Ectodysplasin-A signaling is a key integrator in the lacrimal gland–cornea feedback loop
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ABSTRACT
A lack of ectodysplasin-A (Eda) signaling leads to dry eye symptoms, which have so far only been associated with altered Meibomian glands. Here, we used loss-of-function (Eda<sup>−/−</sup>) mutant mice to unravel the impact of Eda signaling on lacrimal gland formation, maturation and subsequent physiological function. Our study demonstrates that Eda activity is dispensable during lacrimal gland embryonic development. However, using a transcriptomic approach, we show that the Eda pathway is necessary for proper cell terminal differentiation in lacrimal gland epithelium and correlated with modified expression of secreted factors commonly found in the tear film. Finally, we discovered that lacrimal glands present a bilateral reduction of Eda signaling activity in response to unilateral corneal injury. This observation hints towards a role for the Eda pathway in controlling the switch from basal to reflex response to unilateral corneal injury. Our results suggest that Eda downstream targets could help alleviate dry eye symptoms.

KEY WORDS: Ectodysplasin, Lacrimal gland, Tear film, Cornea, Wound healing

INTRODUCTION
The lacrimal apparatus is responsible for producing the tear film, which protects the cornea from the external environment (Zieske, 2004). The tear film is composed of three layers, each of them produced by a different element of the lacrimal apparatus. Goblet cells secrete the inner or mucous layer, directly in contact with the cornea. Lacrimal glands (LGs) secrete the central or aqueous layer. Meibomian glands secrete the outer or lipid layer. Lastly, the Harderian gland produces an oily component necessary for the nictitating membrane movements. The oily part of the lacrimal film is known to be important for maintaining the film stability, but, based on observations in mouse models, other potential roles remain unclear (Miletich, 2018). Together, the different elements of the lacrimal apparatus secrete three types of tears, namely basal, reflex and psycho-emotional tears. Basal tears supply the avascular corneal epithelium with growth factors necessary for its maturation and maintenance (for reviews, see Klenkler et al., 2007; Zieske, 2004). Some growth factors can originate from the aqueous humor, through the endothelium following injury (Chandler et al., 2019). Epidermal growth factor (EGF), for instance, is one of the most characterized LG-secreted factor and is known to positively regulate proliferation of corneal epithelial cells (Xiao et al., 2012). Transforming growth factor-beta (TGFβ) and hepatocyte growth factor (HGF), among others, are likewise produced by LGs and play pleiotropic roles in corneal epithelium homeostasis (Wilson et al., 1999). Reflex tear production results from eye injury and supports corneal regeneration. Finally, psycho-emotional tears have so far only been found in human and seem to be related to social interactions (Murube, 2009).

Similarly to cornea, LG epithelium originates from the embryonic ectoderm. After a budding and ductal elongation period starting at embryonic day (E) 14 in mouse, LG branching morphogenesis begins around E16 and continues after birth. Our previous work showed that the LG continues its growth in postnatal stages, reaching its full size by postnatal day (P) 50 (Kuony and Michon, 2017). Numerous signaling pathways are involved in LG morphogenesis, such as fibroblast growth factor (FGF), Notch and Wnt pathways (Chen et al., 2014; Dean et al., 2005; Dvoriantchikova et al., 2017; Makarenkova et al., 2000; Tsau et al., 2011).

The ectodysplasin-A (Eda) pathway is known to be involved in the formation of several ectodermal organs, including hair and mammary glands (Mikkola, 2008), but has not been extensively studied in the LG context (Pispa et al., 2003). Eda loss-of-function (Eda<sup>−/−</sup>) mutation in mammals, which leads to X-linked hypohidrotic ectodermal dysplasia (XLHED, MIM 305100) in humans (Bayès et al., 1998; Headon et al., 2001; Kowalczyk et al., 2011; Srivastava et al., 1997), is characterized by severe symptoms, including hair, tooth, sweat gland and salivary gland hypoplasia (for reviews, see LeFebvre and Mikkola, 2014; Reyes-Reali et al., 2018). Moreover, LG defects in XLHED patients lead to dry eye diseases (Dietz et al., 2013).

In mouse, Eda<sup>−/−</sup> (tabby) mutants present atrophied LGs during embryogenesis (Grüneberg, 1971), and a dry eye phenotype in postnatal stages (Wang et al., 2016). However, this last report disregarded the effect of Eda mutation on LG formation and function, attributing the related dry eye phenotype to atrophied or missing Meibomian glands, and subsequent tear film-accelerated evaporation (Wang et al., 2016). The lack of thorough study on Eda<sup>−/−</sup> LGs has precluded a full understanding of all aspects of dry eye symptoms experienced by XLHED patients.

Corneal abrasion represents one of the most common eye injuries (Kalha et al., 2018a). In a healthy context, cornea wound healing occurs spontaneously and is known to be supported by an increase in secretion of LG-specific factors in reflex tears (Petznick et al., 2013). Corneal repair is characterized by a cell migration period followed by a stratification step. This well-conserved mechanism results in a fully re-stratified corneal epithelium 7 days post-injury in cat (Petznick et al., 2013). Interestingly in mouse, Eda<sup>−/−</sup> mutants...
exhibit delayed corneal wound healing in comparison with control animals (Li et al., 2017).

In this study, we characterized physiological and molecular defects of LGs resulting from an Eda loss-of-function mutation. After showing Eda and Edar expression patterns during LG formation, we localized Eda pathway activity in the epithelium, from E16 to 13 weeks old (wo). As expected, Eda activity was absent in Eda−/− LGs. Eda−/− animals displayed a lower basal tear secretion volume, reflecting defective LG function. We demonstrated that, despite normal morphology, Eda−/− LGs exhibited altered terminal differentiation in comparison with control. Moreover, 1.8% of the Eda−/− LG transcriptomic signature was significantly different from control. Finally, we showed that corneal injury leads to an inhibition of Eda signaling in both LGs. Subsequently, a set of key genes reacting to corneal injury responded differently in Eda−/− LGs, illustrating the role of Eda signaling in LG–cornea crosstalk and reflex tear composition.

RESULTS

The Eda pathway is active in embryonic and adult LG epithelium

To study Eda pathway activity, we first confirmed the expression of the ligand Eda and its receptor Edar in LGs using RNAscope technology (Kalha et al., 2018b; Wang et al., 2012). Edar was previously reported in the LG epithelial pre-bud at E14 (Pispa et al., 2003). However, LG branching morphogenesis seemed delayed and did not initiate before E16 in mice from the C57BL/6J background, compared with E15 in ICR mice (Kuony and Michon, 2017) (Fig. S1). Therefore, as the mouse lines used in this study were maintained in a C57BL/6JRccHsd background, we focused here on the morphogenesis events happening between E16 and E18. During this period, Eda expression was mostly detected in the LG mesenchymal compartment, whereas Edar was found in the epithelium (Fig. 1A). As the ligand and receptor were present, we aimed at visualizing Eda pathway activity. For that purpose, we used.

**Fig. 1. The Eda pathway is active in embryonic and postnatal LG epithelium.** (A) RNAscope assay shows Eda (red) and Edar (blue) expression pattern (single mRNA molecules) from E16 to E18. Low magnification (upper panels) allows the visualization of LG global morphology. The boxed areas in the upper panels indicate the magnified region in the lower panels, in which Eda and Edar signals are replaced with false colors for clarity. White lines delimit the epithelial compartment. (B) X-gal staining (blue) shows β-galactosidase activity in NF-κB-gal reporter samples, from E16 to E18. NF-κB-gal reporter unravels Eda pathway activation. Dashed black lines delimit the epithelial compartment. (C) X-gal staining (blue) shows β-galactosidase activity in NF-κB-gal reporter samples, from P7 to 13 wo, in different portions of the LGs (beginning, center and end). At postnatal stages, the Eda pathway is active in single acinar cells (black arrowheads). n>3 animals per time point. Scale bars: 100 µm.
Eda signaling has little to no impact on LG embryonic morphogenesis. Together, these data suggest that Eda signaling has little to no impact on cell terminal differentiation and LG maturation from P0 to 13 wo. First, we studied the effect of the mutation on cell terminal differentiation by measuring tear production and blinking rate in adult animals. Our results indicated that adult Eda\(^{-/-}\) animals (13 wo) had a lower rate of tear production and a higher blinking rate than control animals (0.38-fold and 12-fold, respectively) (Fig. 5A,B). The higher blinking rate remained consistently higher even for one-year-old animals (data not shown), indicating a long-lasting LG physiological defect in Eda\(^{-/-}\) mutants.

We checked the effect of the Eda mutation on the Meibomian glands in our mouse model, and saw a possible abnormal organ differentiation level of lactotransferrin \((Ltf)\), a marker of mature acini (Janssen and van Bijsterveld, 1983), basic helix-loop-helix family member a15 \((BhhA15,\text{ also known as } Mist1)\), which regulates the secretory program (Pin et al., 2001, 2000), and aquaporin 5 \((Aqp5)\), which is involved in fluid secretion (Ishida et al., 1997). From P0 to P21, these three genes were constantly upregulated in Eda\(^{-/-}\) LG, except for Ltf at P0, and BhhA15 and Aqp5 at P7. At 13 wo, Ltf expression was significantly reduced in Eda\(^{-/-}\) compared with control samples. However, BhhA15 and Aqp5 were similarly expressed in both mutant and control LGs in the adult stage (Fig. 4). Then, we analyzed the ductal compartment by investigating the expression levels of keratin 19 \((Krt19)\) (Kuony and Michon, 2017), solute carrier family 12 member 2 \((Slc12a2)\) and keratin 17 \((Krt17)\) (Farmer et al., 2017; Kuony and Michon, 2017; Kurokawa et al., 2010; Walcott et al., 2005). Differently from acinar markers, ductal markers were not as strongly misregulated during Eda\(^{-/-}\) LG maturation. Nevertheless, by P21, Slc12a2 and Krt17 were enriched in Eda\(^{-/-}\) compared with control LGs. At 13 wo, Slc12a2 was downregulated, whereas Krt19 and Krt17 were not differently expressed in the mutant compared with the control (Fig. 4). Finally, we monitored myoepithelial cell (MEC) terminal differentiation via alpha 2 actin \((Acta2,\text{ encoding for alpha-smooth muscle actin})\), alpha 2 smooth muscle actin \((\alpha SMA)\) (Makarenkova and Datt, 2015), transformation related protein 63 \((Trp63)\) and calponin 1 \((Cnn1)\) expression (Farmer et al., 2017). From P0 to 13 wo, these three genes were either similarly expressed or downregulated in Eda\(^{-/-}\) LG. By 13 wo, Trp63 and Acta2 were downregulated in Eda\(^{-/-}\) LG, and Cnn1 was similarly expressed in Eda\(^{-/-}\) and control LGs (Fig. 4). Taken together, four of the nine considered markers were significantly misregulated in adult Eda\(^{-/-}\) LGs. This observation points towards altered cell terminal differentiation in all compartments of the Eda\(^{-/-}\) LG.

Eda\(^{-/-}\) LGs present defective LG secretory functions

Proper terminal differentiation is crucial for physiological LG secretion. Here, we assessed Eda\(^{-/-}\) LG function and eye dryness by measuring tear production and blinking rate in adult animals. Our results indicated that adult Eda\(^{-/-}\) animals (13 wo) had a lower rate of tear production and a higher blinking rate than control animals (0.38-fold and 12-fold, respectively) (Fig. 5A,B). The higher blinking rate remained consistently higher even for one-year-old animals (data not shown), indicating a long-lasting LG physiological defect in Eda\(^{-/-}\) mutants.

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morphology (data not shown), as previously reported in another strain of Eda−/− mutants (Wang et al., 2016). This observation could explain the higher blinking rate, through tear evaporation, but not the lower tear production. Furthermore, although the full body weight of Eda−/− animals was comparable to controls (Fig. 5C), we noticed that Eda−/− LGs were significantly heavier than the control ones (1.76-fold at 13 wo; Fig. 5D, Fig. S4). Therefore, we checked the acinar morphology. Despite a possible increased compaction in Eda−/− acinar compartment, explaining heavier LGs, both control and mutant LGs exhibited a similar histology (Fig. 5E), hinting towards a possible cumulative phenotype, translated to affected LG physiology in the Eda loss-of-function adult mutants.

To understand further the impact of Eda deficiency on LG, we used RNA sequencing to compare control and Eda−/− LG transcriptomic signatures in 13 wo animals (Fig. 6A). We chose to analyze the genes presenting at least 30% expression level difference compared with the control. We found that 1.8% of the total transcriptomic signature (916 from the 50,687 transcripts sequenced) were different in Eda−/− LGs. Of these, 412 transcripts (0.80% of the transcriptomic signature) were significantly enriched in Eda−/− LGs compared with controls. Additionally, 504 transcripts (1%) were depleted in Eda−/− LGs compared with control (Fig. 6B). Among the differently expressed transcripts, some of the terminal differentiation markers that we investigated during the maturation process were misregulated in the Eda−/− adult transcriptomic signature.

In addition, the RNA sequencing results highlighted a misregulation of growth factors known to be secreted in basal tears (for a review, see Klenkler et al., 2007). As expected from the RNA sequencing, quantitative PCR analyses confirmed that Egf expression levels are similar in control and Eda−/− LGs (Fig. 6C). In addition, C-X-C motif chemokine ligand 10 (Cxcl10), previously associated with dry eye diseases (DEDs) (Yoon et al., 2010),
appeared to be downregulated in Eda<sup>−/−</sup> LGs compared with controls. In comparison, growth differentiation factor 5 (Gdf5), involved in the inhibition of corneal epithelial cells proliferation (You et al., 1999), was strikingly enriched in Eda<sup>−/−</sup> LGs compared with control (Fig. 6C).

Previous studies reported decreased cell proliferation in Eda<sup>−/−</sup> corneas, along with impaired wound healing (Li et al., 2017). As our results indicate a misregulation of the growth factors produced by the LG, we hypothesized a defective response of Eda<sup>−/−</sup> LG to corneal insult, resulting in an impaired wound healing process. Therefore, we investigated LG response to corneal abrasion.

**Eda<sup>−/−</sup> LGs show misregulation of specific genes upon corneal abrasion**

Previous reports demonstrated modulation of LG-secreted factors following corneal insult, supporting the wound-healing process (Nagano et al., 2003; Wilson et al., 1999). We recently reported that corneal epithelium is in an active wound-healing process 18 h after abrasion (Kalha et al., 2018b). Moreover, it was shown that Eda<sup>−/−</sup> animals present high corneal dystrophy, along with impaired corneal epithelium barrier function and delayed cornea wound healing (Li et al., 2018, 2017; Wang et al., 2016). Our Eda<sup>−/−</sup> mouse strain did not exhibit corneal dystrophy in young animals (13 wo), but an epithelial thinning in old ones (52-64 wo) (Fig. S5A). Nevertheless, we confirmed a delay in our corneal abrasion model (Kalha et al., 2018a) (Fig. S5B), and analyzed the expression of several factors known to play a role during corneal wound healing (Klenkler et al., 2007; Yan et al., 2016) (Fig. 7A). Notably, we assessed the expression level of Tgfb1, Fgf7 (also known as Kgf) and Hgf in the unwounded situation (‘pre’, in both control and mutant LGs), and compared it with expression levels in post-wounded situations [wounded side (WS) and unwounded contralateral side (CLS) at 18 h post-wound, in both control and mutant LGs]. We noticed an increase in Tgfb1 expression after cornea injury, in both control and Eda<sup>−/−</sup> LGs. However, the level of Tgfb1 expression in post-wounded samples was higher in control compared with Eda<sup>−/−</sup> LGs. Fgf7 was downregulated in both control and Eda<sup>−/−</sup> LGs at 18 h post-injury. However, the level of Fgf7 expression in post-wounded samples was
higher in control compared with Eda−/− LGs. In contrast, our results showed reduced Hgf expression after the wound only in Eda−/− samples. Together, our results suggest that the maturation defects observed in Eda−/− LGs result in an altered response to cornea injury.

The Eda pathway participates in an LG integrated response following corneal abrasion

We monitored the impact of corneal abrasion on Eda pathway activity. Despite the presence of Eda and Edar transcripts in the corneal epithelium at all stages examined (E16-E18 and 13 wo; Fig. S6), Eda signaling was inactive in the cornea at all these stages, both in physiological and in wounded conditions (Fig. 8A, Fig. S6). However, whereas LGs exhibited an active Eda pathway in unwounded samples, we observed an inhibition of the pathway 18 h after cornea wounding (Fig. 8A,B). Moreover, qPCR confirmed an important diminution of Eda and Edar expression levels in the LG, 18 h after a unilateral corneal abrasion (Fig. 8C). Strikingly, Eda pathway inhibition was extended to the CLS LG, on
the unwounded eye side (Fig. 8B,C). We quantified these results, and demonstrated that at 18 h post-cornea wounding, 29 cells on average presented an active Eda pathway in the LG on the wounded side (Kels et al., 2015), and four cells on average in the contralateral gland, compared with 155 on average in the unwounded situation (Kels et al., 2015), and four cells on average in the contralateral average presented an active Eda pathway in the LG on the wounded and demonstrated that at 18 h post-cornea wounding, 29 cells on the unwounded eye side (Fig. 8B,C). We quantified these results, and

Following this last observation and our previous results showing an impaired modulation of LG factors upon corneal abrasion (Fig. 7A), we analyzed the expression of Tgfb1, Fgf7 and Hgf in the contralateral LG after corneal wounding (Fig. 7B). Interestingly, the expression of Tgfb1 and Fgf7 was similarly affected by the corneal injury in both WS and CLS LGs (Fig. 7), independently of the Eda loss-of-function mutation. In contrast, whereas Hgf expression was unaffected in the LG on the wounded side in controls (Fig. 7A), Hgf expression was downregulated in the contralateral LG in controls (Fig. 7B). However, Hgf expression was not significantly reduced in the CLS LG in Eda−/− samples (Fig. 7B).

These last results confirm crosstalk between the LG and the cornea, but most importantly, indicate communication between the two LGs. In addition to impacting the LG maturation process and its related function, our results suggest a role for the Eda pathway during the cornea wound-healing process.

**DISCUSSION**

The Eda−/− mouse strain provides an interesting model in which to study DEDs. Although LG implication in XLHED-related dry eye phenotype was reported in dog (Casal et al., 2007), and suggested in mice (Grüneberg, 1971; Kowalczyk-Quintas et al., 2015), dry eye symptoms have so far been attributed to atrophy or agenesis of Meibomian glands, resulting in an accelerated tear film evaporation in the murine model (Wang et al., 2016). In contradiction to a recent study (Wang et al., 2016), our tabby strain displayed a lower tear film volume, hinting at LG involvement in XLHED-related dry eye phenotype in our model. This discrepancy could result from the use of mouse models exhibiting different mutations in the Eda gene in our study versus the previously published study. The differences might also come from the different genetic background used in these two studies. Indeed, whereas we used C57BL/6JrcHsd in our study, the other study used C57BL/6J from the Jackson Laboratory, corresponding to a different strain (Zurita et al., 2011). We cannot conclude which of these reasons could explain the phenotype discrepancies. Here, we present the first extensive study of the Eda−/− LG phenotype, and the impact of Eda mutation on the cornea–LG feedback loop.

In the past 20 years, the Eda−/− mouse model has been used to study the impact of the ectodysplasin pathway on ectodermal organ morphogenesis. Striking morphological defects have been reported in hair (Laurellkala et al., 2002), tooth (Pispa et al., 1999), sweat glands (Cui et al., 2009), salivary glands (Häärä et al., 2011) and mammary glands (Voutilainen et al., 2012). To date, the LG appears to be the only ectodermal organ not displaying any major morphological defects upon Eda loss of function. Therefore, we hypothesize either that Eda is not involved in LG early development, or that LG early morphogenesis is based on a more robust genetic network, which is able to compensate for the absence of Eda pathway activity.

Although Eda signaling does not play a pivotal role in LG early morphogenesis, our analysis shows that the Eda−/− LG is significantly heavier in the adult, and that cell terminal differentiation is affected. These defects impacted the expression of several factors in basal tears that were shown to have an impact on corneal epithelium maintenance and renewal (Klenkler et al., 2007). Finally, Gdf5 increased expression is of interest, as it was shown to inhibit corneal epithelial cell proliferation (You et al., 1999). The increase of Gdf5 expression might partially explain the delayed corneal wound healing in Eda−/− animals (Li et al., 2017) although we expect that a combination of factors is responsible for this delay. Collectively, our results demonstrate that Eda signaling in LGs plays a crucial role in corneal physiology.

Previous work suggested a modulation of tear film composition when reflex tears are produced, to support corneal wound healing (for a review, see Klenkler et al., 2007), and Eda−/− animals were recently shown to have a slower corneal epithelium wound healing (Li et al., 2017). Here, we showed that Cxcl10, Hgf, Fgf7 and Tgfb1, all involved in corneal wound healing (Tervo et al., 1997; Tuominen et al., 2001; Wilson et al., 1999), were misregulated in Eda−/− LGs. Interestingly, our results showed that the initiation of reflex tear production is concomitant to an inhibition of Eda pathway activity. This event seems to be key in the genetic network regulating tear film composition, although several factors were properly regulated in Eda−/− LGs. The modulation of gland excretion upon external stimulus is not uncommon as salivary glands were reported to regulate their production upon oral wounds (Bodner, 1991), and eccrine sweat glands contribute to epithelial wound closure (Rittié et al., 2013). In a similar manner, the regulation of secreted factors during the switch from basal to reflex tears is a key element supporting corneal homeostasis and wound healing.

Together, these observations demonstrate the importance of Eda signaling in cornea wound healing, via LG factor secretion, as well as its crucial role in an LG–cornea feedback loop, reflected by the tear film modulation supporting corneal epithelium regeneration (Fig. 9).

Finally, the resulting Eda pathway inhibition in LGs upon unilateral corneal insult appeared bilateral. This demonstrates an integration of corneal stress signal, leading to a coordinated systemic response in both eyes and in both LGs, as previously reported (Sagga et al., 2018). It is known that the cornea is highly innervated, and that corneal innervation originates from the trigeminal nerve. Interestingly, previous reports showed that the cornea integrates nociceptive stimuli at the level of the spinal trigeminal spinals, resulting in a modification of LG secretory activities (Meng and Kurose, 2013). For instance, studies in Sjögren’s syndrome mouse models revealed altered LG innervation in this autoimmune response, subsequently affecting LG secretory functions (Chen et al., 2018; Dartt, 2004). To date, our work represents the first study directly linking a specific pathway activity, here Eda, to cornea–LG feedback loop activation. However, at this stage, we can only speculate on the advantage of a bilateral response. One possibility could be that the bilateral response stems from an evolutionary inherited feature. When digging, fossorial animals have exposed eyes, and they are likely to experience bilateral ocular wounds. Therefore, we hypothesize that it might be beneficial to anticipate and simultaneously support corneal regeneration in both eyes.

Collectively, our study demonstrates a minimal role for Eda signaling in early LG morphogenesis, but a pivotal role in LG maturation and function. Indeed, we showed that the Eda pathway acts both on proper cell differentiation, and on the expression of protective secreted factors found in the tear film and directed to the corneal epithelium (Fig. 9) (for a review, see Klenkler et al., 2007). Moreover, inhibiting Eda signaling in LG epithelium seems to be part of a feedback loop between cornea and LG, which allows the secretion of reflex tears supporting cornea wound healing. Therefore, we propose that not only that Eda signaling is crucial for tear film composition in physiological and pathophysiological conditions, but also that XLHED dry eye symptoms result from a complex combination of factors, establishing the Eda−/− mutant as a multifactorial dry eye model.
MATERIALS AND METHODS

Animals and tissue sample processing
All aspects of the animal studies were approved by the Finnish National Board of Animal Experimentation (ESAVI/1284/04.10.07/2016). Eda−/− (tabby, allele B6CBACa-Aw−J/A-Ta) (Pispa et al., 1999) and NF-κB-gal (Eda pathway activity reporter) (Bhakar et al., 2002) mouse strains were used in this study. These mouse lines and corresponding genotyping protocols have been previously described (Bhakar et al., 2002; Pispa et al., 1999). To assess Eda pathway activity in the loss-of-function situation, Eda−/− animals were crossed with NF-κB-gal reporter mice (Bhakar et al., 2002). All mice were kept in C57BL/6 background. C57BL/6 (control) mice were obtained from the Helsinki animal facility. The plug day was considered as E0 and the date of birth as P0.

Collected adult LGs and E16-E18 heads were fixed overnight in 4% paraformaldehyde (PFA) at 4°C, except for the NF-κB-gal samples. E17 and E18 heads were decalcified in 0.5M EDTA. Samples were dehydrated, embedded in paraffin, and sectioned at 5 µm. Sections were used for histological staining and for in situ hybridization. For whole-mount samples, dissected LGs were fixed for 25 min in 4% PFA at room temperature (RT).

In situ hybridization
RNAsecope in situ hybridization (Wang et al., 2012) was performed using the RNAsecope 2.5 HD Duplex chromogenic kit (322430, Advanced Cell Diagnostics). During the pretreatment step, the sections were preheated for 1 h at 60°C, rehydrated in successive xylene and decreasing percentage of ethanol baths, and air-dried at RT. The slides were then incubated for 10 min with hydrogen peroxide (from the kit, 322430, Advanced Cell Diagnostics) and rinsed with H2O. Next, the slides were incubated at 100°C in the target retrieval reagent for 17 min, rinsed with H2O, immersed briefly in ethanol and air-dried at RT. The samples were stored overnight at RT. Following this step, the samples were incubated for 20-30 min with protease (20 min for cornea samples, 30 min for LG samples; from the kit, 322430, Advanced Cell Diagnostics). The following steps, including the hybridization of the probes, amplification and detection of the signals, were performed according to the manufacturer’s protocol with small changes (AMP5 step for 15 min, and red substrate incubation for 25-30 min). Finally, samples were counterstained with a 50% Hematoxylin solution, dried at 60°C on a hotplate for 45 min, and mounted in VectaMount. The probes used included Edar (Mm-Edar, 423011, Advanced Cell Diagnostics) and Eda (Mm-Eda-C2, 506211-C2, Advanced Cell Diagnostics), which were revealed with HRP-based Green in Channel 1 and AP-based Fast Red in Channel 2, respectively.

X-gal staining
For β-galactosidase (β-gal) detection under the NF-xB promoter, embryos (E16-E18) and dissected adult tissues (including eyes, LGs and tongues for positive control) were fixed for 20-30 min in 2% PFA and 0.2% glutaraldehyde in PBS at +4°C. Staining was performed either overnight at RT using an X-gal-staining solution [1 mg/ml X-gal, 5 mM K3Fe(CN)6, 5 mM K4Fe(CN)6, 2 mM MgCl2, 0.1% NP-40 and 0.2% sodium deoxycholate in PBS], or using the β-gal staining kit (11828673001, Thermo Fisher Scientific). Then, the samples were counterstained with Nuclear Fast Red (Fluka).

Immunohistochemical staining and histology
Whole-mount staining and staining on paraffin-embedded sections were performed as previously described (Kuony and Michon, 2017). Non-specific staining was removed by incubating organs for 1-2 h in an ex vivo culture setup (Munne et al., 2013) prior to fixation.
KRT19 and ECAD were used to assess the ductal volume and the overall LG epithelial volume, respectively. Phospho-H3 was used to study the proliferation. Primary antibodies used were: rabbit anti-KRT19 (1:100, Abcam, ab5694), rabbit anti-pH3 (1:100, Abcam, ab5176), mouse anti-ECAD (1:500, BD Biosciences, 610182). Secondary antibodies used were: goat anti-rabbit Alexa Fluor 488 (1:500, Life Technologies, A11008) and goat anti-mouse Alexa Fluor 568 (1:500, Life Technologies, A11004). In addition, samples were counterstained with Hoechst 33342 (1:2000, Life Technologies, H3570), and then mounted in Vectashield (Vector Laboratories).

Hematoxylin-Eosin staining was performed according to standard protocols. Briefly, paraffin sections were dehydrated in successive xylene baths, followed by decreasing percentage of ethanol baths. Slides were kept in Hematoxylin solution for 3 min, followed by Eosin staining for 1 min. Both Hematoxylin and Eosin liquid solutions were filtered prior to usage. To finish, samples were dehydrated, and mounted with coverslip 2000 (Pertex).

**RNA extraction, cDNA synthesis and multiplex qPCR**

Biological triplicates of samples were collected from P0 to 13 wo of C57BL/6 and Eda<sup>-/-</sup> animals. The RNEasy microkit (Qiagen) was used to extract total RNA from dissected LGs, and Quantitect Reverse Transcription Kit (Qiagen, 205310) to generate the corresponding cDNAs.

Specific gene expression was assayed by Multiplex qRT-PCR (CFX96 Touch Real-Time PCR Detection System, Bio-Rad), allowing the study of four genes simultaneously; 10 µl of iTag universal probe super mix (Bio-Rad, 1725130) and 15 ng of cDNA were used per reaction. The following probes (PrimePCR Probe Assay mouse, Bio-Rad) were used: Edar-Hex, Eda-Tex615; Acta2-Cy5.5; BhlhA15-TEX615; Ltf-HEX; gapdh-Cy5 and Hprt-HEX. Gene expression levels were normalized using Slc12a2-FAM; Fgf7-FAM; Egf-HEX; Gdf5-TEX615; Mmp2-FAM; Eda-TEX615; Aqp5-FAM; Acta2-Cy5.5; BhlhA15-TEX615; Ltf-HEX; probed (PrimePCR Probe Assay mouse, Bio-Rad) were used: Edar-HEX; Rad, 1725130) and 15 ng of cDNA were used per reaction. The following probes (PrimePCR Probe Assay mouse, Bio-Rad) were used: Edar-Hex, Eda-Tex615; Acta2-Cy5.5; BhlhA15-TEX615; Ltf-HEX; Slc12a2-FAM; Efp7-FAM; Egf-Hex; Gdf5-Tex615; Mmp2-FAM; Igf1-Hex; Hgf-Tex615; Bmp1-FAM; Tgfβ1-Hex; Gm20410-Cy5.5 (Exp34); Krt19-Fam; Cxcl11-Tex615; Dgat2-Hex; Wnt4-FAM; Lin28a-FAM; Gapdh-Cy5 and Hprt-HEX. Gene expression levels were normalized using Gapdh, except for Krt19 gene expression levels, which were normalized using Hprt.

**RNA sequencing**

RNA sequencing analysis included samples from whole dissected LGs (mesenchyme and epithelium) of 13 wo C57BL/6 or Eda<sup>-/-</sup> animals. Biological triplicates were used in the analysis. RNA extraction and transcriptomic analysis were performed by the Functional Genomics Unit (FuGU, Helsinki, Finland). Briefly, total RNA was extracted from each sample using Precellsy hard-tissue beads and TRizol (Life Technologies), and purified using RNEasy Mini Kit (Qiagen). Ribo-Zero treatment (Illumina) was used to remove ribosomal RNA (rRNA). The rRNA-depleted sample was purified with RNeasy mini Elite columns (Qiagen). The cDNA library for next-generation sequencing was prepared from a sample containing 30 ng of rRNA depleted RNA, using a NEBNext Ultra Directional RNA Library Prep Kit for Illumina. The cDNA libraries prepared from the different samples were then sequenced using an Illumina NextSeq 500 system, generating FASTQ files.

**Spontaneous blinking rate, weight analysis, and tear volume measurements**

Spontaneous blinks were counted for 3 min. Measures were repeated five times at 10 min intervals, and the two extreme values were left out for final statistical analysis. Blinking was monitored for both eyes at the same time. LGs were collected, weighed both before and after dehydration. The total body weight of the mice was also assessed. For tear production measurement, tear secretion was stimulated by applying 10 µl of 0.1 mM menthol solution to the mouse ocular surface (Hirayama et al., 2012). After 1 min, the solution was removed with disposable wipes by lightly touching the tear meniscus on the side of the eye, avoiding the cornea surface. Tear collection was performed 10 min after menthol stimulation, without anesthesia, for 15 s per eye. Tears were collected at the margin of the eyelid using 0.5 µl glass micro-capillaries (Drummond Scientific).

For menthol solution preparation, menthol (Sigma) was dissolved in 40% ethanol to make a 10 mM stock. Stock solution was further diluted in freshly opened vials of commercially available artificial tears (ATs) (Ratiopharm) to obtain the working dilution. The final concentration of ethanol was 0.4% in the menthol solution (Robbins et al., 2012).

**Cornea injury**

Cornea injuries were performed on anesthetized 13 wo animals as previously described (Kalha et al., 2018a). In brief, an ocular burr (Algerbrush II, BR2-5 0.5 mm, Alger company) was used to remove an area of the central cornea epithelium, avoiding peripheral and limbal regions. This experimental procedure allows the corneal stroma and endothelium to remain intact. Only one eye was injured; the other one was kept as contralateral control. A fluorescein solution (1% in PBS, Sigma-Aldrich) was used to visualize the wound under cobalt blue light. Injured mice were collected at 18 h post-wound for transcriptomic analysis.

**Imaging, data analysis and statistics**

Counterstained RNAscope and X-gal sections were imaged using a Leica DM6000 or a DMS5000 microscope. Whole-mounts of X-gal-stained embryos were imaged using an Olympus SXZ9 binocular microscope. Immunohistochemical staining was imaged using a Leica TCS SP8 confocal microscope. The acquired z-stack images (KRT19, ECAD and phospho-H3) were processed for volume rendering, and qualitative and quantitative analyses, using Imaris 8.4.1 (Bitplane) software. Phospho-H3 positive cells, depicting proliferative cells, were counted using Imaris using the spot detection feature. All statistical analyses were performed on at least three biological replicates (n≥3). Technical triplicates were used to validate the qPCR experiments. Data are shown as mean±s.d.

For the comparison of Eda<sup>-/-</sup> and control samples in the embryonic study, littermate controls were preferentially used when possible. For quantitative analysis, at least three LGs from separate individuals of each developmental stage and each genotype were analyzed. A minimum of three TEBs/LG were used for the cell cycle study. Ductal volume was calculated by dividing the volume occupied by KRT19+ cells by the global volume of the gland (ECAD volume) (Kuony and Michon, 2017). TEB quantification was performed as previously described (Kuony and Michon, 2017).

Student’s t-test (unpaired, two-tailed) was used to determine the P-values for statistical significance. Owing to the increased number of t-tests in Figs 4, 6, 7, 8, Fig. 5E and Fig S4, the Bonferroni correction method was applied to determine the acceptable P-values for each set of data (Table 1). The Bonferroni correction method is used to overcome the problem of multiple comparisons (several t-tests performed) and results in an increase in statistical significance stringency. It is calculated using the formula: α=0.05/m; with m the number of tested hypothesis, and 0.05 the standard significant P-value. For Fig. 3, Fig. 5A,B,D and Fig S3, significant P-values were categorized as *P<0.05, **P<0.005 and ***P<0.001.

For the RNA sequencing analysis, data analysis was performed with R, using the DESeq2 package. Corrected P-values (P-adjusted) were used, and P<0.05 was considered as significant. The fold change was arbitrarily chosen as below 0.7 or above 1.3. All images were processed with Photoshop CC and Illustrator CC (Adobe Systems).

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**Competing interests**

The authors declare no competing or financial interests.

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**Table 1. Significant P-values and Bonferroni correction method (α calculation)**

<table>
<thead>
<tr>
<th>Figure</th>
<th>Calculated α</th>
<th>Significant P-value used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fig. 4</td>
<td>α&lt;0.05/9</td>
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<td>Fig. 5</td>
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<tr>
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<tr>
<td>Fig. S3</td>
<td>α&lt;0.05/2</td>
<td>&lt;0.025</td>
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Data availability
RNA sequencing data have been deposited in Gene Expression Omnibus under accession number GSE130965.

Supplementary information
Supplementary information available online at http://dev.biologists.orglookup DOI/10.1242/dev.176693.supplemental

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