Sox9 marks limbal stem cells and is required for asymmetric cell fate switch in the corneal epithelium

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Abstract

Adult tissues with high cellular turnover require a balance between stem cell renewal and differentiation, yet the mechanisms underlying this equilibrium are unclear. The cornea exhibits a polarized lateral flow of progenitors from the peripheral stem cell niche to the center; attributed to differences in cellular fate. To identify genes that are critical for regulating the asymmetric fates of limbal stem cells and their transient amplified progeny in the central cornea, we utilized an in vivo cell cycle reporter to isolate proliferating basal cells across the anterior ocular surface epithelium and perform single-cell transcriptional analysis. This strategy greatly increased the resolution and revealed distinct basal cell identities with unique expression profiles of structural genes and transcription factors. We focused on Sox9; a transcription factor implicated in stem cell regulation across various organs. Sox9 was found to be differentially expressed between limbal stem cells and their progeny in the central corneal. Lineage tracing analysis confirmed that Sox9 marks long-lived limbal stem cells and conditional deletion led to abnormal differentiation and squamous metaplasia in the central cornea. These data suggest a requirement for Sox9 to brake cell division symmetry as transient cells exit the limbal niche. By inhibiting terminal differentiation of corneal progenitors, forcing them into perpetual symmetric divisions, we replicated the Sox9 loss-of-function phenotype. Our findings reveal an essential role for Sox9 for the spatial regulation of asymmetric fate in the corneal epithelium that is required to sustain tissue homeostasis.

Introduction

The corneal epithelium is a stratified, non-keratinizing tissue that serves as an external barrier to protect the eye against environmental insults, similar to the barrier function attributed to the skin epidermis. Like all stratified epithelia, the cornea undergoes constant cellular turnover due to terminal differentiation of cells, that commences with their departure from the proliferative basal layer and ends with their desquamation and shedding from the tissue surface (Creamer et al., 1961; Haddad, 2000; Halprin, 1972; Potten et al., 1987). To match this constant cellular loss and maintain homeostasis, the tissue needs a continuous supply of new cells, which are produced by proliferating stem cells and their immediate progeny (Cotsarelis et al., 1989; Schermer et al., 1986; Schlötzer-Schrehardt & Kruse, 2005).

In vivo lineage tracing analyses have demonstrated that long-lived limbal stem cells (LSCs), located in the periphery of the tissue, give rise to transient amplified cells (TACs), which exit the niche and grow centripetally over several weeks before reaching the central cornea (Di Girolamo et al. 2015, Dora et al. 2015, Lobo, et al. 2016, Nasser et al. 2018, Park et al, 2019). By tracking individual cells in the mouse cornea, using two-photon live imaging, we previously performed quantitative clonal analysis which revealed the spatiotemporal organization of cell fates across the entire corneal epithelium (Farrelly et al. 2021). LSCs were found to undergo mostly symmetric cell divisions to expand their progeny. Subsequently, these short-lived TACs divided in an increasingly asymmetric manner and their probability of undergoing terminal differentiation increased as they moved closer to the center of the cornea. Despite this continuous flux of progenitor cells (peripheral/basal-to-central/basal and basal-to-suprabasal) the tissue exhibits a remarkable ability to maintain a stable number of cells and organization to support its barrier function. While this phenomenon suggests a mechanism that instructs cells to undergo a specific mode of cell division (symmetric vs. asymmetric) depending on their precise location across the ocular epithelium, the genes involved in such regulatory network remain elusive.

A major challenge to resolve the underlying mechanism of cellular fate is the dynamic nature of high turnover tissues, like the cornea, which is not sufficiently resolved by static experimental approaches. This is particularly important when studying the regulation of stem cell and progenitor populations which constitute only a minor fraction of the total cellular content in a

given tissue. Recent advancements in single-cell genomics have revealed a previously underappreciated heterogeneity among LSCs that may be critical for maintenance and repair within the ocular surface (Altshuler et al., 2021; Català et al., 2021; Collin et al., 2021; Dou et al., 2021; Kaplan et al., 2019; D. Q. Li et al., 2021; J. M. Li et al., 2021). These studies have not only challenged the conventional paradigm of a homogenous stem cell population but have also led to the identification of specific markers, such as GPHA2, that distinguish between quiescent and actively dividing LSC subsets (Altshuler et al., 2021, Collin et al., 2021). This highlights the intricate regulatory mechanisms governing LSC activity, which can enable novel regenerative medicine strategies to treat diseases of the ocular surface. However, discrepancies in the expression of new, as well as established markers across the different studies underscore the complex interplay between LSCs, their progeny and their microenvironment, necessitating higher resolution genomic approaches for clearer delineation.

Advanced imaging techniques and cell-marking strategies are pivotal in this endeavor, as they allow for the direct observation of cellular dynamics in vivo. This capability is essential for distinguishing between symmetric and asymmetric cell fates and for assessing their respective contributions to tissue architecture and function. Consequently, the need to effectively visualize and capture cell proliferation in real-time, in vivo, within these dynamic systems stands as a critical step toward our understanding of diseases that afflict the ocular surface, which largely are characterized by abnormal cell proliferation and/or differentiation. The quest to elucidate the dynamics of stem cells and their transiently amplified progeny has long posed a challenge due to the difficulty in isolating pure populations of proliferating cells from high-turnover tissues. In vivo reporter models have enabled the observation and capturing of actively cycling cells, offering novel insights into the regulation of cell fate during cell division (Klochendler et al., 2012). These innovative tools address the limitations of static and post-mortem analyses, providing a dynamic view of cell-cycle processes and their consequences for tissue function.

Here, building on recent advances in genomics and mouse genetics, we ventured to identify the genes and regulatory networks that control the asymmetry of cell fate in the ocular surface epithelium. Using live imaging and single cell transcriptomic analysis, we identified Sox9 as a key transcription factor that is preferentially expressed in LSCs and demonstrate its necessity

for proper regulation of asymmetric cell fate, which is critical to maintain corneal epithelial homeostasis.

Results

A high-fidelity reporter for analyzing cell cycle dynamics in the live cornea.

Since LSCs and their progenitors (TACs) represent only a fraction of cells that make up the epithelium, we sought to enrich for these populations to interrogate the mechanisms which regulate the mode of cell division and therefore their fate. To do this, we implemented a dynamic in vivo reporter mouse model (CycB1-GFP) that enabled us to visualize the activity of actively cycling cells in the corneas of live mice (**Fig. 1A**). CycB1-GFP transgenic animals express an enhanced green fluorescent protein (EGFP) that is fused to the N-terminus of the Cyclin B1 (Ccnb1) gene (Klochendler et al., 2012). Since the N-terminus is targeted for degradation in a cell-cycle dependent manner, only cells that are actively cycling (S-G2-M stages) express the CycB1-GFP protein.

We performed time-lapse imaging in the corneas of live mice to validate reporter dynamics in actively cycling cells. We confirmed that only cells in the basal layer were GFP+ as only basal-layer cells in the corneal epithelium are capable of dividing (**Fig. 1B-D; Movie S1**). Moreover, by real-time imaging we visualized cells in the live cornea undergoing mitoses, which validated the fast kinetics of the in vivo cell cycle reporter (**Fig. 1C; Movie S2**). To further test the fidelity of the reporter we treated mouse corneal with 12-O-Tetradecanoylphorbol 13-acetate (TPA), which has a strong mitogenic effect on epithelial tissues (Taketani et al, 1983). Only one day after treatment we saw massive increases in the number of GFP+ cells across the ocular epithelium, but more pronounced in the limbus and central cornea (**Fig. 1E**). After six days of treatment the number of GFP+ cells returned to normal levels, comparable to those in untreated eyes. Taken together, we demonstrate here a powerful tool for visualizing cell proliferation dynamics in a living eye, and the ability to capture aberrations in response to physiological and pathological stimuli.

To capture possible imbalances in the rate of cell proliferation between the epithelial compartments, we performed image analysis to quantify the number of GFP+ cells in the different areas of the ocular surface epithelium. Our data showed that actively cycling stem cells

and progenitors are distributed uniformly within the basal layer of the limbus and cornea (**Fig. 1F-H**). This suggests that the rate of proliferation alone, does not impose a growth pressure between these compartments. To this point, from all our time-lapse imaging data from live corneas of CycB1-GFP mice, we observed that all cell divisions are planar (**Fig. 1C, Movie S2**), with both daughter cells separating within the basal layer. This implies that the orientation of the mitotic spindle is likely not the mechanism by which corneal epithelial cells regulate their asymmetric fate and terminal differentiation. We then asked if the orientation of the mitoses, in respect to the radial axis connecting the limbus to the center of the cornea plays a role in the centripetal flux of progenitors within the basal layer. Our analysis showed that even though there were measurable biases for specific angles, on average cell divisions in the limbus and cornea were oblique to the radial axis of the cornea (**Fig. 1I)**. From these data, we infer that the mechanism of mitosis does not directly determine the fate of LSCs and TACs in the cornea.

Single-cell transcriptomic analysis of actively cycling cells identifies key transcription factors in distinct progenitor populations in the ocular surface epithelium.

To develop a greater understanding of the genes and molecular pathways that regulate these cells, we harvested corneas from adult mice and dissociated the corneal epithelium. Corneal epithelial cells were then sorted by Fluorescence Activated Cell Sorting (FACS), and only GFP+ cells were sorted for (**Fig. 2A**). Cell filtering and unbiased clustering (R package Seurat (Hao et al., 2023) defined 8 cell populations (**Fig. B, C**). To verify the faithfulness with which we capture cycling cells from our CycB1-GFP reporter animals based on GFP expression, we performed cell cycle analysis on our single cell dataset (**Fig. S1**). The vast majority (~88%) of cells within our dataset exhibited a signature of cell cycle markers indicative of S and G2/M phases (R package Seurat (Kowalczyk et al., 2015)) and expressed cell proliferation genes (**Fig. S1**). Furthermore, the high expression of basal layer genes (Krt14, Krt5, Trp63) (Chen et al., 2010; Pellegrini et al., 2001) coupled with the lack or low expression of suprabasal markers (Cld4, Dsg1a) (King et al., 1997; Y. Yoshida et al., 2009) confirms that our single cell dataset primarily consists of cycling cells from the basal layer of the corneal, limbal, and conjunctival epithelium (**Fig. S1**). Direct comparison with published single-cell RNA sequencing datasets, of mouse corneal epithelial cells highlighted our ability to specifically interrogate LSCs and TACs, with high resolution.

Unique cell clusters were classified on the basis of gene expression, which revealed clusters of conjunctival (1) limbal (1), corneal (5) and a minor immune cell (1) cluster. Conjunctival cells were primarily identified based on the expression of Keratin 13 (Krt13), Keratin 19, and Keratin 4 (Altshuler et al., 2021; Kasper et al., 1988), as well as the lack or low expression of corneal and limbal markers Keratin 12 (Krt12) (Kasper et al., 1988; Kurpakus et al., 1994; Liu et al., 1993), Aldehyde Dehydrogenase 3 Family Member A1 (Aldh3a1), and Keratin 15 (Krt15) and Glycoprotein Hormone Subunit Alpha 2 (Gpha2) (Altshuler et al., 2021), respectively (**Fig. B, Fig. S1**). Similarly, the limbal cluster was defined on the basis of Krt15 and Gdpha2 expression (Altshuler et al., 2021; S. Yoshida et al., 2006) and the lack, or lower expression, of both conjunctival and corneal markers (**Fig. B, Fig. S1)**. The corneal-specific Keratin 12 (Krt12), was detectable in 5 distinct clusters (**Fig. B, Fig. S1**).

The smallest of those clusters displayed hallmarks of the previously described compound corneal niches, such as expression of Krt12, Krt19 and Mucin genes (Pajoohesh et al., 2013). These compound niches were shown to give rise to goblet cells in the corneal epithelium that are distinct from those in the conjunctiva and play a role in the pathology of limbal stem cell deficiency. Other unique gene, associated with this cluster are Krt6b, Krt78, Mal, Nccrp1, Plac8, Psca and the transcription factor Hopx (**Fig S1**). Another distinct cell cluster within the cornea supergroup was characterize by the expression of Urah, a gene which was previously described to be highly expressed in a group of distinct corneal progenitors proposed to play a role in “adaptive regeneration” (Lin et al, PNAS 2023) (**Fig. 2B)**. Similarly, this cluster expresses Fabp5, Sparc and Krt16, consistent with previous reports. In addition to K12 progenitor cells in all the corneal clusters express high levels of Tkt, Slurp1, Mgarp and Aqp5 (**Fig. S1**).

To investigate the relationships between our identified cell clusters and resolve the differentiation trajectory of cells that maintain the corneal epithelium we performed trajectory inference analysis using the Monocle 3 workflow. A clear trajectory emanating from the limbal cluster progressed through three of the corneal groups, suggesting that these represent consecutive stages of terminal differentiation of the TACs that emerge from the limbus to commence their centripetal flow (**Fig. 2D**). The trajectory from the Limbal cluster also showed distinct branching points towards the “goblet niche” and “adaptive” corneal clusters. Next, we asked what are the key transcriptional regulators of the distinct cellular identities, found within

the stem and progenitor cell pools of the corneal epithelium. For that, we performed differential expression analysis of genes that encode for transcription factors. Our data showed that LSCs express high levels of Sox9, Sox4, Barx2, Nfib1 and Osr2 (**Fig. 2E)**. Consequently, expression of these genes decreases as the TACs exit the niche and in turn they upregulate expression of Klf4, Fos, Hif, Jun, Junb and Klf3 (**Fig. 2E)**. While the five corneal clusters showed similar expression of the same set of transcription factors that were unique compared to the limbal cells, the "goblet niche" progenitors additionally expressed high levels of Elf3, Mxd1 and Zbtb7a. Expression of all these transcription factors waned in the last corneal cluster suggesting that this group may be the one closest to terminal differentiation as also indicated by the trajectory analysis.

To address our original question to identify the key genes that regulate the switch from a symmetric cell fate of LSCs to an asymmetric one in the TACs, we focused on Sox9, one of the transcription factors that were found to be differentially expressed between the limbal and the corneal clusters (**Fig 2E, F**). For this we performed differential gene expression analysis, comparing the progenitors with high and low expression of Sox9. We confirmed that Sox9-high cells express all the typical limbal markers including Gpha2 and Krt15 (**Fig. 2G)**. Other highly expressed genes within the Sox9-high population are Wfdc18, Tppp3, Tmem17a, Tmem17b, Igfbp2 and Lgals7 (**Fig. 2G)**.

Identification of the transcription factor Sox9 as a putative limbal stem cell marker.

The transcription factor Sox9 has long been known for its role as a regulator of stem and progenitor cell activity in other ectodermally-derived appendages (Kadaja et al., 2014; Poché et al., 2008; Scott et al., 2010; Vidal et al., 2005). Given the many parallels in gene expression patterns between the cornea and the hair follicle, where Sox9 has an established role in regulating hair follicle stem cell fate, we sought to explore the expression of Sox9 within our single cell dataset. Within our dataset, Sox9 expression was highest within the limbal cluster (**Fig. 2F, G**). Although transcript levels are not necessarily indicative of functional activity, we predicted that Sox9 may be functionally more important in LSCs than in corneal epithelial cells.

To interrogate the role of Sox9 in homeostatic maintenance of the corneal epithelium, we utilized a two-photon-microscopy-based system to visualize whole eyes in live mice without compromising the structural integrity of the tissue (Farrelly et al., 2021). To validate Sox9 expression within the murine cornea in vivo, we imaged the corneas of adult Sox9IRES-EGFP (Sox9-EGFP) mice (Chan et al., 2011), which express EGFP from the endogenous Sox9 locus (**Fig. 3A, B**). In these animals, GFP expression is almost exclusively restricted to the limbal compartment. Sox9-EGFP levels have been previously shown to correlate with endogenous Sox9 levels (Formeister et al., 2009). Here, we observe Sox9-EGFP is expressed at two steady-state levels whereby Sox9 activity is highest in LSCs residing in the niche and lowest in progenitors that have exited the niche (**Fig, 3B**).

We next combined intravital imaging with a Cre-recombinase-based in vivo lineage tracing strategy to resolve the activity and stemness potential of Sox9-expressing limbal cells in corneal homeostasis by longitudinal live imaging. These mice express a tamoxifen-inducible Cre recombinase from the endogenous Sox9 locus and a ROSA26LoxP-STOP-LoxP-tdTomato (R26-tdTom) Cre-reporter (Sox9CreERT2;tdTom) (**Fig. 3A**). To directly track the activity of Sox9CreERT2;tdTom lineage-traced cells, we acquired high resolution, full-thickness serial optical sections of the whole eye, including the limbus and central cornea, and then reimaged the same eyes longitudinally with the same acquisition parameters (**Fig. 3A, C**). By 1-month post-induction, we observed the emergence of long-lived lineages emanating from the limbus (**Fig. 3A**). Given that these lineages can only be sustained by LSCs (Amitai-Lange et al., 2015; Di Girolamo et al., 2015; Dorà et al., 2015), we concluded that Sox9 marks a population of LSCs as evidenced by their ability to contribute to long-term corneal homeostasis.

Conditional deletion of Sox9 disrupts corneal epithelial homeostasis.

To explore the tissue-specific role of Sox9 within the corneal epithelium, we implemented an inducible conditional gene targeting strategy (**Fig. 4A**). Sox9 floxed mice (Akiyama et al., 2002) were crossed to p63CreERT2 reporter animals (p63CreERT2;tdTom) to selectively delete Sox9 from basal-layer epithelial cells, the only cells that retain proliferative capacity and stemness potential (Cotsarelis et al., 1989), in adult corneas. By 2 months post induction (MPI), Sox9-deleted corneas developed hyperplastic growths of varying size and severity at the central cornea, while the epithelium at the periphery remained normal (**Fig. 4B, D**). Histologically, Sox9-

deleted corneas displayed extensive thickening and keratinization in the epithelium compared to control animals (**Fig. 4C/C', E/E', F-G**). Furthermore, the formation of these keratinized plaques always coincided with stromal thickening throughout the tissue (**Fig. 4H**). Altogether, about two-thirds of Sox9-deleted corneas (14/21) presented with moderate to severe corneal thickening compared to control corneas (0/25) (**Fig. 4I**). Based on these observations, we hypothesized Sox9 may play an important role in regulating the integrity of the corneal epithelium. To begin characterizing our phenotype, we first validated the efficiency of our floxed system by staining for Sox9 (Figure 4.5). In control eyes, nuclear Sox9 staining could be observed in basal layer cells within the limbus, whereas at the central cornea, Sox9 staining could also be observed in the suprabasal layers (**Fig. S2A/A', C/C'**). In Sox9-deleted animals, Sox9 was not detected in either the limbus or central cornea, indicating efficient deletion of the Sox9 gene (**Fig. S2B/B', D/D**'). Furthermore, we validated the epithelial specificity of our system by staining for Sox9 in retinal Müller glial cells, which are not of epithelial origin (**Fig. S2E/E', F/F'**) (Poché et al., 2008).

In stratified epithelia, like the epidermis and corneal epithelium, keratins exhibit unique expression patterns that are regulated by the differentiation program of the tissue (Fuchs & Green, 1980; Kasper et al., 1988; Kurpakus et al., 1994). Keratin 14 (K14) is a classic marker of basal-layer keratinocytes, the proliferative layer of the epithelium (Coulombe et al., 1993; Moll et al., 1982). Unlike control corneas, which exhibited a single K14+ basal layer, Sox9-deleted corneas displayed an expanded K14 layer in which K14 could be detected ectopically in the suprabasal and superficial layers of the epithelium (**Fig. S3A-D'**). Unlike the epidermis of the skin, the corneal epithelium is non-keratinized and mostly defined by the expression of Keratin 12 (K12) (Liu et al., 1993). In control animals, K12 expression can be detected throughout the suprabasal and superficial layers of the epithelium, however, it is not expressed within the limbus. In Sox9-deleted corneas, K12 is noticeably absent within the plaques, however, its expression can still be detected in the periphery of the corneal epithelium outside the plaques while its expression at the periphery is comparable to control eyes (**Fig. S3E-H'**). As K12 expression is unique to the corneal epithelium, its absence within the corneal plaques indicated squamous metaplasia due to a loss of corneal identity or cell fate switch.

Histologically, Sox9-deleted corneas displayed evidence of a keratinized surface, which had a striking resemblance to the stratum corneum of the epidermis (**Fig. S4**). Staining for Loricrin

(Lor), a major structural protein of the epidermal cornified cell envelope, confirmed our suspicion that Sox9-deleted corneas were starting to take on an epidermal-like fate (**Fig. S4**). Within Sox9-deleted corneas that displayed severe thickening and plaque growth (n = 5 corneas [4 mice]), Loricrin could be detected within the plaque growths, but not at the corneal periphery or limbus. This is consistent with the observation that K12 expression was properly patterned within Sox9-deleted corneas outside of the plaques. These observations suggested that Sox9 plays an important role in regulating corneal epithelial cell fate and identity as its loss resulted in a corneal-to-epidermal cell fate switch.

Loss of Sox9 induces aberrant proliferation in the corneal epithelium.

Within the corneal epithelium, cell fate decisions are largely influenced by the position of the cell within the tissue. At the limbus, LSCs divide with fairly low frequency. As they divide, these stem cells will self-renew, to maintain the stem cell pool, and give rise to differentiating progeny. As these progeny exit the niche, they begin to proliferate with increasing frequency giving rise to progenitors that will either terminally differentiate or divide, giving rise to daughter cells with asymmetric fates where one terminally differentiates and the other is retained as a progenitor with proliferative capacity (Altshuler et al., 2021; Farrelly et al., 2021). Compared to controls, Sox9-deleted corneas with severe plaque development showed extensive hyperproliferation (**Fig. S5A-D'**). Staining for KI67 revealed almost all basal cells, as well as cells in the suprabasal layers, were actively proliferating throughout the limbus and corneal epithelium compared to controls. We postulated that the increased proliferative activity throughout the corneal epithelium in the absence of Sox9 explains, at least in part, the observed thickening of the epithelium.

Maintenance of the corneal epithelium requires a tightly regulated balance between proliferation, differentiation, and migration. Given that Sox9-deleted corneas displayed extensive hyperproliferation, we wanted to further investigate how differentiation and cell migration might also be implicated. To assess changes in differentiation, we administered the nucleotide analog, 5-Bromo-2'-deoxyuridine (BrdU), to control and Sox9 conditional knockout (cKO) animals 2 weeks after inducing floxed deletion of Sox9 and collected eyes after 72 hours. While we did not observe a significant difference in the number of BrdU+ cells in the basal layer at the peripheral cornea (~limbus), the number of BrdU+ cells in the suprabasal layers were trending towards significance ($p < 0.0882$) (**Fig. S5E**). At the central cornea, there were significantly more BrdU+

cells in the suprabasal layers of Sox9-deleted corneas compared to controls ($p < 0.0009$) while in the basal layer Brdu+ cell counts were trending towards significance in Sox9-deleted corneas compared to controls ($p < 0.1008$) (**Fig. S5B**). These observations suggested that a greater number of cells in Sox9-deleted corneas were proliferating, expanding their numbers and delaminating as compared to controls.

Inhibition of terminal differentiation in corneal TACs phenocopies the loss of Sox9

To this end we discovered evidence that the transcription factor Sox9 is preferentially highly expressed in LSCs and that conditional ablation of Sox9 from the basal layer leads to loss of homeostasis and ultimately to squamous metaplasia in the central cornea. This presents a conundrum since loss of Sox9 was predicted to affect LSCs the most due to its high expression within this population. Instead, the most prominent phenotype of Sox9 cKO mice is the aberrant growths developing at the center of the cornea. To explain this phenomenon, we drew from our previous observations which showed a switch from mostly symmetrical cell divisions in the limbus to increasingly asymmetrical divisions and propensity for terminal differentiation in the central cornea (Farrelly et al. 2021). Based on this we hypothesized that the transition from Sox9-high to Sox9-low expression as the TACs exit the limbal niche is required for their normal differentiation. Based on this, the prediction is that loss of Sox9 deprives TACs from an essential switch to an asymmetric cell fate, without which they continue to divide symmetrically. Consequently, the unchecked centripetal expansion of TACs leads to cell crowding at the center of the cornea.

To test this hypothesis , we developed an inducible dual Cre and Tet system that would enable us to suppress differentiation (Differentiation-OFF) in single clones that we could visualize with a co-dependent H2BGFP reporter and track their behavior over time by live imaging (**Fig. 5**) (Farrelly et al., 2019). The premise of this genetic system is that active Notch signaling is required for the commitment to terminal differentiation of progenitors in stratified epithelia. Therefore, by enforcing total inhibition of canonical Notch, using the dominant negative MAML1 allele (Differentiation-OFF), we predicted that cells would be locked in a symmetric fate and undergo perpetual self-renewal. We found that while H2BGFP labelled clones in control corneas

could be observed differentiating normally and moving from the basal to the suprabasal layers of the epithelium over time, labelled clones in “Differentiation-OFF” corneas remained in the basal layer (**Fig. 5B**). To further validate that suppression of asymmetric cell fate and differentiation of TACs leads to unchecked self-renewal we performed quantitative clonal analysis. As expected, basal clones from normal corneas decayed over time, consistent with the short-lived nature of corneal TACs (**Fig. 5C**). In contrast, “Differentiation-OFF” clones continued to grow. While delamination of “Differentiation-OFF” cells was predictably suppressed for many days, it eventually started increasing after several days. This is likely due to crowding of cells which compete for space leading to cell extrusion from the basal layer (**Fig. 5B** see right panel, **Movie S3**).

Next, we performed live imaging of the central corneas from mice in which we clonally suppressed differentiation of TACs to test if that impacts their centripetal growth. Despite being unable to differentiate, “Differentiation-OFF” clones retained their ability to clonally expand and transit centripetally, leading to increasing crowding in the central cornea (**Fig. 5D**; **Movie S4**). Moreover, several weeks after induction we observed plaques developing in the central cornea that phenocopied those we previously saw in the Sox9 cKO mice. Altogether, these data provide support for a model whereby plaque growths in Sox9-deleted corneas are the result of TACs failure to switch to an asymmetric cell fate (cells trapped in basal cell identity), leading to their unchecked growth and crowding at the central cornea (**Fig. 6**). Therefore, Sox9 is a key transcription factor that regulates progenitor cell fate and is required to maintain corneal epithelial homeostasis.

Discussion

The corneal epithelium is a self-renewing tissue that is maintained by a population of tissue-resident stem cells (Cotsarelis et al., 1989). However, the molecular mechanisms which regulate the activity of LSCs and their progenitors are not fully known. The limbal niche is the specialized microenvironment in the limbus which regulates LSC function (H. S. Dua et al., 2005; Grieve et al., 2015; Tseng et al., 2016). As stem cells from the limbus divide symmetrically they give rise to TACs, which migrate out from the limbal niche and transit

centripetally towards the central cornea where they are eventually shed and washed away in the tear film (Lobo, et al. 2016, Nasser et al. 2018, Park et al, 2019, Farrelly, et al. 2021). This coordinated activity is achieved through a combination of cues from the niche which include niche factors like extracellular matrix, corneal nerve fibers, and coordinated signaling of various pathways (Gesteira et al., 2017; W. Li et al., 2007; Mei et al., 2012; Xu et al., 2023). Amongst these signaling pathways, several transcription factors have been shown to play a critical role in LSC maintenance and function (Bhattacharya et al., 2019; González et al., 2019; G. Li et al., 2015; Lu et al., 2012; Nakatsu et al., 2011; Sartaj et al., 2017; Shwartz et al., 2020; Vauclair et al., 2007). Our results in this study identified the transcription factor Sox9 as a putative marker of LSCs. Based on our single cell dataset and lineage tracing experiments, we determined that Sox9 is also expressed within the conjunctiva, a vascularized mucosal epithelium that is physically separated from the corneal epithelium via the limbus (Wei et al., 1996). It is well-established that the conjunctiva does not contribute to homeostatic maintenance of the corneal epithelium (S. H. Dua, 1998; Kruse et al., 1990; Shapiro et al., 1981; Wei et al., 1993, 1996). Thus, we had no reason to believe that any of our Sox9 lineage-traced cells were of conjunctival origin.

The transcription factor Sox9 has been reported to be essential for cell proliferation and lineage commitment of adult human corneal epithelial stem cells in ex vivo cultures (Menzel-Severing et al., 2018). Sox9 is known to have a critical role in the maintenance of several adult tissues (Blache et al., 2004; Furuyama et al., 2011; Poché et al., 2008). Functionally, Sox9 has been described as a regulator of stem/progenitor cell maintenance and as having a role in cell fate specification (Aoki et al., 2003; Bastide et al., 2007; Kadaja et al., 2014; Passeron et al., 2007; Scott et al., 2010). However, the role that Sox9 plays in regulating corneal stem cell activity and fate in vivo was not previously addressed. By conditional deletion of Sox9 in basal epithelial progenitors, we showed that in the absence of Sox9, the corneal epithelium becomes hyperproliferative, which suggests Sox9 primarily functions as a suppressor of symmetric cell division in the epithelium. Furthermore, the loss of Sox9 results in improper differentiation and keratinization of the corneal epithelium leading to the development of squamous metaplasia in the central cornea.

Previously, cKOs of Notch1 from the corneal epithelium were reported to develop a similar epidermal cell fate switch (Movahedan et al., 2013; Vauclair et al., 2007). In addition to the epidermal-like plaques, these animals had also displayed swollen eyelids. Thus, meibomian gland dysfunction was thought to be a contributing factor, at least indirectly, to the epidermal cell fate switch. While meibomian glands in Notch1-deleted corneas developed into cyst-like structures and were devoid of meibum, the lipid secretion produced by the glands, meibomian gland dysfunction alone was not enough to produce the epidermal cell fate switch in Notch1-deficient corneas. Under homeostatic conditions, meibomian glands synthesize and secrete lipids that make up the protective tear film which covers the ocular surface (McCulley & Shine, 2003). Disruptions to proper meibomian gland activity can lead to a disrupted tear film, making the corneal epithelium more susceptible to chronic irritation and injury (Chhadva et al., 2017).

While minor injuries are normally repaired by an LSC-mediated injury repair response, Notch1-deficient corneal cells could not properly repair the wounded epithelium and instead generated epidermal plaques at the central cornea (Vauclair et al., 2007). On the other hand, corneas that were protected from injury by having their eyelids stitched shut were never observed developing epidermal growths on their eyes, providing further support for the role of Notch1 as a mediator of wound repair. Although we did not explore changes in meibomian gland physiology or wound repair in our Sox9-deleted corneas, it is not inconceivable to propose that the role of Sox9 in the cornea might also be one involved in injury repair rather than homeostatic maintenance. If this is the case, it would provide a reasonable explanation for as to why the epidermal growths in Sox9-deleted corneas vary so much in frequency and severity after 2 months. Perhaps the corneas that developed plaques had somehow been injured, resulting in a failed wound repair response and the growth of the epidermal plaques.

Cell fate and behavior is largely influenced by the cellular microenvironment. In most, but not all, self-renewing tissues, stem cells reside in a specialized microenvironment (niche) where they receive a mixture of intrinsic and extrinsic signals to preserve their unique ability to self-renew and give rise to progenitor populations (Fuchs et al., 2004). Within the cornea, the activity and fate of LSCs and their progenitors is tightly regulated to ensure proper maintenance of the epithelium. As progenitors migrate out of the limbal niche, they embark on a differentiation trajectory that is influenced by their intrinsic differentiation potential and the cues they receive

from the local microenvironment. For every cornea that developed epidermal growths, we were intrigued to see that these growths were always accompanied by thickening of the underlying stroma. Previously, Vauclair et al., also showed that conditional ablation of Notch1 from the corneal epithelium resulted in stromal remodeling and the development of epidermal-like growths at the central cornea (Vauclair et al., 2007). While Vauclair et al., noted that the corneal-to-epidermal cell fate switch always developed after corneal injury, the authors acknowledged this cell fate switch likely involved cell non-autonomous signals mediated by the underlying stroma, which became largely vascularized as a result of increased FGF-2 signaling in the epithelium following Notch1 deletion. Thus, given the striking phenotypic similarities of Notch1-deleted corneas, it is plausible for us to propose that the observed cell fate switch in Sox9-deleted corneas may also be driven, in part, by cell non-autonomous signals from the underlying stroma.

Acknowledgments

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Author contributions: G.R. and P.R. conceptualized the study, designed the experiments and wrote the manuscript. G.R. performed most experiments. L.O. and E.C. performed histological analyses. O.F.,S.H., L.N. and C.L. performed the scRNAseq experiment. All authors discussed results and participated in the manuscript preparation and editing. P.R. supervised the study.

Competing interests: The author(s) report no conflicts of interest in this work.

Data and materials availability: Genomic data are available in GEO (GSE263151). Additional data sets and reagents presented in this study are available from the corresponding author upon request.

Figure 1

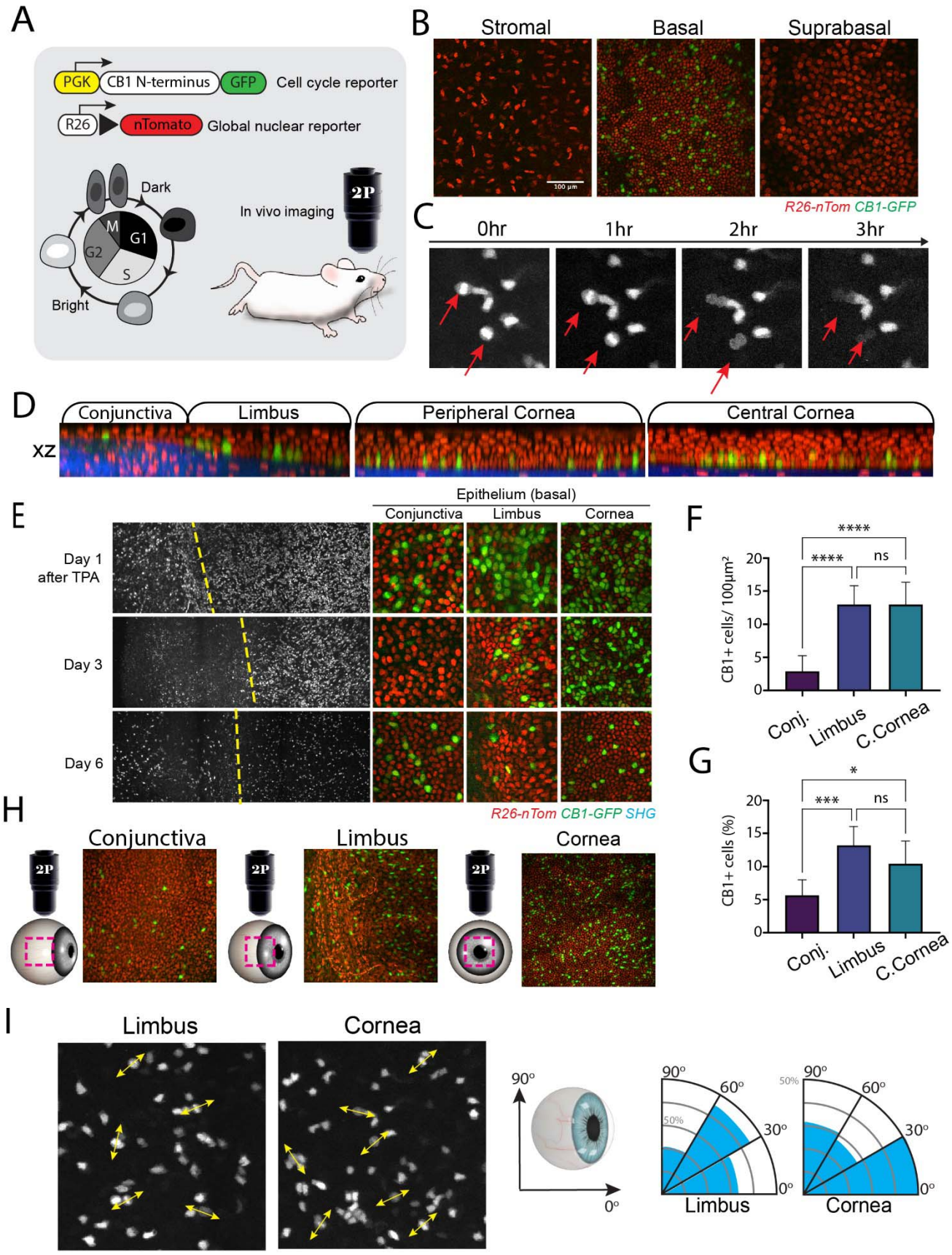

A
PGK
CB1 N-terminus
GFP
Cell cycle reporter
R26
nTomato
Global nuclear reporter
Dark
Bright
M
G1
G2
S
In vivo imaging
2P
B
Stromal
Basal
Suprabasal
100 µm
R26-nTom CB1-GFP
C
0hr
1hr
2hr
3hr
D
Conjunctiva
Limbus
Peripheral Cornea
Central Cornea
xz
E
Epithelium (basal)
Conjunctiva
Limbus
Cornea
Day 1 after TPA
Day 3
Day 6
R26-nTom CB1-GFP SHG
F
****
****
ns
CB1+ cells/ 100µm²
20
15
10
5
0
Conj.
Limbus
C.Cornea
G
*
***
ns
CB1+ cells (%)
20
15
10
5
0
Conj.
Limbus
C.Cornea
H
Conjunctiva
Limbus
Cornea
2P
I
Limbus
Cornea
90°
0°
90°
60°
30°
0°
50%
Limbus
90°
60°
30°
0°
50%
Cornea

Figure 1. Single-cell sequencing identifies Sox9 as a differentially expressed marker between LSCs and the central cornea.

(A) Genetic alleles of in vivo cell cycle reporter (Cyclin B1-GFP) used to sort stem and progenitor cells in the corneal epithelium. Cell cycle diagram displays how brightness of the GFP reporter changes throughout the cell cycle. (B) Individual planes from a series of optical sections of the CB1-GFP mouse cornea taken at the indicated depths. Also see Movie S1. (C) Representative snapshots from live time-lapse imaging of the cornea from a CB1-GFP mouse. Only actively cycling cells are labelled with GFP (Also see Movie S2). (D) Reconstructed side view (XZ) of the conjunctiva, limbus and cornea from a CB1-GFP mouse, build from a series of optical sections and acquired by two-photon microscopy. (E) Panel shows representative snapshots from a time course live imaging experiment. The limbus and peripheral cornea were imaged at high magnification at the indicated times after treatment with TPA. Dotted line indicates the limbal-corneal border. (F-H) Optical sections of a CB1-GFP mouse eye imaged at the indicated epithelial compartments. The globally expressed nuclear tomato reporter allowed for visualization and quantification of total cell numbers. Graphs show quantification of the number of CB1-GFP+ cells (top panel) and percentage of CB1-GFP+ cells in each epithelial compartment (n = 6 [conjunctiva] or 8 [limbus + cornea] sampled images per epithelial compartment from 2 mice, one-way ANOVA). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

Figure 2

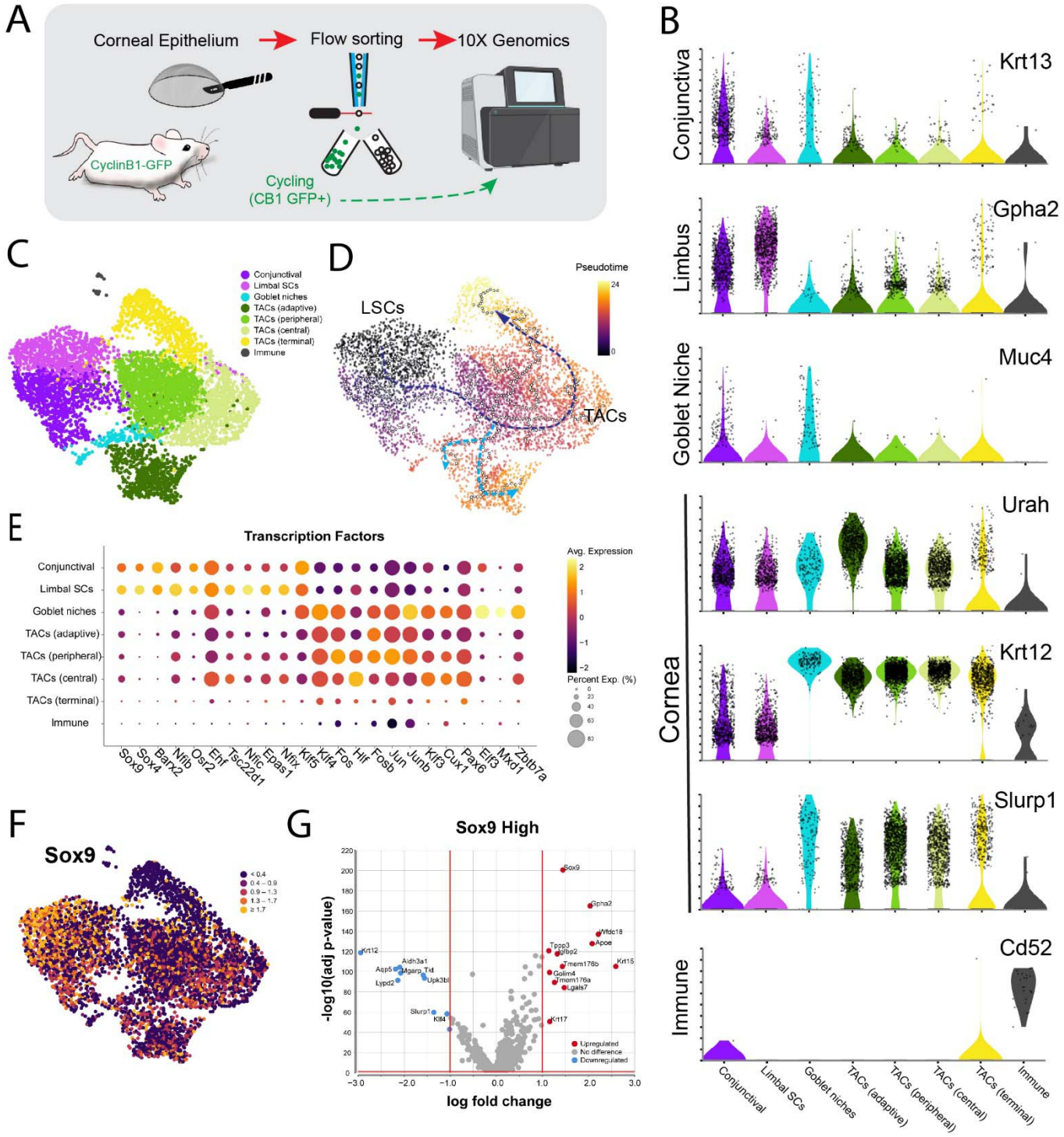

A
Corneal Epithelium
Flow sorting
10X Genomics
CyclinB1-GFP
Cycling
(CB1 GFP+)
B
Conjunctiva
Krt13
Limbus
Gpha2
Goblet Niche
Muc4
Cornea
Urah
Krt12
Slurp1
Immune
Cd52
Conjunctival
Limbal SCs
Goblet niches
TACs (adaptive)
TACs (peripheral)
TACs (central)
TACs (terminal)
Immune
C
Conjunctival
Limbal SCs
Goblet niches
TACs (adaptive)
TACs (peripheral)
TACs (central)
TACs (terminal)
Immune
D
Pseudotime
24
0
LSCs
TACs
E
Transcription Factors
Conjunctival
Limbal SCs
Goblet niches
TACs (adaptive)
TACs (peripheral)
TACs (central)
TACs (terminal)
Immune
Sox9
Sox4
Barx2
Nfib
Osr2
Ehf
Tsc22d1
Nfic
Epas1
Nfix
Klf5
Klf4
Fos
Hif
Fosb
Jun
Junb
Klf3
Cux1
Pax6
Elf3
Mxd1
Zbtb7a
Avg. Expression
2
1
0
−1
−2
Percent Exp. (%)
0
20
40
60
80
F
Sox9
< 0.4
0.4 – 0.9
0.9 – 1.3
1.3 – 1.7
≥ 1.7
G
Sox9 High
-log10(adj p-value)
log fold change
−3.0
−2.0
−1.0
0.0
1.0
2.0
3.0
Sox9
Gpha2
Wfdc18
Apoe
Tpp3
Gjb2
Tmem176b
Krt15
Golim4
Tmem176a
Lgals7
Krt17
Krt12
Aldh3a1
Aqp5
Mgarp
Tkt
Upk3bl
Lypd2
Slurp1
Klf4
Upregulated
No difference
Downregulated

Figure 2. Single cell transcriptomic analysis of corneal stem and progenitor cells. (A) Experimental design for isolating and profiling actively cycling cells in the mouse ocular epithelium. (B) Stacked violin plots of differentially expressed markers used to define cluster identity. The y-axes represent expression level. (C) UMAP plot presentation of GFP+ cells sorted from corneas of CB1-GFP mice. Unbiased clustering of 5545 cells (2 pooled samples, 30 and 22 corneas total) revealed 8 distinct groups: Conjunctiva (860 cells), Limbus (823) , Goblet niche (150 cells), Cornea – TACs (adaptive) (702 cells), Cornea – TACs (peripheral) (1443 cells), Cornea – TACs (central) (875), Cornea – TACs (terminal) (668), Immune (24 cells). (D) Inference trajectory analysis. Dotted lines show inferred pseudotime trajectories of corneal progenitors.(E) Dot plot of top differentially expressed transcription factors. (F) Umap plot for Sox9. (G) Volcano plot comparing differentially expressed genes between Sox9-high (>1.3 log) or low (<1 log) expressing cells.

Figure 3

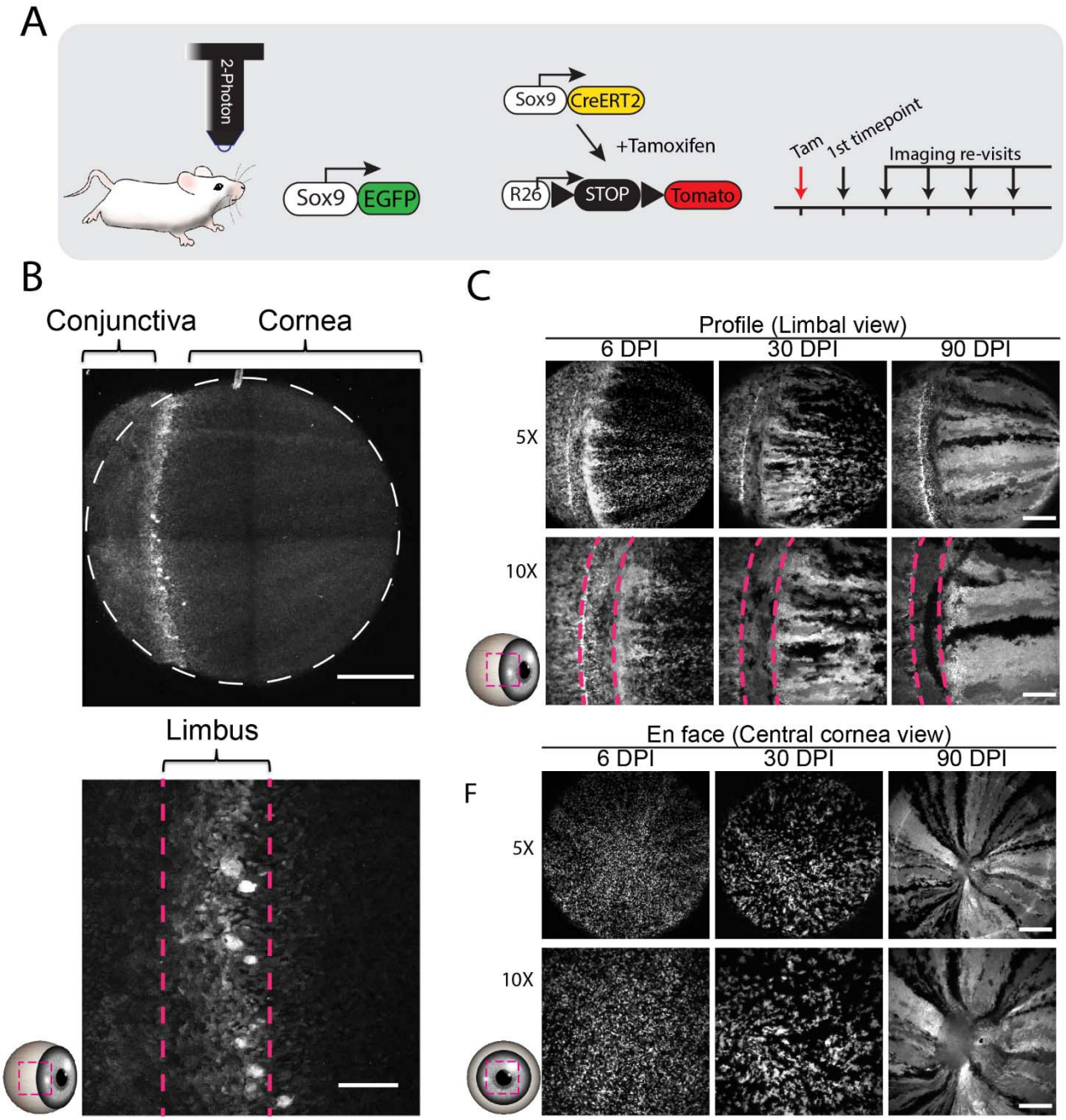
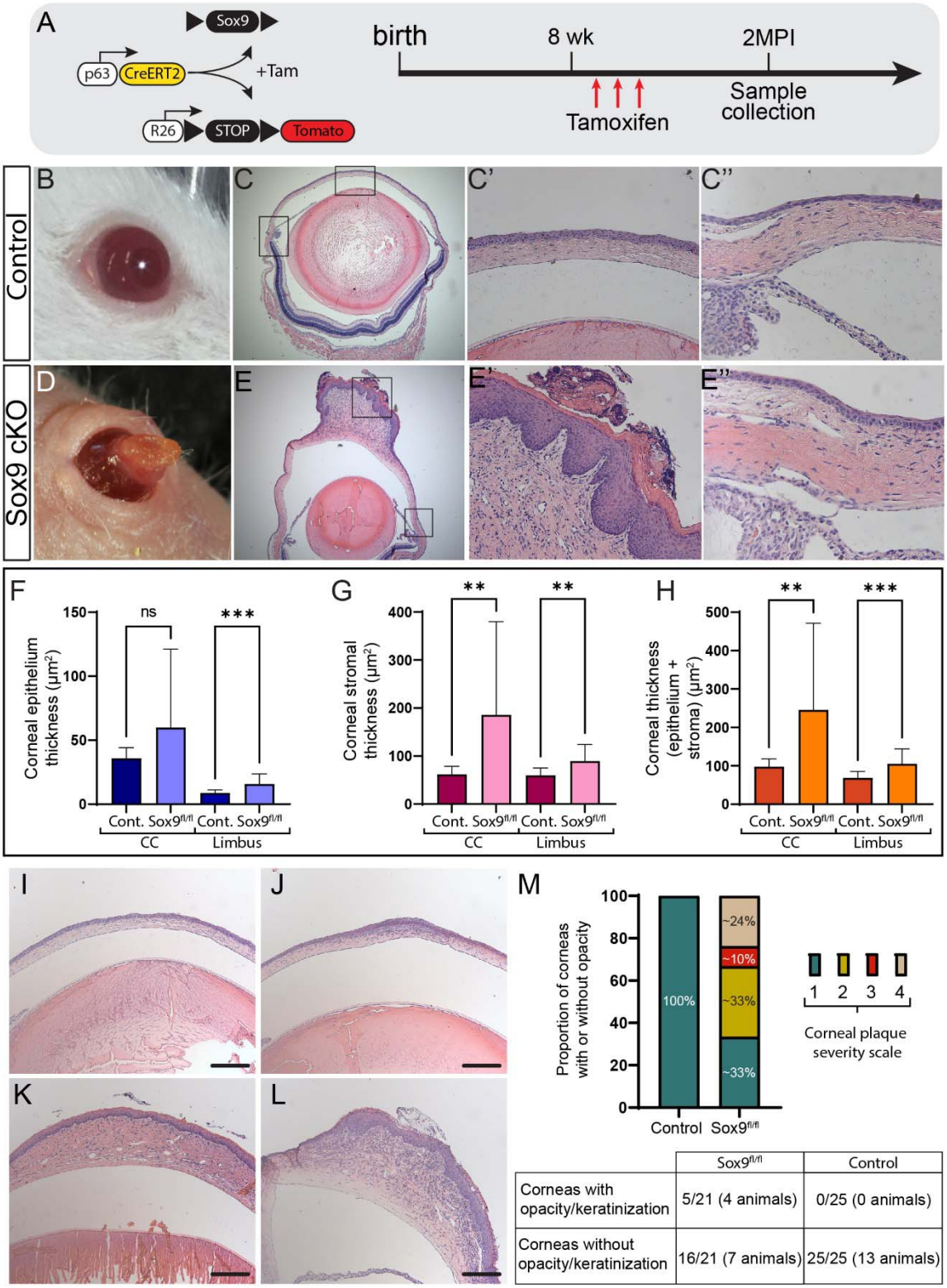

A
2-Photon
Sox9
EGFP
Sox9
CreERT2
+Tamoxifen
R26
STOP
Tomato
Tam
1st timepoint
Imaging re-visits
B
Conjunctiva
Cornea
Limbus
C
Profile (Limbal view)
6 DPI
30 DPI
90 DPI
5X
10X
F
En face (Central cornea view)
6 DPI
30 DPI
90 DPI
5X
10X

Figure 3. Sox9 marks long-lived limbal stem cells that maintain the cornea.

(A) Genetic alleles for in vivo imaging. (B) Global and high-magnification views of the eye imaged at the indicated epithelial compartments show GFP expression predominantly at the limbus (n = 3 animals). Scale bars represent 500µm (global view) and 100µm (high magnification). (C) In vivo lineage tracing of Sox9CreER;tdTom cells by longitudinal live imaging. Representative tracing time series generated by reimaging the same eye at the indicated time points after tamoxifen administration (n = 5 mice). Lower panels show representative magnified views of the same compartment. Dotted lines indicate the margins of the limbus. Scale bars represent 500µm (panel B and 5x panels) and 250µm (panel C and10x panels). Abbreviations: DPI, Days Post Induction.

Figure 4



	Sox9fl/fl	Control
Corneas with opacity/keratinization	5/21 (4 animals)	0/25 (0 animals)
Corneas without opacity/keratinization	16/21 (7 animals)	25/25 (13 animals)

Figure 4. Sox9 cKO corneas develop squamous metaplasia.

(A) Experimental timeline. Adult mice (p63CreER; Sox9 fl/fl cKO and control littermates) were treated with Tamoxifen daily for 3 days and analyzed 2 months post tamoxifen induction (MPI). (B-I) Gross morphology and representative histological sections of the central cornea (D, H) and limbus (E, I) of a control and Sox9 cKO cornea at 2MPI. (F-H) Quantifications of tissue thickness for the epithelium, stroma, and total corneal thickness in each epithelial compartment (control corneas: n = 13 animals [25 eyes]; Sox9 cKO corneas: n = 11 animals [21 eyes], Welch's t test). (I-L) Representative histological sections depicting phenotype severity. Phenotype severity was scored from 1-4 as follows: 1) No gross morphological/molecular abnormalities (appear like controls), n = 7/21 corneas (6/11 animals); 2) Mild thickening of the epithelium and/or stroma, n = 7/21 corneas (6/11 animals); 3) Moderate stromal thickening with mild to moderate epithelial thickening, n = 2/21 corneas (1/11 animals); 4) Severe thickening of stroma, evidence of keratinization (loricrin expression), n = 5/21 corneas (4/11 animals). (M) Bar graph and table depicting distribution of corneas across phenotype severity categories and the proportion of corneas that developed opacities, respectively. Scale bars are 200 µm.

Figure 5

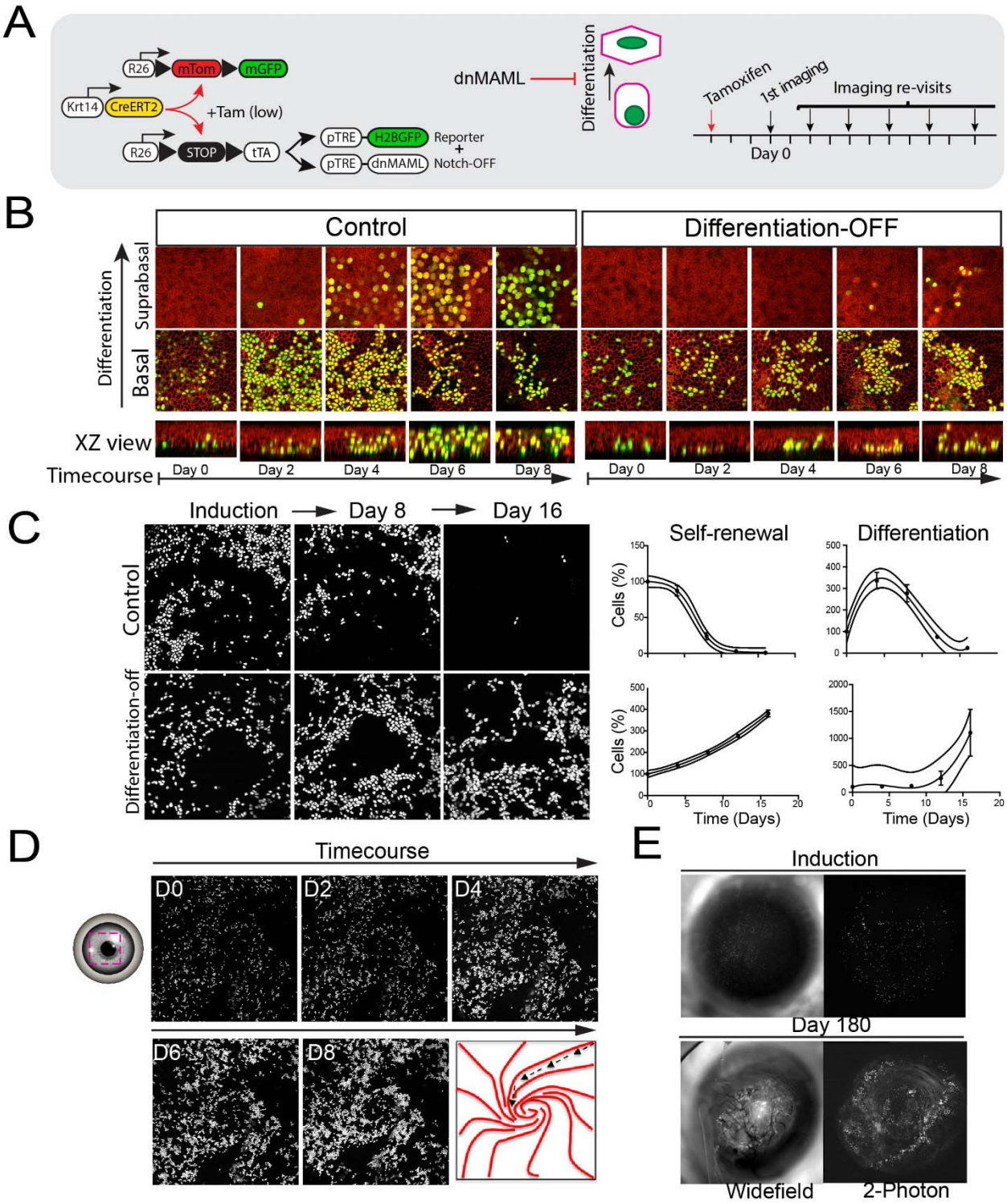
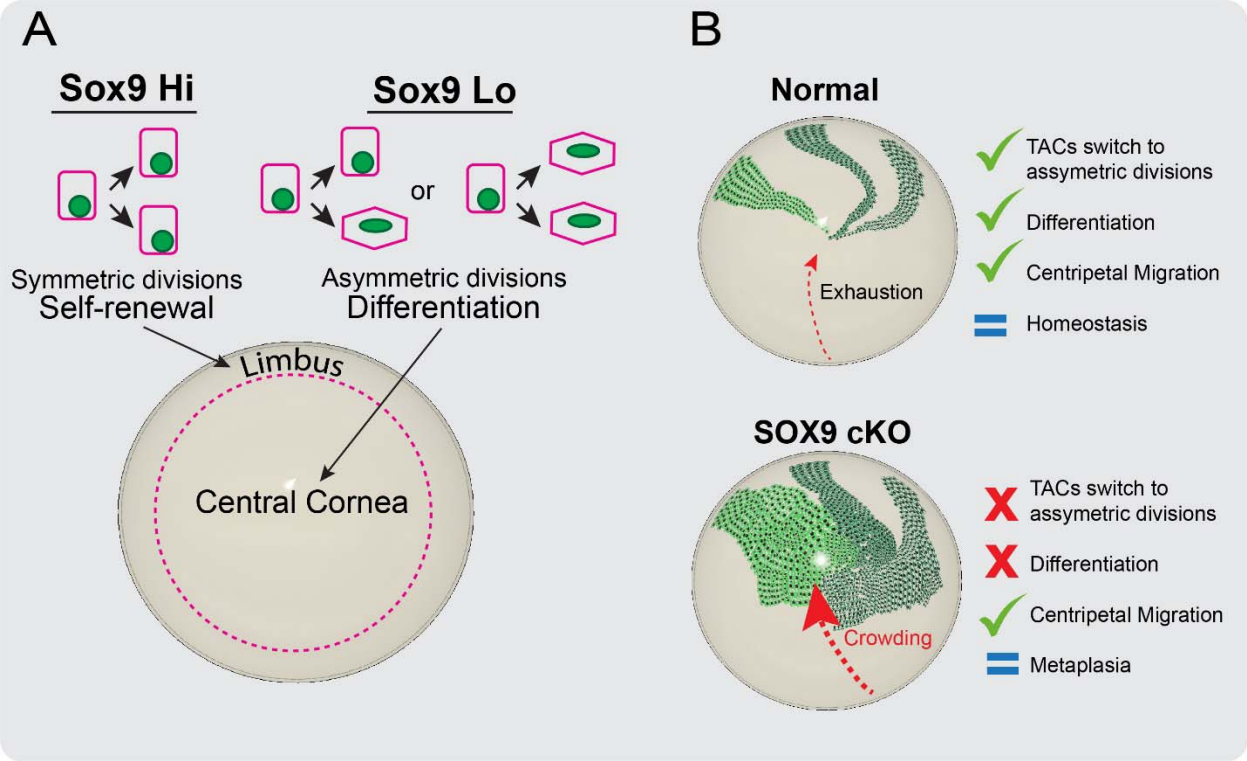

A
R26
mTom
mGFP
Krt14
CreERT2
+Tam (low)
R26
STOP
tTA
pTRE
H2BGFP
Reporter
+
pTRE
dnMAML
Notch-OFF
dnMAML
Differentiation
Tamoxifen
1st imaging
Imaging re-visits
Day 0
B
Control
Differentiation-OFF
Differentiation
Suprabasal
Basal
XZ view
Timecourse
Day 0
Day 2
Day 4
Day 6
Day 8
C
Induction
Day 8
Day 16
Control
Differentiation-off
Self-renewal
Differentiation
Cells (%)
Time (Days)
D
Timecourse
D0
D2
D4
D6
D8
E
Induction
Day 180
Widefield
2-Photon

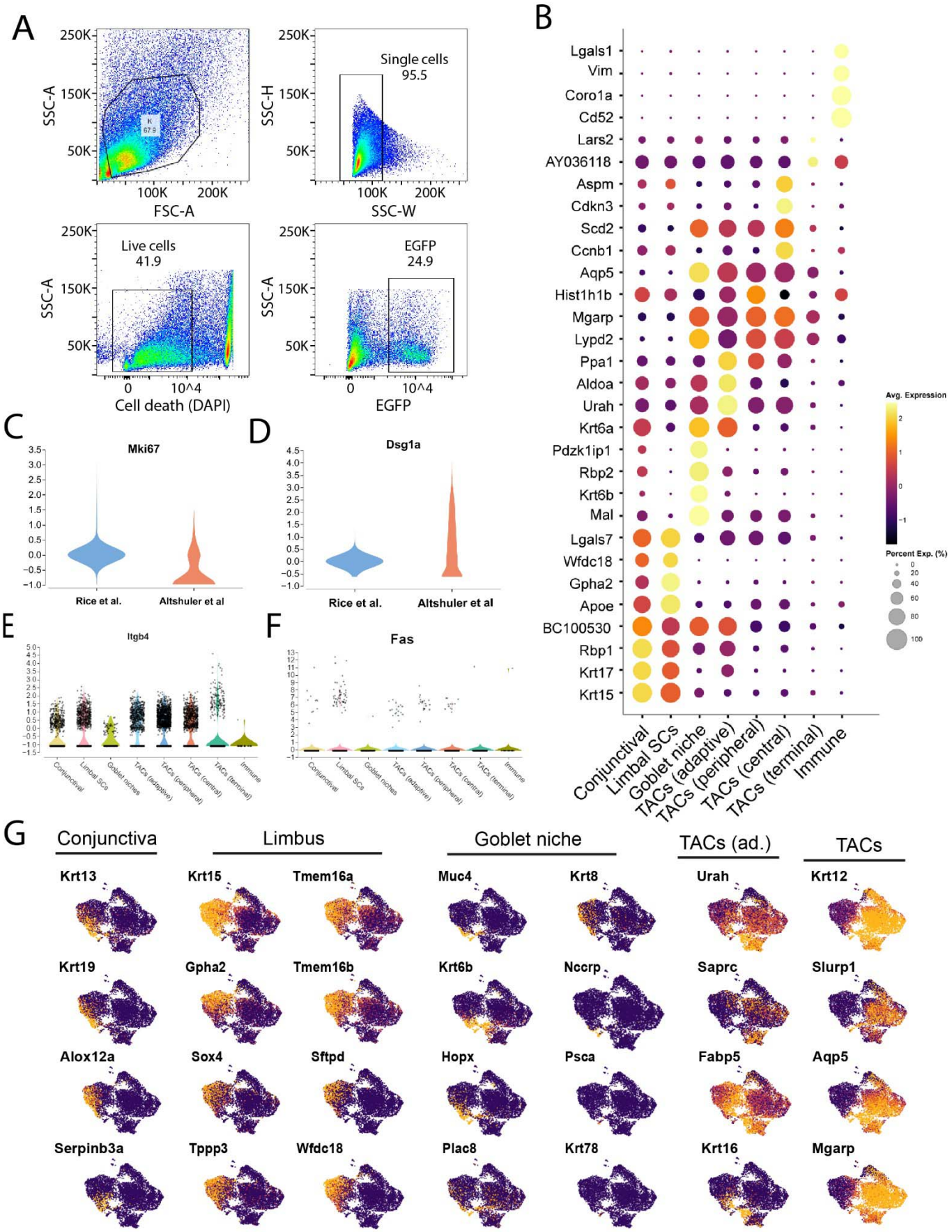
Figure 5. Centripetal growth and improper differentiation drive cellular crowding at the central cornea.

(A) Allelic system and experimental strategy used to generate single, Differentiation-OFF clones in the basal layer of the corneal epithelium. Tamoxifen administration in low doses enables Cre-mediated excision of loxP-flanked sequences in a reporter allele and a tetracycline transactivator allele (tTA) in Keratin14-expressing cells. Membrane-localized GFP (mGFP) becomes expressed after excision of membrane-localized tdTomato (mTom) in single clones while excision of a STOP cassette in the ROSA26 locus allows tTA expression. tTA expression in single clones enables expression of two TetO alleles: a dominant negative MAML allele to suppress Notch signaling (Differentiation-OFF) and a reporter allele (TetO-H2BGFP) to label the single “Differentiation-OFF” clones with nuclear GFP. Control animals have only the one TetO-H2BGFP reporter allele. Labelled cells will be followed over time by re-imaging the same areas of the cornea. (B) Labelled clones in control animals (n = 5) were tracked differentiating into the suprabasal and superficial layers of the cornea. Differentiation-OFF (n = 6 animals) clones were observed primarily in the basal layer with very few cells observed differentiating into the suprabasal and superficial layers. (D) Over time, Differentiation-OFF clones could be observed clonally expanding and migrating radially towards the central cornea. (E) Representative examples of corneas from Differentiation-OFF mice that developed squamous metaplasia in the central cornea.

Figure 6. Working model for the role of Sox9 in the regulation of cellular fate in the corneal epithelium.

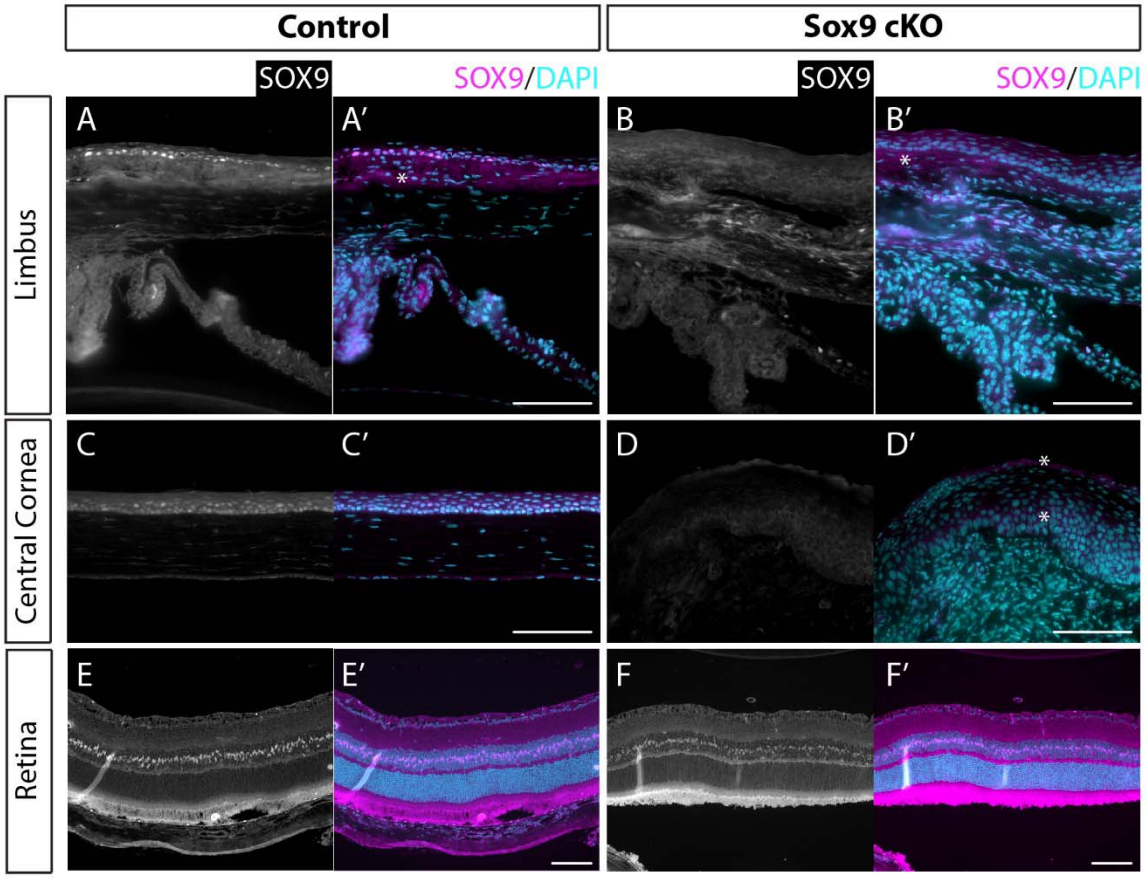


Supplementary Figure 1



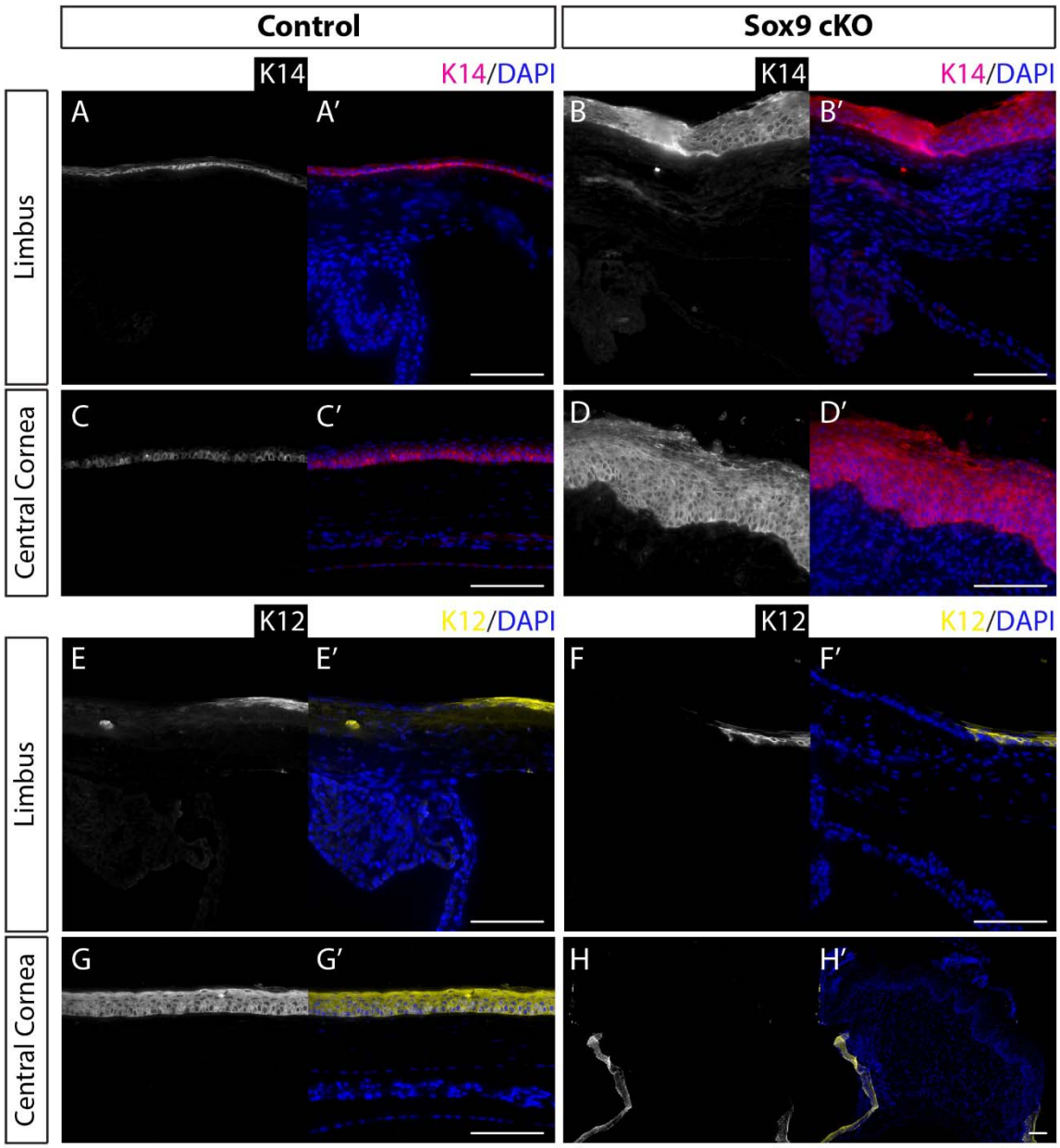
Supplementary Figure 1. Single cell sequencing reveals CycB1-GFP sorted cells from the corneal epithelium have a mitotic, basal cell signature. (A) Representative images of FACS gating strategy used to sort for GFP+ cells from Cyclin B1-GFP animals. (B) Dot plot of top differentially expressed genes between the identified cell clusters. (C-E) Violin plots of proliferation (Mki67) and differentiation (Dsg1a) markers, comparing this study with a published study of single cell transcriptomic analysis of bulk corneal epithelial cells (*Altshuler et al. 2021*). (E-F) Violin plots basal cell identity (Itgb4) and apoptosis (Fas) markers used to validate our dataset. (G) Umap plots of differentially expressed genes in each of the identified cell clusters.

Supplementary Figure 2



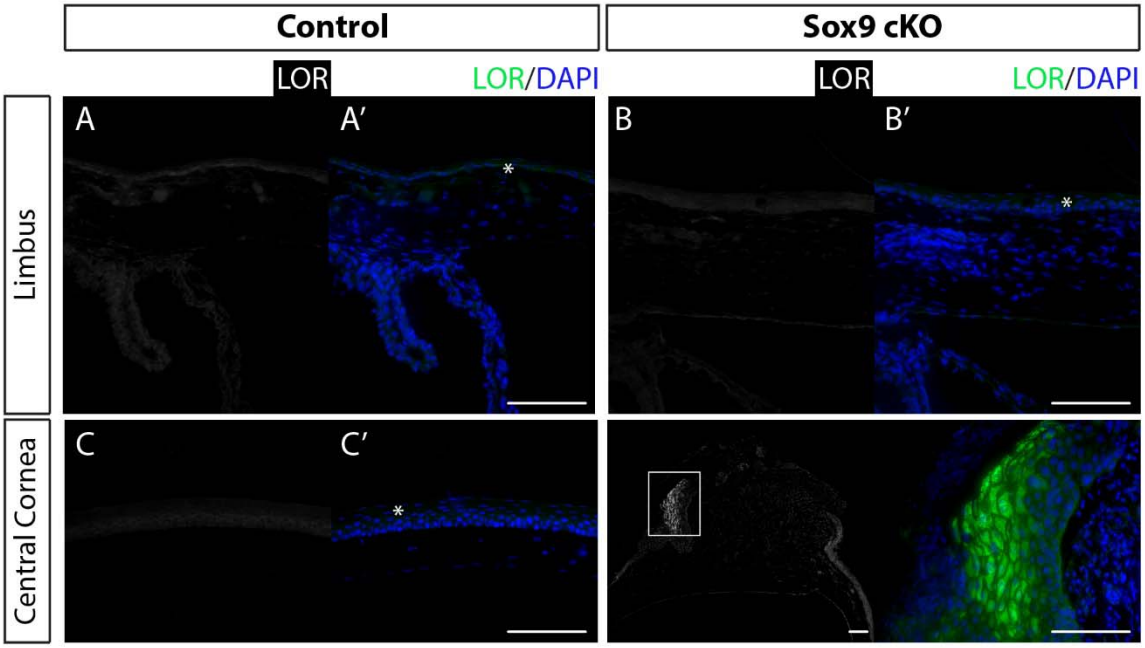
Supplementary Figure 2. Sox9 Expression in the mouse eye. (A/A') Immunofluorescence images of Sox9 in the limbus and central cornea (C/C') of control corneas (n = 25 corneas [13 animals]). (B/B') Immunofluorescence images of Sox9 in the limbus and central cornea (D/D') in Sox9 cKO corneas (n = 21 corneas [11 animals]). (E/E') Immunofluorescence images of control and Sox9 cKO (F/F') eyes stained for Sox9 in the retina. Muller glial cells in the retina are non-epithelial cells that also express Sox9 but are not targeted for deletion by Cre recombinase. Asterisks indicate non-specific background staining. Scale bars are 100 µm.

Supplementary Figure 3



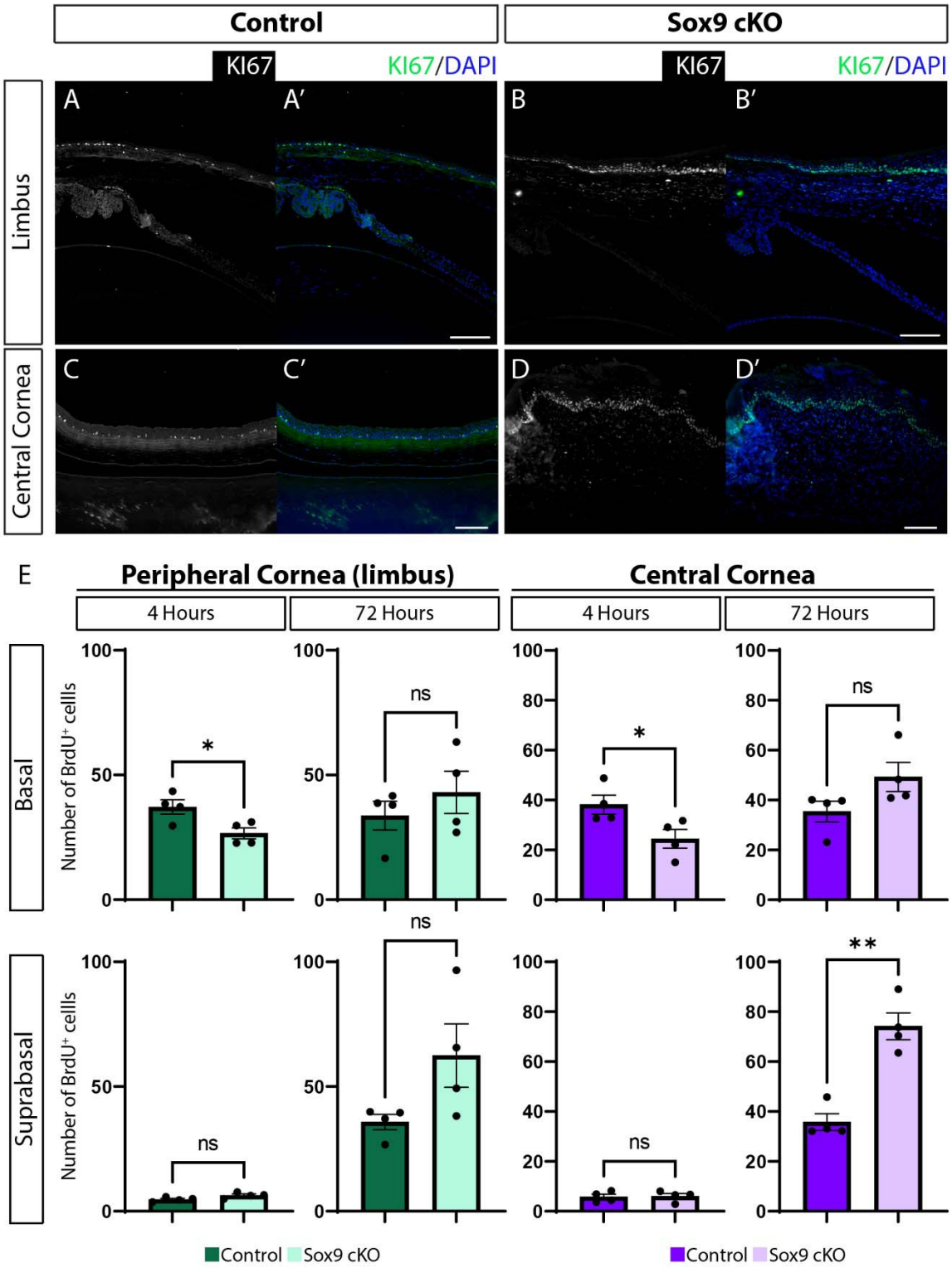
Supplementary Figure 3. Keratin expression is abnormal in Sox9 cKO corneas. (A/A') Immunofluorescence images of K14 in the limbus and central cornea (C/C') of control corneas (n = 25 corneas [13 animals]). (B/B') Immunofluorescence images of K14 in the limbus and central cornea (D/D') of Sox9 cKO corneas (n = 21 corneas [11 mice]). (E/E') Immunofluorescence images of K12 in the limbus and central cornea (G/G') of control corneas (n = 25 corneas [13 animals]). (F/F') Immunofluorescence images of K12 in the limbus and central cornea (H/H') of Sox9 cKO corneas (n = 21 corneas [11 animals]). Keratin 14 (K14), Keratin 12 (K12); Scale bars are 100 µm.

Supplementary Figure 4



Supplementary Figure 4. Sox9 cKO corneas express the cornification marker, loricrin. (A/A') Immunofluorescence images of Loricrin in the limbus and central cornea (C/C') of control corneas (n = 25 corneas [13 animals]). (B/B') Immunofluorescence images of Loricrin in the limbus and central cornea (D/D') in Sox9 cKO corneas (n = 21 corneas [11 animals]). Loricrin (LOR); Asterisks indicate non-specific background staining. Scale bars are 100 µm.

Supplementary Figure 5



Supplementary Figure 5. Sox9 loss leads to unchecked proliferation throughout the corneal epithelium. (A/A') Immunofluorescence images of KI67 in the limbus and central cornea (C/C') of control corneas (n = 8 corneas [7 mice]). (B/B') Immunofluorescence images of KI67 in the limbus and central cornea (D/D') in Sox9 cKO corneas (n = 7 corneas [4 animals]). (E) Quantifications of the number of BrdU+ cells in basal and suprabasal/superficial layers in each epithelial compartment from corneas collected at 4 and 72 hours (n = 4 corneas [2 mice]/genotype), unpaired Student's T-Test. Scale bars in A', B', C', D' are 100 µm and 25 µm in H.

Materials and Methods

Mice

All procedures involving animal subjects were performed with the approval of the Institutional Animal Care and Use Committee (IACUC) of the University of Pennsylvania and were consistent with the guidelines set forth by the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. p63CreERT2 mice were created by J. Xu (Baylor College of Medicine) and obtained from A. Vaughan (University of Pennsylvania). K14CreERT mice were created by Valeri Vasioukhin (University of Chicago) and obtained from Elaine Fuchs (The Rockefeller University). R26loxp-stop-loxp-tdTom, R26loxp-stop-loxp-tTA, Ptre-H2BGFP, TetO-dnMAML-GFP, and Sox9IRES-EGFP mice were obtained from The Jackson Laboratory. Sox9fl/fl mice come from The Jackson Laboratory and were obtained from V. Lefebvre (University of Pennsylvania). All mice used in this study were bred for multiple generations into a Crl:CD1(ICR) mixed background. Adult mice (8 weeks old) were used for experiments. Mice were housed in a temperature and light-controlled environment and received food and water ad libitum. Up to 5 mice of the same sex and similar age were housed in a cage. Mice were provided Bed-o'Cobs (The Andersons Lab Bedding), a porous cob material, as bedding and Shred-n'Rich nestlets (The Andersons Lab Bedding) for nesting and enrichment.

Tamoxifen induction

To induce Cre-recombinase activation, mice were injected intraperitoneally (IP) with Tamoxifen (Sigma) dissolved in corn oil (Sigma). For lineage tracing experiments, Sox9-CreERT2;R26-tdTom mice were administered a single dose of 2 mg Tamoxifen. For lineage tracing experiments in Notch-suppressed K14Cre-ERT;R26-tTA;TetOS-dnMAML-GFP and control K14Cre-ERT;R26-tTA;pTRE-H2BGFP mice, a single dose of 0.2 mg Tamoxifen was administered. To induce floxed deletion of Sox9, p63-CreERT2;Sox9 fl/fl;R26-tdTom mice and littermate controls (Sox9 fl/fl;R26-tdTom) were administered a single dose of 2 mg Tamoxifen daily for 3 days. Subsequent experiments were conducted at the indicated times after induction.

Intravital imaging of the mouse eye

Preparation of the mice for intravital imaging of the eye was performed with the following amendments to the previously described protocol (Rompolas et al., 2016). Mice were initially anesthetized with IP injection of ketamine/xylazine cocktail (0.1 mL / 20 g bodyweight: 87.5 mg / kg Ketamine, 12.5 mg / kg Xylazine). A deep plane of anesthesia was verified by checking pedal reflexes. The mouse head was stabilized with a custom-made stereotaxic apparatus that includes palate bar and nose clamp but no ear bars. Precision, 3-axis micro-manipulators are used to adjust the head tilt so that the eye to be imaged is facing up. A drop of eye gel (0.3% Hypromellose) was used as an optically neutral interface between the eye and a glass coverslip, and to prevent dryness and irritation to the tissue during the anesthesia and imaging procedure. After preparation and mounting is complete, the stage is placed on the microscope platform under the objective lens. A heating pad is used to keep a stable body temperature and vaporized isoflurane is delivered through a nose cone to maintain anesthesia for the duration of the imaging process. After each imaging session, the eyes were rinsed with PBS and the mice were monitored and allowed to recover in a warm chamber before being returned to their housing facility.

Imaging equipment and acquisition settings

Image acquisition was performed with an upright Olympus FV1200MPE microscope, equipped with a Chameleon Vision II Ti:Sapphire laser. The laser beam was focused through 5X, 10X, 20X or 25X objective lenses (ZEISS FLUAR 440125-0000-000, N.A. 0.25; Olympus UPLSAPO10X2, N.A. 0.40; Olympus UPLSAPO20X, N.A. 0.75; Olympus XLPLN25XWMP2, N.A. 1.05). Emitted fluorescence was collected by two multi-alkali and two gallium arsenide phosphide (GaAsP) non-descanned detectors (NDD). The following wavelengths were collected by each detector: NDD1 419-458 nm; NDD1 458–495 nm; GaAsP-NDD1 495–540 nm; GaAsP-NDD2 575–630 nm. GFP and Tomato reporters were excited at 930 nm and their signal was collected by GaAsP-NDD1 and GaAsP-NDD2, respectively. Second harmonic generation (SHG) signal was generated using 850 nm or 930 nm excitation wavelengths and detected by NDD1 or NDD2, respectively. Serial optical sections were acquired in 10 µm steps, starting from the surface of the eye and capturing the entire thickness of the cornea (epithelium ~40 µm, stroma/endothelium ~80 µm). Expanded views of the cornea and limbus were obtained by acquiring a grid of sequential optical fields-of-view that were automatically stitched into one high-resolution tiled image using the microscope manufacturer software. Multi-day tracing experiments were done by re-imaging the same field of view or the entire eye at the indicated

times after the initial acquisition. For each time point, inherent landmarks within the cornea, including the organization of the vasculature and collagen fibers (SHG), were used to consistently identify the limbus and navigate back to the original regions. Macroscopic images of the mouse eye were acquired under brightfield and fluorescence with an Olympus MVX10 Fluorescent Macro Zoom microscope fitted with Hamamatsu Orca CCD camera for digital imaging.

Tissue collection and processing

Following euthanasia by CO2 narcosis and cervical dislocation in accordance with the American Veterinary Medical Association guidelines, whole globes were dissected and directly fixed in 1.3% paraformaldehyde in PBS for 1 hour at room temperature. Whole globes were washed in 1X PBS 2x10 minutes, placed in fresh 1X PBS, and stored at 4°C until further processing.

Immunofluorescence staining

For section staining, fixed globes were submitted to the Penn Skin Biology and Diseases Resource-based Center for paraffin embedding and sectioning. Slides were heated for 5-10 minutes at 70°C, deparaffinized using Everclear xylene substitute (2 x 5 minutes), and then rehydrated using a graded ethanol series (95% EtOH 2 x 2 minutes, 70% EtOH for 2 minutes, ddH2O 2 x 2 minutes). For antigen retrieval, slides were places in a 1:100 dilution of Antigen Unmasking Solution (Vector Laboratories) and heated in a boiling bath for 20 minutes and then left to sit at room temperature until solution cooled. Sections were washed as follows: ddH2O 1 x 5 minutes, 1 X PBS 2 x 3 minutes, 0.1% Triton X-100 in 1X PBS 2 x 3 minutes. Sections were incubated in blocking solution (10% normal goat serum, 0.1% Triton X-100 in 1X PBS) for 1 hour at room temperature and then incubated with primary antibody diluted in staining solution (2% normal goat serum 0.1% Triton X-100 PBS) overnight at 4°C. After 4 x 5-minute washes in 0.1% Triton X-100 in 1X PBS, tissue sections were incubated with secondary antibody and DAPI diluted in staining solution for 1 hour at room temperature. Secondary antibody incubation was followed by 4 x 5-minute washes in 0.1% Triton X-100 in 1X PBS and slide mounting using Fluor-Gel mounting medium (Electron Microscopy Sciences).

The following primary antibodies were used: Anti-KI67 (1:100, ab16667, Abcam), Anti-Sox9 (1:500, ab185966, Abcam), Anti-KERATIN 17 (1:300, 4543, Cell Signaling Technology), anti-

hair cortex cytokeratin/K40 [AE13] (1:50, ab16113, Abcam), and anti-RFP (1:500, Rockland Immunochemicals, Cat # 600-401-379).

The following secondary antibodies were used: Alexa Fluor 488 Goat anti-Mouse (1:500, A11001, ThermoFisher Scientific), Alexa Fluor 488 Goat anti-Rabbit (1:500, A11008, ThermoFisher Scientific), Alexa Fluor 594 Goat anti-Rabbit (1:500, A11012, ThermoFisher Scientific), Alexa Fluor 594 Goat anti-Mouse (1:500, A11005, ThermoFisher Scientific), and DAPI (1:1000, 40043, Biotium).

BrdU preparation and administration

BrdU solutions were prepared at a concentration of 10mg/ml in 1X PBS. BrdU was administered via IP injections at a concentration of 0.1mg/g bodyweight. Animals were euthanized after 4 and 72 hours and whole globes were dissected for fixation and further processing (see 3.6.6 Tissue collection and processing).

Wholemount BrdU staining

Following fixation of the whole globe (see 3.6.6 Tissue collection and processing), corneas were dissected, petaled, permeabilized (2N HCl for 30 minutes at 37°C), washed (TBE, 3 x 5 minutes), washed again (1X PBS, 4 x 10 minutes), blocked (0.3% Triton X-100 in 10% goat serum, 1 hour), blocked again (3% BSA in 1x PBS, 10 minutes), and incubated with primary antibody (0.3% Triton X-100 in 1X PBS, Rat-anti BrdU 1:500) on a rocker at 4°C for 3 days. Corneas were then washed (1X PBS, 4 x 15 minutes), incubated with secondary antibodies (0.3% Triton X-100 in 1X PBS, Alexa Fluor 594 Goat anti-Rat 1:500, DAPI 1:1000) on a rocker at 4°C for 2 days). Secondary antibody incubation was followed by 4 x 15-minute washes in 1X PBS and slide mounting using Fluor-Gel mounting medium (Electron Microscopy Sciences). Tissues were mounted epithelium-side up.

Image acquisition of stained tissue sections

Images of stained tissue sections were acquired using an Olympus BX51 equipped with a Hamamatsu Orca CCD camera or a Leica DM6 B equipped with a Leica DFC9000 GT fluorescent camera and a Leica DMC2900 brightfield camera.

Corneal epithelial cell isolation for flow cytometry and sorting

Dissected corneas (n = 6) were placed into 2.4 units/µl of Dispase prepared in Keratinocyte Serum Free Medium and incubated overnight at 4°C. For each cornea, the epithelium was gently peeled off, washed in 1 X PBS, and incubated in Trypsin-EDTA (0.25%) at 37°C for 20 minutes. Following incubation, tissue was processed into a cell suspension via successive rounds of manual pipetting with a P1000 tip. 2 ml of FBS and 6 ml of 1 X PBS were added to the cell suspension and cells were spun down at 1000-1500 rpm for 5 minutes. Following centrifugation, the supernatant was aspirated, cells were resuspended in 1X PBS, and the entire volume was filtered through a 40 µm cell strainer to obtain a single cell suspension. For cell staining, DAPI (1:1000) was added directly to the cell suspension and incubated for 10 minutes on ice. Following incubation, cells were spun down at 1000-1500 rpm for 5 minutes, supernatant was aspirated, and cells were resuspended for sorting in 2% FBS 5 mM EDTA in 1X PBS. Flow cytometry was performed on a BD LSRII cytometer (BD Biosciences), sorting on a BD FACS Aria II sorter (BD Biosciences). Only GFP+ live cells were collected from sorting. Flow cytometry data was collected and exported using BD FACs Diva software (BD Biosciences) and analyzed and plotted using FlowJo software.

Library preparation and data generation

The FACS-generated cell suspension was used to generate a single-cell RNA-seq library according to the 10x Genomics protocol (Chromium Single Cell 3' Library & Gel Bead Kit v3, PN-1000075). Single cell separation was performed using the Chromium Single Cell B Chip Kit (PN-1000074). The library was sequenced on an Illumina NextSeq500 instrument with a high-output mode (Illumina, 20024907) 150bp paired-end reading strategy according to 10X recommendations.

Processing of 10X Genomics data

Cell Ranger (version 3.1.0) was used for primary data analysis (alignment, filtering, barcode and UMI counting) to determine transcript counts per cell (producing a gene-barcode matrix). Transcripts were mapped to the mouse reference genome, GRCm38 (refdata-cellranger-mm10–2.1.0/GRCm38.84)

Downstream analysis of single-cell RNA sequencing data

Downstream analysis was performed using the R Package Seurat, version 5.0.0 (Hao et al., 2023) for clustering and differential expression analyses. Low quality cells (>5% mitochondrial UMI counts, <1000 or >3000 features) were excluded to produce a dataset with 14,525 features across 2,370 total cells that were analyzed.

Cell cycle scoring

Cell cycle scores were assigned using Seurat's CellCycleScoring function. Cell cycle scores were assigned based on the expression of genes associated with G1, S, or G2/M phase (Kowalczyk et al., 2015).

Quantitative image analysis of CB1-GFP+ cells

Raw digital files from 2-photon imaging were acquired and saved in the OIB format using the microscope manufacturer's software (FluoView, Olympus USA). Extended fields-of-view that encompass the entire ocular surface epithelium, including the cornea, limbus, and conjunctiva, were captured using a tiling method to reconstruct a single image from multiple full-thickness serial optical sections (FluoView, Olympus USA). To image the entire eye with the 10X objective lens, a matrix area of 2 × 2 (XY) field-of-view with 10% overlap between tiles is defined. Using the motorized platform, the microscope automatically acquires the four fields-of-view in a sequential pattern and uses information from the overlapping margins to stitch the individual fields-of-view into a single image. Raw image files were imported into ImageJ/Fiji (NIH) using Bio-Formats for further analysis. For cell counts, supervised image segmentation and blob detection was performed on individual optical sections. Identified blobs were manually validated and their number, size, and signal intensity as mean gray values were measured. Images shown in figures typically represent maximum projections or single optical sections selected from the z stacks unless otherwise specified.

Quantitative image analysis of BrdU+ cells

Images of BrdU-stained whole-mounted corneas were acquired with the upright Olympus FV1200MPE microscope described in 3.6.5. The laser beam was focused through the 25X objective lens (Olympus XLPLN25XWMP2, N.A. 1.05). Blue (DAPI) and red (Alexa Fluor 594) fluorophores were excited at 930 nm and 850 nm and their signal was collected by NDD1 and GaAsP-NDD1, respectively. Serial optical sections were acquired in 1 µm steps, capturing the entire thickness of the corneal epithelium (~50 µm). Raw digital files were saved in the OIB format using the microscope manufacturer's software (FluoView, Olympus USA). For each cornea, 2 fields were imaged to represent the central cornea (CC1/CC2) and 4 fields were imaged to represent the peripheral cornea (PC3-6). Raw image files were imported into ImageJ/FIJI (NIH) using Bio-Formats for further analysis. For each image, 5 150x150µm regions of interest were defined, roughly representing 4 corners and the center, and used to perform cell quantifications of brdu-positive cells in basal versus suprabasal layers. Quantifications were performed manually with the cell counter plugin in Fiji/ImageJ. BrdU+ cells in the basal layer were marked as 1s while BrdU+ cells in suprabasal/superficial layers were marked as 2s.

Quantitative image analysis of corneal epithelium thickness

Images at the central cornea and limbus of stained H&E tissue sections were acquired using an Olympus BX51 equipped with a Hamamatsu Orca CCD camera or a Leica DM6 B equipped with a Leica DFC9000 GT fluorescent camera and a Leica DMC2900 brightfield camera. Length measurements were recorded using the straight line and Set Measurements functions in ImageJ/FIJI. Epithelial and stromal compartments were measured separately and then combined to generate values for total corneal thickness.

Statistical analysis

Sample sizes were not pre-determined. Data were collected and quantified randomly, and their distribution was assumed normal, but this was not formally tested. Lineage tracing experiments were successfully reproduced under similar conditions using different mouse cohorts. The values of ''n'' (sample size) refer to number of corneal samples, unless otherwise indicated in the figure legends. Statistical calculations and graphical representation of the data were performed using the Prism software package (GraphPad). Data are expressed as percentages or mean ± SEM and two-tailed Student's t test was used to analyze datasets with two groups,

unless otherwise stated in the figure legends. For all analyses, p-values < 0.05 were designated as significant and symbolized in figure plots as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$, with precise values supplied in figure legends. No data were excluded from the analyses.

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