Endogenous retinoic acid signaling is required for maintenance and regeneration of cornea

Sandeep Kumar[1,*], **Pascal Dollé**[2], **Norbert B. Ghyselinck**[2], and **Gregg Duester**[1,*]

[1]Development, Aging, and Regeneration Program, Sanford Burnham Prebys Medical Discovery Institute, La Jolla, CA 92037, USA

[2]Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC), Illkirch, France; Centre National de la Recherche Scientifique (CNRS), UMR 7104, Illkirch, France; Institut National de la Santé et de la Recherche Médicale (Inserm), U 964, Illkirch, France; Université de Strasbourg, Illkirch, France

Abstract

Retinoic acid (RA) is a biologically active metabolite of vitamin A (retinol) that serves as an important signaling molecule in orchestrating diverse developmental processes including multiple roles during ocular development. Loss-of-function studies using gene knockouts of RA-synthesizing enzymes encoded by *Aldh1a1*, *Aldh1a2*, and *Aldh1a3* (also known as *Raldh1, Raldh2,* and *Raldh3*) have provided valuable insight into how RA controls eye morphogenesis including corneal development, however it is unclear whether endogenous RA is required for maintenance and regeneration of adult cornea. Here, we investigated the role of *Aldh1a* genes in the adult cornea using a novel conditional *Aldh1a1,2,3-flox/flox*;Rosa26-CreERT2 loss-of-function mouse model to determine the biological function of RA. Our findings indicate that loss of RA synthesis results in corneal thinning characterized by reduced thickness of the stromal layer, impaired corneal epithelial cell proliferation, and increased apoptosis. Corneal thinning in *Aldh1a*-deficient mice was significantly rescued by RA administration, indicating an important role of endogenous RA signaling in adult corneal homeostasis and regeneration. Thus, *Aldh1a1,2,3-flox/flox*;Rosa26-CreERT2 mice provide a useful model for investigating the mechanistic role of RA signaling in adult corneal maintenance and could provide new insights into therapeutic approaches for controlling corneal repair to prevent vision loss.

*Corresponding Authors: duester@sbpdiscovery.org (Gregg Duester), sandeep@sbpdiscovery.org (Sandeep Kumar).

Disclosure

The authors report no conflicts of interest.

Retinoic acid (RA) is a small lipophilic signaling molecule derived from vitamin A (retinol). RA regulates diverse biological processes including cellular proliferation, differentiation, and apoptosis throughout embryonic organogenesis including ocular development, although its role in adult tissue maintenance is much less understood. RA serves as a ligand for nuclear RA receptors (RARs) which bind to RA response elements (RAREs) as heterodimers with retinoid X receptors (RXRs) to regulate target genes (Mark et al., 2006). Genetic studies in mouse have shown that RA synthesis is carried out by three retinaldehyde dehydrogenase enzymes encoded by *Aldh1a1, Aldh1a2, and Aldh1a3* (also known as *Raldh1*, *Raldh2*, and *Raldh3*), all three conserved in human (Cunningham and Duester, 2015; Niederreither and Dolle, 2008). During embryogenesis, ocular *Aldh1a1* and *Aldh1a3* expression is limited to the dorsal and ventral retina respectively, where it functions as a paracrine source of RA that diffuses to the perioptic mesenchyme to control anterior eye development (Cunningham and Duester, 2015). A functional role for RA has been very well established during ocular morphogenesis based upon genetic loss-of-function studies on *Aldh1a* genes and RAR genes (Lohnes et al., 1994; Ghyselinck et al., 1997; Wagner et al., 2000; Mic et al., 2004; Matt et al., 2005; Molotkov et al., 2006; Matt et al., 2008; Kumar et al., 2010). However, the functional role of these enzymes in the adult eye and cornea has not been explored, primarily due to the fact that deletion of *Aldh1a2* is lethal early in development (Niederreither et al., 1999), whereas *Aldh1a3* null mutants die at birth (Dupé et al., 2003); *Aldh1a1* mutants are viable but do not exhibit obvious eye defects (Fan et al., 2003). Recent studies on expression patterns of RA signaling components in the human eye show that ALDH1A enzymes are expressed in adult eye tissues including cornea, conjunctiva, and choroid (Nezzar et al., 2007; Harper et al., 2015). Also, ALDH1A1 expression in the cornea and lens has been proposed to protect inner ocular tissues from ultraviolet radiation and reactive oxygen-induced damage as ALDH1A1 can metabolize reactive aldehydes as well as retinaldehyde (Lassen et al., 2007).

In the adult, vitamin A plays a vital role in ocular surface maintenance and visual function as has been long known from vitamin A deficiency studies (Wolbach and Howe, 1925; Dowling and Wald, 1958). Lack of vitamin A causes xerophthalmia characterized by abnormal differentiation of the ocular surface that results in corneal and conjunctival keratinization, ulceration, epithelial squamous metaplasia, and loss of conjunctival goblet cells, all of which contribute to vision impairment and blindness (Hatchell et al., 1984; Macsai et al., 1998; Sommer, 1998; Combs et al., 1998). Also, retinoic acid treatment has been shown to reverse corneal xerophthalmia and keratinization in vitamin A deficient animal models (Ubels et al., 1984). Much of what is currently known about the role of RA in the adult cornea comes from recent studies suggesting that RA treatment regulates differentiation of corneal epithelial cells to maintain epithelial barrier function (Kim et al., 2012) and to stimulate re-epithelialization of cornea (Keino et al., 2011). RA treatment also increases stromal keratocyte numbers in vitro to promote corneal wound healing (Gouveia et

al., 2013), and RA helps reverse corneal epithelial keratinization (Ubels et al., 1987; Johansen et al., 1998). However, little is known about the effect of endogenous RA in maintaining corneal function, and the mechanism of endogenous RA action in the adult eye is not established.

The aim of the present study is to determine the function of endogenous RA in corneal maintenance and regeneration in adult eye using *Aldh1a1, Aldh1a2* and *Aldh1a3* loss-of-function studies. As germ-line knockouts of *Aldh1a2* and *Aldh1a3* are lethal (Niederreither et al., 1999; Dupé et al., 2003), we came up with a conditional knockout strategy to investigate the functions of RA-synthesizing enzymes in the adult cornea. We used a novel genetic loss-of-function approach which allows time-controlled knockdown of *Aldh1a1, Aldh1a2* and *Aldh1a3* in an adult mouse strain carrying homozygous flox alleles for all three genes, referred to here as *Aldh1a1,2,3-flox/flox* (Vermot et al., 2006; Raverdeau et al., 2012). We generated *Aldh1a1,2,3-flox/flox;Rosa26-Cre/ERT2* mice by crossing *Aldh1a1,2,3-flox/flox* mice with mice carrying the *Rosa26-Cre/ERT2* transgene that allows widespread induction of Cre following tamoxifen treatment (Ventura et al., 2007). Heterozygous offspring were back-crossed to *Aldh1a1,2,3-flox/flox* to obtain adult *Aldh1a1,2,3-flox/flox;Rosa26-cre/ERT2* mice, referred to here as *Aldh1a1,2,3-f/f;Cre+* mice. All experimental procedures were approved by the Institutional Animal Care and Use Committee at the Sanford Burnham Prebys Medical Discovery Institute (Animal Welfare Assurance Number A3053-01) and all animals were treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

For induction of Cre-mediated recombination, *Aldh1a1,2,3-f/f;Cre+* mice carrying the *Rosa26-Cre/ERT2* transgene and *Aldh1a1,2,3-f/f;Cre−* littermates were fed a tamoxifen-containing diet (TD130858, Harlan-Teklad; 0.5 mg tamoxifen per g of diet) for 2–12 weeks starting at the age of 6–8 weeks to analyze inactivation of *Aldh1a1, Aldh1a2* and *Aldh1a3*. In order to assess ablation of *Aldh1a* genes in the cornea, mice on tamoxifen diet were sacrificed at different time points from 2 to 12 weeks and PCR genotyping of whole cornea was performed using gene specific primers to assess inactivation of all three *Aldh1a* genes (primers for *Aldh1a1*, ADX318: ACAGGATCAGGCATCAGGAG, ADX319: GATTCCAGCAAACGGTAGGA; primers for *Aldh1a2*, ACH91: GTTTCATAGTTGGATATCATAATTTAAACAAGCAAAACC, ACM11:GTGGGACTCTGCCAGAAGTACTTC, ABI118: GTGTTGTGTTCACTCAGTCTC, ACF204:GGCAGCTGTGGAGAACATTTTATATCCATGTTG; primers for *Aldh1a3*: ACCM223: AAACCAGCACCACCTCCATA, ACCM 224: GACCAGCTTTCCAACCTTCA, ACCM225: AAACAACACAGAACCTCCTT); PCR conditions were previously reported (Vermot et al., 2006; Raverdeau et al., 2012). We found that after 2, 4, or 8 weeks on the tamoxifen diet, Cre-mediated recombination of *Aldh1a* genes was not complete (data not shown), but after 12 weeks we observed that recombination of *Aldh1a1* and *Aldh1a2* flox alleles was essentially complete, whereas for *Aldh1a3* excision was only ~80% complete (Fig. 1A). Also, at 12 weeks of tamoxifen treatment (but not at 2, 4, or 8 weeks) we observed that *Aldh1a1,2,3-f/f;Cre+* mice displayed symptoms of xerophthalmia (inability to open eyes and consistent eye twitching), whereas *Aldh1a1,2,3-f/f;Cre−* control mice with or without 12 weeks of tamoxifen treatment did not

exhibit these symptoms (Fig. 1B). At 12 weeks of tamoxifen treatment, none of the *Aldh1a1,2,3-f/f;Cre+* mice displayed a keratoconus-like phenotype. For histological and immunohistochemical evaluation of cornea, eyes from *Aldh1a1,2,3-f/f;Cre+* and control mice were enucleated and prefixed in 4% paraformaldehyde (Sigma-Aldrich) in PBS (pH 7.4), embedded in Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA) and then stored at −80°C. Adult cornea tissues were cryo-sectioned at 10 μm in the central region parallel to the optic nerve (to obtain the correct section plane), postfixed in 4% paraformaldehyde and processed for hematoxylin-eosin (H&E) or immunohistochemical staining. Histological analysis of eyes from adult mice revealed significant corneal thinning characterized by reduced thickness of the stromal layer in the central region of *Aldh1a1,2,3-f/f;Cre+* cornea compared to *Aldh1a1,2,3-f/f;Cre−* control mice treated with or without tamoxifen (Fig. 1C). The corneas of *Aldh1a1,2,3-f/f;Cre−* control mice with and without 12 weeks of tamoxifen treatment both had normal corneal morphology, demonstrating that tamoxifen treatment does not lead to corneal defects. Stromal thickness measurements were performed in H&E stained cryosections of cornea of *Aldh1a1,2,3-f/f;Cre+* and control mice using NIH ImageJ software. Stromal thickness measurements from the central region of the cornea were analyzed by averaging three sampled locations per image. The mean central corneal thickness of tamoxifen-treated adult *Aldh1a1,2,3-f/f;Cre+* mice was 66% that of the *Aldh1a1,2,3-f/f;Cre−* tamoxifen control cornea. No statistically significant difference in the thickness of the stromal layer was observed for *Aldh1a1,2,3-f/f;Cre−* control mice treated with or without tamoxifen (Fig. 1E). Also, a reduced thickness of the corneal epithelium was observed in tamoxifen-treated adult *Aldh1a1,2,3-f/f;Cre+* cornea compared with controls. By quantitating DAPI positive cells in the stroma layer, we observed a significant reduction in the number of keratocytes (Fig. 1D,F). These results indicate that *Aldh1a1, Aldh1a2* and *Aldh1a3* loss-of-function in the adult eye leads to xerophthalmia and corneal defects characterized by reduced thickness of the corneal stroma and epithelium. As *Aldh1a3* was not completely inactivated by Cre-recombination, it is possible that it does not contribute to the maintenance of cornea.

In order to maintain corneal integrity, basal epithelial cells divide and stratify upward to replenish outer epithelial cells that are regularly shed off, providing a dynamic process that is essential for normal vision (Meek and Knupp, 2015). Next, we explored if the loss of *Aldh1a* function in the adult cornea alters corneal epithelial maintenance. Immunostaining of Ki67, a marker of cell proliferation that is expressed in active cycling cells, was performed on corneal cryosections from *Aldh1a1,2,3-f/f;Cre+* and control mice using a Ki67 antibody (RM-9106-R7, Thermofisher). We found that Ki67-positive cells were markedly reduced in the basal layer of the central corneal epithelium in *Aldh1a1,2,3-f/f;Cre+* eyes when compared to *Aldh1a1,2,3-f/f;Cre−* control mice treated with tamoxifen or without the treatment (Fig. 1G,H). This observation indicates that *Aldh1a* deficiency leads to reduced cell proliferation. To further investigate whether loss of *Aldh1a* function in *Aldh1a1,2,3-f/f;Cre+* cornea leads to apoptosis in the corneal epithelial layer, TUNEL assay was performed on corneal cryosections using the In Situ Cell Death Detection Kit, Fluorescein (Roche) following the manufacturer's instructions; TUNEL also detects necrosis, however as we did not observe any scarring or clouding of *Aldh1a1,2,3-f/f;Cre+* corneas at 12 weeks, it is unlikely that TUNEL staining detected necrotic cells. We observed a drastic increase in

TUNEL-positive cells in the central corneal epithelial layer of *Aldh1a1,2,3-f/f;Cre+* mice treated with tamoxifen compared to *Aldh1a1,2,3-f/f;Cre−* control with or without the tamoxifen administration (Fig. 1I,J). In contrast, we did not observe any Ki67-positive cells or TUNEL-positive cells in the corneal stroma or endothelium in *Aldh1a1,2,3-f/f;Cre+* or *Aldh1a1,2,3-f/f;Cre−* mice, suggesting that dysregulation of corneal stromal-epithelial interactions could be a contributing factor to the corneal thinning phenotype.

Developmental defects observed in *Aldh1a2−/−* and *Aldh1a3−/−* knockout mouse embryos can be rescued with dietary RA supplementation to further understand the role of these RA-synthesizing enzymes during later stages of development (Molotkov et al., 2006). To examine if the corneal defect observed in *Aldh1a1,2,3-f/f;Cre+* mice upon induction of Cre by tamoxifen treatment is due to loss of RA, we treated *Aldh1a1,2,3-f/f;Cre+* mice with exogenous RA to evaluate if RA treatment can rescue the corneal thinning phenotype. RA treatment of adult mice was performed by dietary supplementation of all-*trans*-RA doses previously demonstrated to provide an amount of RA in the normal physiological range (Molotkov et al., 2006). All-*trans*-RA (Sigma-Aldrich) was dissolved in ethanol, diluted in corn oil then mixed with powdered standard mouse chow to provide a final concentration of 0.25 mg RA per g diet. Mice were provided fresh chow containing RA twice a day. For RA rescue experiments, *Aldh1a1,2,3-f/f;Cre+* mice were treated with tamoxifen for 12 weeks to stimulate corneal defects followed by RA treatment for 5 weeks. *Aldh1a1,2,3-f/f;Cre+* and *Aldh1a1,2,3-f/f;Cre−* control mice treated with tamoxifen but not RA were used as controls. We found that *Aldh1a1,2,3-f/f;Cre+* mice treated with tamoxifen and RA showed significant rescue of the corneal thinning phenotype compared to *Aldh1a1,2,3-f/f;Cre+* mice treated with tamoxifen but not RA (Fig. 2A,B); *Aldh1a1,2,3-f/f;Cre−* mice treated with tamoxifen but not RA were also used as a control to observe normal corneal morphology. We examined whether RA rescue of the corneal thinning phenotype observed in *Aldh1a1,2,3-f/f;Cre+* mice may be due to a restoration of corneal epithelial proliferation. Interestingly, *Aldh1a1,2,3-f/f;Cre+* mice treated with both tamoxifen and RA exhibited a normal level of Ki67-positive cells in the basal corneal epithelial layer compared to *Aldh1a1,2,3-f/f;Cre+* with tamoxifen but without RA treatment (Fig. 2C,D). Also, in contrast to *Aldh1a1,2,3-f/f;Cre+* mice treated with tamoxifen but not RA that shows a higher number of TUNEL-positive cells in the corneal epithelium, *Aldh1a1,2,3-f/f;Cre+* mice treated with both tamoxifen and RA showed a drastic reduction of TUNEL-positive cells comparable to *Aldh1a1,2,3-f/f;Cre−* control mice treated with tamoxifen but not RA (Fig. 2E,F). These results indicate that 5 weeks of RA treatment partially rescues the corneal thinning phenotype in *Aldh1a*-deficient mice, suggesting that RA plays an important role in mediating the proliferative function of corneal basal epithelial cells for maintaining corneal transparency and normal vision.

In this study, we found that mice with targeted deletions of RA-generating enzymes encoded by *Aldh1a* genes exhibit xerophthalmia and corneal thinning defects. Adult vitamin A deficiency has been implicated in abnormal corneal structure and function, and has been suggested to contribute to the onset and progression of corneal diseases such as xerophthalmia (McLaren and Frigg, 2001) and keratoconus (Mutch and Richards, 1939). However, a requirement for the endogenous vitamin A metabolite RA in corneal maintenance has not previously been reported. Corneal stromal thinning and disorganization

are common hallmarks of vitamin A deficiency, but the functional role of endogenous RA in corneal stromal biology has not been established. Our results indicate that RA deficiency leads to reduced corneal stromal thickness and directly affects cell proliferation and apoptosis in the corneal epithelium. Over the years, several corneal modulators of stromal-epithelial interactions such as cytokines and growth factors have been implicated in normal corneal maintenance, regeneration and wound healing. Our finding on the endogenous role of RA in the adult cornea provides further insight into signaling mechanisms that may lead to alternative corneal therapies, perhaps the ability to convert human pluripotent stem cells to corneal cells for tissue engineering, transplant therapy, and clinical applications. It will be interesting to further explore how RA affects stromal-epithelial interactions and how deficiency of RA modulates stromal keratocyte homeostasis to affect the integrity of the stromal layer and result in corneal degeneration and disease. Further studies using our *Aldh1a1,2,3-flox/flox*;Rosa26-creERT2 mouse model will allow us to better understand the mechanistic action of RA signaling in corneal maintenance, regeneration and wound healing.

Acknowledgments

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Highlights

- Retinoic acid (RA) synthesis by ALDH1A enzymes is essential for corneal maintenance.

- *Aldh1a* genetic loss-of-function in adult eye results in corneal thinning.

- RA treatment of *Aldh1a*-deficient mice rescues the corneal thinning phenotype.

- RA deficiency reduces cell proliferation & increases apoptosis in corneal epithelium.
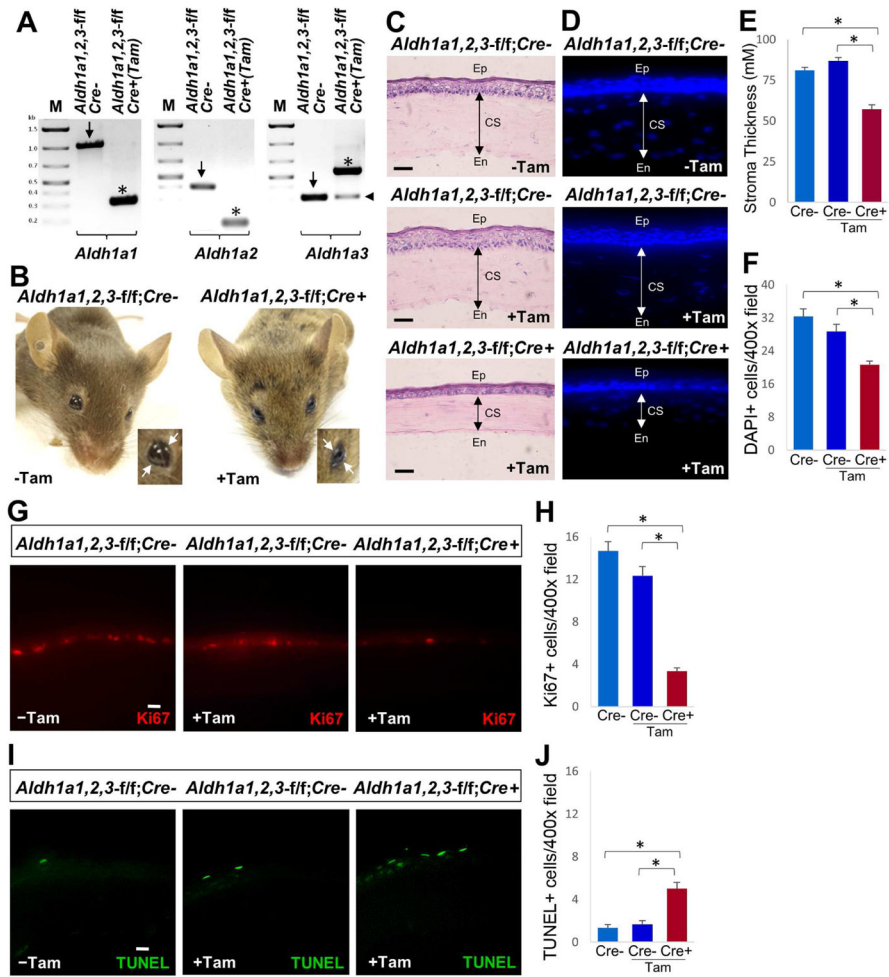
Fig. 1. Ablation of *Aldh1a* genes leads to corneal defects

(A) PCR genotyping of whole cornea for *Aldh1a1, Aldh1a2* and *Aldh1a3* in untreated *Aldh1a1,2,3-f/f;Cre−* mice and *Aldh1a1,2,3-f/f;Cre+* mice treated with tamoxifen for 12 weeks; arrows mark the flox allele while asterisks mark the deleted allele that inactivates the gene. In the case of *Aldh1a3*, the combination of PCR primers results in a band identifying the deleted allele that is larger than the flox allele; for *Aldh1a3*, Cre-mediated excision is not complete as indicated by an arrowhead next to the band detecting the remaining portion of DNA carrying the flox allele. (B) *Aldh1a1,2,3-f/f;Cre+* mutant mice treated with tamoxifen display symptoms of xerophthalmia and inability to open eyes (marked by arrows). (C) Comparison of corneal thickness in hematoxylin/eosin (H&E)-stained sections from adult *Aldh1a1,2,3-f/f;Cre−* mice with or without tamoxifen treatment and *Aldh1a1,2,3-f/f;Cre+* mice with tamoxifen treatment; corneal sectioning can be difficult, however our observation of a consistently thinner cornea in all sections of Cre+ mice treated with tamoxifen indicates that we did not experience significant variations in section quality. (D) DAPI (4′,6-diamidino-2-phenylindole) staining of cell nuclei in central corneal cross-sections to detect stromal keratocytes. (E) Quantitation of corneal stromal thickness in *Aldh1a1,2,3-f/f;Cre+* mice with tamoxifen compared with Cre- controls with or without tamoxifen treatment. (F) Quantitation of DAPI+ cells (keratocytes) in corneal stroma. (G) Comparison of Ki67-

positive cells in the corneal basal epithelial layer of *Aldh1a1,2,3-f/f;Cre+* mice treated with tamoxifen compared to Cre- controls. (H) Quantitation of Ki67-positive cells in corneal epithelium. (I) Comparison of TUNEL-positive cells in the corneal epithelium of *Aldh1a1,2,3-f/f;Cre+* cornea with tamoxifen compared to Cre- controls. (J) Quantitation of TUNEL-positive cells in corneal epithelium. For all panels, scale bar is 20 μm. Number of DAPI+, Ki67+ and TUNEL+ cells per microscopic field was counted at 400X magnification. For each condition examined, data are expressed as mean ± SEM, $n = 3$ (*, $p < 0.05$; Student's *t*-test, two-tailed). CS, corneal stroma; En, endothelium; Ep, epithelium.
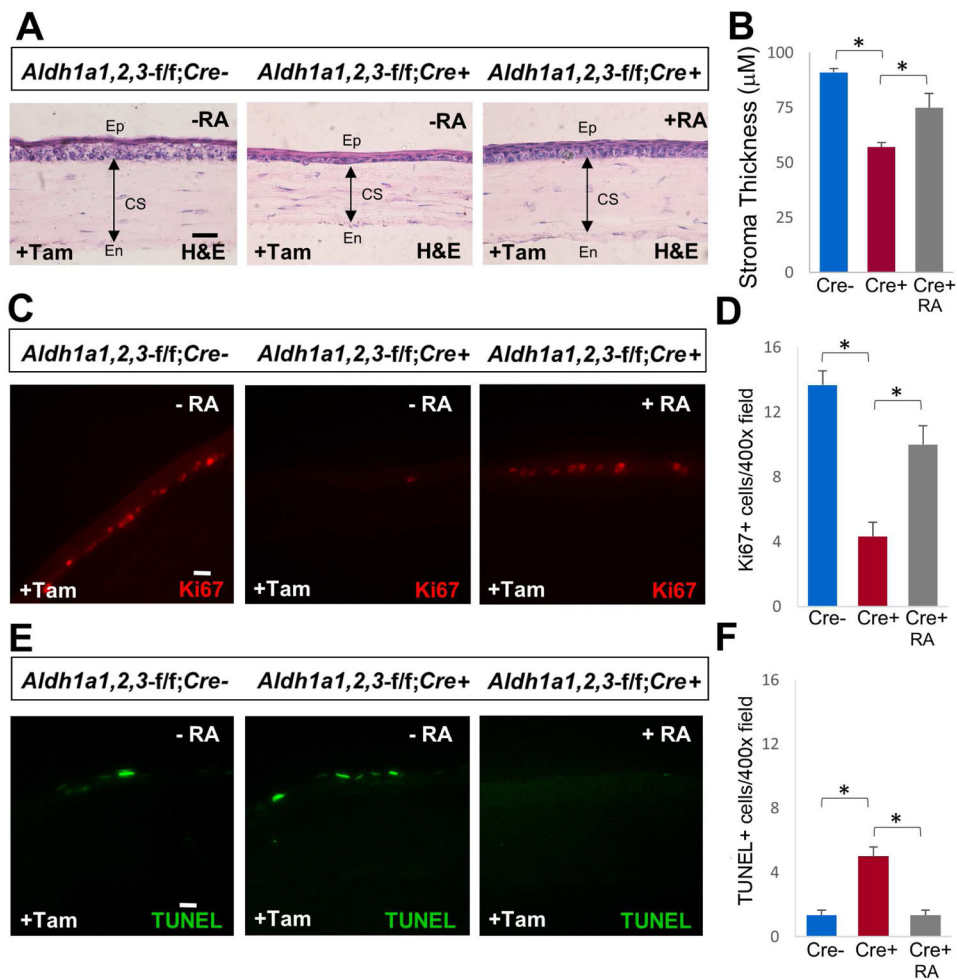
Fig. 2. RA rescues the corneal thinning phenotype in *Aldh1a*-deficient mice
(A) Comparison of corneal stroma thickness in hematoxylin/eosin (H&E)-stained sections of *Aldh1a1,2,3-f/f;Cre+* mice treated with tamoxifen only or treated with both tamoxifen and RA; *Aldh1a1,2,3-f/f;Cre−* mice treated with tamoxifen only were used as a control. (B) Quantitation of corneal stromal thickness. (C) Comparison of Ki67-positive cells in basal corneal epithelial layer. (D) Quantitation of Ki67-positive cells in corneal epithelium. (E) Comparison of TUNEL-positive cells in corneal epithelium. (F) Quantitation of TUNEL-positive cells in corneal epithelium. For all panels, scale bar is 20 μm. Number of DAPI+, Ki67+ and TUNEL+ cells per microscopic field was counted at 400X magnification. For each condition examined, data are expressed as mean ± SEM, $n = 3$ (*, $p < 0.05$; Student's *t*-test, two-tailed). CS, corneal stroma; En, endothelium; Ep, epithelium.