A Spatiotemporal Requirement for Prickle 1-Mediated PCP Signaling in Eyelid Morphogenesis and Homeostasis

Dianlei Guo,1 Zhaohui Yuan,1 Jiali Ru,1 Xinyu Gu,1 Wang Zhang,1 Fuxiang Mao,1 Hong Ouyang,1 Kaili Wu,1 Yizhi Liu,1 and Chunqiao Liu 1,2

1State Key Laboratory of Ophthalmology, Zhongshan Ophthalmic Center, Sun Yat-sen University, Guangzhou, China
2Guangdong Province Key Laboratory of Brain Function and Disease, Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou, China

Correspondence: Chunqiao Liu, State Key Laboratory of Ophthalmology, Zhongshan Ophthalmic Center, Sun Yat-sen University, Guangzhou 510060, China; liuchunq3@mail.sysu.edu.cn.

DG and ZY contributed equally to the work presented here and should therefore be regarded as equivalent authors

Submitted: September 8, 2017
Accepted: January 16, 2018


PURPOSE. Tissue closure/fusion is a fundamental process during organogenesis, driven in part by the Wnt/planar cell polarity (Wnt/PCP) pathway. This study explored the spatial and temporal aspects of PCP signaling in eyelid development through analysis of mice lacking Prickle 1, a core PCP component, and the Prickle1-dependent signaling networks underlying eyelid development.

METHODS. Wild type and Prickle 1 compound mutant mice with a hypomorphic and a null allele were bred and used to study eyelid morphogenesis. The time course of embryonic eyelid fusion and postnatal reopening was examined by light microscopy of tissue sections and scanning electron microscopy. Immunohistochemistry was conducted to monitor cell proliferation, death, and molecular identities through pre- and postnatal eyelid development.

RESULTS. Prickle 1 mutant embryos exhibited a profound delay in eyelid closure at embryonic ages, but manifested precocious eyelid reopening postnatally, with ensuing cornea malformation. Mutant embryonic showed downregulation of phosphorylated c-Jun, and upregulation of increased b-catenin in separate cell populations of the eyelid front area. Increased cell death and decreased mesenchymal infiltration was observed in postnatal mutant eyelid prior to eyelid reopening. While broadly expressed in many tissues, Prickle 1 was spatially restricted to the eyelid front at E15.5, a location where c-Jun and b-catenin expression was altered in Prickle 1 mutants.

CONCLUSIONS. The study demonstrates a spatiotemporal requirement for Prickle 1-mediated PCP signaling during eyelid morphogenesis and homeostasis. The study links Prickle 1-mediated PCP signaling to existing networks, and provides a useful animal model for studying congenital ocular surface diseases.

Keywords: eyelid closure, prickle 1, planar cell polarity, eyelid homeostasis, c-Jun, beta-catenin

The formation of the eyelid provides a physical barrier for the anterior ocular surfaces from potential harms from the external environments. Interruption of the eyelid development would lead to a series of anterior ocular anomalies, including cornea erosion and conjunctiva inflammation. A common feature of eyelid development to all mammals is the closure and subsequent reopening of the eyelid to ensure a relative isolated niche for cornea and eye globe development. While eyelid closure uniformly occurs in utero, eyelid reopening varies between species either before or after birth. Closure and reopening of the human eyelids are both accomplished in utero,1,2 whereas they occur in mouse pre- and postnatally, respectively.3

The mouse eyelid development starts from embryonic day 11.5 (E11.5), with the centripetal invagination and elongation of the dorsal and ventral periocular ectoderm forming the eyelid folds. The eyelid folds then grow toward each other across the primitive cornea surface at approximately E15.5.4,5 Complete eyelid closure occurs between E16 and E17, and two lids remain closed until postnatal day 12 (P12) and separate completely at approximately P14.4,5,6 Studies over the years indicate that multiple signaling pathways are involved in eyelid development.5 For example, crosstalk between FGF and bone morphogenetic protein (BMP) pathways is required for conjunctiva fate and eye closure9; TGF α/β acts upstream of the c-Jun pathway to regulate EGFR-mediated actin polymerization, which is required for epithelial sheet migration during eyelid closure.10–12 The Wnt/b-catenin signaling pathway appears to alter the timing of eyelid opening, as disruption using the Wnt antagonist Dkk2 led to opened eyelid at birth.13 Gain-of-function of b-catenin in cornea or skin epidermis in mouse disrupts cornea and eyelid morphogenesis by perturbation of cell proliferation and mesenchymal structure.14,15 On the other hand, b-catenin independent Wnt signaling promotes eyelid closure, notably evidenced by genetic disruption of two planar cell polarity (PCP) genes, Fz3 and Fz6, causing opened eyelids at E16.16 Moreover, antagonism was also observed between Wnt/PCP and Wnt/b-catenin pathways.17–21 Wnt5a, a component of PCP pathway,12 is able to activate or repress b-catenin in either cultured cells or developing embryos, depending on Wnt receptor context.17,21 Additionally, PCP
activity inversely correlates with β-catenin protein levels in multiple cultured cell lines in vitro. 20

Originally identified in Drosophila hair cells, PCP signaling module consists of a set of six proteins, including Frizzled, Disheveled, Vangl, Prickle, Diego, and Flamingo. 25 The pathway is critical for cytoskeleton remodeling and oriented cell migration and arrangement in convergent extension during embryogenesis. 24,25 Our previous study showed that mutation of one of the PCP components, Prickle 1, led to open atrial septum, shortened snout and limbs, resembling Robinow syndrome. These tissue morphogenetic defects were associated with compromised cell arrangement and migration. 3 Additionally, eyelid and eyelash abnormalities in Prickle 1 mutant mice were apparent. 5 To further investigate the role Prickle 1 and thus Prickle 1 mediated-PCP signaling in development and pathogenesis of ocular surface, we performed a detailed histologic analysis of Prickle 1 mutant eyelid tissues at different time points of development. We found biphasic eyelid morphogenetic defects, in which a delayed eyelid closure was observed within a narrow embryonic window, yet a precocious postnatal eyelid reopening occurred at P10. Further investigations connected Prickle 1 to existing signaling networks involving c-Jun and β-catenin.

**Materials and Methods**

**Mice and Genotyping**

All procedures involving the use of mice were approved by the Animal Care and Use Committee, Zhejiang Ophthalmic Center, and adhered to ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Prickle 1 gene-trap mutant strain was generated as described by Liu et al. 3,26 Straight knockout allele was created upon excision by Cre recombinase. 3 A knock-in EYFP reporter under the control of endogenous Prickle 1 promoter was used to monitor Prickle 1 gene expression.

**Histology**

Mouse embryos and postnatal mice were killed by decapitation and cervical dislocation, respectively. Eyeballs with attached intact eyelids were dissected from the eye sockets, and fixed in 4% paraformaldehyde for 24 hours at 4°C. For paraffin sections, samples were washed three times in PBS, dehydrated in a series of alcohols and two times in xylene, then embedded in paraffin and sectioned at 8 μm with a microtome (RM 223; Leica, Wetzlar, Hesse-Darmstadt, Germany). Sections were stained with hematoxylin and eosin (H&E) according to the Leica, Wetzlar, Hesse-Darmstadt, Germany). Sections were deparaffinized with xylene, hydrated with a series of alcohols, and rehydrated with PBS and water, then immersed in 30% sucrose for cryoprotection. Cryosections were cut at 15 μm with a cryostat (M1950; Leica) and mounted on 3% gelatin-coated slides. Cryosections were stained with hematoxylin and eosin (H&E) according to the Dey et al. 27

**Immunohistochemistry, Apoptotic Assay, Imaging, and Antibodies**

For immunohistochemistry, tissue sections were dewaxed and rehydrated using a standard protocol. Antigen retrieval was performed by boiling slides in 10 mM sodium citrate buffer (pH 6.02) with microwaves for 8 minutes. The sections were blocked with 10% donkey serum/0.1% Triton X-100/ PBS) for 30 minutes at room temperature, then incubated with primary antibodies at 4°C overnight. After washing with PBST, sections were incubated with fluorescent dye-conjugated secondary antibodies, and mounted with Fluoromount-G (Southern Biotech, Birmingham, AL, USA). Phalloidin (A12380; Thermo Fisher, Waltham, MA, USA) staining was performed using the same immunohistochemistry protocol except for the omission of secondary antibodies.

**For cell death detection, deparaffinized sections were subjected to TUNEL staining (Cat. 11684795910; Roche, Basel, Switzerland). Tissue sections were treated with proteinase K (20 μg/mL in PBS) for 15 minutes at room temperature and incubated in labeling solution at 4°C overnight. Sections were washed with PBS, stained with 4,6-diamidino-2-phenylindole (DAPI), and mounted. Fluorescence microscopy using a Zeiss 700 confocal microscope and Imager.Z2 (Zeiss, Oberkochen, Germany) equipped with ApoTome (Zeiss) were used to acquire images.

Primary antibodies used in this study were: anti-Ki67 (ab15580; Abcam, Shanghai, China), anti-CD45 antibody (30-F11; BD, Franklin Lakes, NJ, USA), anti-mucin SAC (ab3649; Abcam), anti-Cytokeratin 1 (ab185629; Abcam), anti-Cytokeratin 4 (C5176; Sigma, Saint Louis, MO, USA), anti-Cytokeratin 12 (ab124975; Abcam), anti-Cytokeratin 14 (ab78000; Abcam), anti-GFP (TP-401; Torrey Pines Biolabs, Secaucus, NJ, USA), anti-β-catenin (610155; BD), anti-phosphorylated c-Jun (P-c-jun) (AP3072a; Abgene). 28

**Quantification and Statistics**

For all quantifications below, statistics was performed by Student’s t-test.

For analysis of embryonic eyelid closure or reopening, maximal vertical distance between the upper and lower eyelid edges was measured to evaluate progress of eyelid closure. More than five embryos were used for each genotype. For analysis of postnatal eyelid reopening, distance between upper and lower eyelid was measured on multiple sections (>3) of each animal. Multiple animals (>3) were used through ages.

For measuring the eyelid thickness, because of the uneven thickness along different parts of the eyelid, a thickness index was generated as follows: (1) line “a” with definitive length was drawn from the apex of the eyelid wedging epithelium; (2) line “b” was drawn to skin surface vertical to “a”; (3) line “c” was drawn along skin surface connecting “a” and “b”; (4) the closed area contained within “a,” “b,” and “c” were measured by ImageJ software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA); and (5) measured areas were divided by length of “a” to generate thickness indices. Multiple sections (n > 5) from meridian region of each eyelid were measured. Three or more embryos were used for each genotype.

For analysis of cell proliferation of the embryonic eyelid, a total of three embryos and five vertical sections from each embryo were analyzed. A tip region of an eyelid comprising peridermal, epithelial, and stroma cells was chosen for counting number of Ki67-positive cells, which was then normalized to total number of cells revealed by DAPI. For analysis of postnatal eyelid proliferation, Ki67-positive mesenchymal cells were counted in and normalized to a defined area. Five sections of each embryo were subjected to counting. More than three embryos for each genotype were used.

For analysis of eyelid cell infiltration, Prickle 1/enhanced yellow fluorescent protein (EYFP)-positive mesenchymal cells

**Scanning Electron Microscopy (SEM)**

The embryonic heads from E13.5 to E17.5 were fixed with 2% glutaraldehyde in 0.1 M sodium phosphate buffer. After dehydration through a graded alcohol series, the samples were dried, sputter-coated and imaged with SEM (Quanta 2000; Sun Yat-sen University Histology Core, Guangzhou, China).
were counted from E17.5 to P15. Because cell infiltration is positively correlated with the area of eyelid pocket on static two-dimensional sections, but not with the superficial circumference of the eyelid pocket, we normalized counted cells on each section to the length of surficial circumference. ImageJ was used to find the length of surficial circumference and area of each eyelid section. Multiple sections (>3) from multiple embryos (>3) were counted for all ages.

For analysis of cell death, a total of three animals and three sections from each were subjected to counting. Eyelid junction (before open) and tip regions (after open) excluding hair follicles, Meibomian gland, and cornea were analyzed.

For measuring cell size, staining of beta-catenin was used to demarcate the cell boundary. Cell size indices were generated by total measured area divided by total cell number. Multiple mice (>3) and multiple sections (>5) from each mouse were used for counting.

For quantification of apoptotic cells, eyelid-edge mesenchymal and sloughed dead cells underneath the eyelid were counted (hair follicles were excluded from counting). Counted cell numbers were normalized to the counting areas of the eyelid tip for E15.5 eyelids. For E18.5, total numbers of apoptotic cells were simply potted. Multiple mice (>3) and multiple sections (>5) from each mouse were subjected to counting. Peridermal cells revealed by DAPI staining at E15.5 were counted and analyzed the same way as apoptotic cells.

For quantification of P-c-Jun cells, defined areas in front of the eyelid wedging epithelium (E15.5) and in the junction tissue of the eyelid (E17.5 and P1) were subjected to counting. P-c-Jun-positive cells were plotted as fractions of total cells within the counted areas. Multiple mice (>3) and multiple sections (>5) from each mouse were counted.

For quantification of beta-catenin expression, the eyelid front area was divided into two subdomains, the wedging epithelium and the protruding front cells. Fluorescence intensity from these two domains was quantified separately using ImageJ. Intensity ratios of wedging epithelium to protruding front cells were plotted. Multiple mice (>3) and multiple sections (>3) from each mouse were used for counting.

For quantification of cell polarity/orientation and shape, the orientation of a bisecting line through the apex of the wedging eyelid epithelium is defined as 0°. Cell orientations are measured as angles (degrees) of long axes referring to the bisecting line (0°). Data were binned as average cell numbers per 30°. Cell shapes were quantified in two-dimensional space based on index ratios of long axis to short axes. Data were plotted as means ± SD with statistics.

**RESULTS**

**Disruption of Prickle 1 Led to Delayed Eyelid Closure Within a Narrow Time Window**

To gain insights into the role of Prickle 1 in ocular development, we examined the embryonic eyelid closure process in both wild-type and Prickle 1 mutant mice. To circumvent embryonic lethality of complete null mutants, Prickle 1 compound mutant embryos or mice with one hypomorphic (Prickle 1<sup>a/b</sup>) and one null allele (Prickle 1<sup>b</sup>) were bred (Supplementary Fig. S1A, from Liu et al. 3). Loss of Prickle 1 protein expression was detected by western blotting for each genotype (Supplementary Fig. S1B). SEM was used to study the eyelid morphogenesis.

Primordial eyelids began to form from E11.5 to E13.5 with the commencement of invagination of periorcular ectodermal cells. 9 We observed grossly normal eyelid development in the mutant embryos at E13.5 and E14.5 (Figs. 1A–D). Ocular surface morphology revealed by SEM showed minor differences in coverage of the cornea surface between wild-type and mutant embryos (dashed circles in Figs. 1A, 1B). H&E-stained tissue sections exhibited virtually same ectodermal invagination in both wild-type and mutant embryos (Figs. 1C, 1D). However, the mutant eyelid outgrowth was stunted between E15.5 and E16.5 (Fig. 2, Supplementary Fig. S2A). Measurement of the distance between the upper and lower eyelids demonstrated an average delay of 120.78 ± 12.36 μm ($P = 0.0005$) and 62.07 ± 4.05 μm ($P = 0.0021$) in mutant eyelid outgrowth for E15.5 and E16.5 embryos, respectively (Supplementary Fig. S2A). At E15.5, SEM showed a larger open cornea.
FIGURE 2. Delayed eyelid closure in *Prickle 1<sup>ab</sup>* embryos. SEM and H&E staining of embryonic eyelid. *Arrows* point to the peridermal eyelid epithelial cells for all panels. *Boxed areas* were magnified in panels below. (A–D) Mutant cornea area was less covered by the growing eyelid at E15.5, and the peridermal epithelial cells were barely developed. (E–H) Wild-type eyelid completely fused at E16.5 and covered the cornea, whereas the mutant eyelid remained open. The mutant eyelid morphology was much like that of E15.5 wild type (compare *left panels* of [A–D] to corresponding *right panels* of [E–H]). (I–L) At E17.5, mutant eyelid development was comparable with wild type, and the eyelid is entirely fused.
The Role of Prickle 1 in Eyelid Morphogenesis

The number of leading edge peridermal cells was significantly reduced in the mutants (Supplementary Fig. S2B, Figs. 2C, 2D, compare right and left panels). The mean difference in peridermal cell counts between wild-type and the mutant eyelids is 9 ± 2.94 cells (P = 0.0061; Supplementary Fig. S2B). At E16.5, while the upper and lower lids of wild-type embryos were completely joined (Figs. 2E–H, left panels), the mutant lids remained separate (Figs. 2E–H, right panels), resembling those of wild-type embryos at E15.5 (compare Figs. 2E, 2F right panels with Figs. 2A, 2B left panels, respectively). Despite the prominent eyelid outgrowth defect, both the mutant and wild-type eyelids were completely closed at E17.5 (Figs. 2J, 2L). Statistical type eyelids were completely closed at E17.5 (Figs. 2I–L). Intriguingly, the eyelid thickness of wild-type and the mutant embryos appeared different at E17.5 (Figs. 2I, 2L). Statistical analysis demonstrated a mean difference of 55.12 ± 10.34 μm (thickness index; P = 0.0005) in eyelid thickness between E17.5 wild type and mutants. No significant difference was detected at E15.5 or E16.5 between wild-type and the mutant embryos (Supplementary Fig. S2C). The embryonic eyelid phenotypes of the Prickle 1 mutants (null alleles) were very similar to that of Prickle 1 (b/b) (null alleles) mutants (Supplementary Fig. S3). Together, the data illustrated a critical role of Prickle 1 in early eyelid growth.

Disruption of Prickle 1 Did Not Alter Ocular Surface Fate Nor Did it Alter Proliferation and Cell Death in Prenatal Eyelids

We next asked whether ocular surface fate specification was altered concurrently with or preceding the embryonic eyelid defect. At E15.5, mutant eyelids were largely open (Figs. 2A–D, 3A). Staining for keratin14 (K14), a pan ocular surface marker, did not reveal differences between wild-type and mutant embryos (Fig. 3A). K14 continued to be expressed in the correct domains at E17.5, when both wild-type and mutant eyelids were completely closed (Fig. 3B). We further examined expression of other keratin proteins, Keratin 1, 4, and 12, which were seen above the cornea (Fig. 5D). Like in the early-phase eyelid dysgenesis, mutant eyelid fate was not altered at several postnatal ages examined. K1 and K4 were expressed correctly in complementary domains of skin and conjunctiva, respectively (Supplementary Fig. S5, also K1 staining in Fig. 5). K1 expression appeared expanded in the inner lid of the P5 and P8 mutants (Supplementary Figs. S5A–E, Figs. 5A, 5B). However, measurement of K1 expression domain did not show significant difference between the wild-type and mutant eyelids (Supplementary Fig. S5M). In contrast, K4 expression was expanded from palpebral conjunctiva toward the cornea epithelium at P10 and P15 (Supplementary Figs. S5H, S5I, S5K, S5L), which was probably secondary to the observed cornea pathology.

The prominent postnatal eyelid changes led us to examine the eyelid thickness. The mutant eyelid was significantly thinner than the wild type at P5 (mutant versus wild type: 80 ± 4.32 vs. 165.34 ± 12.68, P = 0.009), P8 (mutant versus wild type: 108.34 ± 6.13 vs. 204 ± 6.98, P = 0.001), and P10 (mutant versus wild type: 220 ± 4.97 vs. 303.34 ± 16.76, P = 0.0025) (Supplementary Fig. S6A, Fig. 5F). Further measurement of the number of Prickle 1-positive mesenchymal cells per surface circumference demonstrated less infiltrated mutant eyelid pockets from P1 to P8 (Supplementary Fig. S6B, Fig. 5G). The reduction of mesenchymal cell number of the mutant eyelids was most significant at P8 among all ages examined (Fig. 5G). In contrast, cell size, another possible contributor to eyelid morphology, did not alter significantly at the junction epithelium of P5 or P8 eyelid (Supplementary Figs. S6C, S6D). Taken together, disruption of Prickle 1 led to precocious eyelid reopening and dysgenesis with decreased mesenchymal cell infiltration.

We next sought to examine whether proliferation and/or cell death might contribute to the eyelid dysgenesis of the mutants. Ki67-positive cells were counted in the mutants before and after eyelid closure (Figs. 4A–C). Similarly, TUNEL-labeled apoptotic cell numbers, though few in the eyelid area at E15.5 and E17.5, were comparable between mutant and wild-type embryos (Figs. 4D–F, Supplementary Fig. S4A). These results suggested that ocular surface fate specification, proliferation and cell death were largely unaffected upon loss of Prickle 1, and as such they cannot be primary causes of mutant eyelid closure defect.

Disruption of Prickle 1 Led to Precocious Eyelid Reopening and Thinner Eyelid With Decreased Mesenchymal Cell Infiltration and Increased Apoptosis of the Eyelid Tissue

The observation that mutant eyelid closure was delayed prompted us to investigate whether late-stage eyelid morphogenesis would also be affected. Similar to E17.5, P5 and P8 mutant eyelids were thinner visualized by staining of K1 and DAPI (Figs. 5A, 5B). The mutant eyelid lip exhibited a “V” rather than a “U” shape normally seen in wild type at P5 and P8 (Figs. 5A, 5B). At P8, there were only a few threads of connective tissue bridging the mutant lids (Fig. 5B). As a result, the mutant eyelid opened at P10 (Fig. 5C), far earlier than wild type, which normally opens between P12 and P14. Measurement of the amount of eyelid opening demonstrated that the distance between upper and lower mutant eyelids was 625.77 ± 58.27 μm at P10, approximately half of that of wild type at P15 (1033.32 ± 64.57 μm), a time point of which eyelids were fully opened. Surprisingly, the distance between mutant two eyelids was less at P15 compared with P10 (~278.73 ± 30.02 μm, P = 0.0114; Supplementary Fig. S4B). The less opening of P15 mutant eyelids might be a result of protective responses of the eyelids to the inflammatory ocular irritations. Consistent with this speculation, cornea disorganization was also observed in the mutants (Figs. 5C–E), with tissue debris often seen above the cornea (Fig. 5D).

Like in the early-phase eyelid dysgenesis, mutant eyelid fate was not altered at several postnatal ages examined. Ki1 and Ki4 were expressed correctly in complementary domains of skin and conjunctiva, respectively (Supplementary Fig. S5, also Ki staining in Fig. 5). Ki1 expression appeared expanded in the inner lid of the P5 and P8 mutants (Supplementary Figs. S5A–E, Figs. 5A, 5B). However, measurement of Ki1 expression domain did not show significant difference between the wild-type and mutant eyelids (Supplementary Fig. S5M). In contrast, Ki4 expression was expanded from palpebral conjunctiva toward the cornea epithelium at P10 and P15 (Supplementary Figs. S5H, S5I, S5K, S5L), which was probably secondary to the observed cornea pathology.

The prominent postnatal eyelid changes led us to examine the eyelid thickness. The mutant eyelid was significantly thinner than the wild type at P5 (mutant versus wild type: 80 ± 4.32 vs. 165.34 ± 12.68, P = 0.009), P8 (mutant versus wild type: 108.34 ± 6.13 vs. 204 ± 6.98, P = 0.001), and P10 (mutant versus wild type: 220 ± 4.97 vs. 303.34 ± 16.76, P = 0.0025) (Supplementary Fig. S6A, Fig. 5F). Further measurement of the number of Prickle 1-positive mesenchymal cells per surface circumference demonstrated less infiltrated mutant eyelid pockets from P1 to P8 (Supplementary Fig. S6B, Fig. 5G). The reduction of mesenchymal cell number of the mutant eyelids was most significant at P8 among all ages examined (Fig. 5G). In contrast, cell size, another possible contributor to eyelid morphology, did not alter significantly at the junction epithelium of P5 or P8 eyelid (Supplementary Figs. S6C, S6D). Taken together, disruption of Prickle 1 led to precocious eyelid reopening and dysgenesis with decreased mesenchymal cell infiltration.
FIGURE 3. Unaltered ocular surface fate of Prickle 1<sup>+/−</sup> embryos. Keratinocyte markers K14, K1, K4, and K12 were used to immunostain sagital sections through the vertical meridian of the eyelids. For all panels, red, yellow, and white arrows point to skin, conjunctiva, and corea, respectively. (A, B) Immunostaining of K14, a marker for all ocular surface epithium at E15.5 and E17.5. (C, D) Immunostaining of K1, a marker for skin epithelial sheet at E15.5 and E17.5. (E, F) Immunostaining of K4, a marker for nonkeratinized, conjunctival epithelial cells at E15.5 and E17.5. (G, H) Immunostaining of K12, a corneal-type epithelial marker at E15.5 and E17.5. Ocular surface territories remained normal from all examined keratinocyte markers.
tip further confirmed the observation of the TUNEL labeling (Fig. 6J). Thus, together with the compromised mesenchymal cell infiltration, apoptosis may have also contributed to precocious eyelid reopening in Prickle 1 mutant mouse.

**Disruption of Prickle 1 Altered Cell Orientation of Embryonic Eyelid Front Cell**

Open eyelid is seen in many PCP mutants during embryogenesis. As a core PCP component, Prickle 1 is crucial for oriented cell migration in tissue morphogenesis. We therefore examined whether cell orientation, which generates physical forces pulling the eyelid epithelial sheet together, altered the front most cells of the eyelids. Assisted with E-cadherin staining on static tissue sections, we observed alteration in orientation of the front most cells of the mutant eyelid (Fig. 7). At E15.5, wild-type front cells elongated with their long axis toward the direction of eyelid closure (Figs. 7A, 7A’, 7B). In contrast, many Prickle 1 mutant cells tended to orient to the direction perpendicular to eyelid closure (Figs. 7C, 7C’, 7D). Quantification and statistical analysis demonstrated significant differences in cell orientation and shape changes between wild-type and mutant embryos (Figs. 7E, 7F). At E17.5, after eyelids had closed, most eyelid junction cells of wild type embryos were columnar (Figs. 7G, 7G’), whereas they elongated in spindle shape in the mutants (Figs. 7I, 7I’). Statistical analysis again revealed significant differences in cell orientations and shapes between wild-type and the mutant cells (Figs. 7H, 7J, 7K, 7L). The result suggested that altered cell orientation and shape might contribute to the delayed eyelid closure in Prickle 1 mutants.

**Loss of Prickle 1 Altered Phosphorylated c-Jun and β-Catenin in Distinct Cell Populations of Mutant Embryonic Eyelids**

To pinpoint the exact role of Prickle 1 in the early phase of eyelid morphogenesis, we investigated the potential connections of Prickle 1 with known signaling cascades for eyelid closure. Because actomyosin system is crucial for eyelid closure,^27,28^ and a primary downstream target of Wnt/PCP, we examined whether actin polymerization was disrupted in Prickle 1 mutants. Phalloidin-staining appeared similar between the wild-type and mutant eyelid edge at E14.5 (Figs. 8A, 8B). In contrast, less staining was observed in the mutant eyelid edge at E15.5 (Figs. 8C, 8D). On vertical sections, mutant eyelid front cells showed apparent reduction in actin staining compared with wild type (Figs. 8E, 8F). It was reported that c-Jun, downstream of the JNK pathway, was required for actin organization of the leading edge of the eyelids.11,12 We therefore tested whether the active, phosphorylated form of c-Jun was altered upon disruption of Prickle 1. P-c-Jun was located at eyelid front adjacent to wedging epithelial sheet, but not any of the peridermal cells (Figs. 8G, 8H). At E15.5, the fraction of P-c-Jun cells

![Figure 4](https://example.com/image4.png)

**FIGURE 4.** Normal proliferation and cell death of the Prickle 1<sup>+/−</sup> embryonic eyelids. (A, B) Ki67 staining demonstrates that proliferating cells of the eyelid in both mutant and wild-type embryos before (E15.5) and after (E17.5) the eyelid was entirely closed. (C) Quantification of Ki67-positive cells. Cells were counted in defined areas described in Supplementary Figure 2C, and plotted as fractions of total cells of the counted areas. No significant difference was detected in cell proliferation between wild-type and the mutant eyelids at E15.5 and E17.5. (D, E) TUNEL-labeled apoptotic cells in the mutant and wild-type embryos at E15.5 and E17.5. White dashed circles demarcate eyelid front apoptotic cells or cell debris (green); brackets demarcate dermal apoptotic mesenchymal cells. (F) Quantification of apoptotic cells at eyelid lip and junctions at E15.5 and E17.5. Apoptotic cells were counted and plotted as total numbers. Counted area was defined as described in Supplementary Figure 4A. No significant difference of cell death was observed between wild-type and the mutant eyelids. Five sections of each embryo were subjected to counting. More than three embryos for each genotype were used. P value was detected by Student’s t test.
Supplementary Fig. 8A) was significantly reduced in either upper or lower lids of the mutants (mutant versus wild type: 22.28% ± 0.024 vs. 34.51% ± 0.0089, P = 0.0028; Fig. 8K).

However, a similar number of P-c-Jun cells were observed in the eyelid front at E17.5 (Figs. 8I, 8K). P-c-Jun expression in the mutant eyelids was retained until P1, a time point at which its expression entirely disappeared in wild type (Figs. 8J, K). Other than the delay, P-c-Jun expression profile largely resembled that of the wild type (Figs. 8G–J). Thus, P-c-Jun expression in the eyelid front was also delayed in the mutants, but occurred prior to the embryologically apparent delay of eyelid closure.

Wnt/β-catenin signaling provides a counterbalance to eyelid outgrowth.13 Several studies revealed antagonism between β-catenin dependent and independent Wnt pathways.17–20 Because Prickle 1 is a mediator of noncanonical Wnt5a signaling,4 we asked whether loss of Prickle 1 would bias Wnt-signaling toward β-catenin-dependent canonical pathway. At E15.5, β-catenin is broadly expressed in all epithelial cells of the eyelid, with relatively lower expression at the eyelid wedging epithelium (dashed lines in left panels of Figs. 8L, 8M) compared with the eyelid front cells (asterisks in left panels of Figs. 8L, 8M). In contrast, immunostaining of β-catenin was enhanced in the wedging epithelium of the mutant eyelid (Figs. 8L, 8M, right panels). Quantification of fluorescent intensity ratio of β-catenin expression in wedging epithelium to that in the eyelid front cells (Supplementary Figs. 8B, 8C) demonstrated a significant difference between wild type and the mutants (Fig. 8P). The result suggested that there might be a locally increased canonical Wnt signaling in the mutants upon ablation of Prickle 1. Like P-c-Jun, β-catenin level also returned to normal after mutant eyelid closure at E17.5 (Figs. 8N, 8O). Thus, changes in β-catenin occurred prior to the embryologically apparent delay of eyelid closure.

Restricted Expression of Prickle 1 in Eyelid Protrusion and Its Possible Integrative Signaling Networks

To further define a role of Prickle 1 in eyelid morphogenesis, we investigated normal Prickle 1 expression by immunostain-
ing of a knock-in EYFP reporter. 

Prickleb/a/b reporter mouse showed no difference in eyelid morphology and closure, and appeared indistinguishable from wild-type mouse in previous studies. Three cell populations observed expressing Prickle 1 might be highly relevant to the protrusive activities of the eyelid: the front cells, the wedging epithelium, and the dermal cells (Fig. 9A). P-c-Jun was downregulated and β-catenin was upregulated in front cell and wedging epithelium, respectively, in the mutant embryos (Figs. 8E–J). Prickle 1 expression overlapped with both β-catenin and P-c-Jun (Figs. 9B–F). By E17.5, Prickle 1 was uniformly distributed to eyelid epidermal epithelium and eyelid junction cells (Supplementary Fig. 9A). From P1 to P5, Prickle 1 was downregulated in epidermal cells (Supplementary Figs. 9B, 9C), while remaining highly expressed in mesenchyme-derived connective and glandular tissues, and hair follicles.

A schematic illustration of expression dynamics of Prickle 1, β-catenin and P-c-Jun at critical ages of embryonic eyelid closure were shown in Figure 10 (Figs. 10A, 10B). Disruption of Prickle 1 altered β-catenin and P-c-Jun expression dynamics, which together could interfere the progresses of eyelid closure (compare Fig. 10A with 10B). Prickle 1 may antagonize Wnt/β-catenin signaling in eyelid wedging epithelium, while promoting c-Jun activity in eyelid front cells. Additionally, Wnt/β-catenin may interact with c-Jun pathway during eyelid closure (Figs. 10C, 10D). The dermal expression of Prickle 1 may be related to the mesenchymal cell infiltration of the eyelid pockets, which is currently under investigation.

**Figure 6.** Increased apoptosis in Prickle 1+/- postnatal eyelids. (A–D) Ki67 staining of the postnatal eyelids from P5 to P15. (E) Quantification of Ki67-positive cells in the eyelids from selected postnatal ages. Cell counts normalized to chosen areas (see Supplementary Fig. S7A) were presented as graph with error bars and P values detected by t-test. (F–I) TUNEL-labeled apoptotic cells of postnatal eyelids. (E G) Increased apoptotic cells detected in the mutant eyelid junction tissues at P5 and P8. Brackets demarcate eyelid junctional tissue and apoptotic eyelid areas; circles demarcate apoptotic cells falling beneath the eyelids and above the cornea surface. (H) Eyelid tissue debris (asterisk, right panel) and the cornea of the mutant mice (arrow, right panel) exhibited extensive apoptosis at P10. Note the reduced TUNEL labeling in the mutant eyelid tips at P10 (dashed brackets) in comparison with that from the same age of wild-type eyelids (left panel). (I) The eyelid tip apoptotic events were comparable between wild-type and the mutant mice at P15 (brackets). The mutant cornea was under erosion (arrow). (J) Quantification of cell death from (F–I) confirmed the observation of increased apoptosis in the Prickle 1 mutants from P5 to P8. Apoptotic mesenchymal cells of the eyelid edge and sloughed dead cells underneath the eyelids were counted (see Supplementary Fig. S7F). Cells are plotted as total numbers. Five sections of each embryo were subjected to counting. More than three embryos for each genotype were used. Student’s t-test was performed to detect P values.
DISCUSSION

Tissue closure is a recurrent theme orchestrating organogenesis seen in heart septum, neural tube, optic fissure, palate, body wall, and eyelid closure. Failure of tissue closure would lead to severe organ malformations. Notably, a few of these defects often occur concurrently in genetically engineered animal models bearing mutations in key components of the Wnt/PCP pathway, suggesting a common mechanism underlying tissue closure/fusion. The current study aims to elucidate the role of Wnt/PCP pathway in ocular development by focusing on Prickle 1, a core PCP component, which is extensively involved in tissue morphogenesis.

In this study, we show: (1) a crucial role of Prickle 1 in a specific time window during embryonic eyelid closure, (2) a late-phase role of Prickle 1 in eyelid tissue homeostasis, and (3) a complex signaling network for Prickle 1-mediated, PCP-directed cell polarity in eyelid closure.

Spatiotemporal Requirement for Prickle 1-Executed PCP Signaling During Embryonic Eyelid Closure

In contrast to many genetic mutations causing open eyelid at birth, Prickle 1 mutants showed delayed eyelid closure at embryonic ages. Before E14.5, Prickle 1 had a negligible effect on eyelid morphogenesis. At E17.5 and beyond, both wild-type and Prickle 1 mutant eyelids were closed completely. The mutant eyelid defect fell within a narrow window between E14.5 and E17.5, suggesting a critical timing of eyelid closure requiring Prickle 1 function. After the mutant eyelid eventually closed, a precocious eyelid reopening followed postnatally. The latter phenotype does not appear to be a direct extension of the early eyelid closure deficit, because premature but not delayed eyelid reopening was observed in Prickle 1 mutant pups.

Eyelid of Prickle 1 mutants opened at P10, approximately 4 days earlier than normal. Mutant eyelids are thinner starting from E17.5 until to P10, when they are
Figure 8. Reduction of P-c-Jun and accumulation of β-catenin in Prickle 1+/b embryonic eyelid. (A, B) Phalloidin-stained flat mount eyelid actin fibers at E14.5. Bracket areas were magnified in (B). (B) Actin cable of the eyelid (arrows) and cortical actins from the epithelial sheet (asterisks) did not differ between the wild-type and mutant embryos. (C) Phalloidin-stained E15.5 flat mount eyelids. Brackets indicate the approximate regions that were magnified in (D). (D) Arrows indicate the eyelid front actin cables. Mutant actin cable was significantly less intense than the wild type. (E) Upper eyelid: vertical sections roughly through the areas in (D) (arrow) demonstrated reduced assembled actin fibers in the mutant eyelid front ridge (right panel compared with the left, dashed lines). (F) Lower mutant eyelid: actin fibers showed diminished assembled area (right panel compared with the left, dashed lines). (G, H) Immunostaining of P-c-Jun in the upper and lower eyelid. Note that the number of P-c-Jun–positive cells (above dashed lines) decreased in the mutant eyelid front. (H) At E17.5, P-c-Jun expression (red, above dashed lines) was similar in both wild-type and mutant eyelids. (J) At P1, after eyelid closure was completed, P-c-Jun was no more expressed in wild-type eyelid, and also downregulated in the mutant eyelid (J) (right panel). (K) Quantification of P-c-Jun–positive eyelid front cells. Significant reduction of P-c-Jun–positive cells in the mutant embryos was observed at E15.5. (L, M) Immunostaining of β-catenin in E15.5 eyelids. Compared with wild type, the mutant β-catenin expression was enhanced in the region of wedging epithelium (dashed lines) of the eyelid. (N, O) Immunostaining of β-catenin in E17.5 eyelids. At these two ages, β-catenin expression was similar between wild type and the mutants. (P) Quantification of β-catenin expression in eyelid wedging epithelium. Relative fluorescent intensity was generated by ratio of fluorescent signal in the wedging epithelium to that in eyelid front cells (asterisks) (see Supplementary Fig. 8I). Statistics was performed with Student’s t-test for all measurements in this figure. Multiple sections (>3) from each of three embryos were counted.
fully open. Yet, statistically no significant altered cell proliferation was detected. Further examinations demonstrated a significant decrease of mesenchymal cell infiltration of the mutant eyelid pocket, and an increase of apoptosis in the eyelid lips at P8. These changes combined could have altered the course of eyelid opening. In addition to the eyelid edge, morphogenesis in the rest of the eyelid or beyond may also have indirectly contributed precocious postnatal eyelid reopening in the mutants. Detailed analyses to address the role of Prickle 1 in specific cell populations of the eyelid are under way. Nonetheless, the pre- and postnatal eyelid phenotypes in Prickle 1 mutants imply distinct temporal requirements for Prickle 1 during eyelid development.

The eyelid closure defect in Prickle 1 mutants was not caused by perturbed cell proliferation or cell death in the eyelid lip. Rather, it is the changes in cell shape/polarity in mutant eyelid front and the wedging epithelia that likely played a key role. Consistent with this notion, actin polymerization, a well-recognized PCP target, was also compromised in Prickle 1 mutants. A recent study demonstrated that cell intercalation generated primary towing forces perpendicularly to the epithelial sheet during eyelid closure. As cell intercalation is a consequence of execution of PCP, the study implies that eyelid closure may be driven by PCP. Defective cell orientation in eyelid front cells of the Prickle 1 mutants is expected to disrupt coordinated cell intercalation, and would thus compromise the towing forces required for eyelid closure. However, we could not exclude the possibility that regions other than eyelid lips also contributed to the delay of the lid closure in Prickle 1 mutants. Because Prickle 1 is highly expressed in eyelid mesenchymal cells, it may also play roles in migratory activities of the mesenchymal cells, which may in turn cause changes in cell shape, crowdedness, and/or polarity. Taken together, our data suggest Prickle 1-mediated PCP activity is required for the normal timing of eyelid development and morphogenesis.

**Figure 9.** Prickle 1, β-catenin and P-c-Jun expression during eyelid development. Immunostained knock-in EYFP reporter for monitoring Prickle 1 expression in E15.5 eyelid. (A) In the eyelid lip, Prickle 1 was expressed in dermal mesenchymal cells (Derm, solid white line), eyelid wedging epithelium (We, yellow dashed bracket), and eyelid front cells (Fc, green dashed lines). Note the exclusion of Prickle 1 expression from peridermal cells (asterisk). (B) β-catenin was generally expressed throughout eyelid lip, but in lower level in the eyelid wedging epithelium (yellow bracket). (C) Expression of P-c-Jun in eyelid front cells (green dashed lines). (D) Merged image of Prickle 1 and P-c-Jun expression demonstrating a strong overlap in some front cells (yellow), but a weak overlap in others. (E) Merged image of Prickle 1 and β-catenin expression. Prickle 1 showed relative higher expression in the wedging epithelium compared with the front cells. (F) Merged image of P-c-Jun and β-catenin expression.
signals converge on actomyosin contractile system. Notably, JNK/c-Jun, which directly affects actin polymerization, acts as a connecting hub to integrate multiple signaling inputs. The compromised actin expression in Prickle 1 mutant eyelid edge suggests that Prickle 1-mediated Wnt/PCP might connect to JNK/c-Jun pathway as well. Examination of P-c-Jun demonstrated a regulatory role of Prickle 1 in c-Jun expression. Moreover, expression of Prickle 1 in eyelid front cells overlaps with that of P-c-Jun further supporting the connection between PCP and JNK/c-Jun. Overall, our data suggested that Prickle 1-executed PCP act independently of other mitogenic signaling on JNK/c-Jun.

Recent studies suggest that Wnt/β-catenin activity is tightly controlled during eyelid closure. Wnt/β-catenin activity was detected at the eyelid junction where the front meets the surrounding epidermis by a BATGAL reporter. Additionally, epithelial overexpression of Wnt/β-catenin disrupted cornea and eyelid morphogenesis. The current study indicates an inverse correlation of β-catenin expression with the extent of eyelid protrusion. In wild type, as the eyelid becomes more pointed at E15.5, a lower level of β-catenin expression is detected at the eyelid wedging epithelium than the front cells.
The Role of Prickle 1 in Eyelid Morphogenesis

In contrast, mutant eyelid shows significantly increased wedging epithelial β-catenin expression and rounder lip. Together with previous studies, the data implies that aberrant Wnt/β-catenin signaling contributes to the delayed eyelid outgrowth in Prickle 1 mutant embryos. It further suggests that Prickle 1 counterbalances Wnt/β-catenin activity during eyelid morphogenesis. This notion is consistent with the observed antagonism between Wnt/PCP and Wnt/β-catenin.\textsuperscript{17-20} However, we did not find perturbed basement membrane or cell proliferation, which was observed previously by Wnt/β-catenin overexpression.\textsuperscript{14,15} A plausible interpretation is that in Prickle 1 mutants, PCP driven events for eyelid closure are dominant rather than Wnt/β-catenin signaling. Altogether, Wnt/PCP directs cell orientation/polarity and counterbalances Wnt/β-catenin activities to regulate proper timing of eyelid closure in development.

CONCLUSIONS

In summary, our study suggests that a spatiotemporal execution of PCP signaling is crucial for eyelid morphogenesis and homeostasis. PCP signaling controls P-c-Jun and β-catenin expression, and regulates cell orientation/polarity and shape. The resultant corneal pathology may serve as a model system for the study of congenital ocular surface diseases. Future work will aim to understand the role of PCP in the eyelid reopening process, which is more relevant to human ocular diseases.

Acknowledgments

The authors thank Mengqing Xiang, Xuri Li, and Rong Ju for providing imaging facilities and reagent helps. We are indebted to Tiansen Li and Anand Swaroop from the National Eye Institute for providing valuable reagents and mouse models. We thank our lab members, Shiyongzhu, Shanzhen Peng, and Sifeng Wang for critical reading the manuscript and helpful comments.

Supported by grants from the Guangzhou City Sciences and Technologies Innovation Project (201707020009; Guangzhou, Guangdong Province, China); the National Natural Science Foundation of China (NSFC: 31571077; Beijing, China), “100 People Plan” from Sun Yat-sen University (8300-18821104; Guangzhou, Guangdong Province, China), and research funding from State Key Laboratory of Ophthalmology at Zhongshan Ophthalmic Center (303606202400339; Guangzhou, Guangdong Province, China).

Disclosure: D. Guo, None; Z. Yuan, None; J. Ru, None; X. Gu, None; W. Zhang, None; F. Mao, None; H. Ouyang, None; K. Wu, None; Y. Liu, None; C. Liu, None

References


36. Toonen J, Liang L, Sidjanin DJ. Waved with open eyelids 2 (woe2) is a novel spontaneous mouse mutation in the protein phosphatase 1, regulatory (inhibitor) subunit 13 like (Ppp1r13l) gene. BMC Genet. 2012;13:76.


