequencing. Importantly, additional recombination events were identified in family C15 that further refined the PPCD4 locus to between chr8:hg38:101,411,163 and 109,214,442 excluding the two intergenic variants as candidates (Figure S1).

Interrogation of ENCODE data to identify potential enhancer and promoter regions of GRHL2 revealed a cluster of transcription factor binding sites upstream of GRHL2, and spanning the 5’ UTR, first exon, and partial region of intron 1. The transcription factor binding prediction tools MatInspector and AliBaba 2.1 predict that the c.20+544G>T variant disrupts binding sites, leading to loss, or gain, of multiple transcription factors that are expressed in the corneal endothelium (Table 2 and Figure S1). Further analysis of c.20+544G>T in ENCODE data (Ensembl) identified this precise base location in intron 1 as a bivalent histone modification site, with

Table 1. Unique Variants within the PPCD4 Locus, chr8:hg38:100,821,039–119,725,923, Identified by WGS in Family C15

| Variant No. | Coordinates (hg19) | Coordinates (hg38) | Reference Allele | Observed Allele | Location | Closest Transcript | CADD score |
|------------|--------------------|--------------------|------------------|-----------------|----------|-------------------|------------|
| 1          | 102,121,864        | 101,109,636        | C                | T               | intergenic| –                 | 7.875      |
| 2          | 102,505,561        | 101,493,333        | G                | T               | intronic | GRHL2             | 10.76      |
| 3          | 115,648,021        | 114,635,792        | A                | T               | intergenic| –                 | 6.33       |

Three novel variants were identified within the mapped PPCD4 locus from four WGS datasets (C15; VI:7, VI:9, VII:8, VIII:1) filtered by (1) removal of all variants located outside refined locus, (2) all variants with a MAF ≥ 0.005 in publicly available Kaviar, gnomAD, 1000G, UK10K, GoNL datasets, and (3) that were shared between the four affected individuals. Abbreviations are as follows: Kaviar, Kaviar Genomic Variant database; gnomAD, The Genome Aggregation Database; 1000G, 1000 Genomes Project; UK10K, UK10K Rare Genetic Variants in Health and Disease; GoNL, Genomes of the Netherlands. One variant (G>T) is within the GRHL2 gene (intronic) whereas the remaining two were intergenic.
histone H3 lysine 4 trimethylation (H3K4me3) and histone H3 lysine 27 trimethylation (H3K27me3) modifications in different cell types, which are associated with gene activation and repression, respectively (Table 2 and Figure S2). In addition, this location marks a DNase I hypersensitive site and CTCF binding site, commonly associated with accessible chromatin and transcription factor binding and for forming local chromatin loops necessary for the tethering of promoters with associated regulatory elements, respectively (Table 2 and Figure S2).

**Targeted Screening of GRHL2 Regulatory Regions in Unsolved PPCD-Affected Families**

Given that the c.20+544G>T variant lies within a regulatory region of GRHL2, a 2,728-bp region encompassing the 5’ UTR, exon 1, and partial intron 1 of GRHL2 containing predicted regulatory regions and transcription factor binding sites (Figure S2), was therefore PCR amplified and Sanger sequenced in unsolved PPCD cases. The same variant, c.20+544G>T, was identified in three additional families of Czech origin (C23, C26, and C33, Figure 2). None of the probands were knowingly related to the original pedigree or to each other. In two families (C23 and C26), the variant was shown to segregate with disease; however, in family C33 the proband was the only affected individual, and the variant was absent in both parents (Figure 2). Paternity testing confirmed the identity of the proband’s biological father, thereby suggesting that the variant occurred de novo in this individual.

To investigate potential ancestral haplotypes in families of Czech origin, rare variants identified in the WGS data from family C15 that refined the PPCD4 locus were genotyped by Sanger sequencing. The same mini-haplotype was identified in families C23 and C26, with an additional recombination event refining the haplotype (chr8:hg38:101,492,922del), was identified in the proband of Czech origin, rare variants identified in the WGS data from family C15 that refined the PPCD4 locus were genotyped by Sanger sequencing. The same mini-haplotype was identified in families C23 and C26, with an additional recombination event refining the haplotype (chr8:hg38:101,492,922del), suggesting that the GRHL2 variant in these families arose in a common ancestor (Figure S1). This analysis also confirmed the lack of an ancestral haplotype in family C33, further supporting the finding that this variant arose independently.

**Screening the 2,728-bp GRHL2 region in 19 genetically unsolved, unrelated PPCD-affected case subjects from the UK cohort identified two further variants. A single-nucleotide deletion c.20+257delT (chr8:hg38:101,493,046del; Figure 2) in intron 1 of GRHL2 was identified in a proband from family B4 (Table 2). In family B5, a 1-bp deletion, also situated within the first intron of GRHL2, c.20+133delA (chr8:hg38:101,492,922del), was identified in the proband (II:4) and her affected brother (II:1) and was absent in her unaffected sister (II:6) (Figure 2 and Table 2). Both variants were absent from the control databases gnomAD, Kaviar, 1000G, GoNL, and UK10X (Figure 2 and Table 2).**

To further verify the pathogenicity of the GRHL2 variants (Table 2), a genomic region encompassing the Czech c.20+544G>T variant and the two other variants identified in individuals with PPCD, was Sanger sequenced in 210 Czech control samples (420 alleles). None of the PPCD-associated variants were detected in the control cohort. Interestingly, only a single heterozygous variant (rs548346355) was identified in a single individual in the control cohort, suggesting that this region is a highly conserved region. Similar to the variant identified in the Czech families, the c.20+257delT and c.20+133delA variants occur within regions rich in transcription factor
Figure 2. Additional PPCD-Affected Families with GRHL2 Regulatory Region Mutations

Presence of a GRHL2 variant, shown in the electropherograms, is indicated by +/− in each family and absence by −/−. The heterozygous variant in intron 1 of GRHL2, c.20+544G>T, was found to segregate with disease in families C23 and C26 of Czech origin who share an ancestral haplotype with C15. The same mutation was identified in an affected individual in family C33 that occurred de novo. Two other mutations in intron 1 of GRHL2 were identified; a 1-bp deletion, c.20+257delT (chr8:hg38:101,493,046delT), was identified in the proband in family B4, and a 1-bp deletion, c.20+133delA (chr8:hg38:101,492,922delA), in affected individuals in family B5. GRHL2 variants are annotated according to transcript GenBank: NM_024915 and Ensembl ENST00000251808.7.

Clinical Characterization of PPCD4

In this cohort, PPCD4 was found to display both inter- and intra-familial phenotypic variation. In the 27 affected individuals from families of Czech origin, harboring the same GRHL2 mutation, 26 had typical corneal signs of PPCD, with an irregular posterior corneal surface and occasional opacities of variable size and shape clinically described as bands or geographic or vesicular lesions (Figures 3A, 3B, and 3G). The disease presented subjectively as blurred vision due to corneal edema in four individuals (Figure 3D). In two children, corneal edema and associated irritation of the eye was noted at 2 and 3 months after birth. Five individuals initially presented with a diagnosis of secondary glaucoma, either during a regular check-up, familial screening, or due to the development of corectopia (Figure 3C), prompting a visit to an eye specialist. Five individuals had low vision in one or both eyes since childhood and have not reported subsequent major changes of their visual function. One subject noticed decrease in visual acuity in the second decade of life. Nine individuals were asymptomatic at their last examination, and in one individual, information about the subjective onset was not available.

At the last follow-up, best corrected visual acuity ranged from 1.0 bilaterally in five individuals to light perception in a 55-year-old male with secondary glaucoma and bullous keratopathy. Corneal transplantation was performed in 7 out of 27 (25.9%) individuals, and of these, 3 had bilateral surgery. The mean age of the first surgery was 34.9 ± 17.9 years (range 8.5 to 59 years). Glaucoma was diagnosed in seven individuals (25.9%), unilaterally in one male. The mean age of a diagnosis of glaucoma was 46.4 ± 17.1 years (range 20 to 63 years), but two subjects developed glaucoma after penetrating keratoplasty, and in these individuals, glaucoma may have been precipitated by surgery. Two subjects had an enucleation of a painful blind eye, one at the age of 25 years and the second at 70 years. Corectopia was noted in four eyes of three individuals and was associated with secondary glaucoma in all case subjects. Secondary corneal calcification (band keratopathy) developed bilaterally in two individuals (Figure 4C).

Specular microscopy and SD-OCT imaging documented a reduced endothelial cell density, with both normal and abnormal morphology and irregularities of the posterior corneal surface (Figures 3I–3K). In a 79-year-old individual, the disease status was unknown because the corneal periphery was obscured by age-related stromal haze. Although the corneal center was clear, the endothelial cell density count was 1,295 cells/mm² in the right eye and 1,309 cells/mm² in the left eye (normal range 2,400–2,600 cells/mm²).16 H&E staining of a full-thickness corneal sample (individual III:2, family C23) revealed an oedematous cornea with variation in endothelial cell size and shape and focal multilayering of the cells (Figure 3H).

The proband of B4 had an unusually prominent fold of Descemet membrane (Figure 3E). There was no family history of eye disease and the other family members were unavailable for examination.

Proband B5 (II:4) had markedly asymmetric disease with diffuse geographic endothelial changes restricted to her left eye (Figures 3A and 3F). She also had left amblyopia and a decompensated left exotropia. Intraocular pressures were normal in both eyes and there were no iris abnormalities. The endothelial cell density was markedly reduced in her affected eye (871 cells/mm²) compared to 3,165 cells/mm² in her right eye. Her brother (II:1) carried the same GRHL2 variant but had a significantly different phenotype. Although the endothelial cell count was lower than expected (1,900 cells/mm² both eyes), there were no changes in cell morphology. Notably, numerous elevated...
Hassal-Henle bodies were present in the far periphery of the cornea.

Expression of GRHL2 in Healthy and Diseased Corneal Endothelium

Our interrogation of publicly available RNA-seq data from healthy adult and fetal human corneal endothelial tissue revealed no evidence of GRHL2 expression (Figure S4A). Examination of additional publicly available RNA-seq data also confirmed lack of GRHL2 expression in corneal stromal cells, whereas high levels of expression were detected in the corneal epithelium (Figure S4A). We therefore further defined corneal expression of GRHL2 in cultured cells by RT-PCR and the distribution of GRHL2 in corneal tissue by immunohistochemistry (IHC).

GRHL2 expression was detected in cultured human corneal epithelial cells derived from limbal epithelial stem cells and in a spontaneously immortalized human corneal epithelial cell line with progenitor-like characteristics, but was absent in corneal endothelial tissue, an immortalized cell line of human corneal endothelial origin, and stromal fibroblasts by RT-PCR (Figure S4B).

GRHL2 encodes a transcription factor that is a direct transcriptional repressor of ZEB1.49 Given this role, in addition to the lack of GRHL2 expression in the corneal endothelium, we hypothesized that the putative regulatory mutations could lead to inappropriate transcriptional activation and ectopic expression of GRHL2 in the corneal endothelium, similar to the mechanism we proposed for the variants in OVOL2.2 To explore this hypothesis further, a full-thickness corneal sample from individual II:1 from family C23, with the GRHL2 c.20 +544G>T variant, was analyzed by IHC and compared to control tissue. First, we tested for presence of GRHL2 in different cell layers. GRHL2 was detected in the nuclei of epithelial cells in control tissue, consistent with its role as a transcription factor, and was absent from the stroma and endothelium, concordant with the transcriptomic data (Figure 4A). Strikingly, in the diseased cornea, endothelial cell nuclei were positive for GRHL2, suggesting that the c.20+544G>T GRHL2 variant induces ectopic expression of GRHL2 resulting in detection of GRHL2 protein in the corneal endothelium.

Differences in the levels of epithelial, mesenchymal, and endothelial markers were also observed between the diseased and control endothelial cells (Figure 4C). N-Cadherin, which is normally detected in corneal endothelial and epithelium cells,50,51 was detected in the control endothelium and the diseased tissue (Figure 4C). In contrast,
E-Cadherin, a component of adherens junctions and marker of epithelial cell status, is not detected in healthy corneal endothelium; however, regions of positive staining for E-Cadherin were evident in the PPCD4 endothelium, which was negative in the control, indicating that the cells had diverged from their normal identity (Figure 4C). This upregulation of E-Cadherin is consistent with cells undergoing MET.

Previous IHC studies of PPCD1 and PPCD3 samples have shown inappropriate positive staining for keratins in diseased tissue. CK7, a marker of corneal epithelial cells, was positive in the diseased endothelium and negative in the control sample (Figure 4C). Collectively, these data indicate that the PPCD4 endothelial cells were in transition to an epithelial-like cell type or had already diverged. We hypothesize that this diseased cell state transition is due to ectopic expression of GRHL2 in the corneal endothelium, induced by the c.20+544G>T variant.

**Promoter Mutations Result in Increased Expression of GRHL2**

Given the striking ectopic detection of GRHL2 in diseased PPCD4 corneal endothelial cells (Figure 4), and that in silico analysis of all three PPCD4 variants identified are predicted to alter transcriptional activity (Table 2 and Figure S3), we experimentally tested how each of these variants alter GRHL2 promoter activity in vitro. A 2,728-bp fragment encompassing the position of all three variants and predicted surrounding regulatory regions was cloned into a promoter-less firefly luciferase reporter vector. The PPCD4-associated variants were independently introduced by site-directed mutagenesis. HEK293 cells were co-transfected with each of the GRHL2 promoter constructs to test promoter activity, in combination with Renilla luciferase for normalization purposes. The wild-type GRHL2 construct was an active promoter region driving expression of firefly luciferase (Figure 5). Each of the three GRHL2 PPCD4-associated mutations were found to significantly (p ≤ 0.001) increase the promoter activity of the region compared to the wild-type sequence (Figure 5).

**Discussion**

In this study we identified a locus for autosomal-dominant PPCD, PPCD4, on chromosome 8q22.3–q24.12. WGS
revealed three unique non-coding variants within the linked region in the index family (C15), one of which, c.20+544G>T, mapped within a potential regulatory region of GRHL2. Additional recombination events were identified by genotyping in the extended family, that refined the PPCD4 locus (chr8:hg38:101,411,163–109,214,442) and excluded two of the variants, leaving c.20+544G>T as the outstanding candidate disease-causing variant.

The same variant was found in two additional unsolved PPCD-affected families of Czech origin that shared an ancestral haplotype with family C15. A de novo occurrence of this variant was identified in another PPCD-affected individual, suggesting that this is a recurrent mutation. Two further unique variants were found in intron 1 of GRHL2 (c.20+257delT and c.20+133delA) in additional unrelated individuals affected with PPCD.

All three GRHL2 mutations were located in a conserved uncharacterized regulatory region. We hypothesize that the mechanism of disease is similar to the mechanism we and others proposed for OVOL2 promoter mutations that cause PPCD1, whereby mutations lead to an overactive promoter that drives inappropriate ectopic expression of OVOL2 in the corneal endothelium. To understand further how the promoter mutations affect expression of GRHL2 in corneal endothelial cells and contribute to the pathogenesis of PPCD4, we performed IHC on a corneal sample from a PPCD4-affected individual. GRHL2 is not normally expressed in the corneal endothelium in vivo; however, in PPCD4-diseased corneal tissue, we detect GRHL2 in the corneal “endothelium” supporting the hypothesis that the PPCD4 variants result in inappropriate activation and ectopic expression of GRHL2. Furthermore, we demonstrate that this putative regulatory region of GRHL2 is transcriptionally active and that all PPCD4-associated variants significantly increased GRHL2 promoter activity compared to wild-type.

Interestingly, mutations leading to presumed haploinsufficiency of GRHL2 cause autosomal-dominant non-syndromic hearing impairment (DNFA28 [MIM: 608641]) and homozygous missense mutations have been associated with autosomal-recessive ectodermal dysplasia syndrome with hearing loss (ECTDS [MIM: 616029]). None of the PPCD-affected individuals with GRHL2 promoter mutations in our study reported hearing loss or other features of ectodermal dysplasia syndrome. This is not unexpected given that neither of the previously described GRHL2-associated disease mechanisms are predicted to result in the aberrant upregulation and ectopic expression of GRHL2.

In addition to GRHL2 in the PPCD4 corneal “endothelium,” we also detected E-Cadherin and Cytokeratin 7, consistent with cellular state transition as the mechanism of disease for PPCD4, through the MET pathway. This is also consistent with the abnormal “endothelial” cell morphology detected in PPCD4-affected case subjects using specular microscopy and examination of histological sections.

GRHL2 has an important role in epithelial morphogenesis through trans-activation of genes required for the formation of the apical junctional complex and repression of EMT. In duct cells of the kidney, GRHL2 regulates lumen expansion and epithelial barrier formation by trans-activating OVOL2 expression, which in turn activates the expression of E-Cadherin, claudin 4 (epidermal tight junctions), and Rab25 (apical trafficking). OVOL2 maintains the transcriptional program of human corneal epithelium cells by repressing expression of mesenchymal genes such as ZEB1. Similarly, GRHL2 is known to be a direct transcriptional repressor of ZEB1. Importantly, haploinsufficiency of ZEB1 causes PPCD3, and ectopic expression of OVOL2 in the corneal endothelium caused by promoter mutations, leading to repression of ZEB1 transcription, is the mechanism proposed for PPCD1. Therefore, we propose that ZEB1, OVOL2, and GRHL2 form a finely balanced mutually inhibitory EMT/MET pathway that controls specific cell characteristics and intermediate cell states.
methods employed, revealed a significant decrease in ZEB1 expression compared to controls. Furthermore, transcriptomic data of the corneal endothelium of an individual with PPCD of undefined genetic cause identified GRHL2 as the most differentially expressed gene (upregulated) compared to controls.

Collectively, these data support the disease mechanism of ectopic expression of GRHL2 in PPCD4 endothelial cells as a result of mutations in a regulatory region and that MET is a convergent pathogenic mechanism leading to intermediate cell states and dysfunction of the endothelial barrier and disease in PPCD (Figure 6).

Acknowledgments

We thank all the families for participating in this research. This work was funded by awards from GACR 17-12355S (P.L., L.D., P.S.), Fight for Sight, the Rosetrees Trust, Moorfields Eye Charity (A.J.H., S.J.T., A.E.D., K.E.R.L., and C.J.E.), The Academy of Medical Sciences (A.E.D.), and the Wellcome Trust (M.E.C.). S.J.T., N.P., and A.J.H. were supported by the National Institute for Health Research Biomedical Research Centre based at Moorfields Eye Hospital NHS Foundation Trust and UCL Institute of Ophthalmology. Institutional support was provided by UNCE 204064 (P.L., L.D., S.K., V.S.) and Progres-Q26/LF1. P.S. was also funded by GAUK 250361/2017 and SVV 260367/2017 and S.K. and V.S. by grant 15-28208A from the Ministry of Health of the Czech Republic and the project LQ1604 NPU II from the Ministry of Education, Youth, and Sports of the Czech Republic.

We thank The National Center for Medical Genomics (LM2015091) for providing allele frequencies in an ethnically matched population (project CZ.02.1.01/0.0/0.0/16_013/0001634). This study was performed within the framework of ERN-EYE. The pGL3-Control vector was a kind gift from Dr. Stephanie Halford. We would like to thank Ales Horinek for paternity testing and Erik Kykal for basic bioinformatic analysis.
Web Resources

1000 Genomes, http://www.internationalgenome.org/
Alibaba 2.1, http://gene-regulation.com/pub/programs/alibaba2/index.html
ClinVar, https://www.ncbi.nlm.nih.gov/clinvar/dbSNP, https://www.ncbi.nlm.nih.gov/projects/SNP/
ENCODE, https://genome.ucsc.edu/ENCODE/Ensembl Genome Browser, http://www.ensembl.org/index.html
ExAC Browser, http://exac.broadinstitute.org/
ExomeDepth, https://cran.r-project.org/web/packages/ExomeDepth/index.html
GATK, https://software.broadinstitute.org/gatk/
gnomAD Browser, http://gnomad.broadinstitute.org/
GoNL (Genomes of the Netherlands), http://www.nlgenome.nl/search/
IGV, http://www.broadinstitute.org/igv/
Kaviar Browser, http://db.systemsbiology.net/kaviar/
MatInspector, http://www.genomatix.de/index.html
NCBI Genome build GRCh38, https://www.ncbi.nlm.nih.gov/assembly?term=GRCh38&ecd=DetailsSearch
NHLBI Exome Sequencing Project (ESP) Exome Variant Server, http://evs.gs.washington.edu/EVS/
Novoalign, http://www.novocraft.com/products/novoalign/
OMIM, http://www.omim.org/
RefSeq, http://www.ncbi.nlm.nih.gov/RefSeq
SAMTools, http://www.htslib.org/
SeattleSeq Annotation, https://snp.gs.washington.edu/SeattleSeqAnnotation150/
Sequence Variant Nomenclature, http://varnomen.hgvs.org/
UCSC Genome Browser, http://genome.ucsc.edu
UK10K Consortium, http://www.uk10k.org/
Variant Effect Predictor, http://useast.ensembl.org/Homo_sapiens/Tools/VEP

References

1. Krachmer, J.H. (1985). Posterior polymorphous corneal dystrophy: a disease characterized by epithelial-like endothelial cells which influence management and prognosis. Trans. Am. Ophthalmol. Soc. 83, 413–475.
2. Davidson, A.E., Liskova, P., Evans, C.J., Dudakova, L., Nosková, L., Pontikos, N., Hartmannová, H., Hodanova, K., Stranecký, V., Kozmík, Z., et al. (2016). Autosomal-dominant corneal endothelial dystrophies CHED1 and PCCD1 are allelic disorders caused by non-coding mutations in the promoter of OVOL2. Am. J. Hum. Genet. 98, 75–89.
3. Evans, C.J., Liskova, P., Dudakova, L., Hrabciokova, P., Horinek, A., Jirsova, K., Filipec, M., Hardcastle, A.J., Davidson, A.E., and Tuft, S.J. (2015). Identification of six novel mutations in ZEB1 and description of the associated phenotypes in patients with posterior polymorphous corneal dystrophy 3. Ann. Hum. Genet. 79, 1–9.
4. Weiss, J.S., Moller, H.U., Aldave, A.J., Seitz, B., Bredrup, C., Kivelä, T., Munier, F.L., Rupuano, C.J., Nischal, K.K., Kim, E.K., et al. (2015). IC3D classification of corneal dystrophies–edition 2. Cornea 34, 117–159.
5. Jirsova, K., Merjava, S., Martinova, R., Gwilliam, R., Ebenezer, N.D., Liskova, P., and Filipec, M. (2007). Immunohistochemical characterization of cytokeratins in the abnormal corneal endothelium of posterior polymorphous corneal dystrophy patients. Exp. Eye Res. 84, 680–686.
6. Rodrigues, M.M., Sun, T.T., Krachmer, J., and Greensmoe, D. (1980). Epithelialization of the corneal endothelium in posterior polymorphous dystrophy. Invest. Ophthalmol. Vis. Sci. 19, 832–835.
7. Henriquez, A.S., Kenyon, K.R., Dohlman, C.H., Borouchoff, S.A., Forstot, S.L., Meyer, R.F., and Hanninen, L.A. (1984). Morphologic characteristics of posterior polymorphous dystrophy. A study of nine corneas and review of the literature. Surv. Ophthalmol. 29, 139–147.
8. Merjava, S., Malinova, E., Liskova, P., Filipec, M., Zemanova, Z., Michalova, K., and Jirsova, K. (2011). Recurrence of posterior polymorphous corneal dystrophy is caused by the overgrowth of the original diseased host endothelium. Histochim. Cell Biol. 136, 93–101.
9. Krafchak, C.M., Pawar, H., Moroi, S.E., Sugar, A., Lichter, P.R., Mackey, D.A., Mian, S., Nairus, T., Elner, V., Schteingart, M.T., et al. (2005). Mutations in TCF8 cause posterior polymorphous corneal dystrophy and ectopic expression of COIL4A3 by corneal endothelial cells. Am. J. Hum. Genet. 77, 694–708.
10. Liskova, P., Evans, C.J., Davidson, A.E., Zaliova, M., Dudakova, L., Trkova, M., Stranecky, V., Carnt, N., Plagnol, V., Vincent, A.L., et al. (2016). Heterozygous deletions at the ZEB1 locus verify haploinsufficiency as the mechanism of disease for posterior polymorphous corneal dystrophy type 3. Eur. J. Hum. Genet. 24, 985–991.
11. Liskova, P., Tuf, S.J., Gwilliam, R., Ebenezer, N.D., Jirsova, K., Prescott, Q., Martinova, R., Pretorius, M., Sinclair, N., Boase, D.L., et al. (2007). Novel mutations in the ZEB1 gene identified in Czech and British patients with posterior polymorphous corneal dystrophy. Hum. Mutat. 28, 638.
12. Chung, D.D., Frausto, R.E., Cervantes, A.E., Gee, K.M., Zakharievich, M., Hanser, E.M., Stone, E.M., Heon, E., and Aldave, A.J. (2017). Confirmation of the OVOL2 promoter mutation c.-307T>C in posterior polymorphous corneal dystrophy 1. PLoS ONE 12, e0169215.
13. Hong, T., Watanabe, K., Ta, C.H., Villarreal-Ponce, A., Nie, Q., and Dai, X. (2015). An Ovol2-Zeb1 mutual inhibitory circuit governs bidirectional and multi-step transition between epithelial and mesenchymal states. PLoS Comput. Biol. 11, e1004569.
14. Kitazawa, K., Hikichi, T., Nakamura, T., Mitsunaga, K., Tanaka, A., Nakamura, M., Yamakawa, T., Furukawa, S., Takasaka, M., Goshima, N., et al. (2016). OVOL2 maintains the transcriptional program of human corneal epithelium by suppressing epithelial-to-mesenchymal transition. Cell Rep. 15, 1359–1368.
15. Kalluri, R., and Weinberg, R.A. (2009). The basics of epithelial–mesenchymal transition. J. Clin. Invest. 119, 1420–1428.
16. Chen, T., You, Y., Jiang, H., and Wang, Z.Z. (2017). Epithelial-mesenchymal transition (EMT): A biological process in the development, stem cell differentiation, and tumorigenesis. J. Cell. Physiol. 232, 3261–3272.
17. Prada, D., Naro, C., Sette, C., and Ghigna, C. (2017). EMT and stemness: flexible processes tuned by alternative
splicing in development and cancer progression. Mol. Cancer 16, 8.

18. Singh, A.J., Ramsey, S.A., Filtz, T.M., and Kioussi, C. (2017). Differential gene regulatory networks in development and disease. Cell. Mol. Life Sci. Published online October 10, 2017. https://doi.org/10.1007/s00018-017-2679-6.

19. Eghrari, A.O., Riazuddin, S.A., and Gottsch, J.D. (2015). Overview of the cornea: structure, function, and development. Prog. Mol. Biol. Transl. Sci. 134, 7–23.

20. Srinivas, S.P. (2012). Cell signaling in regulation of the barrier integrity of the corneal endothelium. Exp. Eye Res. 95, 8–15.

21. Chung, D.D., Frausto, R.F., Lin, B.R., Hanser, E.M., Cohen, Z., and Aldave, A.J. (2017). Transcriptomic profiling of posterior polymorphous corneal dystrophy. Invest. Ophthalmol. Vis. Sci. 58, 3202–3214.

22. Zakharevich, M., Kattan, J.M., Chen, J.L., Lin, B.R., Cervantes, A.E., Chung, D.D., Frausto, R.F., and Aldave, A.J. (2017). Elucidating the molecular basis of PPCD: Effects of decreased ZEB1 expression on corneal endothelial cell function. Mol. Vis. 23, 740–752.

23. Liskova, P., Gwilliam, R., Filipec, M., Jirsova, K., Reinstein Merjava, S., Deloukas, P., Webb, T.R., Bhattacharya, S.S., Ebenezer, N.D., Harris, A.G., and Hardcastle, A.J. (2012). High prevalence of posterior polymorphous corneal dystrophy in the Czech Republic: linkage disequilibrium mapping and dating an ancestral mutation. PLoS ONE 7, e45495.

24. Abecasis, G.R., Cherny, S.S., Cookson, W.O., and Cardon, L.R. (2002). Merlin—rapid analysis of dense genetic maps using sparse gene flow trees. Nat. Genet. 30, 97–101.

25. Pontikos, N., Yu, J., Moghul, L., Withington, L., Blanco-Kelly, F., Vulliamy, T., Wong, T.L.E., Murphy, C., Cipriani, V., Fiorentino, A., et al.; UKIRDC (2017). Phenopolis: an open platform for harmonization and analysis of genetic and phenotypic data. Bioinformatics 33, 2421–2423.

26. Plagnol, V., Curtis, J., Epstein, M., Mok, K.Y., Stebbings, E., Grigoridiou, S., Wood, N.W., Hambleton, S., Burns, S.O., Thrasher, A.J., et al. (2012). A robust model for read count data in exome sequencing experiments and implications for copy number variant calling. Bioinformatics 28, 2747–2754.

27. McKenna, A., Hanna, M., Banks, E., Sivachenko, A., Cibulskis, K., Kernytsky, A., Garimella, K., Altshuler, D., Gabriel, S., Daly, M., and DePristo, M.A. (2010). The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res. 20, 1297–1303.

28. McLaren, W., Gil, L., Hunt, S.E., Riat, H.S., Ritchie, G.R., Thor- mann, A., Flicek, P., and Cunningham, F. (2016). The Ensembl variant effect predictor. Genome Biol. 17, 122.

29. Desmet, E.O., Hamroun, D., Lalande, M., Collod-Béroud, G., Claustres, M., and Béroud, C. (2009). Human Splicing Finder: an online bioinformatics tool to predict splicing signals. Nucleic Acids Res. 37, e67.

30. Reese, M.G., Eckstein, F.H., Kulp, D., and Haussler, D. (1997). Improved splice site detection in Genie. J. Comput. Biol. 4, 311–323.

31. Eng, L., Coutinho, G., Nahas, S., Yeo, G., Tanouye, R., Babaei, M., Dörk, T., Burge, C., and Gatti, R.A. (2004). Nonclassical splicing mutations in the coding and noncoding regions of the ATM Gene: maximum entropy estimates of splice junction strengths. Hum. Mutat. 23, 67–76.

32. Brunak, S., Engelbrecht, J., and Knudsen, S. (1991). Prediction of human mRNA donor and acceptor sites from the DNA sequence. J. Mol. Biol. 220, 49–65.

33. ENCODE Project Consortium (2004). The ENCODE (ENCyclopedia Of DNA Elements) Project. Science 306, 636–640.

34. Grabe, N. (2002). AliBaba2: context specific identification of transcription factor binding sites. In Silico Biol. (Gedrukt) 2, S1–S15.

35. Cartharius, K., Frech, K., Grote, K., Klocke, B., Haltmeier, M., Klingenhoff, A., Frisch, M., Bayerlein, M., and Werner, T. (2005). MatInspector and beyond: promoter analysis based on transcription factor binding sites. Bioinformatics 21, 2933–2942.

36. Wingender, E. (2008). The TRANSFAC project as an example of framework technology that supports the analysis of genomic regulation. Brief. Bioinform. 9, 326–332.

37. Valtink, M., Gruschwitz, R., Funk, R.H., and Engelmann, K. (2008). Two clonal cell lines of immortalized human corneal endothelial cells show either differentiated or precursor cell characteristics. Cells Tissues Organs (Print) 187, 286–294.

38. Massie, I., Dziasko, M., Kureshi, A., Levis, H.J., Morgan, L., Neale, M., Sheth, R., Tovell, V.E., Vernon, A.J., Funderburgh, J.L., and Daniels, J.T. (2015). Advanced imaging and tissue engineering of the human limbal epithelial stem cell niche. Methods Mol. Biol. 1235, 179–202.

39. Harikumar, A., and Mesheror, E. (2015). Chromatin remodeling and bivalent histone modifications in embryonic stem cells. EMBO Rep. 16, 1609–1619.

40. Vastenhouw, N.L., Zhang, Y., Woods, I.G., Imam, F., Regev, A., Liu, X.S., Rinn, J., and Schier, A.F. (2010). Chromatin signature of embryonic pluripotency is established during genome activation. Nature 464, 922–926.

41. Kundaje, A., Meuleman, W., Ernst, J., Bilenky, M., Yen, A., Herr- avi-Moussavi, A., Kheradpour, P., Zhang, Z., Wang, J., Ziller, M.J., et al.; Roadmap Epigenomics Consortium (2015). Integrative analysis of 111 reference human epigenomes. Nature 518, 317–330.

42. Nakashashi, H., Kieffer Kwon, K.R., Resch, W., Vian, L., Dose, M., Stavreva, D., Hakim, O., Pruett, N., Nelson, S., Yaman, A., et al. (2013). A genome-wide map of CTCF multivalency redefines the CTCF code. Cell Rep. 3, 1678–1689.

43. Xiang, J., Fu, X., Ran, W., and Wang, Z. (2017). Grhl2 reduces invasion and migration through inhibition of TGFiβ-induced EMT in gastric cancer. Oncogene 6, e284.

44. Frisch, S.M., Farris, J.C., and Pifer, P.M. (2017). Roles of Grainyhead-like transcription factors in cancer. Oncogene 36, 6067–6073.

45. Aue, A., Hinze, C., Valentin, K., Ruffert, J., Yurtadas, Y., Werth, M., Chen, W., Rabien, A., Kilic, E., Schulzke, J.D., et al. (2015). A grainyhead-like 2/Ovo-like 2 pathway regulates renal epithelial barrier function and lumen expansion. J. Am. Soc. Nephrol. 26, 2704–2715.

46. McCoy, B.E., Edelhauser, H.F., and Lynn, M.J. (2008). Review of corneal endothelial specular microscopy for FDA clinical trials of refractive procedures, surgical devices, and new intraocular drugs and solutions. Cornea 27, 1–16.

47. Chen, Y., Huang, K., Nakatsu, M.N., Xue, Z., Deng, S.X., and Fan, G. (2013). Identification of novel molecular markers through transcriptomic analysis in human fetal and adult corneal endothelial cells. Hum. Mol. Genet. 22, 1271–1279.
48. Bath, C., Muttuvelu, D., Emmersen, J., Vorum, H., Hjortdal, J., and Zachar, V. (2013). Transcriptional dissection of human limbal niche compartments by massive parallel sequencing. PLoS ONE 8, e64244.

49. Cieply, B., Riley, P., 4th, Piéfer, P.M., Widmeyer, J., Addison, J.B., Ivanov, A.V., Denvir, J., and Frisch, S.M. (2012). Suppression of the epithelial-mesenchymal transition by Grainyhead-like-2. Cancer Res. 72, 2440–2453.

50. He, Z., Forest, F., Gain, P., Rageade, D., Bernard, A., Acquart, S., Peoc’h, M., Defoe, D.M., and Thuret, G. (2016). 3D map of the human corneal endothelial cell. Sci. Rep. 6, 29047.

51. Wang, H.S., Hung, S.C., Peng, S.T., Huang, C.C., Wei, H.M., Guo, Y.J., Fu, Y.S., Lai, M.C., and Chen, C.C. (2004). Mesenchymal stem cells in the Wharton’s jelly of the human umbilical cord. Stem Cells 22, 1330–1337.

52. Kim, Y.R., Kim, M.A., Sagong, B., Bae, S.H., Lee, H.J., Kim, H.J., Choi, J.Y., Lee, K.Y., and Kim, U.K. (2015). Evaluation of the contribution of the EYA4 and GRHL2 genes in Korean patients with autosomal dominant non-syndromic hearing loss. PLoS ONE 10, e0119443.

53. Van Laer, L., Van Eyken, E., Fransen, E., Huyghe, J.R., Topsakal, V., Hendrickx, J.J., Hannula, S., Mäki-Torkko, E., Jensen, M., Demeester, K., et al. (2008). The grainyhead like 2 gene (GRHL2), alias TFCP2L3, is associated with age-related hearing impairment. Hum. Mol. Genet. 17, 159–169.

54. Vona, B., Nanda, I., Neuner, C., Müller, T., and Haaf, T. (2013). Confirmation of GRHL2 as the gene for the DFNA28 locus. Am. J. Med. Genet. A. 161A, 2060–2065.

55. Petrof, G., Nanda, A., Howden, J., Takeichi, T., McMillan, J.R., Aristodemou, S., Ozoemena, L., Liu, L., South, A.P., Pourreyron, C., et al. (2014). Mutations in GRHL2 result in an autosomal-recessive ectodermal Dysplasia syndrome. Am. J. Hum. Genet. 95, 308–314.

56. Boivin, F.J., and Schmidt-Ott, K.M. (2017). Transcriptional mechanisms coordinating tight junction assembly during epithelial differentiation. Ann. N Y Acad. Sci. 1397, 80–99.