TITLE: *Sox9* prevents retinal degeneration and is required for limbal stem cell differentiation in the adult mouse eye

Running title: Retinal and limbal defects in adult *Sox9* mutant mice

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ABSTRACT

Sox9 is a transcription factor with multiple roles during development and in adult organ homeostasis. In the adult eye, *Sox9* expression persists in several cell types, including the retinal pigmented epithelium cells and the Müller glial cells, as well as in the limbal and corneal basal epithelia. To uncover the role of *Sox9* in these cell types, we induced the deletion of the gene in adult mice. We found that, after *Sox9* ablation, mutant mice undergo a severe process of retinal degeneration characterized by the loss of Müller glial cells and complete depletion of the photoreceptors layer. Moreover, by combining single-cell RNA sequencing and *Sox9* lineage tracing, we found that *Sox9* is expressed in a basal limbal stem cell population with the ability to form two types of long-lived cell clones involved in stem cell maintenance and homeostasis. Mosaic analysis of *Sox9* positive and negative cells confirmed that the gene is essential for limbal stem cell differentiation. Our results show that *Sox9* is required for the maintenance of retinal integrity and for limbal stem cell differentiation in the adult mouse eye.

INTRODUCTION

The retina and cornea represent indispensable components of the vertebrate visual system that ensure proper vision (Casey et al., 2023). The retina, a multilayered sensory tissue at the back of the eye, converts light into neural signals, initiating visual perception. It consists of various specialized cells organized into three nuclear layers and two synaptic layers. Photoreceptors, including rods and cones, are located in the outer nuclear layer (ONL) and are responsible for converting light stimuli into electrical signals. These signals are then transmitted to bipolar cells in the inner nuclear layer (INL), which, in turn, establish synapsis with retinal ganglion cells (RGCs), whose axons form the optic nerve that send the electrical impulse to the brain. Additionally, horizontal and amacrine cells, also positioned in the INL, provide lateral connections, offering feedback and feedforward signals. Finally, Müller glial (MG) cells, with nuclei located in the INL and bodies extending across the entire retina, provide structural, metabolic, and functional support to the other retinal cells, thus maintaining retinal function and integrity (Baden et al., 2020). The cornea, positioned at the forefront of the eye, is a transparent and refractive structure responsible for transmitting and focusing light onto the retina, which is devoid of blood vessels. The outermost layer of the cornea is a stratified squamous epithelium that undergoes continuous regeneration. Stem cells responsible for this replenishment reside within a specialized niche located at the periphery, adjacent to the conjunctiva, known as the limbus (Lavker et al., 2004; West et al., 2015). Within this limbal niche, long-lived limbal epithelial stem cells (LESCs) give rise to a more differentiated progeny responsible for the regeneration of the entire corneal epithelium throughout two distinct pathways of cell migration: one within the basal layer,

moving from the limbus to the center of the cornea, and another from the basal layer to the terminally differentiated, desquamated cells at the epithelial surface (Yazdanpanah et al., 2017).

SOX9 is a transcription factor involved in the regulation of multiple developmental processes (Jo et al., 2014). Mutations affecting *SOX9* cause campomelic dysplasia (CD), a syndrome characterized by skeletal malformations and XY sex reversal (Wagner et al., 1994; Foster et al., 1994). In the adult, SOX9 has been shown to play a role in the homeostasis of neural, liver, pancreas, intestine and nail stem cells (Scott et al., 2010; Furuyama et al., 2011; Lao et al., 2022), as well as in the maintenance of Sertoli cells (Barrionuevo et al., 2016), among others.

In the context of the mouse retina, *Sox9* is initially expressed in retinal progenitor cells (RPCs), and its expression becomes restricted to Müller glial (MG) cells (Poché et al., 2008) as a result of Notch regulation, maintaining Müller glial differentiation and contributing to the regenerative capacity of retinal tissues (Poché et al., 2008; Muto et al., 2009; Masuda et al., 2014). Additionally, *Sox9* expression also persists in the mature retinal pigment epithelium (RPE), where it collaborates with other transcription factors, such as OTX2 and LHX, to regulate the visual cycle (Masuda et al., 2014). In the cornea, *Sox9* is expressed in LESCs and in corneal epithelial cells (Sartaj et al., 2017; Menzel-Severing et al., 2018). Several *in vitro* experiments have indicated that the gene is involved in limbal stem cell differentiation (Sartaj et al., 2017; Parfitt et al., 2015; Menzel-Severing et al., 2018).

Although several studies have addressed the role of *Sox9* in retinal and corneal development (Poché et al., 2008; Muto et al., 2009; Masuda et al., 2014; Sartaj et al., 2017; Parfitt et al., 2015; Menzel-Severing et al., 2018), its specific *in vivo* contributions to the maintenance and regeneration of these tissues in adult mammals remain unknown.

In this work, we used a tamoxifen-inducible Cre/LoxP system to suppress *Sox9* in adult mice and a lineage-tracing mouse strain to study the fate of *Sox9*-expressing cells. With these tools, in conjunction with scRNAseq analysis, we conducted a comprehensive study of morphological changes, cell-specific marker expression, and lineage tracing to determine the role of *Sox9* in maintaining retinal integrity and corneal stem cell homeostasis.

RESULTS

Deletion of *Sox9* leads to retinal degeneration in adult mice

To evaluate the role of *Sox9* in adult retinal homeostasis, we conditionally deleted the gene in adult mouse cells using a ubiquitous tamoxifen-inducible CAGG-CreER™ recombinase (Hayashi and McMahon, 2002) and a conditional *Sox9*$^{flox/flox}$ allele (Akiyama et al., 2002). Histological examination of retinas from control (Cre-negative *Sox9*$^{flox/flox}$) and *CAGG-CreERTM;Sox9*$^{flox/flox}$ (hereafter *Sox9*$^{\Delta/\Delta}$) mice at different days after TX administration (DATX) revealed that most of the analyzed mutant samples (control, n=7; *Sox9*$^{\Delta/\Delta}$, n = 18/24; Table S1), showed either no relevant morphological defects in the retinal sections or a mild phenotype in which the main retinal nuclear

layers (INL and ONL) occupied a smaller area and their edges did not appear as clearly differentiated as in the control retinas. However, we also observed an extreme phenotype in a reduced number of mutant retinas (n = 6/24), characterized by the loss of the ONL (Fig. 1A). The severity of the phenotype showed no correlation with the time elapsed after tamoxifen administration, as we found mutant retinas with no apparent phenotype at 100 DATX and others with an extreme phenotype at 20 DATX (Table S1). Retinal degeneration was never observed in mice that had not been tamoxifen-treated, nor any other controls, eliminating the formal possibility that the retinal degeneration allele of photoreceptor cGMP phosphodiesterase 6b (*Pde6b*^{rd1}) was present in our mice (Bowes et al., 1990).

During retinal development, *Sox9* is expressed in neural RPCs but once the neural retina is differentiated, *Sox9* expression is restricted to MG and RPE cells (Poché et al., 2008; Masuda et al., 2014). This is, indeed, the SOX9 expression pattern we observed in retinal sections obtained from adult control mice (Fig. 1B). To explain the phenotypic diversity shown by the TX-treated mice, we assessed the efficiency of *Sox9* deletion in mutant mice. For this, we performed SOX9/SOX8 double immunofluorescence, as both transcription factors are normally co-expressed in MG cells (Fig 1B; Muto et al., 2009), and we always observed the presence of many SOX8^{+} cells that lacked SOX9 expression in the retinas of mutant TX-treated mice (Fig. 1B). We calculated the percentage of SOX8^{+} cells that also co-expressed SOX9 in control and in *Sox9*$^{\Delta/\Delta}$ corneas with mild and extreme phenotypes (Fig. 1C; Table S2). In control MG cells, all SOX8^{+} cells also expressed SOX9. In contrast, in mutants with a mild phenotype, the average percentage was 55%, although with a large standard deviation (55% ± 23%),

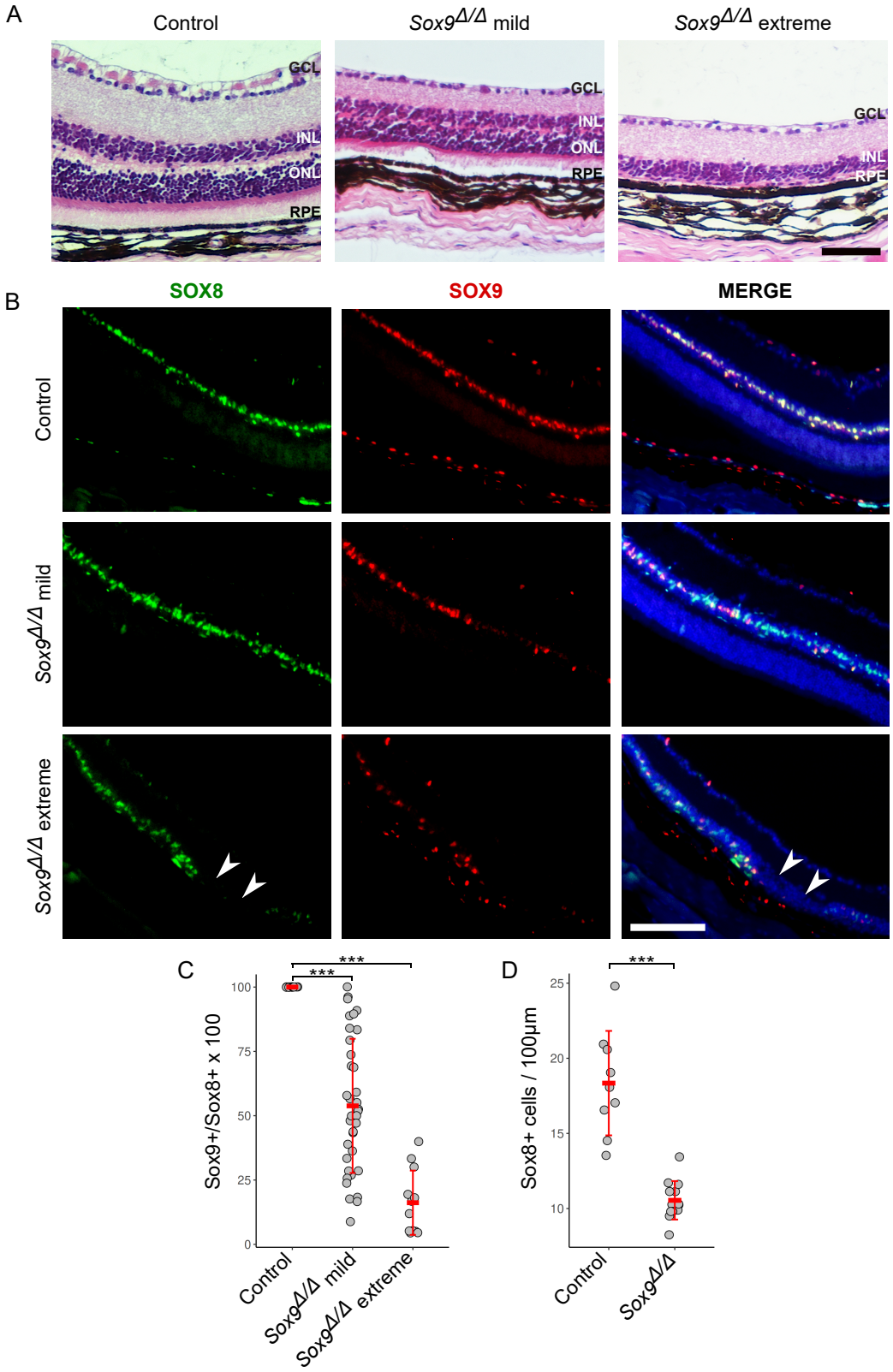

Figure 1. Efficiency and morphological impact of *Sox9* deletion on the adult mouse retina. **(A)** Hematoxylin-eosin stained histological sections of retinas from control and *Sox9*$^{\Delta/\Delta}$ mice. Mutant retinas exhibit variability in phenotype severity, with some showing mild morphological defects and a few displaying an extreme phenotype characterized by the loss of the outer nuclear layer

(ONL). **(B)** Double immunofluorescence for SOX8 (green) and SOX9 (red) in retinal sections from adult control and *Sox9*$^{\Delta/\Delta}$ mice. The severity of phenotypes correlates with the extent of *Sox9* inactivation. Regions completely lacking SOX8^{+} cells are indicated by arrowheads. DAPI (blue) was used to counterstain nuclei. **(C)** Quantification of the percentage of SOX8^{+} cells co-expressing SOX9 in control and *Sox9*$^{\Delta/\Delta}$ retinas with mild and extreme phenotypes. **(D)** Quantification of SOX8^{+} cells per 100 µm of inner nuclear layer (INL) length in control and *Sox9*$^{\Delta/\Delta}$ retinas. The black scale bar represents 50 µm in A, and the white scale bar represents 100 µm in B. GCL, Ganglion cell layer; INL, inner nuclear layer; RPE, retinal pigmented epithelium.

while in extreme mutants, the percentage was significantly lower (16% ± 12%). Altogether, these results indicate that the severity of the phenotype is directly related to the efficiency of *Sox9* inactivation, and that only individuals with *Sox9* inactivation occurring in at least 80% of the cells expressing the gene exhibit the extreme phenotype.

Next, we used cell-specific molecular markers to characterize the most affected *Sox9*-deficient retinas. Initially, we focused on the MG cell layer by means of SOX8 immunofluorescence. We counted the number of SOX8+ cells per 100 µm of INL, and we found a significant reduction of in the number of MG cells in mutant retinas (control, 18.5 ± 3.5, n = 7; mutant, 10.5 ± 1.2 n= 6; p = 0.000107, Mann-Whitney U test; Fig 1D; Table S3). In addition to the reduced number of SOX8^{+} cells, we observed areas within some *Sox9* mutant INL layers that completely lacked SOX8^{+} cells. (Fig. 1B arrowheads), indicating that these regions were entirely devoid of MG cells.

Since the main observable consequence of *Sox9* deletion in the adult mouse retina is the absence of the ONL layer, which harbors both rod and cone photoreceptors, we further examined the status of these cell types by immunofluorescence. Cone photoreceptor cells were assessed by retinal whole-mount staining with two different opsins: OPN1SW (short-wavelength-sensitive opsin, referred to as S opsin) and

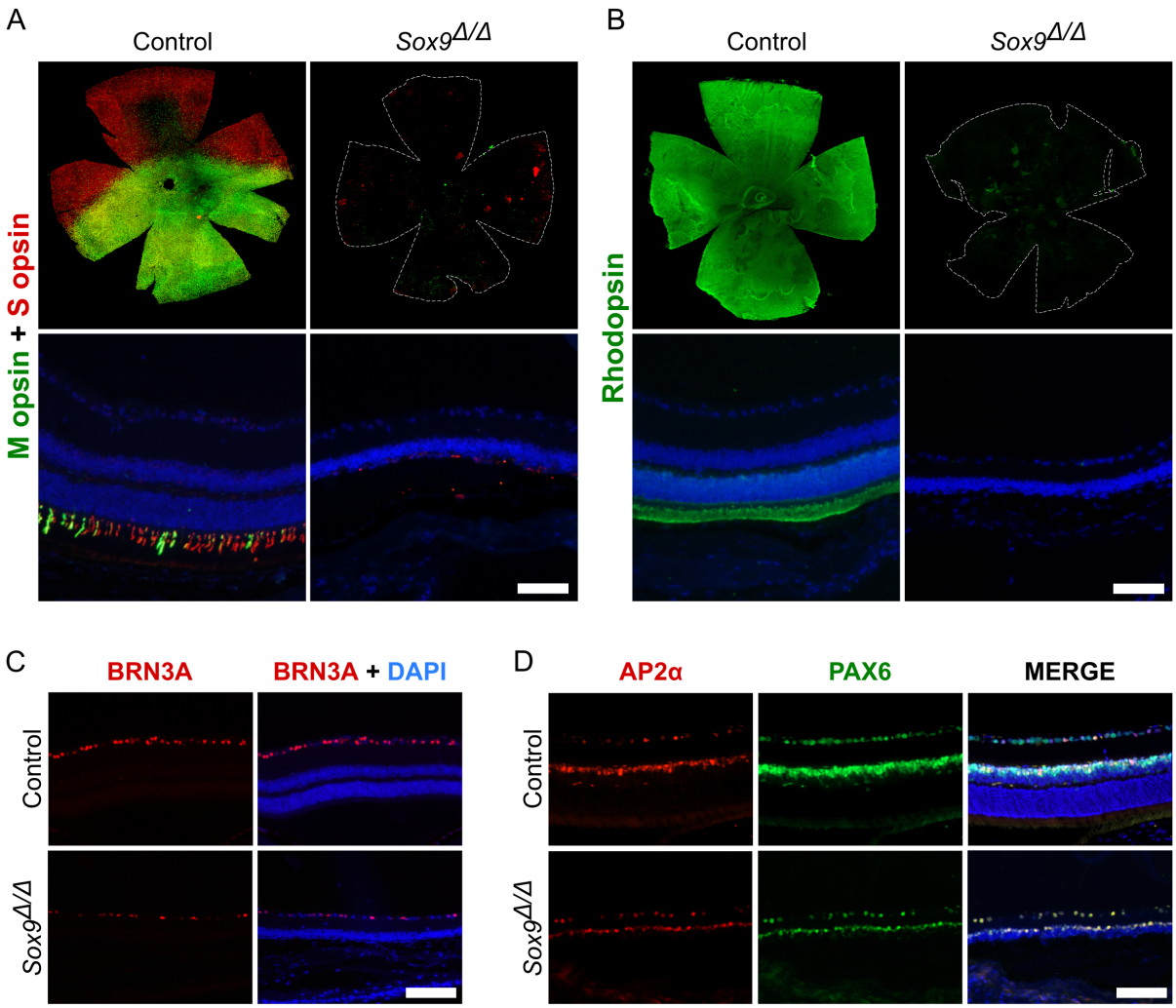

Figure 2. Immunodetection of retinal cell markers in adult retinas from control and *Sox9*$^{\Delta/\Delta}$ mice. **(A)** Immunofluorescence analysis of cone photoreceptor cells in retinal whole-mounts (upper images) and histological sections (lower images) using double staining for OPN1SW (S opsin) and OPN1LW (M opsin). **(B)** Immunofluorescence analysis of rod photoreceptor cells in retinal whole-mounts (upper images) and histological sections (lower images) using Rhodopsin staining. **(C)** Immunofluorescence analysis of ganglion cells in retinal sections using BRN3A staining. **(D)** Immunofluorescence analysis of amacrine and horizontal cells in retinal sections using double staining for PAX6 and AP2α. DAPI (blue) was used to counterstain nuclei. The scale bars in A and B represent 1 mm for the top row and 50 µm for the bottom row; the scale bars in C and D represent 90 µm.

OPN1LW (medium- and long-wavelength-sensitive opsin, referred to as M opsin). In control retinas, we observed uniform staining on the ventral surface and on the entire

surface for S opsin and M opsin, respectively. In contrast, in *Sox9*-deficient retinas we could only identify small isolated immunofluorescent patches (Fig. 2A. upper). Double immunofluorescence on slides confirmed that mutant retinas lacked expression for both cone photoreceptors (Fig. 2A, bottom). We also performed immunofluorescence for RHO, a marker for rod photoreceptor cells, on retinal whole-mounts and slides. We found a similar pattern of expression to that described for M-opsin in both the control and mutant retinas. These results evidence that *Sox9* is required for the maintenance of the two photoreceptor cell types present in the adult mouse retina (Fig. 2B).

We also studied the status of other retinal cell types. The transcription factor BRN3A was used to identify ganglion cells (Nadal-Nicolás et al., 2009), which were shown to decrease in number in the mutant retinas, compared to control ones (Fig. 2C). Similarly, double immunodetection of the transcription factors PAX6 and AP2A was used to identify both amacrine and horizontal cells, as previously described (Marquardt et al., 2001; Barnstable et al., 1985; Edqvist and Hallböök, 2004), showing a similar reduction in both cell types in degenerated retinas (Fig. 2D).

Photoreceptors undergo apoptosis in the absence of *Sox9*

Since photoreceptors are absent in *Sox9*-mutant corneas we conducted TUNEL assays to study the role of cell death in the process of retinal degeneration. In control samples (n=5), almost no TUNEL signal was observed in the retina. In contrast, *Sox9*$^{\Delta/\Delta}$ mice with a mild retinal phenotype (n=5) showed numerous TUNEL+ cells, mainly located in the persisting ONL, indicating that photoreceptor cells were dying. In *Sox9*$^{\Delta/\Delta}$ retinas lacking ONL, we only observed a low number of TUNEL+ cells in the resulting

cell monolayer, indicating that the process of photoreceptor degeneration had already occurred (Fig. 3A).

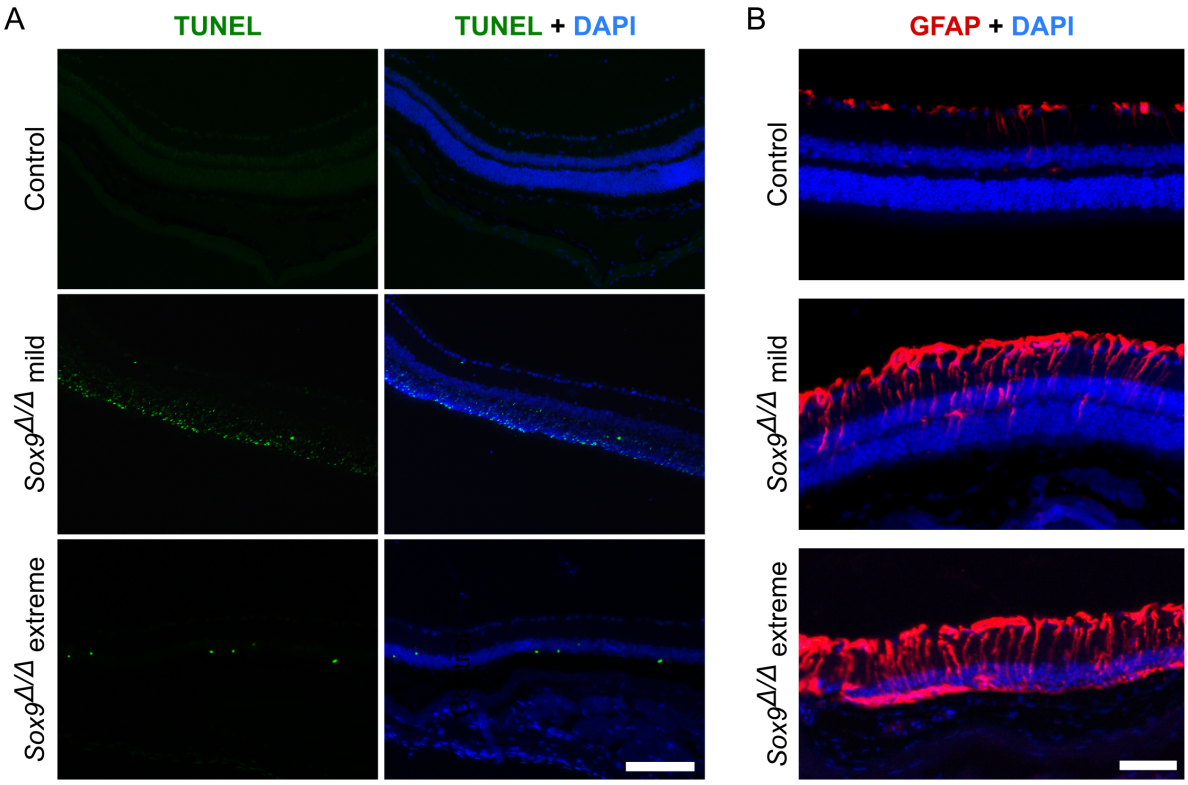

Figure 3. Assessment of retinal damage and apoptosis in *Sox9*-deficient mice. **(A)** TUNEL staining of adult retinas from control and *Sox9*$^{\Delta/\Delta}$ mice with mild and extreme phenotypes. A large number of TUNEL-positive photoreceptor cells is evident in mutant mice with mild phenotypes, indicating extensive apoptotic events affecting the outer nuclear layer. **(B)** Immunofluorescence staining of GFAP in adult retinas from control and *Sox9*$^{\Delta/\Delta}$ mice with mild and extreme phenotypes. Müller glial cell activation is observed in *Sox9*-deficient retinas, with GFAP expression extending across the entire thickness of the retina in extreme phenotypes, suggesting progressive gliosis. The scale bars represent 50 µm.

Müller glial cells undergo reactive gliosis in *Sox9*-deficient retinas

Finally, we decided to study the expression of the glial fibrillary acidic protein (GFAP), whose expression is upregulated in MG cells undergoing reactive gliosis associated with retinal cell injury (Ekström et al., 1988). As previously reported (Fernández-Sánchez et al., 2015), in control mice GFAP immunostaining was mainly limited to the ganglion cell layer (GCL), in the inner margin of the retina. However, in *Sox9*-deficient retinas with a mild phenotype, GFAP-positive processes reached the INL layer. In mutant retinas with an extreme phenotype GFAP-positive MG processes were distributed throughout the entire degenerated retinas. Thus, in the absence of *Sox9*, MG cells seem to undergo a progressive process of gliosis (Fig. 3B).

Lineage tracing of *Sox9*-expressing cells in the limbus and the cornea

We next decided to shed light on the role of S*ox9* in the adult cornea. Previous studies have documented the expression of *Sox9* in the adult mouse limbal and central cornea epithelium (Menzel-Severing et al., 2018; Sartaj et al., 2017). In line with these findings, we also observed the presence of the SOX9 protein in the basal cells of both structures (Fig. 4A and B), which are the sites housing corneal progenitor and stem cells. To assess the stemness potential of these *Sox9*-positive cells, we conducted TX-inducible genetic lineage tracing by breeding mice harboring an *IRES-CreER*^{T2} cassette inserted into the endogenous *Sox9* locus, *Sox9*$^{IRES\text{-}CreERT2}$ (Soeda et al., 2010), to either *R26R-EYFP* (Srinivas et al., 2001) or *R26R-LacZ* (Soriano, 1999) reporter mice. We induced labeling of *Sox9*-expressing cells and their progeny by administering TX to adult mice and analyzed reporter expression at different DATX (Fig. 4C). No X-gal- or EYFP-positive cells were detected at any of the stages analyzed in the absence of TX

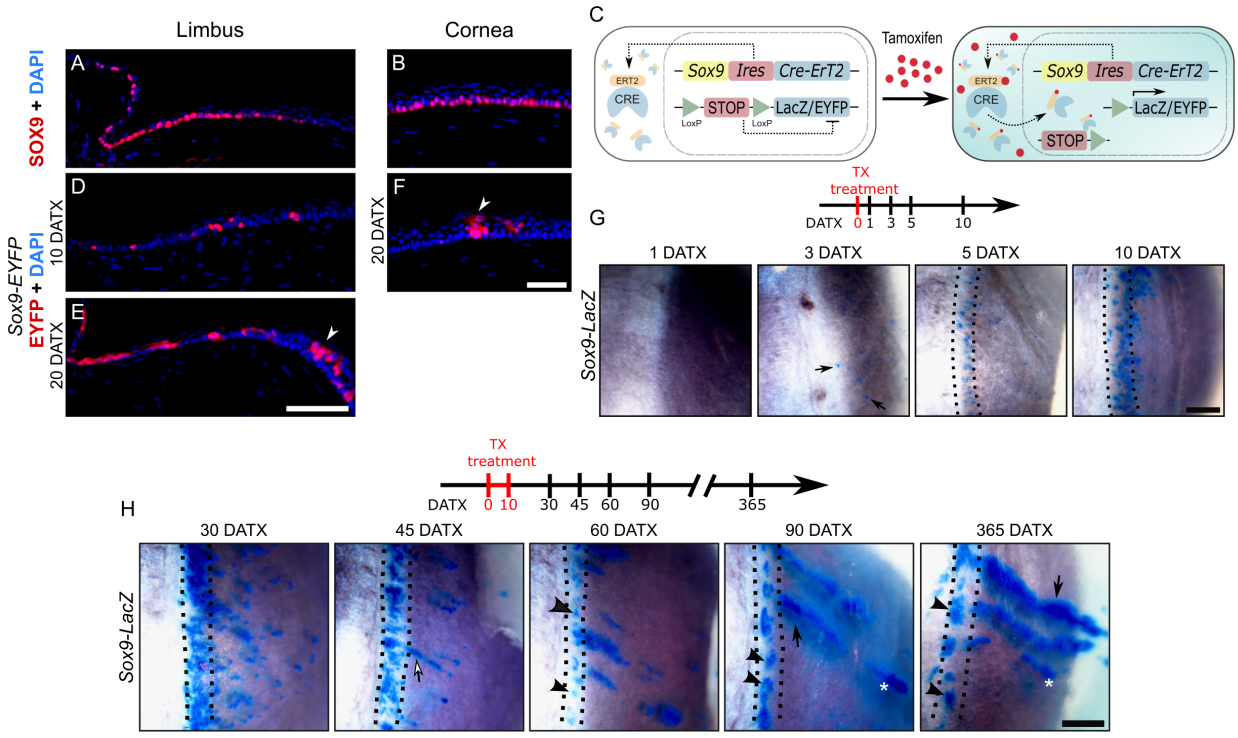

Figure 4. Analysis of the fate of SOX9-expressing cells through lineage tracing experiments. **(A-B)** Immunofluorescence for SOX9 in limbal (A) and corneal (B) sections of adult control mice. DAPI (blue) was used to counterstain nuclei. **(C)** Schematic of the genetic lineage tracing strategy. Tamoxifen (TX) was administered to label SOX9-expressing cells and their progeny. **(D-F)** Analysis of EYFP expression in the limbus (D-E) and cornea (F) of *Sox9*-EYFP adult mice. At 10 days after TX administration (10 DATX), discrete EYFP-positive clones are visible in the limbal region (D). By 20 DATX, these clones expand towards the peripheral cornea, with some cells reaching the outer epithelial layers at the limbus-cornea border (arrowhead in E). In the central cornea, EYFP-positive cells extend along the entire epithelium by 20 DATX (arrowhead in F). **(G)** Short-term whole mount X-gal staining of *Sox9*-LacZ eyes. The first LacZ-positive cells scattered across the limbal and corneal surface appeared at 3 DATX (arrows). **(H)** Long-term whole mount X-gal staining of *Sox9*-LacZ eyes. At 45 DATX, LacZ-positive stripes emerge from the limbus and extend into the peripheral cornea (arrow). At 60 DATX, circumferential clones are observed in the limbus (arrowheads). At 90 DATX, three clone types are visible: circumferential clones in the limbus (arrowheads), stripes from the limbus reaching the central cornea (arrow), and stripes without a base in the limbus (asterisk). At 365 DATX, the first two clone types are mainly observed, though in reduced numbers and larger sizes (see arrowheads and arrow as in the 90 DATX picture), with some stripes without a base in the limbus (asterisk). Scale bars in E and F represent 50 µm; scale bars in G and H represent 500 µm.

(Fig. S1, control panels). Immunofluorescence for EYFP on limbal *Sox9*$^{IRES\text{-}CreERT2}$-*R26R-EYFP (Sox9-EYFP)* sections at 10 DATX unveiled the presence of discrete clones of EYFP-positive cells (Fig. 4D). By 20 DATX, these clones were larger and extended towards the peripheral cornea. At this latter stage, EYFP-positive clones with cells reaching the outer layers of the epithelium were visible at the limbus-cornea border (Fig. 4E, arrowhead). At 20 DATX, we also observed groups of cells spanning the entire apical-basal axis of the central corneal epithelium (Fig. 4F , arrowhead). These results demonstrate that SOX9^{+} cells residing in the basal layer of the limbal and corneal epithelium are progenitors of the terminally differentiated cells of the corneal epithelium.

We then investigated the dynamics of stem cell progression through whole mount Xgal-staining of *Sox9*$^{IRES\text{-}CreERT2}$ *;R26R-LacZ (Sox9-LacZ)* eyes. At first, we conducted a short-term study with a single TX injection and analyzed X-gal-staining for up to ten days after treatment (Figs. 4G and S1). We observed the first X-gal-positive cells at 3 DATX, and they were scattered throughout the entire limbal and corneal surface (Fig. 4G, arrows). At 5 DATX, most X-gal^{+} cells were located in the limbal area, and by 10 DATX, the number of stained cells increased notably in this area. These observations suggest that *Sox9*-expressing descendant cells experience a process of proliferation in the limbus, illustrating their transient cell amplification behavior. To gain insights on the long-term cloning capacity and dynamics of *Sox9*-progenitor cells, we administered tamoxifen for 10 days in the food and performed X-gal staining from 30 DATX to 365 DATX (Figs. 4H and S1). At 30 DATX, LacZ was mostly expressed in the limbus, although lacZ^{+} clones could also be observed throughout the peripheral and central cornea. Overall, these clones were larger than those observed at 10 DATX. Fifteen days

255 later (45 DATX), the limbal staining began to disperse, allowing the observation of discrete clones. Simultaneously, X-gal$^+$ stripes emerging from the limbus and extending into the peripheral cornea became apparent (Fig. 4H, 45 DATX, arrow, and Fig. S1). This situation was evident at 60 DATX, when the thickness and number of the stripes had increased. Additionally, starting at this stage, circumferential clones that remained
260 in the limbus could also be appreciated (Fig. 4H, 60 DATX, arrowheads, and Fig. S1). At 90 DATX, three types of clones were observed: circumferential ones in the limbus (Fig. 4H, 90 DATX, arrowheads, and Fig. S1), stripes originating from the limbus, which often reached the central cornea (Fig. 4H, 90 DATX, arrow, and Fig. S1), and stripes without a base in the limbus (Fig. 4H, 90 DATX, asterisk, and Fig. S1). At 365 DATX, the first
265 two types of clones were mainly observed, albeit in reduced numbers and larger sizes. We also observed some stripes without a base in the limbus (Fig. 4H, 365 DATX, asterisk, and Fig. S1).

Sox9 is involved in limbal stem cell differentiation

To gain insight into the function of *Sox9* as a limbal stem cell marker, we used the
270 single-cell RNA-sequencing dataset of isolated epithelial cells from the limbus (with marginal conjunctiva and corneal periphery) recently generated by Altshuler and colleagues (Altshuler et al., 2021). In this study, the authors describe the co-existence of two separated stem populations in the limbus, the quiescent “outer” limbus (OLB) one, adjacent to the conjunctiva, and the active “inner” limbus (ILB) one, adjacent to the
275 peripheral cornea. Unbiased clustering of the sc-RNAseq dataset and subsequent assignament of cell identity revealed the existence of 10 cell populations equivalent to those identified by Altshuler and colleagues (Figs. 5A and S2A). Density plots based on

gene-weighted kernel density estimation and violin plots of expression levels revealed high expression levels of *Sox9* in OLB cells, and progressive weaker expression in the ILB, corneal basal and corneal suprabasal clusters (Figs. 5B and C). This expression pattern is consistent with our immunofluorescence observations. (Fig. 4A and B). We also detected strong expression for *Sox9* in a mitotic cluster (Figs. 5B). Next, we inferred the trajectory of limbal and corneal cells by using the partition-based graph abstraction (PAGA) algorithm, and reconstructing the temporal order of differentiating cells with the diffusion pseudotime software, using the OLB cluster as “root” (Wolf et al., 2019; Haghverdi et al., 2016). The PAGA graph revealed a strong connection between the OLB node and a mitotic cell population with high expression of OLB and stem cell markers (*Gpha2*, *Ifitm3, Cd63,* and *Krt15)*, which, in turn, was strongly linked to a second mitotic cell cluster, in which the expression of these markers diminished while the expression of corneal markers (*Krt12*, *Ppp1r3c,* and *Slurp*) increased (Figs. 5D and Fig. S2B). Through the PAGA graph, we also observed that the expression levels of *Sox9* correlated with the pseudotime graph (Fig. 5E), indicating that *Sox9* expression decreases as transiently amplifying progenitors undergo progressive differentiation from limbal to peripheral corneal cells. Next, we conducted a comparative analysis of gene expression to examine the differences between cells exhibiting high (>= 0.7) and low expression levels (between 0.7 and 0.15) of *Sox9* (Fig. 5F). We identified 2816 deregulated genes, 137 upregulated, and 2679 downregulated (adjusted p-value < 0.05; |avg_log2FC| > 0.1; Fig. 5G; Table S4). Among the upregulated genes we found OLB markers including *Gpha2*, *Ifitm3* and *Cd63,* in addition to stem cell markers such as *Krt14* (Fig. 5G; Table S4). To uncover biological processes associated to *Sox9*

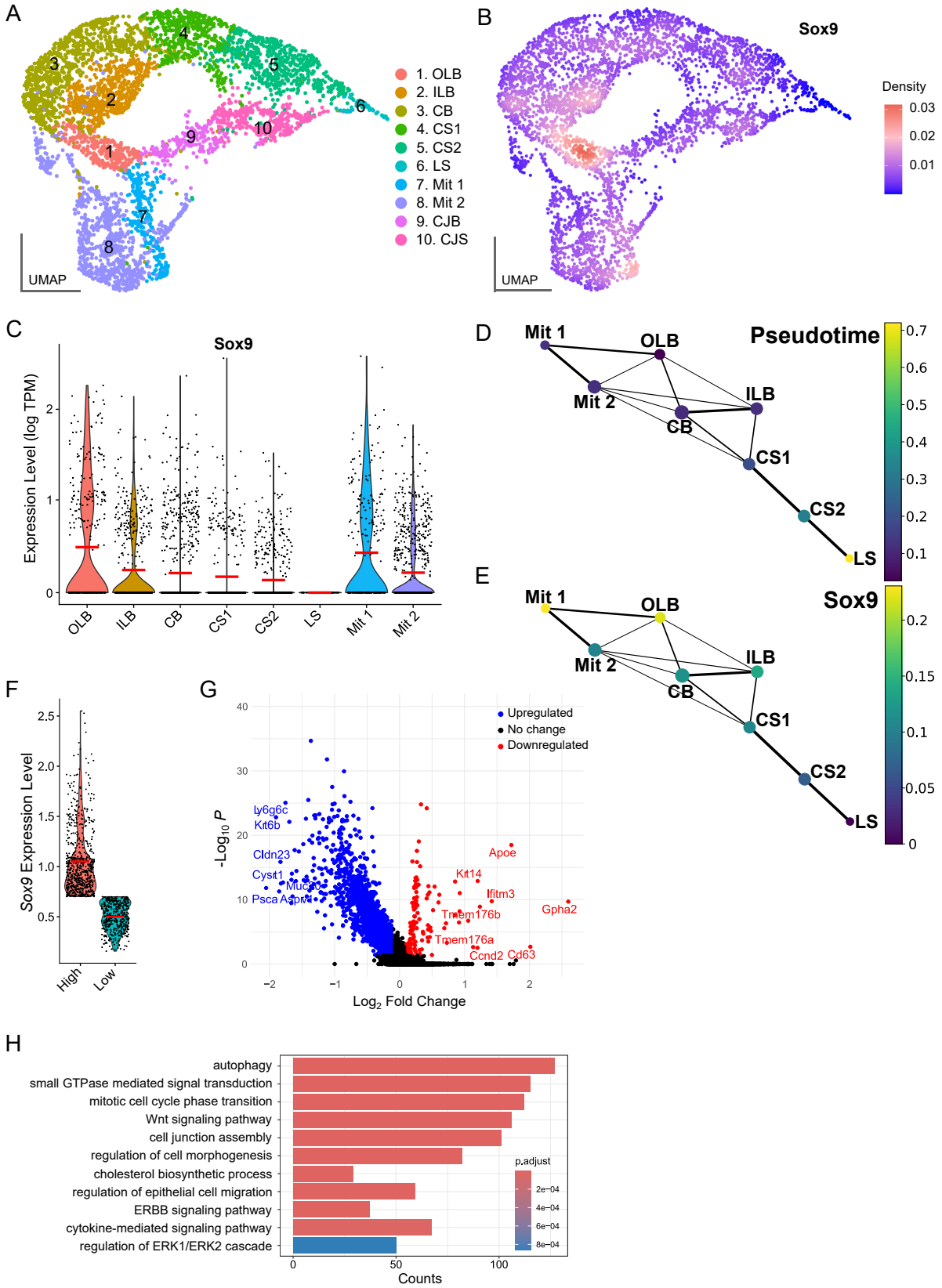
A
1. OLB
2. ILB
3. CB
4. CS1
5. CS2
6. LS
7. Mit 1
8. Mit 2
9. CJB
10. CJS
UMAP
B
Sox9
Density
0.03
0.02
0.01
UMAP
C
Sox9
Expression Level (log TPM)
OLB
ILB
CB
CS1
CS2
LS
Mit 1
Mit 2
D
Pseudotime
Mit 1
OLB
Mit 2
ILB
CB
CS1
CS2
LS
E
Sox9
Mit 1
OLB
Mit 2
ILB
CB
CS1
CS2
LS
F
Sox9 Expression Level
High
Low
G
Upregulated
No change
Downregulated
-Log10 P
Log2 Fold Change
Ly6g6c
Krt6b
Cldn23
Cystt1
Muc20
Psca
Aspm
Apoe
Krt14
Ifitm3
Tmem176b
Tmem176a
Ccnd2
Cd63
Gpha2
H
autophagy
small GTPase mediated signal transduction
mitotic cell cycle phase transition
Wnt signaling pathway
cell junction assembly
regulation of cell morphogenesis
cholesterol biosynthetic process
regulation of epithelial cell migration
ERBB signaling pathway
cytokine-mediated signaling pathway
regulation of ERK1/ERK2 cascade
p.adjust
Counts

Figure 5. Single-cell RNA-sequencing (scRNA-seq) analysis of *Sox9* expression in the mouse limbal and corneal epithelium. **(A)** Uniform manifold approximation and projection (UMAP) visualization of scRNA-seq data of isolated limbal epithelial cells from Altshuler *et al*. (2022). **(B)** Density plot of *Sox9* expression. **(C)** Violin plot of *Sox9* expression levels across the different cell clusters identified in A. **(D)** Pseudotime partition-based graph abstraction (PAGA) graph of the trajectory of limbal and corneal cells. **(E)** *Sox9* expression in the trajectory of cell differentiation described in D. **(F)** Violin plot of *Sox9* expression according to cells exhibiting high and low expression levels. **(G)** Volcano plot of differentially expressed genes (DEG) between cells exhibiting high and low *Sox9* expression levels. **(H)** Gene ontology analysis of DEG identified in G. CB, corneal basal; CJB, conjuntiva basal; CJS, conjuntiva suprabasal; CS1, corneal suprabasal 1; CS2, corneal suprabasal 2; ILB, inner limbal basal; LS, limbal suprabasal; Mit 1, mitiotic 1; Mit 2, mitotic 2; OLB, outer limbal basal.

downregulation in limbal and corneal cells, we performed gene ontology analysis using the downregulated genes, and we found terms related to stem cell differentiation, such as “mitotic cell phase transition” (Knoblich, 2008), “autophagy” (Chang, 2020), “cell-cell junction” (Ning et al., 2021), “regulation of epithelial cell migration” (Puri et al., 2020), “regulation of cell morphogenesis” and “stem cell differentiation”, as well as gene pathways involved in these processes including “Wnt-signaling pathway” (Yu et al., 2012), “ERBB signaling” (Hassan and Seno, 2022), “cytokine mediated signaling pathway” (Korkaya et al., 2011) and “regulation of ERK1 and ERK2 cascade” (Lavoie et al., 2020;Fig. 5H; Table S5).

Sox9$^{\Delta/\Delta}$ progenitor cells lose their clonogenic capacity

Our results suggest that *Sox9* is essential for limbal stem cell differentiation. To test this hypothesis, we used the same tamoxifen-inducible conditionally deleted *Sox9* gene in adult mouse cells that we used in the retina study. However, histological examination of the limbus and cornea from *Sox9*$^{\Delta/\Delta}$ mice at any time up to and including 100 DATX did not reveal any discernible phenotypic effects (Fig. 6A). Immunofluorescence staining for SOX9 failed to reveal any differences in the number of SOX9-expressing cells between mutants and controls. Similar observations were made for corneal and limbal

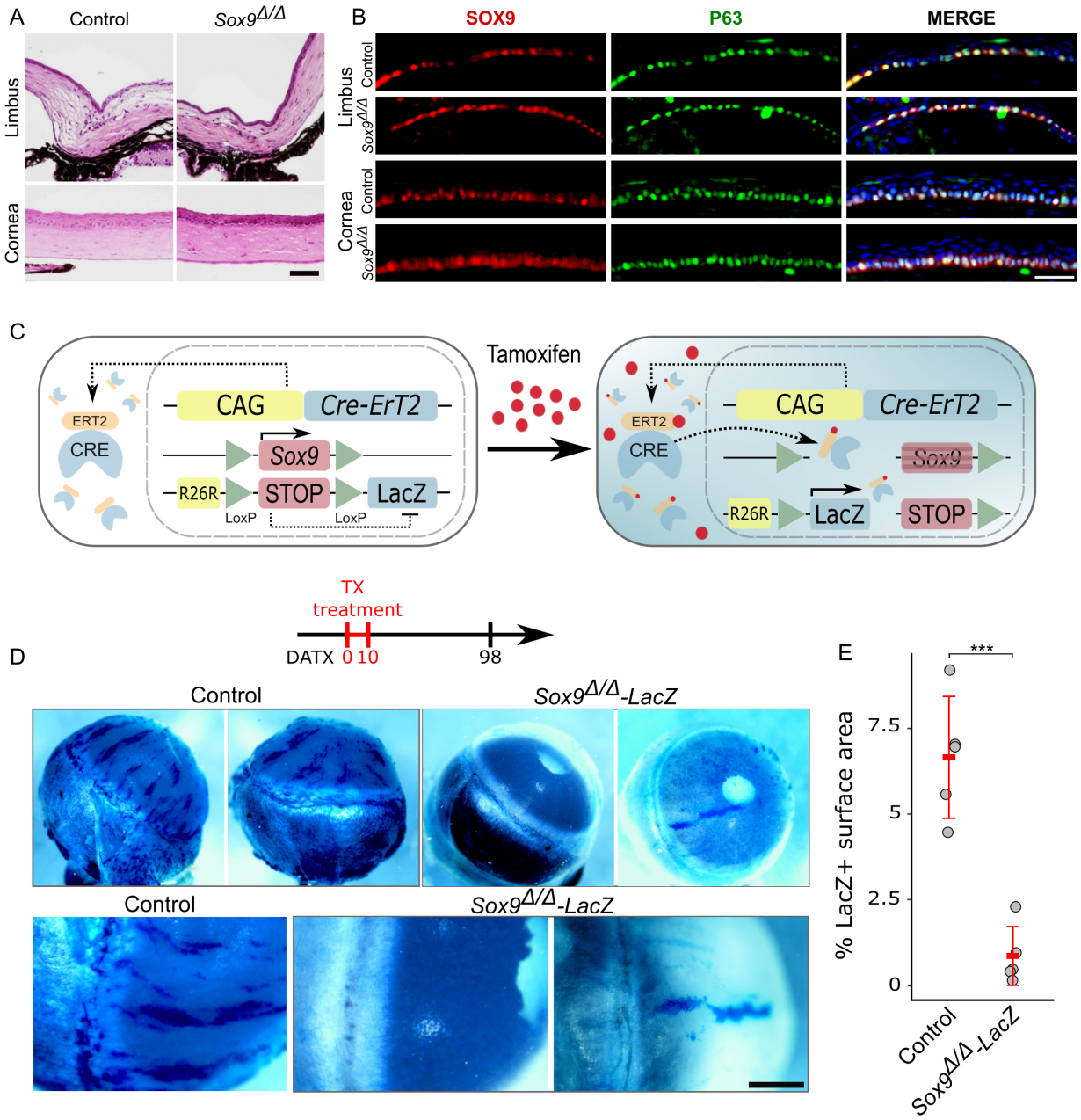

Figure 6. Impact of *Sox9* deletion on limbal stem cell differentiation and clonogenic capacity. **(A)** Hematoxylin-eosin stained histological sections of the limbus and cornea from control and *Sox9*$^{\Delta/\Delta}$ mice. **(B)** Immunofluorescence staining for SOX9 (red) and P63 (green) in the limbus and cornea from control and *Sox9*$^{\Delta/\Delta}$ mice. **(C)** Schematic of the generation of *Sox9*$^{\Delta/\Delta}$-LacZ mice. Tamoxifen (TX) was administered for ten days to label *Sox9*-deleted cells and their progeny. **(D)** X-gal staining of whole eyes from control and *Sox9*$^{\Delta/\Delta}$-LacZ mice at 98 days after TX administration. Control corneas exhibited numerous LacZ^{+} patches and elongated stripes originating from the limbus. In contrast minimal X-gal staining was observed in *Sox9*$^{\Delta/\Delta}$-LacZ corneas, indicating impaired clonogenic capacity in *Sox9*-deleted cells. **(E)** Quantification of X-

gal-stained surface areas in control and *Sox9*$^{\Delta/\Delta}$-LacZ mice. Scale bar in A represents 100 µm; Scale bar in B represents 50 µm; Scale bar in D represents 1 mm for the top row and 500 µm for the bottom row.

marker genes such as *P63* and *Pax6* (Figs. 6B and S3A). These observations are consistent with a model whereby tamoxifen induces mosaic patterns of cell *Sox9*-deleting in the ocular surface, but that the *Sox9*-null cells cannot survive or proliferate as well as their wild-type neighbors, and are hence outcompeted over time, leading to an essentially wild-type cornea. We tested this model by leveraging this mosaicism to investigate the *in vivo* clonal capacity of both *Sox9*-positive and *Sox9*-negative cells. For this, we generated *CAGG-CreERTM;Sox9*$^{flox/flox}$*;R26R-LacZ* mice (*Sox9*$^{\Delta/\Delta}$*-LacZ),* in which *Sox9*-deleted cells express the *LacZ* gene, and therefore, are labelled in blue after X-gal staining (Fig. 6C). As controls, we used *CAGG-CreERTM;Sox9*$^{flox/+}$*;R26R-LacZ* mice (*Sox9*$^{\Delta/+}$*-LacZ*). Mice were fed with a tamoxifen-supplemented diet for 10 days to induce *LacZ* expression followed by a chase period of 14 weeks. This time frame aligns with previous observations reporting the appearance of clear X-gal^{+} stripes in *CAGG-CreERTM;R26R-LacZ* mice *(Dor*à et al., 2015). In control *Sox9*$^{\Delta/+}$*-LacZ* corneas (4 mice, 8 eyes), we could observe numerous discrete X-gal^{+} patches of different sizes spanning over the entire corneal surface. In all cases, we observed a variable number of elongated stripes originating from the limbus, suggesting that they come from limbal stem cells (Figs. 6D and S3B). In contrast, the majority of *Sox9*$^{\Delta/\Delta}$*-LacZ* corneas (5 mice, 10 eyes) exhibited minimal blue staining, with only occasional, small X-gal^{+} spots observed. Notably, only one mutant cornea revealed the presence of an elongated stripe originating from the limbus (Fig. 6D, right panels; Fig. S3B). According to this, the measurement of the percentage of blue-stained surface areas

revealed a significant decrease in the mutant eyes compared to the controls (n = 5; *Sox9*$^{\Delta/+}$*-LacZ:* 6.65±1.77; *Sox9*$^{\Delta/\Delta}$*-LacZ:* 0.85 ± 0.85; paired t-test, p = 0.00017; Fig. 6E; Table S6). These results reveal that during normal *in vivo* homeostasis, epithelial limbal and corneal stem/progenitor cells require *Sox9* to maintain their clonogenic capacity, indicating a key role for this gene in the process.

DISCUSSION

In the present study, we conditionally deleted *Sox9* in the adult mouse retina and observed a phenotype characterized by different degrees of retinal degeneration. In the most affected individuals, this degeneration leads to the disappearance of the ONL layer and almost complete absence of cone and rod photoreceptors. Since *Sox9* is expressed in MG and RPE cells (Poché et al., 2008), it is challenging to determine the contribution of each cell type to the final mutant phenotype. Conditional deletion of *Sox9* during mouse retinal development showed that P20 mutant retinae exhibited normal lamination and nuclear layer thickness comparable to control samples, indicating that *Sox9* alone likely does not affect global retinal fate determination (Poché et al., 2008). In addition, *Sox9* has also been conditionally deleted in RPE cells. In this case, one study reported no obvious morphological abnormalities in the eye (Masuda et al., 2014), while two independent groups found that the gene is necessary for choroid development (Goto et al., 2018; Cohen-Tayar et al., 2018). Finally, no hypoplasia of the choroidal vasculature or lack of pigmentation was found in mice with a MG cell-specific *Sox9* deletion (Goto et al., 2018). Hence, previous studies on *Sox9*-mutant mice have not described any eye phenotype similar to the one we report here. The main reason is probably that these

390 studies focused on the developing retina and are therefore not useful for understanding how the absence of *Sox9* in MG and RPE cells in the adult eye leads to the retinal degeneration observed in our *Sox9*-deficient adult mutants. In this context, we can obtain better insights by examining the physiological response to RPE and MG cell loss after Cre-mediated activation of diphtheria toxin A (DTA) in these cell types. In the first
395 case, the major retinal abnormality was the formation of regions of photoreceptor rosetting with very mild degeneration of the photoreceptors (Longbottom et al., 2009). In mice with a DTA-induced ablation of MG cells, a wave of cell apoptosis was observed in the ONL but not in the other retinal layers (Shen et al., 2012). In our mutant mice, we did not observe photoreceptor rosetting in the ONL, but a massive apoptosis in this
400 retinal layer. In addition, we observed that some regions within the INL layer were devoid of MG cells. Thus, we favor the hypothesis that continuous expression of *Sox9* in MG cells is necessary for normal function and/or survival of this cell type. In the absence of *Sox9*, MG cells undergo massive retinal gliosis and they cannot provide structural, metabolic, and functional support to the other retinal cells, leading to a
405 massive loss of photoreceptors followed by the depletion of other retinal cell types. In agreement with this, *Sox9* expression is upregulated in MG cells after retinal light damage in rats (Wang et al., 2013). However, the retinal phenotype we report here is much more severe than the one observed in both DTA-induced transgenic lines. This could be due to the different efficiency of Cre-induced recombination, but also to the fact
410 that the combined deletion of *Sox9* in both MG and RPE may cause a more severe phenotype than the individual cell type-specific mutants.

To our knowledge, no retinal abnormality has been described in patients with a mutation in *SOX9* (see Hejtmancik and Daiger, 2020). This is probably because CD patients are mostly heterozygous for *SOX9* and show early postnatal lethality, so a retinal degeneration phenotype in adulthood was either overlooked or not addressed. However, *SOX9/Sox9* has a complex regulatory landscape (Bagheri-Fam et al., 2006; Despang et al., 2019), and mutations in its regulatory region have been associated with tissue-specific human phenotypes (Wunderle et al., 1998; Benko et al., 2009; Kim et al., 2015; Kurth et al., 2009). In this context, transgenic mice carrying an enhancer located around 500 kb upstream of *SOX9* revealed enhancer activity in the eye (Sreenivasan et al., 2017). Thus, screening of the *SOX9* regulatory region may provide new molecular mechanisms underlying idiopathic cases of retinal degeneration.

Several expression studies have proposed *Sox9* as a marker of epithelial limbal and corneal stem/progenitor cells, with further support from *in vitro* experiments validating this finding (Sartaj et al., 2017; Parfitt et al., 2015; Menzel-Severing et al., 2018). Through *in vivo* cell lineage tracing and gene targeting strategies in mice, combined with the analysis of sc-RNAseq data, we shed light on the nature, differentiation dynamics and function of these stem/progenitor *Sox9*-positive cells. Trajectory analysis of limbal and corneal scRNAseq data revealed that *Sox9* is highly expressed in a cell population characterized by the expression of stem cell markers (Gpha2, Ifitm3, Cd63, and Krt14), and that its expression gradually decreases as these cells enter mitosis and differentiate into suprabasal corneal cells. Consistent with this, we observed a substantial proliferation of *Sox9*-expressing descendant cells, leading to a significant increase in their numbers within the first 10 days after the initiation of the TX treatment.

Subsequently, these cells originate small clones with a rapid decrease and finally they give rise to few, large, radially oriented clones. The fact that *Sox9* was expressed exclusively in epithelial basal cells, while *Sox9*-expressing descendant cells were observed throughout the entire corneal epithelium indicates that many *Sox9*-expressing descendant cells experience a decline in *Sox9* expression, initiating their differentiation into cells that proliferate, migrate, and differentiate into suprabasal corneal cells, thus contributing to the maintenance of corneal homeostasis.

In this process, we observed that *Sox9* downregulation coincides with the upregulation of several gene pathways implicated in stem cell differentiation, including the *Wnt*-signaling pathway (Yu et al., 2024), ERBB signaling (Hassan and Seno, 2022), the cytokine mediated signaling pathway (Korkaya et al., 2011) and the regulation of ERK1 and ERK2 cascades (Lavoie et al., 2020). Consistent with this, *in vitro* studies have highlighted the pivotal role of *Sox9* as a regulator of proliferation and differentiation in corneal epithelial stem/progenitor cells. Furthermore, increased expression levels of *Sox9* during these processes have been shown to attenuate *Wnt* signaling in the limbal stem cell niche by promoting the degradation of the ß-catenin complex (Menzel-Severing et al., 2018). This is consistent with the well-known notion that *Sox9* acts as an antagonist of the *Wnt* signaling pathway (Sinha et al., 2021; Lao et al., 2022; Barrionuevo et al., 2006; Akiyama et al., 2004). The persistence of some small clones (including a radial stripe) of *LacZ*$^{+}$ *Sox9*$^{\Delta/\Delta}$ cells in the limbal and corneal epithelia of *CAGG-CreERTM;Sox9*$^{flox/flox}$*;R26R-LacZ* mice nevertheless shows that *Sox9* is not absolutely required for contribution of cells to the limbal and ocular epithelium, but that the lack of *Sox9* significantly impairs the cells' ability to compete with wild-type cells.

This competition would explain why, in contrast to the whole mount *LacZ* analysis, *Sox9*-null cells were not detected tissue sections of these eyes. A recent preprint (Rice et al, 2024) describes a study that confirms the stem-clonogenic potential of *Sox9*-positive limbal epithelial cells. That study also reports that conditional deletion of *Sox9* leads to abnormal corneal epithelial differentiation and squamous metaplasia in the central cornea. This phenotype was not observed in our study but is explicable because of the mosaic nature of our model, which may allow for rescue of the normal Wnt and Notch signalling by wild-type cells, preventing transdifferentiation.

We also observed long-lived circumferential clones that never exited the limbus. These latter clones are similar to those produced by OLB cells, as recently shown in lineage-tracing experiments , which predominantly self-renew and minimally contribute, if at all, to the formation of lineages maintaining the corneal epithelium during homeostasis. However, in response to corneal injury, OLB proliferate and play an essential role in wound healing (Altshuler et al., 2021; Farrelly et al., 2021). Consistent with this, we saw that *Sox9* is highly expressed in the OLB cluster. Furthermore, previous studies utilizing an H2B-GFP/K5tTA mouse model have shown that *Sox9* is expressed in a corneal quiescent/slow-cycling cell population (Parfitt et al., 2015) , and wound healing assay using a human corneal organ culture revealed an increased in the number of $SOX9^{+}$ cells in both activated limbal and re-grown corneal epithelial cells (Menzel-Severing et al., 2018). We also observed that the number and extension of the limbal circumferential clones clones diminished in *Sox9*$^{\Delta/\Delta}$-*LacZ* corneas, indicating that *Sox9* may also control the proliferation of OLB stem cells. Altogether, these

observations suggest that *Sox9* may play different roles in the maintenance and homeostasis of limbal stem cells.

MATERIAL AND METHODS

Mouse lines and crosses

To obtain *Sox9* null mutant mice, we crossed *Sox9*$^{flox/flox}$ (*B6.129S7-Sox9*$^{tm2Crm/J}$) (Akiyama et al., 2002) to *CAGG-CreER*^{TM} (*B6.Cg-Tg*$^{(CAG-cre/Esr1*)5Amc}$) (Hayashi and McMahon, 2002) mice, and the resulting double heterozygous offspring was backcrossed to *Sox9*$^{flox/flox}$ mice. Both mouse strains were sourced from The Jackson Laboratory. Mutation occurs after treatment with tamoxifen, as described below. Cre-negative *Sox9*$^{flox/flox}$, tamoxifen-treated mice served as controls. For corneal *Sox9* mosaic analyses we crossed *CAGG-CreER*^{TM}*; Sox9*$^{flox/flox}$ mice *to R26R-LacZ (B6.129S4-Gt(ROSA)26Sor*$^{tm1Sor/J}$*)* (Soriano, 1999), generating *CAGG-CreER*^{TM}*; Sox9*$^{flox/flox}$; *R26R-LacZ* mice. As control we used *CAGG-CreER*^{TM}*; Sox9*$^{flox/+}$; *R26R-LacZ* mice. For genetic lineage tracing assays, we used *Sox9*$^{IRES-CreERT2}$ (*B6.129S7-Sox9*$^{tm1(cre/ERT2)Haak}$) (Soeda et al., 2010) mice obtained from the RIKEN BioResource Research Center (Tsukuba, Japan). These mice were crossed with either *R26R-LacZ* (*B6.129S4-Gt(ROSA)26Sor*$^{tm1Sor/J}$) (Soriano, 1999) or *R26R-EYFP* (*B6.129X1Gt(ROSA)26Sor*$^{tm1(EYFP)Cos/J}$) (Srinivas et al., 2001) reporter mice, resulting in double heterozygotes. Both of them were provided by The Jackson Laboratory (Bar Harbor, ME). All the experiments were performed with adult mice (2 months old). Mice were housed under Specific Pathogen-Free (SPF) conditions in the animal facilities of the Center for Biomedical Research (University of Granada, Granada, Spain). The

animals had *ad libitum* access to food and water and were kept in groups. The occupancy density of the cages (microventilated) was in accordance with legal requirements. The cages were kept at a temperature of 22 +/- 2°C, a humidity of 55 +/- 10%, and a 12-hour/12-hour dark light cycle. Mice were provided with activity elements in the form of nest building material and hiding places. The animal procedures and housing conditions were approved by the University of Granada Ethics Committee for Animal Experimentation and the Consejería de Agricultura, Ganadería, Pesca y Desarrollo Sostenible of the Andalussian government, Junta de Andalucía (reference 12/12/2016/177).

Tamoxifen supplementation

For conditional deletion of *Sox9*, *CAGG-CreERTM;Sox9*$^{flox/flox}$ mice were fed a standard diet (Harlan, 2914) supplemented with tamoxifen (TX; Sigma-Aldrich, St. Louis, MO, C8267) at a concetration of 40 mg TX/100 g diet for 10 days. For short-term tracing of *Sox9*-descendant cells (less than 10 days), TX (Sigma-Aldrich, St. Louis, MO, T5648) was dissolved in corn oil at a concentration of 30 mg/ml and administered at a dose of 0.2 mg per gram of body weight through intraperitoneal injection to *Sox9*$^{IRES-CreERT2}$ mice. For long-term tracing of *Sox9*-positive cells (more than 10 days), *Sox9*$^{IRES-CreERT2}$ mice were administered TX as detailed above for the conditional deletion analyses .

Estimation of the percentage of tamoxifen-induced, Cre-mediated recombination

To estimate the percentage of cells undergoing Cre-mediated recombination in TX-treated *CAGG-CreERTM;Sox9*$^{flox/flox}$ mice, the total number of SOX9+ cells was divided

by the total number of SOX8+ cells in a 20x microphotograph of a retinal section stained for SOX9 and SOX8.

Histology and immunofluorescence

Eyes were dissected out and the crystalline lenses removed in order to facilitate histological sectioning. The eyes were fixed in 4% paraformaldehyde (PFA), dehydrated in increasing EtOH/saline solutions (EtOH + 0.9% Nacl), embedded in paraffin, sectioned, and either stained with hematoxylin-eosin or processed for protein immunodetection. For simple and double immunofluorescence, sections were incubated overnight with the primary antibodies, washed, incubated with the appropriate conjugated secondary antibodies for 1 h at room temperature, and counterstained with 40,6-diamidino-2-phenylindole (DAPI). Several sections of eyes from control and mutant mice were consistently mounted on the same slide and processed together. Parallel negative controls were performed in which the primary antibody was omitted. The following antibodies were used: anti-SOX8 (1/500), kindly provided by Dr. Wegner at the Universität Erlangen-Nurnberg (Germany); anti-OPN1SW (1/200, Santa Cruz, sc-14363); anti-OPN1LW (1/500, Chemicon, AB5405); anti-RHO (1/500, Sigma, O4886); anti-BRN3α (1/100, Chemicon, MAB1585); anti-PAX6 (1/40), anti-AP2α (1/30, kindly provided by the Developmental Studies Hybridoma Bank at the University of Iowa), anti-SOX9 (1/400, Millipore, AB5535), anti-GFP (1/100, Novus Biologicals, NB600-308), anti-p63 (1/500, Master Diagnostica MAD-000479QD).

The two eyes of each mouse were analyzed in all experiments, and there were no notable differences between them (left-right) either histologically or in the expression

pattern of the markers studied. Similarly, we found no differences between the eyes of control mice regardless of whether they were treated with TX or not.

Analysis of retinal and corneal cell death

Retinal and corneal cell death was assessed using the TUNEL Fluorescent In Situ Cell Death Detection Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions.

Retinal flat whole-mount immunofluorescence

Eyes were fixed in 4% PFA and retinas were dissected in TBS containing 0.05% Tween-20 and 0.1% Triton X-100 for 30 minutes at room temperature (RT). Retinas were then washed in TBS with 0.05% Tween-20 (TBS-T) and exposed to primary antibodies diluted PBS with 4% of BSA overnight at 4°C. After washing with TBS-T, the retinas were incubated with secondary antibodies diluted in PBS with 4%BSA for 3 hours at RT. Finally, the retinas were mounted flat for confocal microscopy.

Whole-mount X-gal staining

The eyes were dissected and cut transversely to facilitate solution penetration and whole-mount β-galactosidase staining using X-gal as a substrate. Whole-mount X-gal staining was performed overnight as described by Hogan et al. (1995). After staining, eyes were fixed in 4% paraformaldehyde.

Estimation of the percentage of corneal surface area stained with Xgal

The surface area was calculated using the FIJI (V2.1.0) program based on ImageJ. The entire cornea surface was outlined, and the areas of the X-gal stripes were

manually selected and marked. Then, the percentage of the X-gal marked area relative to the total corneal area was calculated. An automatic threshold could not be applied due to variations in brightness across the cornea.

Imaging

Histological and immunofluorescence images were photographed using a DS-Fi1c camera installed on a Nikon Eclipse Ti microscope (Japan). Retinal flat mount images were obtained using a high-speed spectral confocal microscope Nikon A1 ASHS-1. Subsequently, GIMP2 was used to adjust the color levels and the contrast of the immunofluorescence images. Whole-mount X-gal-stained eyes were photographed using a DP70 digital camera mounted on an Olympus SZX12 stereomicroscope (Japan).

Single-cell RNA-sequencing dataset analyses

We used the single-cell RNA-sequencing datasets of isolated epithelial cells from the limbus (with marginal conjunctiva and corneal periphery) generated by Altshuler and colleagues (Altshuler et al., 2021; Gene Expression Omnibus GSE167992). Bioinformatic analyses were performed with Seurat (version 5.0.3) (Hao et al., 2021) following the author guidelines (https://satijalab.org/seurat/). Cells with unusually high and low numbers of unique feature counts or high mitochondrial counts were filtered out. The data were normalized and subsequently scaled. RunPCA was used for principal component analysis, and cells were clustered at a resolution of 0.6. Uniform manifold approximation and projection was used for nonlinear dimensional reduction. Cluster-specific markers were selected with a minimum average log fold change threshold of 0.25 among genes that were expressed in a minimum of 25% of the cells.

Cluster identity was assigned using the following markers: Conjunctiva: *Krt17, Krt4, Krt19, Krt6a, Krt13, Krt8* (and lack of *Krt12, Slurp1, Ppp1r3c*)*;* cornea: *Krt12, Ppp1r3c, Slurp;* outer limbus: *Cd63, Ifitm3, Gpha2;* inner limbus: *Atf3, Socs3, Mt1;* basal cells: *Itgb1, Itgb4, Ccnd1;* supra-basal cells: *Cldn4, Cdkn1a, Dsg1a;* and mitosis: *Mki67, Top2a, Ccna2.* For differential expression of cells expressing high and low levels of *Sox9*, we selected cells with high *Sox9*-expression levels (>= 0.7) and cells with low *Sox9*-expression levels (between 0.7 and 0.15) with the WhichCells and the SetIdent functions of the Seurat package. Differential expression analysis was performed with the FindMarkers function (min.pct = 0.20, logfc.threshold = 0.01). For trajectory analysis we used the Python scampy suite (Haghverdi et al., 2016; Wolf et al., 2018) following the general PAGA trajectory inference workflow (https://scanpy.readthedocs.io/en/stable/tutorials/trajectories/paga-paul15.html) with a Leiden resolution of 0.6. Cluster identity was assigned as described above, and the conjunctiva cluster was removed. For pseudotime analysis (Wolf et al., 2019), the OLB cluster was chosen as the root. Gene Ontology analyses were done with the enrichGO and compareCluster functions of the clusterProfiler R package (Yu et al., 2012).

Statistical analysis

We analyzed datasets derived from distinct experimental conditions using appropriate statistical tests based on preliminary assessments of normality and variance equality. When these conditions were not met, non-parametric approaches were favored for their robustness to data distribution assumptions. Specifically, we employed the Kruskal-Wallis rank-sum test to identify overall differences among group distributions followed by post-hoc Dunn's multiple comparison tests with Bonferroni adjustment to pinpoint

specific pairwise comparisons that contributed to these differences. When no significant departures from normality or equality of variances were detected, parametric methods were deemed suitable. So, we conducted a standard Student *t*-test to evaluate the hypothesis of equal means between groups. Complete information on these statistical analyses are provided in Supplementary Tables S2, S3 and S6.

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