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Meibomian gland morphogenesis requires developmental eyelid closure and lid fusion^{☆,☆☆}

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Abstract

Background and purpose—Meibomian glands (MGs) play an important role in the maintenance of ocular surface health, but the mechanisms of their development are still poorly understood. The MGs arise from the epithelium at the junction of eyelid fusion, raising the possibility that defective eyelid fusion disturbs the formation of MGs.

Methods—We examined, histologically and functionally, the development of MGs in mice with either normal or defective eyelid fusion, displaying eye-closed at birth (ECB) or eye-open at birth (EOB) phenotypes, respectively.

Results—The Meibomian anlage was detected in the epithelium at the eyelid fusion junction immediately after birth at postnatal day 0 (PD0), and it extended into the eyelid stroma at PD1 and started to branch and produce meibum at PD7 in the ECB mice. In contrast, few if any MG structures were detectable in the EOB mice in the early postnatal periods. The Meibomian gland ductile system was seen aligned along the eyelid margin in the adult ECB mice, but was absent or scarce in that of the EOB mice. While MG abnormalities were found in all EOB mice, the severity varied and corresponded to the position and the size of eye opening but not the genetic defects of the mice.

Conclusion—Proper Meibomian gland formation and development require eyelid closure and fusion.

Keywords

Embryonic eyelid closure; Eye-open at birth; Genetic mutant mice; Meibomian gland morphogenesis; Signaling pathway

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1. Introduction

The Meibomian glands (MGs) are specialized sebaceous glands located in the eyelids. In humans and a wide variety of animal species, MGs are serially arranged within the tarsal plate in the upper and lower eyelids with their ductules open to the cutaneous side of the mucocutaneous junction [1]. They secrete meibum, a special type of oily sebum, to the surface of the eye. The meibum constitutes the outermost layer of the tear film and serves as a lipid barrier to prevent tear evaporation and contamination. The MGs play a pivotal role in the maintenance of eye homeostasis and health, and their dysfunction is the leading cause of ocular surface diseases such as dry eye, blepharitis and punctate keratopathy [1–8].

Meibomian gland dysfunctions (MGDs) are prevalent in the population with diverse etiology [9]. A number of pathological and environmental factors, such as inflammation, autoimmune disorder, aging, cigarette smoking, and contact lens wearing, are considered risk factors for MGDs [10,11]. In addition, rare genetic disorders may cause defective ectodermal development, and thereby MG agenesis, resulting in either partial loss or complete absence of the glands, or coarsening of the acini [12,13]. To date, the origin and pathophysiology of congenital MG disorders remain poorly understood.

MG development undergoes a well-conserved process in mice and humans. In mice, the MG commences at embryonic day 18.5 (E18.5) with the Meibomian anlage emerging from the ectodermal epithelium at the rim of the eyelid [14]. The Meibomian anlage laterally outgrows into the eyelid mesenchyme, continues to elongate, branch and differentiate into connecting ductules, and forms secretory holocrine sebaceous acini. The lipid produced by the sebaceous acini in turn promotes the formation of the central canal that later develops into the central duct between the eyelid folds. The mouse MGs are fully developed 2 weeks after birth. In humans, the Meibomian anlage also develops from the eyelid margin following a process similar to that in mice [15]. Formation of functional MGs is important for maintaining a healthy ocular surface; however, little is understood about the developmental conditions crucial for supporting MG morphogenesis.

Mammalian eye development involves a transient eyelid closure, where the upper and lower eyelids fuse to cover the ocular surface. In humans, the eyelids are first evident at the seventh week post-fertilization. The eyelids fuse at week 9–12, and the upper and lower eyelids separate at 4–6 months of fetal development [15,16]. The mouse eyelids also transiently close and re-open, with closure occurring between embryonic (E) day E15.5 and E16.5 and reopening at 12–14 days after birth [17,18]. Mouse studies have shown that MG development takes place entirely during the time when the eyelids are closed. With the first sign of MG primordia appearing at E18.5 in the epithelial cell aggregates at the eyelid fusion junction, the development is accomplished 2 weeks post-natally concurrent with eyelid re-opening [14].

We have previously reported that MG hypoplasia is observed in a mutant mouse strain defective in embryonic eyelid closure, displaying an Eye-Open at Birth (EOB) phenotype [19]. These observations prompted the idea that embryonic eyelid closure and lid fusion are crucial for the initiation and morphogenesis of MGs. To test the idea, we examined MG

development in a number of genetic mutants with either normal or defective embryonic eyelid closure. The results showed that all the EOB mice displayed abnormal Meibomian anlage as early as post-natal day 1 (PD1). In addition, they exhibited disarrayed arrangement of Meibomian ductules and signs of Meibomian gland atrophy with deficiency in lipid production with age.

2. Material and methods

2.1. Chemical reagents

Triton X-100, dimethyl sulfoxide (DMSO), paraformaldehyde, Oil-red O (ORO), 4',6-diamidino-2-phenylindole (DAPI), glycerol and isopropanol were from Sigma-Aldrich (St. Louis, MO). Alexa-546 or -488 Phalloidin was from Molecular Probes, Invitrogen (Carlsbad, CA). PCR reaction reagents were from Life Science Research (Hercules, CA), and PCR primers were from Integrated DNA Technologies (Coralville, IA).

2.2. Mouse strains, crossings and genotyping

Mice used in this work are listed in Table 1. The wild type C57/BL6 strain was from Jackson Laboratory, and the genetic mutant strains, carrying mutations in *c-Jun*, *Limk2*, *Map3k1*, *Jnk1*, *S1pr2/S1pr3*, were as described [20–24]. The *c-Jun*(*OSE*) mice were derived from crossing *c-Jun-floxed* with *Le-cre* transgenic strain, in which the Cre recombinase was expressed specifically in the ocular surface ectoderm (OSE) [25].

Mice were housed in Laboratory Animal Medical Service facility at the University of Cincinnati, College of Medicine; their genotypes were identified by PCR using gene-specific primers. All animals were treated in adherence to the NIH Guide for the Care and Use of Laboratory Animals and conformed to the ARVO guidelines for humane use of animals. Procedures of animal handling were approved by the Institutional Animal Care and Use Committee at the University of Cincinnati (Cincinnati, OH, USA).

2.3. Examination of eye open/close status

The eyelid open/close status was examined visually at birth and recorded. Photographs were taken using a Leica MZ-16FA microscope of some pups immediately after birth at PD0 and eyelids dissected at PD0, 1, and 7.

The size of open-eye was evaluated by comparing the distances of the inner and outer canthi (D1) and the distance of the inner and outer eyelid margin (D2) as shown in Fig. 1. D2 is zero if the eyelids are fully closed. The relative eye opening (%) was calculated as $D2/D1 \times 100$.

2.4. Morphological examination of the meibomian glands

The heads of PD0–PD7 pups and eyes of PD21 and PD50 mice were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer at 4 °C overnight. The eyelids were dissected, washed extensively in PBS, and subjected to subsequent evaluation.

For morphological evaluation, the eyelids were first incubated in permeabilization/staining buffer (0.25% DMSO, 0.25% Triton-100 and 15% sucrose in PBS) for 30 min at room temperature, then stained with Alexa-546 Phalloidin (1:20) and DAPI in permeabilization/staining buffer at 4 °C overnight. The eyelids were washed with permeabilization/staining buffer twice, transferred to PBS, mounted on glass slides in 50% glycerol and photographed under a fluorescent microscope (Zeiss Observer. Z1).

For functional evaluation of meibum production, the eyelids derived from PD7 pups or older mice were rinsed with 60% Isopropanol for 5 min, followed by incubation with ORO (ORO 300 mg/ml) for 30 min at room temperature. After washing with 60% Isopropanol twice for 3 min each time and rinsing with PBS, the samples were subjected to staining with Alexa-488 Phalloidin and DAPI at 4 °C overnight. The samples were transferred to PBS, mounted on glass slides in 50% glycerol and photographed under a fluorescent microscope (Zeiss Observer. Z1).

2.5. Meibomian glands quantification

The ORO-positive MG branches in the PD7 eyelids were counted under a fluorescent microscope and at least 6 eyes of each phenotype were examined.

2.6. Statistical analyses

Statistical comparisons were performed with Student's two-tailed paired *t*-test and $p < 0.001$ (***) were considered statistically significant.

3. Results

3.1. Abnormal MG morphogenesis is associated with an EOB phenotype

In order to evaluate whether lacking eyelid fusion would affect MG morphogenesis, we employed an array of mutant mice, which carry single or multiple allelic mutations that result in either normal or defective eyelid closure (Tables 1 and 2). Pups with defective eyelid closure were born with EOB phenotype, in a striking contrast to the normal pups with their eyelids closed at birth (ECB; Fig. 2A). By visual examination of the newborn pups (PD0) and genotype analyses, we identified which pups/mice had the ECB and EOB phenotypes under each genetic condition (Table 2).

Immediately after birth (PD0), epithelial placodes were detected at the eyelid fusion junction in the ECB eyes, but they were absent in the EOB eyes (Fig. 2A). In the ECB eyes, the placodes invaginated to form solid cords with no clear lumen at PD1 (Fig. 2B). Conversely, the EOB eyes lacked obvious signs of MG structures in the epithelium at the eyelid margin.

Because lipid production by the immature MGs begins at PD3 [14], we performed ORO staining of the PD7 eyelids. The ORO positive-stained cells, restricted to the inner core of the developing ducts, were readily detectable in the ECB but not the EOB eyelids (Fig. 2C). In the ECB eyelids, the lipid producing MGs were elongated with increased size and extended deeper into the underlying mesenchyme; in the EOB eyelids, however, the MGs were either severely hypoplastic or completely absent. Taken together, the observations

made in 67 ECB and 39 EOB pups at ages ranging from PD0 to PD7 suggest that embryonic eyelid closure and lid fusion are required for early placode formation in MG morphogenesis.

3.2. Meibomian gland abnormalities in adult EOB mice

To assess whether the developmental abnormalities could lead to MG defects in adult mice, we performed ORO staining of the eyelids isolated from the ECB and EOB mice at PD21 and PD50 (Table 1). All eyelids derived from the PD21 ECB eyes displayed ORO staining positive Meibomian gland ducts orderly aligned along the eyelid margin with approximately 10 distinguishable gland modules on each side of the eyelids (Fig. 3A). The glands exhibited extended branches that connected into multiple ductules with lipid production capacity. The eyelids derived from the PD50 ECB eyes had a similar structure of the MG, although the gland size was bigger and the branches fuller with less interglandular spaces (Fig. 3B).

The eyelids derived from the PD21 EOB mice exhibited small dots with weak ORO-positive staining confined to the margin of the eyelids, but lacked detectable MG ducts with discernable branching (Fig. 3A). A similarly weak ORO-stained structure was identified in the P50 EOB eyelids (Fig. 3B). Compared to that at PD21, the structures at PD50 were slightly larger with small extension/branching into the eyelid mesenchyme. Hence, the adult EOB mice lack well-developed MGs with weak or no lipid producing capacity.

3.3. Eyelid closure status, not the mouse genotype, determines MG development

We have previously reported that the EOB phenotype is a developmental threshold trait, induced additively by allelic lesions of genes whose products act in the same biological pathways [26,27]. Recently, the S1P receptors 2 and 3 have been shown to be involved in the regulation of eyelid development [21]. In an attempt to delineate the signaling pathways downstream of S1P receptors, we crossed the receptor mutants with *Jnk1* or *Map3k1* mutants. To our surprise, three of the mice resulting from the crossing displayed dimorphic eyelid phenotypes (Table 2). These mice, two of which are *Map3k1*(+/-)*S1pr2*(-/-)*S1pr3*(+/-) and one is *Jnk1*(-/-) *S1pr2*(+/-)*S1pr3*(-/-), had one eye closed but the other eye remained open at birth.

Taking advantage of the dimorphic phenotypes, we asked whether the MG abnormalities were associated with defective eyelid closure, or alternatively, whether they were the result of global effects of gene mutations on epithelial cell proliferation and differentiation [28,29]. To address this question, we stained with ORO eyelids isolated from ECB and EOB eyes of the same mouse. The lipid-producing cells were abundantly located within the ductile structure of the ECB eyelids, but were absent in the EOB eyelids (Fig. 4). These observations argue that failure of eyelid fusion, but not gene mutation, was the cause of MG defects.

3.4. Lack of eyelid fusion correlates with meibomian gland defects

In PD7 pups, the ORO-red positive MG ductile structures were distinct and readily identifiable (Fig. 2C). There were approximately 20 such structures in each ECB eyelid, but the number was significantly less in the EOB eyelids (Fig. 5A). The EOB eyes examined had an average of 2.7 MG structures per eye; however, there was a large intereye variation

with standard deviation (SD) of 4.27. These observations raised the possibility that the variability of MG defects is due to differences in the severity of eye-opening.

Some genetic conditions produce the EOB phenotype with incomplete or partial penetrance, whereas others produce the phenotype with complete penetrance (Table 3). For instance, while ablation of both *S1pr2* and *S1pr3* genes led to the EOB phenotype, ablation of triple alleles or double alleles of the two genes did not. Nevertheless, the combination of allelic *S1pr2/S1pr3* ablation with either *Map3k1* heterozygous or *Jnk1* knockout led to a “partial eyeopen” phenotype. The open-eye defects, seemingly dependent on the dosage and functional importance of the mutant genes, ranged from 12.5 to 40% of the number of eyes examined under each genetic conditions.

In the compound mutant mice, we compared the size of eye opening and the number of Meibomian glands in each eye and found that indeed they were inversely related (Fig. 5B). Compared to ECB eyelids, the number of MGs was reduced to half in the partially opened eyes, further decreased when the opening was wider, and reduced to zero when the eyelids were completely open. Hence, the severity of MG hypoplasia corresponded to the size of eyelid opening.

Since the partially opened eyes still had MGs, although with reduced numbers, we wondered if the remaining MGs were developed in areas where the eyelids were fused. Close examination of the lipid producing MG structures in partially opened eyes of PD7 pups revealed that, in the same eye, MG structures were detected in regions where the eyelids were fused, but not in regions where eyelids were open (Fig. 5C). This observation lends additional support to the notion that MG morphogenesis requires embryonic eyelid closure and lid fusion.

4. Discussion

4.1. Eyelid closure and meibomian gland development

The MGs play a pivotal role in maintenance of ocular surface health, yet relatively little is known about the molecular and cellular mechanisms underlying their development. By examining 140 mouse eyes derived from 18 genetic conditions associated with either normal (n = 86) or defective (n = 54) eyelid fusion, we conclude that eyelid closure in embryogenesis is essential for the proper development of MGs. In the absence of eyelid fusion, the MG placodes fail to form, and their invagination and epithelial cord branching do not occur at 0–2 postnatal days. Subsequently, the MGs are poorly developed with a few or undetectable acinar regions at PD7, and are either absent or smaller with irregular shape and/or spacing in adult ages.

Of more than 140 genetic mutant strains displaying the EOB phenotype, examination of MGs has been limited to only a few, namely the *Tgfa*- and *Adam17*-mutant mice, deficient in EGFR signaling, mice carrying mutations that result in the loss-of-function of PPP1R13L involved in molecular processes via interactions with p65RelA and/or p53, and mice defective in BARX2, a homeodomain transcription factor, which participates in FGF10 signaling required for branching morphogenesis [30–33]. The genetic conditions in these

mice lead to inactivation of distinct molecular pathways, but all result in the EOB phenotype and abnormalities of MGs. Likewise, the EOB mutants, including *Limk2*($-/-$), *c-Jun* (*OSE*), *Slpr2*($-/-$), *Slpr3*($-/-$) and *Map3k1*($-/-$), that we examined here display either completely missing or severely defective Meibomian glands (Fig. 6). The observations collectively provide a compelling argument that failure of eyelid closure is associated with defective Meibomian gland development.

4.2. Mechanisms of meibomian gland morphogenesis

The MGs originate at E18.5 from the epithelial placode formed at the junction of the eyelid fusion where the epithelium thickens at the gland-forming region [14,34]. Eyelid closure, on the other hand, is the result of the epithelium at the leading edge of the eyelid elongating, spreading, and migrating that ultimately leads to fusion of the opposing eyelids at E16.5 [35,36]. The two events are clearly temporal-spatially separated and mechanistically distinct; however, their outcomes seem to be closely connected.

One of the possibilities is that the two events are connected by the genetic factors, the inactivation of which directly affects not only embryonic eyelid closure, but also MG morphogenesis. By examining mutants that produce the EOB defects with incomplete penetrance and variable severity, we show that the severity of MG defects corresponds to the size of the eye opening, but not the genetic conditions (Fig. 6). In addition, the MGs develop normally if the eyelids are closed, while they are absent if the eyelids are open under the same *Jnk1*($-/-$), *Slpr2*($+/-$), *Slpr3*($-/-$) genotype. Furthermore, even within the same eye, the MGs are completely missing in areas where the eyelids are wide open, but partially developed in areas where the eyelids fuse near canthi. Our data suggest that the MG development is linked more closely to the status of eyelid fusion than to the genetic condition.

The closed eyelids may provide the anchoring site to support the formation of Meibomian placodes. Moreover, MG morphogenesis is triggered by the eyelid mesenchyme supplying the first signal to initiate morphogenesis and the placode epithelial cells responding to those signals [37]. Accordingly, perturbation of the growth factor repertoire in the eyelid mesenchyme and disruption of signal receptors in ectodermal-derived cells have been shown to impair epithelial cell proliferation and differentiation in MG development [38–42]. It is possible that the interactions of the upper and lower eyelid epithelium in the closed eyelids lead to the production of unique signaling molecules, or provide an important structural basis to ensure the communication and transmission of the inductive signals between mesenchyme and epithelium. Eyelid fusion, in this context, is required for proper orchestration and transduction of signals [19,43]. These signals in turn induce placode formation and dictate lineage specification essential for Meibomian gland morphogenesis [14,44].

4.3. The signaling mechanisms of eyelid development

Embryonic eyelid closure is regulated by at least eight major morphogenetic signaling pathways extremely sensitive to genetic modifications [28,29,43]. In the past few years, EOB mice have become valuable tools for the investigation of molecular and signaling

mechanisms of epithelium morphogenesis and for the identification of genetic and environmental risks of developmental diseases [19,28,35,45].

Based on nonallelic noncomplementation, an assay used to identify genes whose products act in the same molecular pathway in a given biological process [46], we have shown that the RhoA-MAP3K1-JNK1/2 pathway is a crucial signaling mechanism in embryonic eyelid closure. Although the susceptible genetic conditions, such as *Map3k1*(+/-) and *RhoA*(-/-), do not cause eyelid abnormalities by themselves, they potentiate eyelid closure defects in the *Jnk1*(-/-) mice [26,27,45]. In the present work, we extend the genetic studies to the S1P receptors, S1PR2 and S1PR3, and identify them as additional players of the same pathway. We show that while the *S1pr2*(+/-)*S1pr3*(-/-) mice have normal eyelid development similar to the *Jnk1*(-/-) and *Map3k1*(+/-) mice, their combinations, i.e. *Jnk1*(-/-)*S1pr2*(+/-)*S1pr3*(-/-) and *Map3k1*(+/-)*S1pr2*(+/-)*S1pr3*(-/-) cause the EOB phenotype in nearly 40% of the offspring (Table 3). To a lesser extent, the *Map3k1*(+/-)*S1pr2*(-/-)*S1pr3*(+/-) mice displayed the EOB phenotype in 27% of the pups, suggesting that S1PR3 makes greater contributions than S1PR2 to the pathway activity. This, to our knowledge, is the first genetic evidence connecting the S1PR2 and 3 to the MAP3K1-JNK pathways [47].

5. Perspective

While the EOB mice lack MG placodes in the early developmental stage, many of them have detectable yet smaller lipid-producing eyelid glands in the adult age. These glands, however, are extremely hypoplastic, suggesting that the EOB mice may not have functionally adequate MGs to maintain normal eye physiology. Our data presented here suggest that defective eyelid closure could be one of the causes underlying MG defects in children. Given that diverse genetic and environmental insults and gene-environment interactions can induce eyelid closure defects, the corresponding MG abnormalities will likely have complex etiology yet to be fully understood.

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Abbreviations

MG	Meibomian gland
EOB	eye-open at birth
ECB	eye-closed at birth
PD	postnatal day
MGD	Meibomian gland dysfunction
ORO	Oil-red O.

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Fig. 1. Measurement of the eye-open size

Graphic illustration of how the size of eye-open was measured. The eyelids are marked in red color. D1: distance between inner and outer canthi, D2: distance between inner and outer eyelid leading edge. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 2. The Meibomian gland development at early post-natal days

The eyelids of ECB and EOB pups at (A) PD0, (B) PD1 and (C) PD7 were photographed directly (top panels) and after staining with Alexa-594 Phalloidin (red) and DAPI (blue) (bottom panels in A and B), or with ORO (red) and DAPI (blue) (bottom panels in C). The epithelial placodes and lipid producing tubules (white arrowheads) were seen only in the ECB eyes. The eyelid fusion line or eyelid margin were marked with dotted lines. n, the number of eyes examined under each condition with similar results. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 3. Characterization of Meibomian glands in adult mice

The eyelids of ECB and EOB mice at (A) PD21 and (B) PD50 were stained with ORO (red), examined using microscope at low (top panels), intermediate (middle panels) and high (bottom panels) magnifications. Areas where the higher magnification photos were taken were marked with boxes (yellow) and the scale bar is 250 μ M. The lipid producing tubules (arrows) were readily detectable in the ECB, but not EOB eyelids. The eyelid margin were marked with dotted lines. n, the number of eyes examined in each condition with similar results. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 4. Meibomian gland development in ECB and EOB eyes of the same genotype
 The eyelids derived from a PD7 *Jnk1*(-/-)*Slp2*(+/-)*Slp3*(-/-) pup, which displayed dimorphic EOB and ECB phenotypes, was examined for ORO-positive MG structures. Fluorescence microscopic pictures were taken at low (top panels), intermediate (middle panels) and high (bottom panels) magnifications. Arrowheads point at the MG structures. The eyelid fusion line or eyelid margin were marked with dotted lines, and areas where the higher magnification photos were focused on were marked with boxes (yellow).

Fig. 5. Relationship between eyelid opening and Meibomian gland defects

The number of MGs was counted in 6 ECB and 6 EOB eyes from PD7 pups and (A) the average MG number was calculated, and (B) the relative size of the eye opening was measured in each eye and compared with the number of MGs. (C) Photographs of a partially opened eyelid from a PD7 *Jnk1*(-/-)*Slp2*(+/-)*Slp3*(-/-) pup stained with Alexa-phalloidin-488 (green) and ORO (red) at low (top left panel), intermediate (a and b, middle and bottom left panels) and high (c and d, right panels) magnifications. Arrowheads point at the MG structures. The red box marked the areas where images were taken at higher magnification, and the eyelid fusion line or eyelid margin were marked with dotted lines. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Molecular pathways regulating embryonic eyelid closure

S1PR2/3 - MAP3K1-JNK1
c-Jun
LIMK2

Genetic conditions affecting pathway activity

Wild type

S1pr2(+/-)*S1pr3*(-/-)

Map3k1(+/-)

Map3k1(+/-)*S1pr2*(+/-)*S1pr3*(+/-)

c-Jun(F/F)

Map3k1(+/-)*S1pr2*(+/-)*S1pr3*(-/-)

Map3k1(+/-)*S1pr2*(-/-)*S1pr3*(+/-)

Limk2(-/-)

Jnk1(-/-)*S1pr2*(+/-)*S1pr3*(-/-)

Map3k1(-/-)
S1pr2(-/-)*S1pr3*(-/-)
c-Jun(ΔOSE)

Fig. 6. Embryonic eyelid closure is required for Meibomian gland formation

Our genetic data suggest that embryonic eyelid closure requires three major molecular pathways, i.e. the S1PR-MAP3K1-JNK, LIMK2 and c-Jun pathways. Gene mutations that attenuate pathway activities lead to complete or partial inhibition of embryonic eyelid closure at E15.5 to E16.5. The degree of attenuation may determine the severity of the open eye phenotype, which in turn leads to complete or partial Meibomian gland deficiency observed at PD0. el, eyelid, E, embryonic day and PD, postnatal day. Eyelid margins are pointed by arrows and epithelium placodes of pre-Meibomian glands are marked by red dots. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1

Genotypes and ages of mice.

Genotype	Developmental days examined					
	PD0	PD1	PD7	PD21	PD50	Total eyes
<i>wild type</i>	8					8
<i>c-Jun(F/F)</i>	18					18
<i>c-Jun (OSE)</i>	2					2
<i>Limk2(-/-)</i>		14				14
<i>Map3k1(+/-)</i>		2	6			8
<i>Map3k1(-/-)</i>		4	6	6	2	18
<i>Slpr2(+/-)Slpr3(+/-)</i>		2	2			4
<i>Slpr2(+/-)Slpr3(-/-)</i>				4		4
<i>Slpr2(-/-)Slpr3(-/-)</i>			4			4
<i>Map3k1(+/-)Jnk1(+/-)</i>				2		2
<i>Map3k1(+/-)Slpr2(+/-)</i>	8	2	2			12
<i>Map3k1(+/-)Slpr2(-/-)</i>	2			2		4
<i>Map3k1(+/-)Slpr2(+/-)Slpr3(+/-)</i>	4			6		10
<i>Map3k1(+/-)Slpr2(+/-)Slpr3(-/-)</i>	4	4	2	2		12
<i>Map3k1(+/-)Slpr2(-/-)Slpr3(+/-)</i>	6			2		8
<i>Jnk1(-/-)Slpr2(+/-)Slpr3(-/-)</i>			4	2		6
<i>Jnk1(+/-)Slpr2(+/-)Slpr3(-/-)</i>				6		6
Total eyes	52	28	26	30	4	140

Table 2

The ECB and EOB mice examined for Meibomian glands.

eyes closed at birth (ECB)		eyes open at birth (EOB)	
Genotype, age	eyes examined	Genotype, age	eyes examined
PD0			
<i>c-Jun(F/F)</i>	18	<i>c-Jun (OSE)</i>	2
<i>wild type</i>	8		
<i>Map3k1(+/-)S1pr2(+/-)S1pr3(+/-)</i>	4		
<i>Map3k1(+/-)S1pr2(+/-)</i>	8		
<i>Map3k1(+/-)S1pr2(-/-)</i>	2		
<i>Map3k1(+/-)S1pr2(+/-)S1pr3(-/-)^a</i>	2	<i>Map3k1(+/-)S1pr2(+/-)S1pr3(-/-)^a</i>	2
<i>Map3k1(+/-)S1pr2(-/-)S1pr3(+/-)^{a,b}</i>	1	<i>Map3k1(+/-)S1pr2(-/-)S1pr3(+/-)^{a,b}</i>	5
total	43		9
PD1			
<i>Limk2(-/-)^{a,b}</i>	3	<i>Limk2(-/-)^{a,b}</i>	11
<i>S1pr2(+/-)S1pr3(+/-)</i>	2		
<i>Map3k1(+/-)S1pr2(+/-)</i>	2		
<i>Map3k1(+/-)S1pr2(+/-)S1pr3(-/-)</i>	2	<i>S1pr2(-/-)S1pr3(-/-)</i>	2
<i>Map3k1(+/-)</i>	2	<i>Map3k1(-/-)</i>	4
total	11		17
PD7			
<i>Map3k1(+/-)</i>	6	<i>Map3k1(-/-)</i>	6
<i>S1pr2(+/-)S1pr3(+/-)</i>	2	<i>S1pr2(-/-)S1pr3(-/-)</i>	4
<i>Map3k1(+/-)S1pr2(+/-)</i>	2		
<i>Map3k1(+/-)S1pr2(+/-) S1p3(-/-)</i>	2		
<i>Jnk1(-/-)S1pr2(+/-)S1pr3(-/-)^{a,b}</i>	1	<i>Jnk1(-/-)S1pr2(+/-)S1pr3(-/-)^{a,b}</i>	3
total	13		13
PD21			
<i>S1pr2(+/-)S1pr3(-/-)</i>	4	<i>Map3k1(-/-)</i>	6
<i>Map3k1(+/-)S1pr2(+/-)S1pr3(+/-)</i>	6	<i>Jnk1(-/-)S1pr2(+/-) S1pr3(-/-)</i>	2
<i>Map3k1(+/-)S1pr2(+/-)S1pr3(-/-)</i>	2	<i>Map3k1(+/-)S1pr2(-/-)</i>	2
<i>Map3k1(+/-)S1pr2(-/-)S1pr3(+/-)^{a,b}</i>	1	<i>Map3k1(+/-)S1pr2(-/-)S1pr3(+/-)^{a,b}</i>	1
<i>Jnk1(+/-)S1pr2(+/-) S1pr3(-/-)^a</i>	4	<i>Jnk1(+/-)S1pr2(+/-) S1pr3(-/-)^a</i>	2
total	17		13
PD50			
<i>Map3k1(+/-)Jnk1(+/-)</i>	2	<i>Map3k1(-/-)</i>	2
total	2	total	2

^aGenotypes producing either ECB or EOB pups.^bA pup with one eye closed and one eye open.

Table 3

Gene dose-dependent contributions to the EOB phenotype.

Compound Genotype	ECB	EOB	total eyes	% EOB
<i>S1pr2</i> (-/-) <i>S1pr3</i> (-/-)	0	28	28	100
<i>S1pr2</i> (-/-) <i>S1pr3</i> (+/-)	18	0	18	0
<i>S1pr2</i> (+/-) <i>S1pr3</i> (-/-)	22	0	22	0
<i>S1pr2</i> (+/-) <i>S1pr3</i> (+/-)	30	0	30	0
<i>Map3k1</i> (+/-) <i>S1pr2</i> (-/-) <i>S1pr3</i> (+/-)	22	8	30	26.66666667
<i>Map3k1</i> (+/-) <i>S1pr2</i> (+/-) <i>S1pr3</i> (-/-)	11	9	22	40.90909091
<i>Map3k1</i> (+/-) <i>S1pr2</i> (+/-) <i>S1pr3</i> (+/-)	21	3	24	12.5
<i>Jnk1</i> (-/-) <i>S1pr2</i> (+/-) <i>S1pr3</i> (-/-)	30	19	49	38.7755102