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Supplemental Information

Keratin-14-Positive Precursor Cells Spawn a Population of Migratory Corneal Epithelia that Maintain Tissue Mass throughout Life

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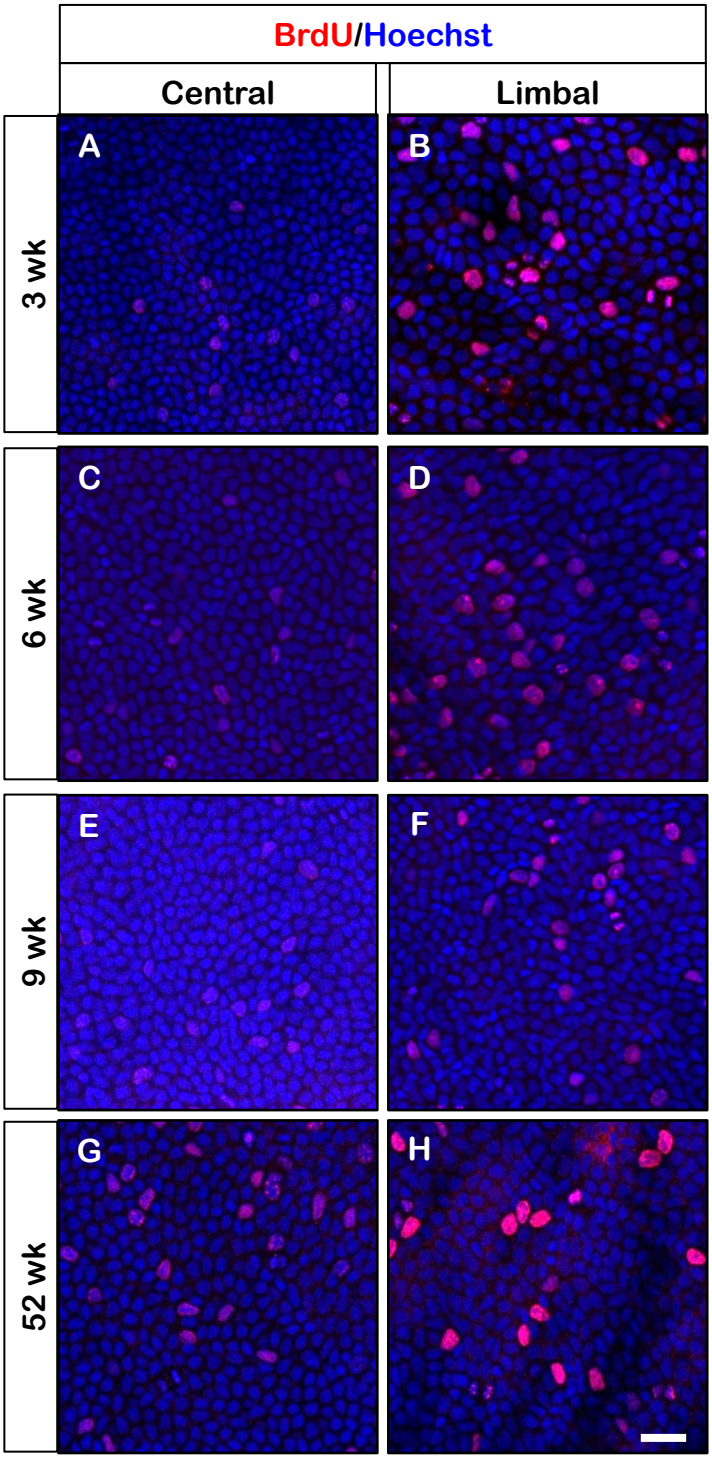
Figure S1


BrdU/Hoechst
Central
Limbal
A
B
C
D
E
F
G
H
3 wk
6 wk
9 wk
52 wk

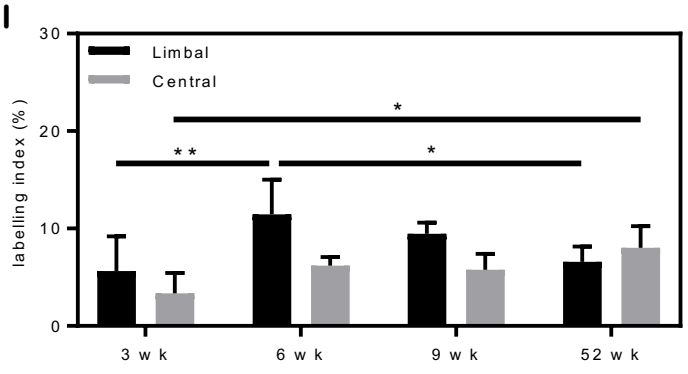

I
Limbal
Central
labelling index (%)
30
20
10
0
*
**
*
3 w k
6 w k
9 w k
52 w k

Figure S2

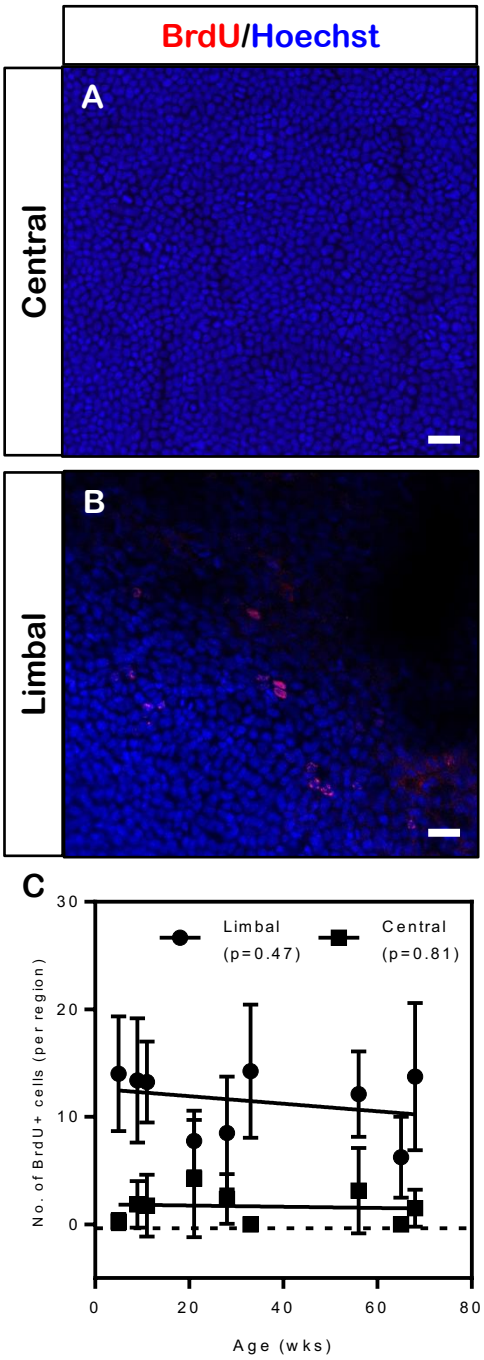

BrdU/Hoechst
A
Central
B
Limbal
C
Limbal
(p=0.47)
Central
(p=0.81)
No. of BrdU+ cells (per region)
30
20
10
0
0
20
40
60
80
Age (wks)

Figure S3

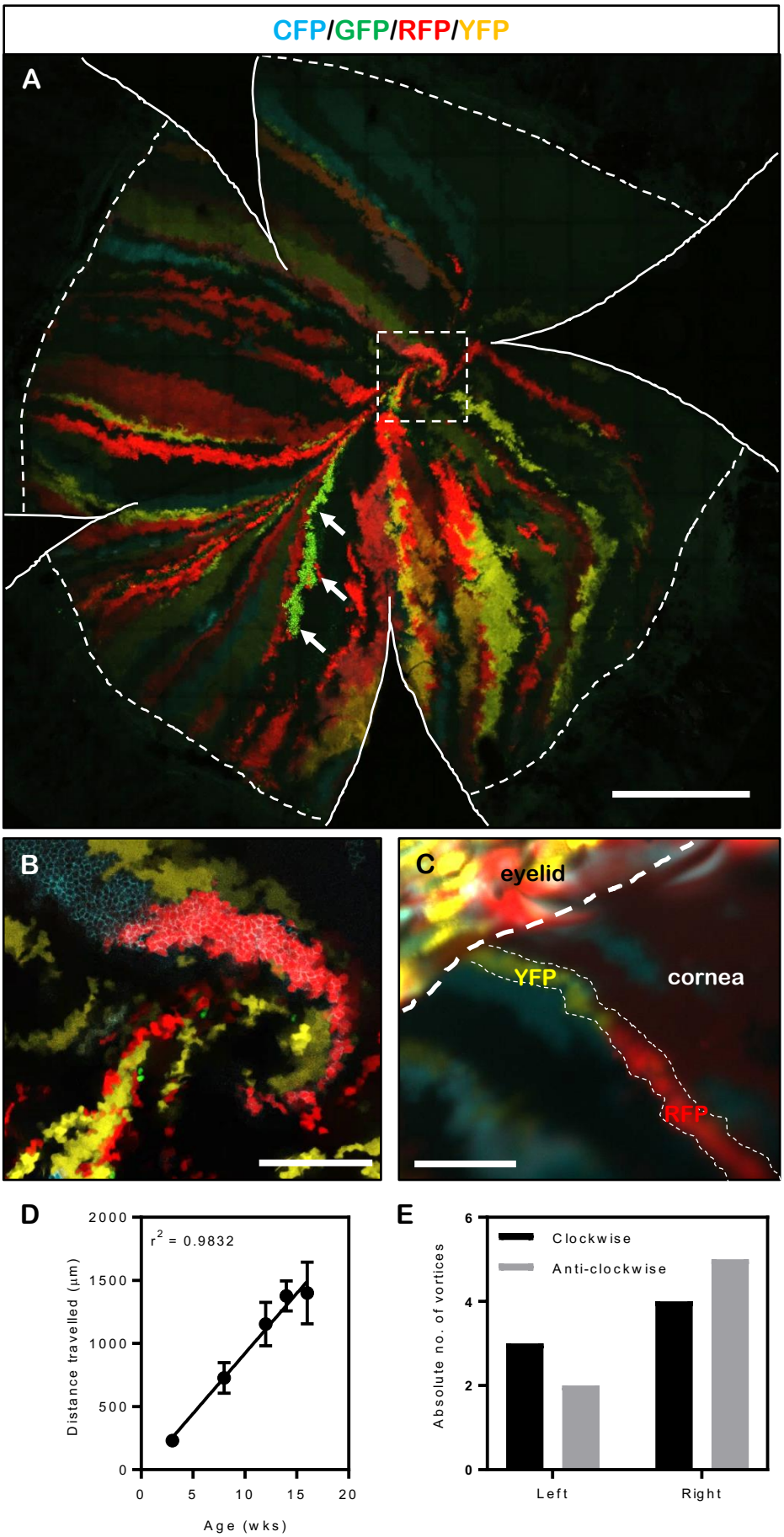
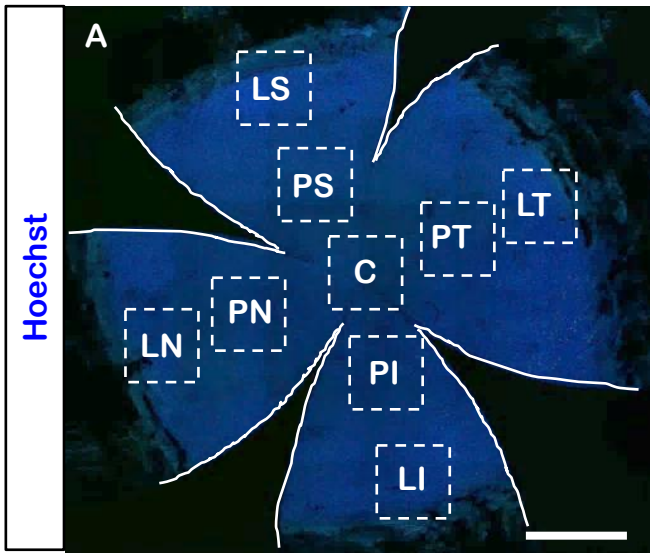
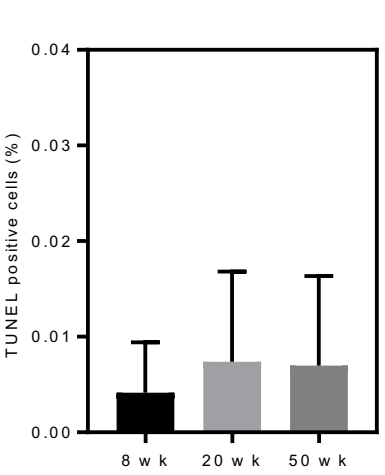

CFP/GFP/RFP/YFP
A
B
C
eyelid
YFP
cornea
RFP
D
r^2 = 0.9832
Distance travelled (µm)
2000
1500
1000
500
0
0
5
10
15
20
Age (wks)
E
Absolute no. of vortices
6
4
2
0
Clockwise
Anti-clockwise
Left
Right

Figure S4


A
Hoechst
LS
PS
LT
PT
C
PN
LN
PI
LI


B
TUNEL positive cells (%)
0.04
0.03
0.02
0.01
0.00
8 w k
20 w k
50 w k

SUPPLEMENTARY FIGURE LEGENDS

Figure S1, related to Figure 2: Proliferating cells within the central corneal and limbal epithelium.

(A-H) WT C57BL/6 mice were pulsed with 2 mg of BrdU, chased for 4 hrs, then euthanized and corneas stained with an anti-BrdU antibody; central cornea (A, C, E, G) and limbus (B, D, F, H) at 3 (A, B), 6 (C, D), 9 (E, F), and 52 (G, H) wks of age.

(I) Labeling index (% $BrdU^+$ cells) in the central cornea and limbus. Bars represent the mean labeling index (*n=4* corneas/group, ± SD, * = $p<0.05$, ** = $p<0.01$, two-way ANOVA with Tukey's multiple comparison test).

Scale bar represents 50 μm.

Figure S2, related to Figure 3: LRC within the central corneal and limbal epithelium during aging.

(A, B) Mice aged between 3 and 68 weeks were administered i.p. BrdU for 7 days then chased for 4 wks. Following euthanasia, corneas were stained for BrdU (red), counter-stained with Hoechst 33342 (blue) and images were acquired from the central cornea (A) and limbus (B).

(C) Quantification of the number of LRC in the central cornea and limbus during aging. Data points represent the mean number of LRC (*n=4* corneas/timepoint, ± SD, regression analysis).

Scale bar A and B = 25 μm.

Figure S3, related to Figures 4 and 5: Age-related clonal patterns observed in the corneal epithelium of Confetti mice.

(A) Whole flat-mounted cornea imaged by confocal microscopy. Solid white lines represents incisions made to facilitate corneal flat-mounting and the hatched lines demarcate the limbal border, as judged by the presence of stromal blood vessels (not shown). Hatched box (A) is enlarged in (B). White arrows point to a green clone that is discontinuous from the limbus.

(B) Central corneal vortex imaged by confocal microscopy.

(C) Intra-vital microscopy of live mouse using 3 channels, one each for CFP, YFP and RFP. The thick hatched line represents the eyelids and the thin hatched lines represent the lateral border of the highlighted streak.

(D) Migration rate of clonal streaks during early monitoring (*n=6* eyes, ± SD, linear regression).

(E) Frequency of the direction of the vortex in both and right eyes during aging (*n=15* eyes, Fisher's exact test)

Scale bars A = 400 μm, B = 50 μm and C = 100 μm.

Figure S4, related to Figure 6: Apoptosis in different regions of the cornea.

(A) Whole flat-mounted WT C57BL/6 cornea stained with Hoechst 33342 nuclear stain. Hatched boxes represent regions from which TUNEL-stained tissue was imaged. C = central, PN = peripheral nasal, PT = peripheral temporal, PS = peripheral superior, PI = peripheral inferior, LN = limbal nasal, LT = limbal temporal, LS = limbal superior, LI = limbal inferior.

(B) Total $TUNEL^+$ cells were divided by the number of cells in each image (330 μm x 250 μm) and the percent positive cells calculated. Bars represent mean (*n=4-5*, ± SD, one-way ANOVA).

Scale bar (A), 500 μm.

Supplementary Methods

5-Bromo-2'-deoxyuridine (BrdU) Staining

BrdU (Sigma-Aldrich, St Louis, MI) was prepared according to the manufacturer's instructions by dissolving it in PBS (10 mg/mL). Confetti mice aged between 3 and 68 wks were injected with either a single i.p. dose of BrdU (2 mg) and euthanized 4 hrs later (*n=4 mice/group*), or injected i.p. with BrdU (2 mg) daily for 7 days and chased for 4 wks prior to euthanizing (*n=5-8* mice/group). Eyes were enucleated, fixed in 4% PFA overnight at 4^{o}C and corneas dissected. Corneas were then stained for BrdU as previously described (Pajoohesh-Ganji et. al., 2006). In brief, tissue was treated with 2N HCl for 25 mins, then washed 4 times (5 mins each) in PBS at RT. Tissue was blocked in 20% goat serum (Sigma-Aldrich), 2% BSA (Thermo Fisher, Waltham, MA) and 0.1% Triton X-100/PBS for 6 hrs at 4^{o}C and a rat anti-BrdU antibody (Abcam, Cambridge, UK) applied for 48 hrs at 4^{o}C in 2% BSA in 0.1% Triton X-100/PBS. Samples were washed thrice (20 mins) shaking at RT in 2% BSA in 0.1% Triton X-100/PBS and a goat anti-rat secondary antibody conjugated to AlexaFluor®-633 (Thermo Fisher) was added for 24 hrs at 4^{o}C. Corneas were again washed thrice in 2% BSA and 0.1% Triton X-100/PBS with Hoechst 33342 nuclear stain (1 mg/mL) (Thermo Fisher), 4 radial incisions were made, corneas were flat-mounted and cover-slipped in Prolong Gold® anti-fade mounting media (Thermo Fisher). Images were obtained on an LSM 780 (Carl Zeiss, Jena, Germany) confocal fluorescent microscope and analyzed using FiJi software (Schindelin et al., 2012). Each image encompasses an area measuring 425 μm x 425 μm and 4 images were obtained from the limbus and central cornea of each tissue sample. The number of cells within each image was counted and an average generated for each cornea at each time point.

Supplementary References

Pajoohesh-Ganji A, Pal-Ghosh S, Simmens SJ and Stepp MA. Integrins in slow-cycling corneal epithelial cells at the limbus in the mouse. Stem Cells 2006;24:1075-1086

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B.*, et al.* (2012). Fiji: an open-source platform for biological-image analysis. Nat Meth *9*, 676-682.