

1 **The core Planar Cell Polarity gene, *Vangl2*, maintains apical-basal organisation of the corneal**  
2 **epithelium.**

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15

16 **ABSTRACT**

17 **The role of the core planar cell polarity (PCP) pathway protein, Vangl2, was investigated in the**  
18 **corneal epithelium of the mammalian eye, a paradigm anatomical model of planar cell migration.**  
19 **The gene was conditionally knocked out in vivo and knocked down by siRNA, followed by**  
20 **immunohistochemical, behavioural and morphological analysis of corneal epithelial cells. The**  
21 **primary defects observed in vivo were of apical-basal organisation of the corneal epithelium, with**  
22 **abnormal stratification throughout life, mislocalisation of the cell membrane protein, Scribble, to**  
23 **the basal side of cells, and partial loss of the epithelial basement membrane. Planar defects in**  
24 **migration after wounding and in presence of an applied electric field were noted. However,**  
25 **knockdown of Vangl2 also retarded cell migration in individual cells that had no contact with their**  
26 **neighbours, which precluded a classic PCP mechanism. It is concluded that some of the planar**  
27 **polarity phenotypes in PCP mutants may arise from disruption of apical-basal polarity.**

28

29 Key words: Cornea, epithelium, planar cell polarity, Vangl2, apical-basal polarity

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## 32 INTRODUCTION

33 The cornea is the main refractive constituent of the anterior segment of the vertebrate eye. Its  
34 tissue organisation is relatively simple: an outer stratified epithelium representing 10-30% of the  
35 corneal thickness (depending on species) sits on a hypocellular collagenous stroma, and an inner  
36 endothelial monolayer (Li et al. 1997; Forrester et al. 2002; Henriksson et al. 2009). The structure  
37 and transparency of the cornea must be maintained throughout adult life for normal sight.

38 Homeostatic maintenance of the corneal epithelium requires tight control of tissue organisation and  
39 cell migration. The corneal and conjunctival epithelia are contiguous and stem cells that maintain  
40 the corneal epithelium are located at the boundary between the two – the limbus – around the  
41 corneal periphery (Cotsarelis et al. 1996). These limbal epithelial stem cells generate transit  
42 amplifying cells that migrate centripetally along the basal layer of the corneal epithelium (Collinson  
43 et al. 2002; Nagasaki and Zhao, 2003; Di Girolamo et al. 2015). Proliferative basal corneal epithelial  
44 cells may divide several times before losing contact with the basement membrane and  
45 differentiating as suprabasal epithelial 'wing cells' (Lehrer & Lavker, 1999). Superficial squamous  
46 cells are continuously lost from the corneal epithelial surface e.g. by abrasion. The balance between  
47 cell migration into the corneal epithelium, basal cell division and apical cell loss from the corneal  
48 surface must be maintained such that the multilayered structure of the corneal epithelium is  
49 preserved (Thoft & Friend, 1983; reviewed in Mort et al. 2012).

50 The corneal epithelium exhibits a rigid apical-basal polarity. Cell proliferation is restricted to the  
51 basal layer of the corneal epithelium, and apoptosis is normally restricted to desquamating  
52 superficial cells (reviewed in Mort et al. 2012). Tight junctions between cells prevent free diffusion  
53 across the epithelium and contribute to its protective function (Yi et al. 2000). In addition,  
54 desmosomes, gap junctions and immunoglobulin-class cell adhesion molecules connect the cells of  
55 the adult corneal epithelium. Hemidesmosomes anchor the basal cells to the underlying basal  
56 lamina (Buck, 1982; Smith et al. 2001; Forrester et al. 2002). The corneal epithelium originates as a  
57 monolayer during development and in mice stratification occurs neonatally concomitant with eye  
58 opening, controlled at least in part by Wnt/ $\beta$ -catenin signalling inhibiting action of bone  
59 morphogenetic protein 4 (Zhang et al. 2015).

60 Studies investigating the control of apical-basal polarity have highlighted three complexes whose  
61 subcellular localisation directs the internal polarity of epithelial cells in vertebrates or invertebrates:  
62 Crumbs and Stardust apically; the Par6/Bazooka/ $\alpha$ PKC complex towards the apical side of the lateral

63 edge of the cell; and Lethal Giant Larvae, Discs Large and Scribble homologues laterally (Xiao et al.  
64 2011; Kumichel & Knust, 2014; Rodriguez-Boulan & Macara, 2014; Whiteman et al. 2014).

65 Planar polarity, i.e. the polarity that epithelial cells exhibit in the plane of their basement membrane,  
66 is directed during embryogenesis by a non-canonical branch of the Wnt signalling pathway – the  
67 planar cell polarity (PCP) pathway. In invertebrate systems at least this derives from interaction  
68 between transmembrane proteins frizzled and van gogh/strabismus, asymmetrically localised to  
69 opposite edges of cells, conferring patterns of epithelial cell directionality in the plane of their  
70 basement membrane (Taylor et al. 1998; Devenport, 2014). Vertebrates have multiple PCP gene  
71 homologues, and PCP pathways have multiple functions during embryogenesis (Lu et al. 2004; Gao  
72 et al. 2011; Mao et al. 2011; Wansleebe & Meijlink, 2011; Wallingford, 2012). Although  
73 extrapolation between vertebrate and invertebrate systems is not straightforward, mutation of the  
74 *van gogh* homologue, *Vangl2*, in vertebrate embryos ablates all PCP signalling and produces heart,  
75 inner ear and neural tube defects consistent with failure of cell directionality and migration (Greene  
76 et al. 1998; Henderson et al. 2001; Kibar et al. 2001; Murdoch et al. 2001; Park & Moon, 2001; Goto  
77 & Keller, 2002; Monticouquiol et al. 2003; Phillips et al. 2005; Roszko et al. 2009; Lei et al. 2010;  
78 Yates et al. 2010; Ramsbottom et al. 2014).

79 There is evidence of interaction between apical-basal and planar polarity pathway components.  
80 Apical localisation of PCP proteins is critical for their function (Das et al., 2014), and protein kinase C  
81 alpha ( $\alpha$ PKC), part of the Par6/Bazooka/ $\alpha$ PKC complex which characterises the apical-lateral side of  
82 the cell, can inhibit Frizzled-PCP signalling in *Drosophila* by phosphorylating Frizzled and thereby  
83 stopping it from signalling (Djiane et al. 2005). Par3 (Bazooka) and Scribble have been linked to  
84 defects in asymmetric cell division and cell polarity defects (Lin et al. 2000). Scribble protein is key to  
85 potential interactions between apical/basal and planar polarity. Within *Drosophila*, scribble binds  
86 van gogh protein (also known as strabismus) via its PDZ domain 3 and cooperates in establishing PCP  
87 (Coubard et al. 2009). In addition heterozygous *scribble* mutations were found to exacerbate PCP  
88 defects of *van gogh* (*vang*) mutants, whereas Discs Large and Lethal Giant Larvae, the other proteins  
89 located with Scribble along the baso-lateral edge of cells, did not, indicating a specific role for  
90 Scribble in *Drosophila* PCP (Coubard et al. 2009). The *scribble*<sup>5</sup> mutation, encoding a protein  
91 truncated before the 3<sup>rd</sup> PDZ domain, exhibited PCP defects in wing cell alignment, but not apical-  
92 basal polarity defects (Coubard et al. 2009).

93 In vertebrates the 3<sup>rd</sup> and 4<sup>th</sup> PDZ domains of Scribble bind the C-terminus of *Vangl2* and this  
94 interaction is required for asymmetric localisation of *Vangl2* in cochlear hair cells (Monticouquiol et

95 al. 2006). *Vangl2*<sup>Lp/+</sup> *Scribble*<sup>Crc/+</sup> double mutants show cochlea PCP defects akin to those observed in  
96 *Vangl2*<sup>Lp/Lp</sup> mutants indicating that Scribble and Vangl2 proteins work in the same genetic pathway  
97 during PCP cell alignment (Monticouquiol et al. 2003). Hence Scribble, although having a canonical  
98 role in establishment of apical/basal polarity, also has a role in the establishment of PCP in  
99 vertebrates, regulating cell cohesion junctional complex maintenance (Yates et al. 2013).

100 Little work has been done on PCP gene function in adult vertebrate tissues. As part of a study  
101 investigating the control of patterning in the corneal epithelium, we conditionally knocked out the  
102 core PCP gene *Vangl2* in the corneal epithelium, and were able to show classic planar polarity  
103 defects – Vangl2 acted through PCP intermediates Dishevelled, DAAM1 and ROCK1/2, modulating  
104 corneal epithelial cell cytoskeletal rearrangement, cell alignment and migration (in vivo and in vitro)  
105 (Findlay et al. 2016). Here we describe also a severe disruption of apical-basal polarity with failure of  
106 basal cells to maintain a normal basement membrane. The data show PCP genes interact with apical-  
107 basal pathways in adult vertebrate system and that Vangl2 activity is required for normal  
108 stratification of the corneal epithelium.

109

## 110 **Materials and Methods**

### 111 **Mice**

112 Hemizygous *Tg(Pax6-cre, GFP)1Pgr* transgenic mice (henceforth '*Le-Cre<sup>Tg/+</sup>*') drive *Cre* recombinase  
113 expression in the lens and corneal epithelia (Williams et al. 1998; Ashery-Padan et al. 2000). *Le-*  
114 *Cre<sup>Tg/-</sup>*, *Vangl2<sup>Lp/+</sup>* and *Vangl2<sup>fl/fl</sup>* floxed mice (Ramsbottom et al. 2013) were maintained on a  
115 congenic CBA/Ca genetic background. All animal procedures were carried out according to Animals  
116 (Scientific Procedures) Act 1986 and were passed by University of Aberdeen Ethical Review Board.

117

118 Due to lethality of the *Vangl2<sup>Lp/Lp</sup>* homozygous mutants (Strong & Hollander, 1949), *Cre-loxP*  
119 technology (Gu et al. 1994) was used to delete *Vangl2* conditionally in the corneal epithelium of *Le-*  
120 *Cre<sup>Tg/-</sup>*; *Vangl2<sup>fl/fl</sup>* animals. *Vangl2<sup>fl/fl</sup>* animals were mated with *Le-Cre<sup>Tg/-</sup>* mice and their genotypes  
121 were confirmed by polymerase chain reaction (PCR) using primers and conditions described in  
122 Findlay et al. (2016). The *Le-Cre* transgene is active from E8.75 in the lens placode and is expressed  
123 continuously throughout the lens and corneal epithelium (Ashery-Padan et al. 2000). *Le-Cre<sup>Tg/-</sup>*;  
124 *Vangl2<sup>fl/+</sup>* from F1 were backcrossed with *Vangl2<sup>fl/fl</sup>* mice to obtain *Le-Cre<sup>Tg/-</sup>*; *Vangl2<sup>fl/fl</sup>* mice and *Le-*  
125 *Cre<sup>Tg/-</sup>*; *Vangl2<sup>fl/+</sup>* littermate controls. *Le-Cre<sup>-/-</sup>* *Vangl2<sup>fl/fl</sup>* animals were normal and exhibited no  
126 looptail defects, indicating the floxed allele was neutral and the *Le-Cre* transgene was not showing  
127 leaky expression in the germline. Mice were killed by cervical dislocation, their eyes enucleated and  
128 fixed for processing.

129

### 130 **Cell Culture**

131 *In vitro* experiments were carried out by using an immortalised human corneal epithelium cell line  
132 (HCE-S) donated by Julie Daniels, Institute of Ophthalmology (Notara & Daniels, 2010). Cells were  
133 maintained in DMEM/F-12 media (Life Technologies), 10% fetal calf serum (FCS) (Invitrogen), 1%  
134 Penicillin Streptomycin solution (10,000 units penicillin and 10 mg streptomycin/ml)(Sigma) at 37°C  
135 in a humidified atmosphere of 5% CO<sub>2</sub>. Culture medium was replenished every 72 hours.

136 A robust knockdown of VANGL2 to ~30% of normal levels was obtained in transfected HCE cells  
137 using 10 nM HPP grade *Vangl2\_5* siRNA 5'-UAGAAUUAGGAAGUACCCAUA-3' as described in Findlay  
138 et al. (2016). In brief, 60,000 cells were seeded in each well of a 24-well plate in 0.1 ml of culture  
139 media. Cells were incubated under their normal growth condition while 75 ng siRNA was diluted  
140 down in 100 µl of serum-free culture medium to a final concentration of 10 nM. The diluted siRNA

141 was mixed with 3  $\mu$ l of HiPerFect transfection reagent and incubated for 10 minutes at room  
142 temperature before adding drop-wise to HCE cells. After 3 hours incubation of with the transfection  
143 complexes 400  $\mu$ l of culture medium were added in each well. Cells were passage 24 h later for  
144 experiments.

#### 145 **Cellular migration studies upon application of electric fields**

146 Following gene silencing with siRNAs HCE cells were exposed to physiological electric field  
147 stimulation to measure forward migration index and directionality of electrotactic movement.  
148 Control and knockdown HCE cells were harvested from 24-well plate by addition of trypsin-EDTA,  
149 spun and re-suspended in 500  $\mu$ l of culture media. Cells were seeded into 'ibidi' 15  $\mu$ m  
150 chamber-slides (Thistle Scientific) and time-lapse video recording performed, using a Nikon Diaphot  
151 inverted phase contrast microscope with a temperature-controlled environment chamber in a direct  
152 current 200 mV/mm electric field by adopting a set-up previously described by Rajnicek et al. (2006).  
153

154 A measure of planar-oriented migration, the forward migration index (FMIX) was measured by using  
155 the 'Manual Tracking' and 'Chemotaxis' plugins (available at  
156 <http://rsbweb.nih.gov/ij/plugins/index.html>) for ImageJ. FMIX is one of the most indicative  
157 measures of migration and is determined by measuring, for each cell, the movement along the x axis  
158 (x) as a proportion of the total migration distance (d), evaluating the magnitude of the directional  
159 cell movement towards the cathode. Conventionally, the cathode is to the left, so the closer the  
160 value of FMIX to -1 the more directed is migration to the cathode on average. FMIX was measured  
161 separately for individual cells in culture and cells as part of confluent sheets.

162

#### 163 **Histology**

164 Eyes were enucleated from adult animals killed by a Schedule 1 procedure and fixed in  
165 paraformaldehyde (4% in phosphate buffered solution, PBS) for 4-6 hours at 4°C prior processing for  
166 paraffin embedding.

167 Eyes were washed 3 times in PBS, 20 minutes, then for 15 minutes in saline solution (0.9% NaCl).  
168 Eyes were then dehydrated through a series of 15 minutes ethanol changes (70%, 85%, 95% and  
169 100%) before being cleared with xylene (2 x 5 minutes washes at room temperature and incubation  
170 overnight in fresh xylene) and embedding in paraffin wax. 7  $\mu$ m-thick sections were cut in the  
171 transverse plane.

**172 Haematoxylin and eosin staining**

173 Wax sections were deparaffinised in HistoClear (HS-200, National Diagnostics), 2 x 10 minutes and  
174 washed 2 x 5 minutes in 100% ethanol. Rehydration in a serial change of ethanol washes for 5  
175 minutes each (95%, 85% and 70% ethanol) followed. Slides were then washed with PBS, incubated in  
176 Gill's haematoxylin solution for 1 minute and washed in tap water. Slides were washed in Scott's tap  
177 water (20g/L MgSO<sub>4</sub>.7H<sub>2</sub>O, 20g/L NaHCO<sub>3</sub>), rinsed with tap water and incubated for 30 seconds in  
178 eosin solution (1% eosin in 50% ethanol-5mM acetic acid). Slides were then dehydrated an ethanol  
179 series, cleared in xylene and mounted with Di-n-butylphthalate in xylene (DPX). Imaging was  
180 performed using a Nikon E400 Eclipse light microscope in bright field.

181

**182 Immunohistochemistry**

183 Detection of PCP proteins in the corneal tissue and HCE cells was carried out by  
184 immunohistochemistry using material and reagents as described in Findlay et al. (2016). Primary  
185 antibodies were: BrdU, ab181664 (Abcam); Scribble sc-28737 (Santa Cruz), PI3K, #4252 (Cell  
186 Signalling Technology); laminin, ab11575 (abcam); PAX6 (Developmental Studies Hybridoma Bank),  
187 mouse monoclonal '4A4' recognizing ΔN-P63 (Santa Cruz); cytokeratin-12 (Santa Cruz). Secondary  
188 antibodies were: (all Molecular Probes) Alexa 488-conjugated goat anti-mouse IgG1 A21121; Alexa  
189 568-conjugated donkey anti-mouse IgG A10037; Alexa 488-conjugated rabbit anti-goat IgG A110178;  
190 Alexa 594-conjugated donkey anti-rabbit IgG A21207. Confocal LSM700-Zeiss Imager M2 Upright  
191 and Nikon 400 Eclipse Microscopes were used to image fluorescent sections.

192

**193 Immunofluorescence on whole-mount corneas**

194 Corneas were dissected from fixed eyeballs of adult animals. Dissected corneas were washed in PBS,  
195 3 x 10 minutes, permeabilised in methanol for 20 minutes at -20°C and washed 3 x 10 minutes with  
196 PBS. Corneas were incubated with 1% pepsin in 10 mM HCl for 15 minutes at 37°C, neutralised in 0.1  
197 M sodium borate buffer, pH 8, for 10 minutes and washed 2 x 10 minutes with PBS. Antigen retrieval  
198 was achieved by treating the corneas with 1% sodium dodecyl sulphate in PBS for 5 minutes and  
199 washed 3 x 5 minutes with PBS. Primary and secondary antibodies incubations followed as described  
200 previously and then corneas were flattened by 4 scalpel incisions and mounted in fluorescence



201 mounting medium with the corneal epithelium facing upwards. Confocal LSM700-Zeiss Imager M2  
202 Upright and Nikon 400 Eclipse Microscopes were used for imaging.

203

#### 204 **Apoptosis assay**

205 The Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) In Situ Cell Death  
206 Detection kit-(fluorescein) (Roche, UK) was used to label apoptotic cells in the corneal epithelium of  
207 tissue sections. Deparaffinised sections were incubated with proteinase K (80 µg/mL) for 5 minutes  
208 at room temperature and then washed in PBS. A positive control slide was treated with DNAase1 [50  
209 units/mL DNAase 1 in 50 mM Tris-HCl pH 7.5, 1mM MgCl<sub>2</sub>, 1 mg/mL bovine serum albumen (BSA)]  
210 for 30 minutes at 37 °C. TUNEL labelling was performed by incubating slides with coverslips with the  
211 TUNEL reaction mixture according to manufacturer's instructions for 60 minutes at 37°C in the dark.  
212 A negative control was included in each experimental set up by incubating fixed and permeabilized  
213 tissue in 50 µL Label solution without enzyme. Slides were then washed 3 x 5 minutes in PBS,  
214 mounted in Vectashield and viewed under a Nikon Eclipse E400 fluorescence microscope.

215

#### 216 **Morphometric measurements of the cornea**

217 Images of sagittal adult eye sections of *Le-Cre<sup>Tg/+</sup>; Vangl2<sup>fl/fl</sup>* and *Le-Cre<sup>Tg/+</sup>; Vangl2<sup>fl/+</sup>* animals  
218 following H&E staining were captured by a digital camera (Qimaging, QICAM Fast1394) at 400x  
219 magnification and morphometric measurements of the corneal epithelium thickness were made  
220 with ImageJ (available online at <http://imagej.nih.gov/ij/>). The method of Ramaesh et al. (2003) was  
221 used to measure the thickness of the corneal epithelium and the whole cornea. In brief,  
222 measurements were made in three different areas (two peripheral and one central) in the five  
223 central serial sections of each adult eye. Mean thicknesses were calculated for the peripheral  
224 regions of each section and used to calculate mean thickness in each eye.

225

226 Corneal diameters were measured using a stereomicroscope. An image of the eye was taken  
227 alongside a calibrated ruler and measurements of the corneal diameter were made.

228

#### 229 **Transmission Electron Microscopy**

230 For transmission electron microscopy, eyes were collected and fixed in 2% gluteraldehyde for 4  
231 hours. The corneas were dissected and the tissue post-fixed in osmium, dehydrated through  
232 increasing concentrations of ethanol and propylene oxide, embedded in plastic and semi-thin  
233 sections cut tetroxide at the Histology Facility (Institute of Medical Sciences, University of  
234 Aberdeen). The sections were mounted on copper grids, stained with lead citrate and uranyl acetate  
235 before imaging on a JEOL 1400 plus transmission electron microscope.

236

### 237 **Cell proliferation study**

238 *Le-Cre<sup>Tg/-</sup>; Vangl2<sup>fl/fl</sup>* and *Le-Cre<sup>Tg/-</sup>; Vangl2<sup>fl/+</sup>* adult littermates were given a single intraperitoneal  
239 injection of 10 mg/mL bromodeoxyuridine (BrdU) in sterile PBS, and killed 2 hours later by cervical  
240 dislocation. Eyes were enucleated and processed for immunohistochemistry as above.

241

### 242 **Statistical analysis**

243 For normally distributed data, a 2-tailed unpaired *t*-test was used in most cases to determine  
244 statistical significance when comparing results obtained from *Le-Cre<sup>Tg/-</sup>; Vangl2<sup>fl/fl</sup>* and *Le-Cre<sup>Tg/-</sup>;*  
245 *Vangl2<sup>fl/+</sup>* littermates or V2\_KD and NT control cells. Mann-Whitney U was used for nonparametric  
246 data to compare between genotypes. One way analysis of variance (ANOVA) was used when three  
247 or more groups of data were compared, with post-hoc Tukey HSD test to identify significant  
248 difference between pairs of groups.

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255 **RESULTS**

256

257 **Ablation of *Vangl2* results in abnormal stratification of the corneal epithelium**

258 Multiple PCP genes including *Vangl2* have been shown by RT-PCR, immunohistochemistry and  
259 western blot to be expressed in the murine corneal epithelium (Findlay et al. 2016). The eyes of  
260 adult 'loop tail' mice that are heterozygous for an inactivating mutation in *Vangl2* (*Vangl2*<sup>Lp/+</sup>) were  
261 grossly normal, but tissue sectioning revealed mild corneal defects, with disruption of epithelial  
262 stratification and irregularities of basal cell nuclei seen in all corneas that were not observed in their  
263 wild-type littermates (Figure 1A, B) (n = 8). *Vangl2*<sup>Lp</sup> is a semi-dominant allele and *Vangl2*<sup>Lp/Lp</sup> mice  
264 die during embryogenesis or shortly after birth with severe neural tube defects (Strong and  
265 Hollander, 1949; Yin et al., 2012; Chen et al., 2013). In order to study the role of *Vangl2* in the adult  
266 corneal epithelium, a conditional knockout was made by breeding *Vangl2*-floxed mice (*Vangl2*<sup>fl/fl</sup>) to  
267 *Le-Cre*<sup>Tg/-</sup> mice expressing Cre in the lens and corneal epithelia (Ashery-Padan et al. 2000;  
268 Ramsbottom et al. 2014). The progeny of these matings were backcrossed onto the *Vangl2*<sup>fl/fl</sup> line to  
269 generate *Le-Cre*<sup>Tg/-</sup> *Vangl2*<sup>fl/+</sup> and *Le-Cre*<sup>Tg/-</sup> *Vangl2*<sup>fl/fl</sup> littermates (as well as *Le-Cre*<sup>-/-</sup> *Vangl2*<sup>fl/+</sup> and *Le-*  
270 *Cre*<sup>-/-</sup> *Vangl2*<sup>fl/fl</sup> control mice). Haematoxylin and eosin staining of adult *Le-Cre*<sup>Tg/-</sup> *Vangl2*<sup>fl/fl</sup> eyes  
271 revealed a highly disrupted stratification of the corneal epithelium that was not observed in *Le-*  
272 *Cre*<sup>Tg/-</sup> *Vangl2*<sup>fl/+</sup> controls (n > 24) (Figure 1C-E). Sections revealed patches of both abnormally thin  
273 (Figure 1D) and abnormally thick corneal epithelium (Figure 1E). Thin regions of the epithelium were  
274 composed of as little as one or two cellular layers with no consistent cellular morphology, and were  
275 sharply juxtaposed with epithelium of normal thickness (5-7 cells) or hypertrophic epithelium up to  
276 10 cells thick. In *Le-Cre*<sup>Tg/-</sup> *Vangl2*<sup>fl/+</sup> controls (n = 24) and all *Le-Cre*<sup>-/-</sup> corneas (n = 40) the epithelium  
277 was uniform and smooth, 5-7 cells thick.

278 Stratification of the corneal epithelium is a postnatal event (Zieske et al. 2004). While the corneal  
279 epithelia of both *Vangl2*-null and control mice were monolayers during embryogenesis, and the  
280 epithelia were morphologically normal to birth examination of corneas from neonatal mice showed  
281 that stratification of the epithelium was delayed in the mutants. At postnatal day 5, in contrast to  
282 *Le-Cre*<sup>-/-</sup> *Vangl2*<sup>fl/+</sup>, *Le-Cre*<sup>-/-</sup> *Vangl2*<sup>fl/fl</sup> and *Le-Cre*<sup>Tg/-</sup> *Vangl2*<sup>fl/+</sup> control mice whose epithelium was  
283 typically 2-3 cells thick, the epithelium of *Le-Cre*<sup>Tg/-</sup> *Vangl2*<sup>fl/fl</sup> mice was a monolayer, and in some  
284 areas only a thin cytoplasmic covering was visible (n = 9) (Figure 2).

### 285 ***Vangl2*-null corneas exhibit reduced or partially absent corneal epithelium basement membrane**

286 Histological analysis (Figure 1) suggested the normal basal/wing/squamous cell apical-basal  
287 arrangement of the epithelium was disrupted in *Le-Cre<sup>Tg/-</sup> Vangl2<sup>fl/fl</sup>* corneas, compared to controls.  
288 The basement membrane of mutant corneal epithelia was disrupted and sometimes undetectable in  
289 mutant corneal epithelia – this was apparent by haematoxylin and eosin staining (n > 25), and  
290 immunohistochemistry using antibodies against extracellular matrix proteins laminin and collagen  
291 IV. Partial or total absence of collagen IV and laminin was reproducibly observed in *Le-Cre<sup>Tg/-</sup>*  
292 *Vangl2<sup>fl/fl</sup>* corneal epithelial (n = 6) but not in the controls (n = 9) (Figure 3; Supplementary Figure 1).  
293 A preliminary transmission electron microscope (TEM) analysis of *Le-Cre<sup>Tg/-</sup> Vangl2<sup>fl/fl</sup>* corneas (n = 2)  
294 was performed alongside control littermates, *Le-Cre<sup>Tg/-</sup> Vangl2<sup>fl/+</sup>* (n = 2) and *Le-Cre<sup>-/-</sup> Vangl2<sup>fl/fl</sup>* (n = 2)  
295 which confirmed disruption of the basement membrane. Control *Le-Cre<sup>Tg/-</sup> Vangl2<sup>fl/+</sup>* and *Le-Cre<sup>-/-</sup>*  
296 *Vangl2<sup>fl/fl</sup>* eyes both exhibited a distinct basement membrane underlying the corneal epithelium and  
297 also a clear morphological difference in cell shape and cytoplasm density between the polarised  
298 basal epithelial cells and the overlying apical cells (Supplementary Figure 2). Strikingly the basement  
299 membrane was also much thinner in the *Le-Cre<sup>Tg/-</sup> Vangl2<sup>fl/fl</sup>* mutant corneas and partially absent.  
300 Cells in the mutant corneas were arranged in a disorganised manner, there was no clear  
301 morphological differentiation between basal and apical cells, and the basal cells showed the same  
302 cytoplasmic density as more superficial cells (Supplementary Figure 2).

303

### 304 **Morphometric analysis of mutant corneas**

305 A morphometric analysis was performed on *Le-Cre<sup>Tg/-</sup> Vangl2<sup>fl/fl</sup>* corneas and controls. Mean  
306 diameter of *Le-Cre<sup>Tg/-</sup> Vangl2<sup>fl/fl</sup>*, *Le-Cre<sup>Tg/-</sup> Vangl2<sup>fl/+</sup>* and *Cre<sup>-/-</sup>* corneas was measured. Consistent with  
307 previous studies showing that Cre expression is not always neutral to phenotype, it was found that  
308 *Le-Cre<sup>Tg/-</sup>* eyes are smaller than Cre-negative eyes, irrespective of *Vangl2*-genotype (Adams and van  
309 der Weyden, 2001; Dorà et al. 2014) (Figure 4A). This is presumed to be either a toxic effect of Cre  
310 or, more likely, a negative effect of reduced Pax6 availability due to Pax6 binding sites in the *Le-Cre*  
311 promoter. For this reason, only *Le-Cre<sup>Tg/-</sup> Vangl2<sup>fl/+</sup>* mice were used as controls for morphometric  
312 analysis.

313 Transverse medial sections from serially sectioned eyes were stained with haematoxylin and eosin  
314 and the thickness of the corneal epithelium was measured in central and peripheral regions as  
315 described in the Methods sections. On average the epithelium of *Le-Cre<sup>Tg/-</sup>; Vangl2<sup>fl/fl</sup>* corneas was  
316 significantly thicker in both central and peripheral regions when compared with *Le-Cre<sup>Tg/-</sup>; Vangl2<sup>fl/+</sup>*

317 controls (central corneal epithelium thickness  $\pm$  SEM: *Le-Cre*<sup>Tg/-</sup>; *Vangl2*<sup>fl/+</sup> 25.85  $\pm$  2.15  $\mu$ m, *Le-Cre*<sup>Tg/-</sup>;  
318 *Vangl2*<sup>fl/fl</sup> 41.61  $\pm$  6.76  $\mu$ m. *t*-test: n = 10; *P* = 0.0396. Peripheral corneal epithelium thickness  $\pm$  SEM:  
319 *Le-Cre*<sup>Tg/-</sup>; *Vangl2*<sup>fl/+</sup> 25.55  $\pm$  1.43  $\mu$ m, *Le-Cre*<sup>Tg/-</sup>; *Vangl2*<sup>fl/fl</sup> 37.62  $\pm$  4.72  $\mu$ m. *t*-test: n = 10; *P* = 0.0157)  
320 (Figure 4B).

321 On the basis of these data it was suggested that one of the primary defects in corneal epithelia that  
322 are null for *Vangl2*, and which should therefore lack all PCP signalling, may in fact be a failure of  
323 normal apical-basal epithelial polarity. Further molecular analysis was performed on mutant corneas  
324 and controls to investigate this.

325

326

### 327 Cell proliferation and cell death in the corneal epithelia of *Vangl2*-deficient mice

328 In normal corneal epithelia, cell proliferation is restricted to the basal cell layer, and apoptotic  
329 events are normally only detected in superficial apical cells prior to desquamation. It was  
330 considered possible that the defects observed above may result from loss of apical and basal identity  
331 of cells in which case patterns of proliferation and apoptosis may be disrupted. TUNEL labelling was  
332 therefore performed to label apoptotic cells in the corneal epithelium of *Le-Cre*<sup>Tg/-</sup>; *Vangl2*<sup>fl/fl</sup> mice  
333 and *Le-Cre*<sup>Tg/-</sup>; *Vangl2*<sup>fl/+</sup> control animals. Apoptotic nuclei were restricted to cells in the superficial  
334 layer of both control and *Le-Cre*<sup>Tg/-</sup>; *Vangl2*<sup>fl/fl</sup> corneas. No TUNEL-positive cells were detected in the  
335 basal or wing layers of the corneal epithelium in either *Vangl2*-deficient or control mice (Figure 5),  
336 which reflects normal shedding from the epithelial surface (Ren and Wilson 1996).

337 To examine whether disrupted stratification of the corneal epithelium in *Vangl2*-deficient corneal  
338 epithelia was linked to defects in epithelial cell proliferation, bromodeoxyuridine (BrdU) staining was  
339 performed in order to observe proliferating cells within the corneal epithelium. Mice were treated  
340 with a single intraperitoneal injection of BrdU, to label cells in S-phase, for 2 hours before the mice  
341 were killed and eyes taken for BrdU immunohistochemistry. In both *Le-Cre*<sup>Tg/-</sup>; *Vangl2*<sup>fl/fl</sup> mice eyes,  
342 *Le-Cre*<sup>Tg/-</sup>; *Vangl2*<sup>fl/+</sup> and all *Le-Cre*<sup>-/-</sup> controls (n = 6-12 of each genotype), BrdU incorporation was  
343 restricted to cells in the basal layer of the epithelium only (Figure 5 D, E). No suprabasal DNA  
344 synthesis was observed within mutant mice and therefore no evidence of loss in apical-basal  
345 controlled mitotic division.

346 These data suggest that although the morphological differentiation between apical and basal  
347 epithelial cells was often lost in *Le-Cre*<sup>Tg/-</sup>; *Vangl2*<sup>fl/fl</sup> corneas, cell identity (basal or apical) was

348 maintained and hence proliferation continued to be restricted to the basal layer and apoptosis to  
349 the superficial cell layers.

350

### 351 **Localisation of apical-basal and cell fate markers in the control and mutant corneal epithelium**

352 The basal lamina is secreted by corneal epithelial cells, so the disruption of the basement membrane  
353 in *Vangl2*-null corneas could result from defective subcellular apical-basal organisation. The  
354 membrane-associated scaffold protein Scribble has been previously described as an important  
355 apical-basal organiser within *Drosophila* cells through its role, along with the other proteins in its  
356 complex, Discs large and Lethal Giant Larvae, in the subcellular localisation of proteins (Bilder and  
357 Perrimon, 2000; Harris & Lim, 2001). *Scribble* has previously been shown to be expressed within the  
358 corneal epithelium of mice (Nguyen et al. 2005). In order to further study apical-basal polarity in the  
359 conditional *Vangl2*-knockout corneas, Scribble immunostaining was performed on *Le-Cre<sup>Tg/-</sup>*  
360 *Vangl2<sup>fl/fl</sup>* eyes and compared to *Le-Cre<sup>Tg/-</sup>* *Vangl2<sup>fl/+</sup>* littermates. Scribble was found to be localised  
361 primarily to the lateral and apical boundaries of the basal epithelial cells of control *Le-Cre<sup>Tg/-</sup>*  
362 *Vangl2<sup>fl/+</sup>* mice, and was generally absent from the basal plasmamembrane of these cells (Figure 6)  
363 (n = 5 corneas). In *Le-Cre<sup>Tg/-</sup>* *Vangl2<sup>fl/fl</sup>* eyes Scribble localisation was moderately but consistently  
364 disrupted, with localisation bleeding into the basal boundary of many cells (n = 5 corneas) (Figure  
365 6B). This suggests **disruption** of apical-basal polarity in those cells, associated with partial or total  
366 loss of the basement membrane described above. Phosphatidylinositol-4,5-bisphosphate 3-kinase  
367 (PI3K) is an important enzyme for corneal epithelial cellular motility and intracellular trafficking  
368 (Zhao et al. 2006). It is activated by growth factors acting upon cells and initiating signalling through  
369 downstream effectors such as protein kinase B (Katso et al. 2001). Xu et al. (2010) grew mammary  
370 epithelial cells upon a laminin-rich ECM; cells grown on the ECM became polarised and displayed a  
371 localisation of PI3K to the basal side. Cells grown in the absence of laminin remained unpolarised  
372 with no localisation of PI3K in the cell. Immunohistochemistry was performed to investigate PI3K  
373 localisation in control and mutant corneal epithelia and to determine whether loss of the basement  
374 membrane affected distribution of the protein. When *Le-Cre<sup>Tg/-</sup>* *Vangl2<sup>fl/fl</sup>* mice (n = 3) were  
375 compared to *Le-Cre<sup>Tg/-</sup>* *Vangl2<sup>fl/+</sup>* littermates (n = 5), **fluorescence was highest in the cytoplasm or cell**  
376 **membranes of basal cells of *Le-Cre<sup>Tg/-</sup>* *Vangl2<sup>fl/+</sup>* mice eyes as expected, however *Le-Cre<sup>Tg/-</sup>* *Vangl2<sup>fl/fl</sup>***  
377 **eyes exhibited only low protein levels in basal cells, similar to that observed in more apical cells**  
378 **(Figure 6).**

379 Further markers of cell fate and apical-basal identity were assayed in mutant corneas.  
380 Immunohistochemical analysis of localisation of the  $\Delta N$  isoform of the tumour suppressor protein  
381 P63, previously shown to be a marker of basal corneal epithelial cells in the mouse (Collinson et al.,  
382 2002; Ramaesh et al., 2005), revealed no difference between control *Le-Cre<sup>Tg/-</sup> Vangl2<sup>fl/+</sup>* (n = 4) and  
383 mutant *Le-Cre<sup>Tg/-</sup> Vangl2<sup>fl/fl</sup>* eyes (n = 3), with basal cells of both genotype exhibiting strong staining  
384 and apical cells very weak or no staining. The definitive corneal epithelial marker, cytokeratin-12,  
385 was localised to cytoplasm of all cells in all corneal epithelia, irrespective of genotype (n = 4 of each),  
386 and the ocular surface marker, Pax6, was localised to nuclei of all cells (n = 4 of each genotype)  
387 (Figure 6). Loss of either of these markers would suggest failure of corneal epithelial identity.

388 These data, together with the defects to the basement membrane described above, suggested that  
389 there was no loss of corneal epithelial identity in the *Le-Cre<sup>Tg/-</sup> Vangl2<sup>fl/fl</sup>* mutants, but that the basal  
390 epithelial cells had partially or totally lost their apical-basal polarity.

391

#### 392 **Loss of *Vangl2* causes cells to exhibit migration defects**

393 Wound healing efficiency in embryonic skin is regulated by a PCP pathway (Caddy et al., 2010). We  
394 previously showed that corneal epithelial cells exhibited slower rates of scratch wound healing when  
395 *Vangl2* was inactivated by a tamoxifen-inducible Cre (Findlay et al. 2016). To determine whether the  
396 *Vangl2*-deficient model employed in this study showed a similar defect, monolayers of *Le-Cre<sup>Tg/-</sup>*  
397 *Vangl2<sup>fl/fl</sup>*, *Le-Cre<sup>Tg/-</sup> Vangl2<sup>fl/+</sup>* and *Le-Cre<sup>-/-</sup> Vangl2<sup>fl/fl</sup>* corneal epithelial cells were cultured as  
398 previously described (Leiper et al. 2006) and scratch-wound assays were performed. *Vangl2*-null *Le-*  
399 *Cre<sup>Tg/-</sup> Vangl2<sup>fl/fl</sup>* cells healed significantly more slowly ( $26.2 \pm 3.5 \mu\text{m/h}$  – mean  $\pm$  s.e.m.; n = 7) than  
400 *Le-Cre<sup>Tg/-</sup> Vangl2<sup>fl/+</sup>* controls ( $39.4 \pm 3.7 \mu\text{m/h}$ ; n = 9) (Figure 7A). These data confirmed a role for  
401 *Vangl2* in the migration during wound healing of corneal epithelial cells.

402 It was considered possible in light of data presented above that the migration defect shown by  
403 *Vangl2*-mutant corneal epithelial cells arises not through a classic PCP pathway but as a secondary  
404 consequence of apical-basal defects through a non-PCP mechanism. This was tested by knocking  
405 down VANGL2 by siRNA in human corneal epithelial cells in vitro and comparing planar migration of  
406 individual isolated cells to that of confluent cells after exposure to a physiological electric field of  
407 200 mV/mm that produces robust planar migration towards the cathode (Soong et al. 1990; Zhao et  
408 al. 1996; Farboud et al. 2000; Findlay et al. 2016). Classic PCP activity requires cell-cell contact so any  
409 effect of VANGL2 knockdown on isolated cells in vitro must represent a non-PCP mechanism of  
410 action – e.g. through cell adhesion defects. Human corneal epithelial cells were transfected with a

411 validated siRNA that causes knockdown of VANGL2 to about 30% of normal levels and which we  
412 showed previously causes a significant reduction in cells' ability to migrate cathodally in an applied  
413 electric field (Findlay et al. 2016). Control cells were transfected with a negative control nonsense  
414 siRNA. Planar migration in an applied electric field was quantified as the Forward Migration Index  
415 (FMIX) representing the mean cosine of the angle  $\theta$  of each cell's direction of travel towards the  
416 cathode, with FMIX = -1 representing perfect cathodal migration and FMIX = 0 representing mean  
417 random migration (see Methods section). It was found that there was no significant different  
418 difference between the behaviour of isolated individual cells compared to that of cells in confluent  
419 sheets (Figure 7B). The fact that planar migration was retarded in VANGL2-knockdown cells even in  
420 absence of cell-cell contact shows that a classic PCP mechanism was not involved. It suggests that  
421 VANGL2 has a non-PCP function, ~~perhaps for normal cell-adhesion~~ associated with apical-basal  
422 polarity, which leads to migration defects in absence of normal VANGL2 dosage.

423

424



425 **Discussion**426 **Disrupted stratification of the *Vangl2*-deficient corneal epithelium.**

427 In this study, the core PCP gene, *Vangl2*, was genetically disrupted in the corneal epithelium and a  
428 previously unsuspected role in apical-basal patterning was identified. The stratification of the  
429 epithelium was delayed in neonates and badly disrupted in adult mice, with a disorganised  
430 arrangement of cells and partial loss of the epithelial basement membrane. Basement membrane  
431 defects were shown by light microscopy, immunohistochemistry and transmission electron  
432 microscopy. Immunohistochemical and TUNEL analysis showed that while the apical-basal polarity  
433 of the individual basal epithelial cells was disrupted (demonstrated by mislocalisation of Scribble  
434 protein to the basal edge of basal cells) and cellular morphology was abnormal, the apical or basal  
435 identity of cells was maintained in mutant epithelia. Hence proliferation was restricted to the basal  
436 layer and apoptosis to the superficial layer of the mutant epithelium. Mutant corneal epithelial cells  
437 showed a defect in corneal epithelium wound healing, but this may be secondary to the disruption  
438 of the basement membrane.

439

440 The role of PCP pathway proteins in adult vertebrate tissues is poorly known. Whereas classically,  
441 the PCP pathway controls the behaviour of epithelial cells in the plane of their basement membrane  
442 during embryological development, examples of PCP genes controlling apical-basal behaviour of  
443 epithelial cells also exist. For example the uterine epithelium of *Vangl2*<sup>Lp/Lp</sup> mutant mouse embryos  
444 is disrupted with loss of apical-basal polarity of columnar epithelial cell (Vandenberg and Sassoon,  
445 2009). The columnar epithelium of *Vangl2*<sup>Lp/Lp</sup> mutant uteri at E18.5 is composed of multiple cell  
446 layers rather than a single monolayer of columnar epithelial cells, reminiscent of the phenotype in  
447 *Vangl2*-null corneal epithelia. Similar findings of apical-basal disruption of epithelia in *Vangl2*  
448 mutants were reported by Yates et al. (2010): airway lumina of the lungs of *Vangl2*<sup>Lp/Lp</sup> homozygotes  
449 at E14.5 were found to be surrounded by disrupted epithelium. In wild-type lung sections, airway  
450 lumina are surrounded by a single layer of epithelium made up of aligned and organized cells. In  
451 contrast, in the lungs of *Vangl2*<sup>Lp/Lp</sup> homozygotes the lumina are demarcated by multi-stratified,  
452 disorganized epithelial cells that are not aligned uniformly (Yates et al. 2010). Dysregulation or loss  
453 of *Vangl2* and PCP activity have been shown to mediate increase of matrix metalloprotease activity,  
454 loss of epithelial morphology and metastasis in some cancers, a phenotype consistent with the  
455 epithelial disruption in *Le-Cre*<sup>Tg/+</sup> *Vangl2*<sup>fl/fl</sup> corneas (Cantrell & Jessen 2009; Puvirajasinghe et al.  
456 2016).

457 The basement membrane is an extracellular matrix (ECM) composed of secretions from the  
458 overlying basal layer of epithelial cells, the basal lamina, and reticular connective tissue (Paulsson,  
459 1992; LeBleu et al. 2007). It was thin and, in patches, absent in *Le-Cre<sup>Tg/+</sup> Vangl2<sup>fl/fl</sup>* mutant eyes.  
460 Basement membrane laminin has been shown to secondarily maintain polarisation in epithelial cells  
461 (Klein et al. 1988; Xu et al. 2010). PI3K has previously been shown to locate to the basal side of  
462 mouse mammary epithelial cells due the influence of laminin in the ECM inducing apical-basal  
463 polarity within the cells (Xu et al. 2010). The results obtained in this study suggest that when *Vangl2*  
464 expression is lost within corneal epithelial cells they lose polarity and exhibit defects in secretion of  
465 basal lamina components from the basal side of cells. The basement membrane is known to be  
466 essential for providing chemical cues that aid in epithelial migration (Abrams et al. 2000; Teixeira et  
467 al. 2003, 2006). Its absence could be the source of the disorganisation and wound healing delay  
468 observed in the *Le-Cre<sup>Tg/+</sup> Vangl2<sup>fl/fl</sup>* mutant eyes.

469 A reduction in cell adhesion may underlie reduced planar migration of *Vangl2*-deficient cells in  
470 response to wounding or an applied electric field. ~~It is also possible that cell adhesion is~~  
471 ~~compromised in *Vangl2*-deficient corneal epithelia.~~ For example, Oteiza et al. (2010) reported  
472 impaired cell adhesion linked to planar cell polarity dysfunction: inhibition of *Wnt11* and *Prickle-1a*  
473 in Zebrafish impaired cell-cell adhesion of the progenitor cells of Kupffer's vesicle. A direct assay of  
474 cell adhesion in conditional knockout cells using single-cell force spectroscopy (Puech et al. 2006)  
475 would be informative in this respect.

476 Apical-basal complexes have previously been shown to interact with PCP components (Djiane et al.  
477 2005; Dollar et al. 2005; Mahaffey et al. 2013). *Vangl2* and *Scribble* have been linked in the  
478 determination of planar polarity in mice: *Scribble<sup>Crc/+</sup> Vangl2<sup>Lp/+</sup>* double mutants exhibit cochlear  
479 disorganisation comparable to that observed in *Vangl2<sup>Lp/Lp</sup>* mutants (Montcouquiou et al. 2003). The  
480 localisation of *Scribble* along the apical-lateral edge of the epithelial cell layer in control corneal  
481 epithelia is consistent with previous observations that *Scribble* localises with the tight junction  
482 protein ZO-1 (Nakagawa & Huibregtse, 2000). The results obtained in this study showed partial  
483 mislocalisation of *Scribble* to the basal side of the innermost layer of the epithelium in *Le-Cre<sup>Tg/-</sup>*  
484 *Vangl2<sup>fl/fl</sup>* mice. This is in contrast to observations made in *Drosophila* by Courbard et al. (2009) who  
485 found no defect in the localisation of *Scribble* in either *Vangl* or *Frizzled* mutants; they also found  
486 that there were no differences in *Vangl* localisation in *Scribble* mutants. Yates et al. (2012) observed  
487 that *Vangl2<sup>Lp/Lp</sup>* mutants exhibited no defects in *Scribble* localisation within the mammalian lung.  
488 The data from this study would therefore suggest that the molecular roles of *Vangl2* may differ in  
489 different tissues.

490 This study has confirmed a novel, sustained role for the core PCP component, Vangl2, in generation  
491 and maintenance of stratified epithelial morphology in an adult vertebrate system. It also provides  
492 preliminary evidence that at least some of the planar defects observed arise through a non-PCP  
493 mechanism, with loss or disruption of the normal epithelial basement membrane. We consider it  
494 possible that some or even all of the planar defects may be secondary to a primary failure of apical-  
495 basal polarity in the epithelial cells. This may represent a more general model for how PCP signalling  
496 is effected in other systems, and warrants further investigation.

497 We previously showed (Dorà et al. 2014) that the *Le-Cre* transgene itself imposes a phenotype on  
498 the mouse eye, independently of the presence or absence of floxed alleles of any gene. The  
499 phenotype varies in severity with genetic background, but *Le-Cre*<sup>Tg/-</sup> mice have a tendency to  
500 microphthalmia, small disorganised lenses and irido-corneal adhesion with small pupillary opening,  
501 though their corneal structure is normal (Dorà et al. 2014). During this study, *Le-Cre*<sup>Tg/-</sup> mice had mild  
502 microphthalmia (Fig. 4A) and variable lens fibre disruption. For this reason, all controls in this study  
503 were *Le-Cre*<sup>Tg/-</sup>, and significant effects of Vangl2 reported here only for comparison of *Le-Cre*<sup>Tg/-</sup>  
504 *Vangl2*<sup>fl/fl</sup> compared to *Le-Cre*<sup>Tg/-</sup> *Vangl2*<sup>fl/+</sup> and/or *Le-Cre*<sup>Tg/-</sup> *Vangl2*<sup>+/+</sup>. We conclude, in agreement  
505 with previous work (Dorà et al. 2014), that *Cre*-negative mice are not valid controls for studies that  
506 have used *Le-Cre* to conditionally inactivate floxed genes in the corneal epithelium. Several previous  
507 studies have however used the *Le-Cre* transgene to knock out floxed genes in adult eyes and claimed  
508 the subsequent defects including microphthalmia, disorganised small lenses or small pupillary  
509 openings were due to the conditionally knocked out gene and not the *Le-Cre* transgene itself. Table  
510 1 lists these studies. With one exception all have used *Le-Cre* negative mice as controls and have  
511 detailed morphological defects in *Le-Cre*-positive *fl/fl* mice that are consistent with the phenotype  
512 caused by the *Le-Cre* transgene expression within the developing eye. A few studies also highlight  
513 the loss of endogenous *Pax6* expression within the *Le-Cre*<sup>Tg/-</sup> mutant cells but claim the cause is the  
514 knocked out gene, whereas Dorà et al. (2014) showed that the eye phenotype in *Le-Cre*<sup>Tg/-</sup> mice was  
515 most likely caused by loss of *Pax6*. In all of these studies it is possible that the phenotype attributed  
516 to the gene knockout was in fact, at least in part, caused by the *Le-Cre* transgene and we would  
517 suggest that the datasets as a whole should be interpreted in this light.

518

519

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525

526 **Author contributions**

527 DAP and ASF both performed experiments, analysed data and contributed equally to writing the  
528 manuscript. RvL performed experiments and contributed to writing the manuscript. JMC conceived  
529 the study, performed experiments and wrote the manuscript.

530

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727 TABLE 1. STUDIES THAT HAVE USED LE-CRE MICE TO STUDY EYE DEVELOPMENT

Publication	Gene	Control	Phenotype Observed
Zhao et al. 2015	<i>ALK3</i>	<i>Le-Cre</i> <sup>-/-</sup> mice	Observed a smaller lens in <i>Le-Cre</i> <sup>Tg/-</sup> mice
Zhang et al. 2015	<i>FGFr2</i>	<i>Le-Cre</i> <sup>-/-</sup> mice	Observed a thinner corneal epithelial layer when compared to wild-type mice, and abnormal Pax6 immunostaining
Choi et al. 2014	<i>Smoothened</i>	<i>Le-Cre</i> <sup>-/-</sup> mice	Observed microphthalmia in <i>Le-Cre</i> <sup>Tg/-</sup> mice.
Li et al. 2014	<i>Frs2</i> and <i>Shp2</i>	<i>Le-Cre</i> <sup>-/-</sup> mice	Found that deletion of either gene caused a decrease in lens size.
Yamben et al. 2013	<i>Scribble</i>	<i>Le-Cre</i> <sup>-/-</sup> mice	Observed small, misshapen lenses with central opacity in <i>Le-Cre</i> <sup>Tg/-</sup> mice.
Gupta et al. 2013	<i>Klf4</i>	<i>Le-Cre</i> <sup>-/-</sup> mice	Observed small lens with central opacity in <i>Le-Cre</i> <sup>Tg/-</sup> mice.
Zhao et al. 2012	<i>ALK3</i>	<i>Le-Cre</i> <sup>-/-</sup> mice	Observed small lens with notable defects in fibre cell differentiation in <i>Le-Cre</i> <sup>Tg/-</sup> mice.
Saravanamuthu et al. 2012	<i>Notch2</i>	<i>Le-Cre</i> <sup>-/-</sup> mice	Observed microphthalmia, and reduced pupillary openings.
Kenchegowda et al. 2011	<i>Klf5</i>	<i>Le-Cre</i> <sup>-/-</sup> mice	Observed microphthalmia and closed eyelids, with small lenses in <i>Le-Cre</i> <sup>Tg/-</sup> mice.
Joo et al. 2010	<i>Pnn</i>	<i>Le-Cre</i> <sup>-/-</sup> mice	Observed microphthalmia.
Chen et al. 2009	<i>Cited2</i>	<i>Le-Cre</i> <sup>-/-</sup> mice	Observed microphthalmia and thin opaque cornea. Found <i>Le-Cre</i> <sup>Tg/-</sup> <i>Cited2</i> <sup>fl/fl</sup> mice have a decrease in <i>Pax6</i> expression.
Le et al. 2009	<i>Jagged1</i>	<i>Le-Cre</i> <sup>Tg/-</sup> <i>Jagged1</i> <sup>fl/+</sup> and <i>Le-Cre</i> <sup>-/-</sup> mice	Although this paper compares homozygous conditional knock-outs to their heterozygous counterparts, they still claim all observations – microphthalmia and decreased pupillary openings – are due to a reduced expression of <i>Jagged1</i> .
Rowan et al. 2008	<i>Rbp-J</i>	<i>Le-Cre</i> <sup>-/-</sup> mice	Microphthalmia, small pupillary opening
Jia et al. 2007	<i>Rbp-J</i>	<i>Le-Cre</i> <sup>-/-</sup> mice	Observed microphthalmia and small lens.
Liu et al. 2006	<i>Six3</i>	<i>Le-Cre</i> <sup>-/-</sup> mice	Observed lens defects, reduced size, cataracts and absence.

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732 **FIGURE LEGENDS**

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734 **FIGURE 1. Corneal epithelial abnormalities in adult *Vangl2*-mutant mice.** Haematoxylin-and eosin  
735 staining of tissue sections of adult mice corneas. **(A)** Wild-type cornea showing stratified epithelium  
736 sitting on hypocellular collagenous corneal stroma. **(B)** Cornea from *Vangl2*<sup>LD/+</sup> mouse (littermate of  
737 A) with mild disruption to apical-basal organisation of epithelium (arrowhead). **(C-E)** Cornea from *Le-*  
738 *Cre*<sup>Tg/-</sup> *Vangl2*<sup>fl/fl</sup> mouse (conditional knockout, cKO) showing irregularity of corneal epithelium,  
739 disruption to normal stratification, and projection of basal cells into the corneal stroma. Corneal  
740 epithelia could be abnormally thin (D) or thick (E) with sharp transitions between the thinner and  
741 thicker domains, phenomena never seen in *Le-Cre*<sup>Tg/-</sup> *Vangl2*<sup>fl/+</sup> or *Le-Cre*<sup>-/-</sup> *Vangl2*<sup>fl/fl</sup> (*n* > 100)  
742 controls. Scale bar represents 50  $\mu$ m (A-C), 20  $\mu$ m (D, E).

743

744 **FIGURE 2. Corneal epithelial abnormalities in neonatal *Vangl2*-mutant mice.** (A) At postnatal day 5,  
745 the corneal epithelium of *Le-Cre*<sup>Tg/-</sup> *Vangl2*<sup>fl/+</sup> control mice is a uniform 2-3 cells thick (*n* = 6). (B) In  
746 contrast, all corneal epithelia of *Le-Cre*<sup>Tg/-</sup> *Vangl2*<sup>fl/+</sup> littermates (*n* = 5) exhibited irregularities of  
747 stratification, with the epithelium being 1-2 cells thick and with areas of cytoplasmic covering only.  
748 Scale bar represents 20  $\mu$ m.

749

750 **FIGURE 3. Corneal epithelial basement membrane deficiency in adult *Vangl2*-mutant mice.**  
751 Haematoxylin and eosin (H&E) staining (top), and immunohistochemistry for ECM proteins laminin  
752 (middle) and collagen IV (bottom) in adult *Le-Cre*<sup>Tg/-</sup> *Vangl2*<sup>fl/+</sup> control mice (left) and *Le-Cre*<sup>Tg/-</sup>  
753 *Vangl2*<sup>fl/+</sup> littermates (right). Arrows point to the basement membrane. In controls, the basement  
754 membrane is visible in H&E-stained controls as a strongly stained lamina immediately underneath  
755 the basal surface of the epithelial cells, sitting on a more weakly stained ECM. This is reproducibly  
756 not visible or patchy in mutants (right). Laminin and collagen IV staining in mutants is absent or  
757 patchy and thin, confirming the light microscopy and Supplementary transmission electron  
758 microscopy. DAPI staining (blue) visualises cell nuclei. Scale bar: 40  $\mu$ m.

759

760 **FIGURE 4. Morphometric analysis of *Vangl2*-mutant corneas.** Corneal epithelial diameter (top) and  
761 thickness (bottom) were measured. *Le-Cre*-positive corneas were slightly but very significantly  
762 smaller than *Le-Cre*-negative corneas, irrespective of *Vangl2* genotype. Conditional knockout of  
763 *Vangl2* lead to increased mean thickness of the corneal epithelium, both centrally and peripherally,  
764 accompanying the cellular, morphological and molecular defects described in this paper.

765

766 **FIGURE 5. Molecular analysis of cell proliferation and apoptosis in *Vangl2*-mutant corneal**  
767 **epithelia. (A-C)** TUNEL analysis of apoptotic cell death. In both *Le-Cre<sup>Tg/-</sup> Vangl2<sup>fl/+</sup>* control corneas  
768 (A) and *Le-Cre<sup>Tg/-</sup> Vangl2<sup>fl/fl</sup>* conditional knockouts (B) apoptosis (green fluorescent labelling) was  
769 restricted to the most superficial cell layer, with no apoptotic events noted in any other layer of the  
770 corneal epithelium (n = 3 of both genotypes). (C) is a positive control - tissue section digested with  
771 DNase1 to create double-stranded breaks in DNA of all cells). **(D, E).** Immunohistochemical analysis  
772 of cell proliferation in corneal epithelium of mice after a single injection of BrdU. In both *Le-Cre<sup>Tg/-</sup>*  
773 *Vangl2<sup>fl/+</sup>* control corneas (E) and *Le-Cre<sup>Tg/-</sup> Vangl2<sup>fl/fl</sup>* conditional knockouts (F), DNA replication was  
774 restricted to the basal layer of the corneal epithelium, with no proliferation events more apically.

775

776 **FIGURE 6. Cell polarity and cell fate markers in corneal epithelia of *Vangl2*-mutants.**  
777 Immunohistochemical labelling of Scribble, PI3K, P63, cytokeratin-12 and Pax6 in *Le-Cre<sup>Tg/-</sup> Vangl2<sup>fl/+</sup>*  
778 control corneas and *Le-Cre<sup>Tg/-</sup> Vangl2<sup>fl/fl</sup>* conditional knockouts. Asterisks denote selected *Vangl2*-  
779 mutant cells with atypical localisation of Scribble to the basal boundary, suggesting disrupted apical-  
780 basal polarity of the basal epithelial cells. PI3K is upregulated in the basal cells of control epithelia  
781 but not of *Vangl2* knockout cells. Other markers of cell fate and apical-basal identity are expressed  
782 normally in mutant epithelia. n = 3-9 for all markers. Scale bar represents 40  $\mu$ m.

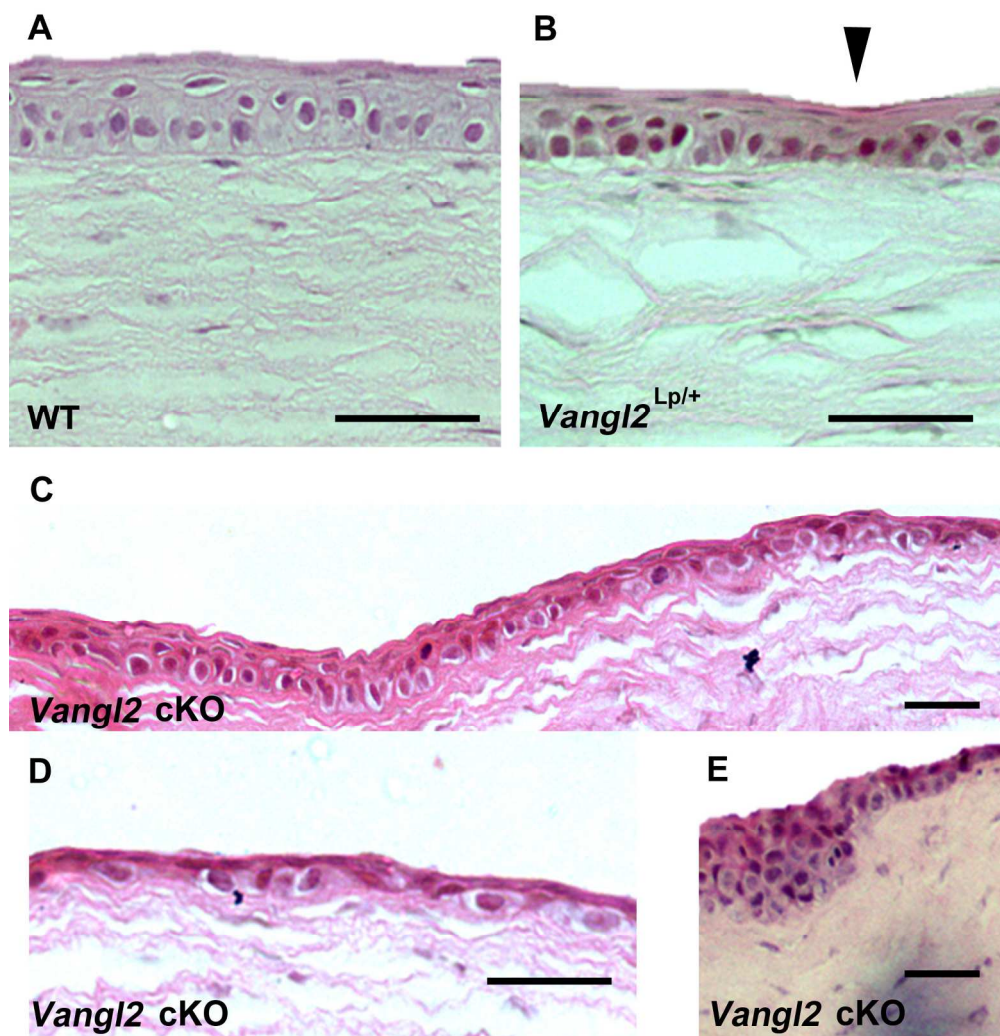
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784 **FIGURE 7. Planar migration defects in *Vangl2*-deficient corneal epithelial cells. (A)** Wound healing  
785 rate of in vitro cultured adult mouse corneal epithelial cells. The rate of migration of *Le-Cre<sup>Tg/-</sup>*  
786 *Vangl2<sup>fl/fl</sup>* cells following scratch-wounding was significantly slower than that of *Le-Cre<sup>Tg/-</sup> Vangl2<sup>fl/+</sup>*  
787 and *Le-Cre<sup>-/-</sup> Vangl2<sup>fl/fl</sup>* cells. (One-way ANOVA: F = 3.720, P = 0.0309). **(B)** Cathodal migration  
788 (expressed as forward migration index – FMIX – as described in Materials and Methods), for human  
789 corneal epithelial cells after VANGL2 knockdown to ~30% of normal protein levels. The response of

790 cell cultured at low density (individual cells) (light grey) was not significantly different from that of  
791 confluent cells (dark grey).

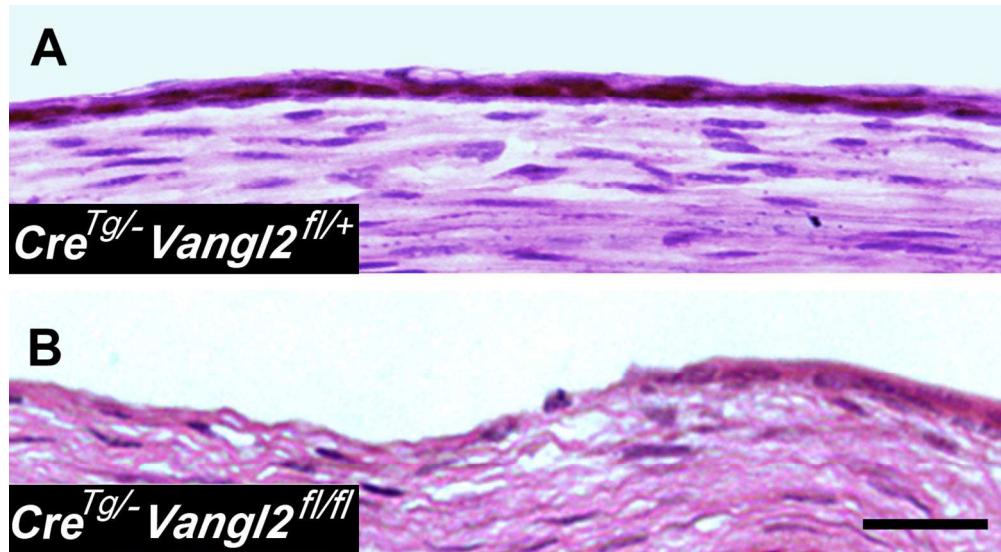
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Corneal epithelial abnormalities in adult *Vangl2*-mutant mice. Haematoxylin-and eosin staining of tissue sections of adult mice corneas. (A) Wild-type cornea showing stratified epithelium sitting on hypocellular collagenous corneal stroma. (B) Cornea from *Vangl2*<sup>Lp/+</sup> mouse (littermate of A) with mild disruption to apical-basal organisation of epithelium (arrowhead). (C-E) Cornea from *Le-CreTg*<sup>-</sup> *Vangl2*<sup>fl/fl</sup> mouse (conditional knockout, cKO) showing irregularity of corneal epithelium, disruption to normal stratification, and projection of basal cells into the corneal stroma. Corneal epithelia could be abnormally thin (D) or thick (E) with sharp transitions between the thinner and thicker domains, phenomena never seen in *Le-CreTg*<sup>-</sup> *Vangl2*<sup>fl/+</sup> or *Le-Cre*<sup>-/-</sup> *Vangl2*<sup>fl/fl</sup> ( $n > 100$ ) controls. Scale bar represents 50  $\mu\text{m}$  (A-C), 20  $\mu\text{m}$  (D, E).

184x193mm (300 x 300 DPI)

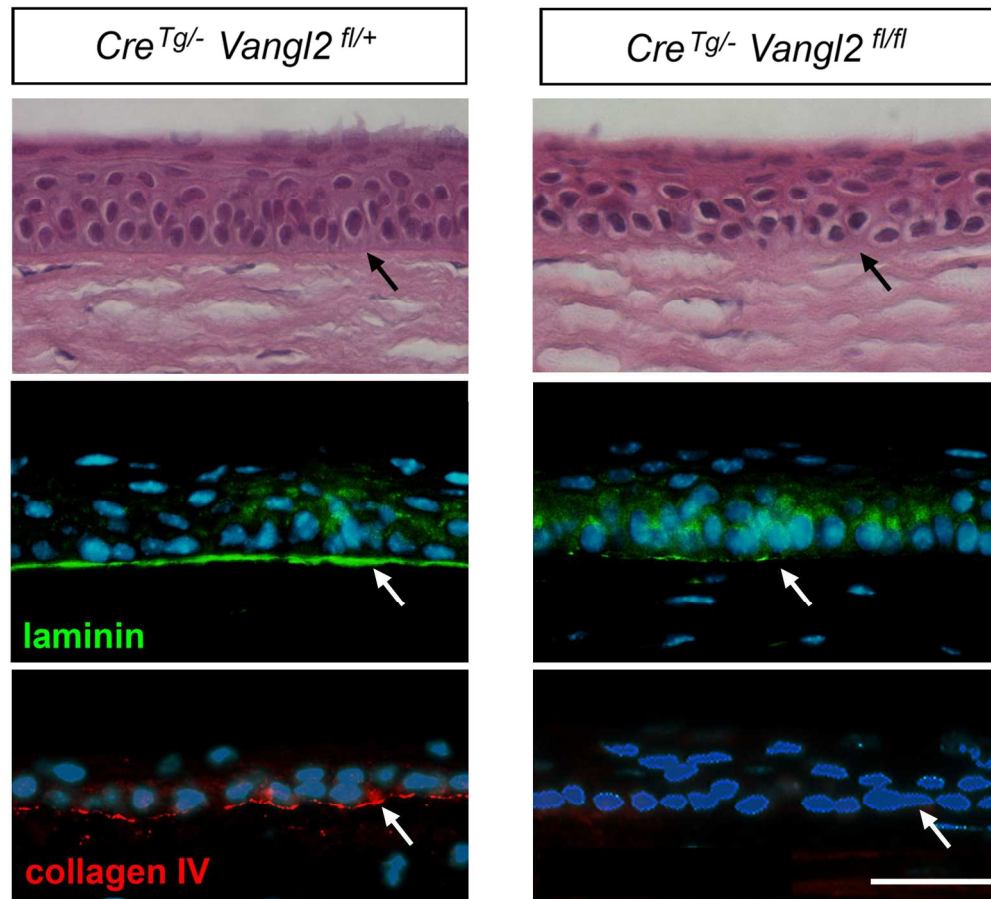


Corneal epithelial abnormalities in neonatal *Vangl2*-mutant mice. (A) At postnatal day 5, the corneal epithelium of  $Le-Cre^{Tg/-} Vangl2^{fl/+}$  control mice is a uniform 2-3 cells thick ( $n = 6$ ). (B) In contrast, all corneal epithelia of  $Le-Cre^{Tg/-} Vangl2^{fl/fl}$  littermates ( $n = 5$ ) exhibited irregularities of stratification, with the epithelium being 1-2 cells thick and with areas of cytoplasmic covering only. Scale bar represents 20  $\mu\text{m}$ .

124x68mm (300 x 300 DPI)

Review Only

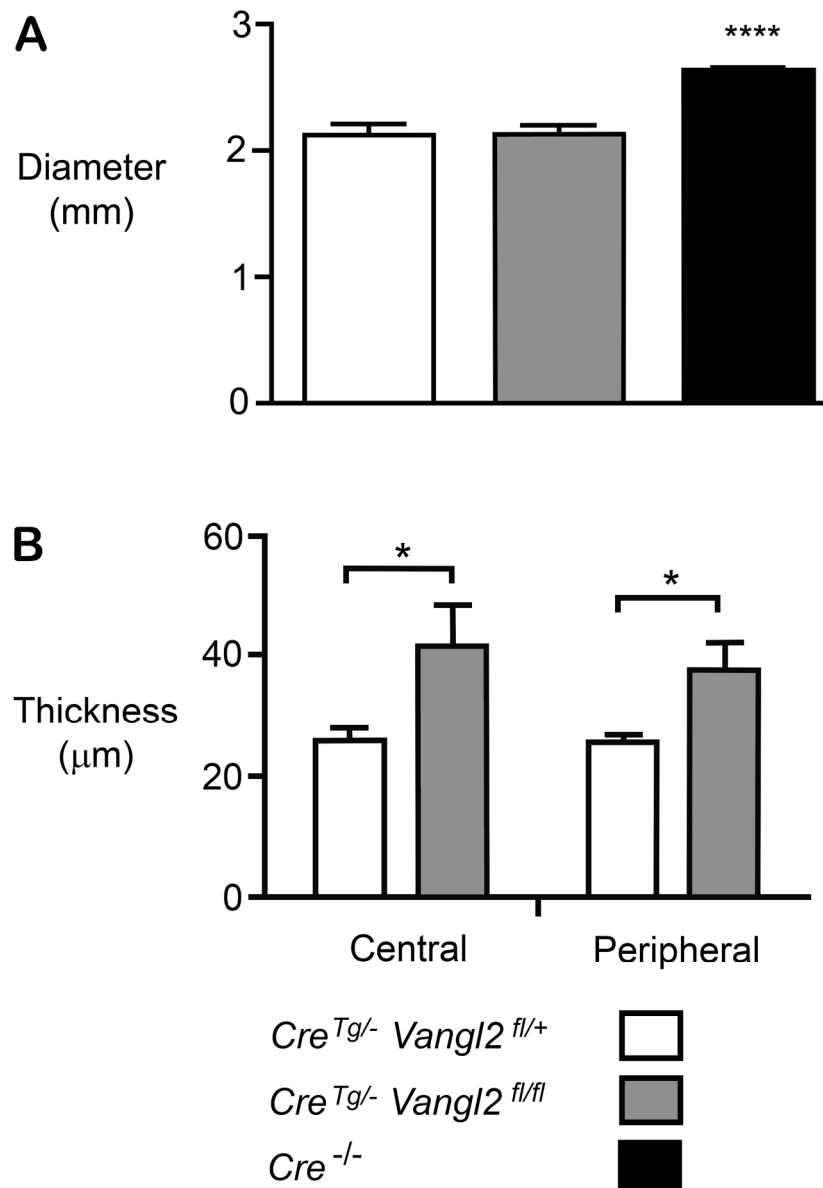




Corneal epithelial basement membrane deficiency in adult *Vangl2*-mutant mice. Haematoxylin and eosin (H&E) staining (top), and immunohistochemistry for ECM proteins laminin (middle) and collagen IV (bottom) in adult *Le-CreTg<sup>-</sup> Vangl2<sup>fl/+</sup>* control mice (left) and *Le-CreTg<sup>-</sup> Vangl2<sup>fl/fl</sup>* littermates (right). Arrows point to the basement membrane. In controls, the basement membrane is visible in H&E-stained controls as a strongly stained lamina immediately underneath the basal surface of the epithelial cells, sitting on a more weakly stained ECM. This is reproducibly not visible or patchy in mutants (right). Laminin and collagen IV staining in mutants is absent or patchy and thin, confirming the light microscopy and Supplementary transmission electron microscopy. DAPI staining (blue) visualises cell nuclei. Scale bar: 40  $\mu$ m.

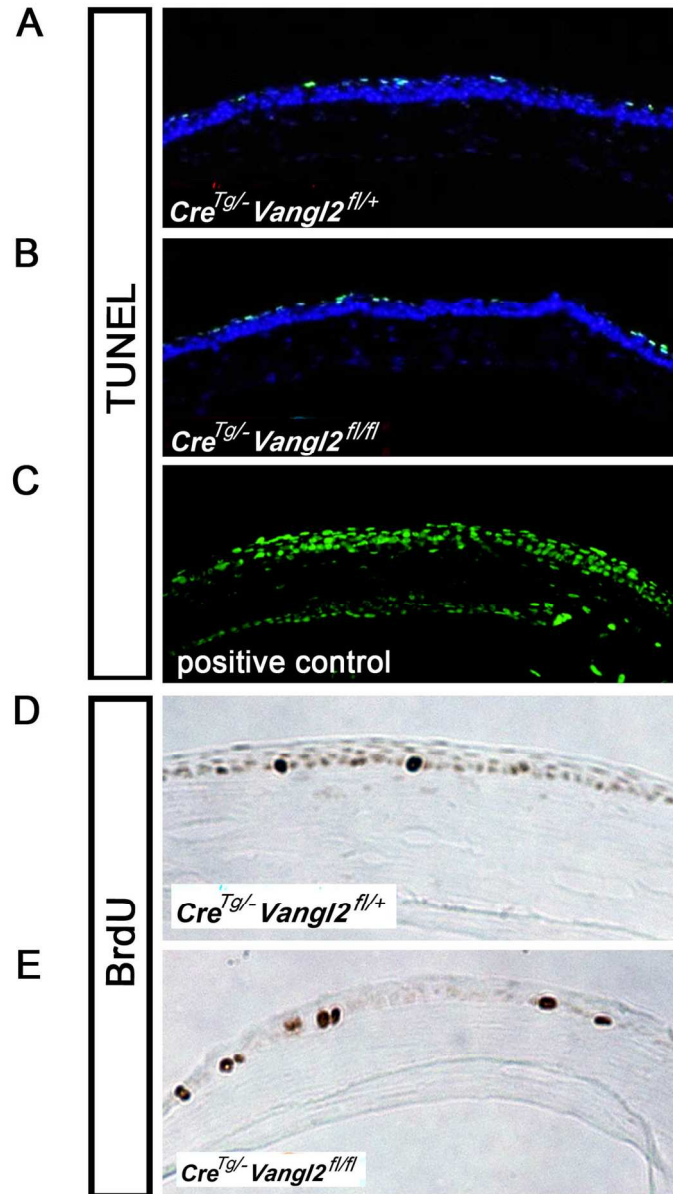
181x164mm (300 x 300 DPI)





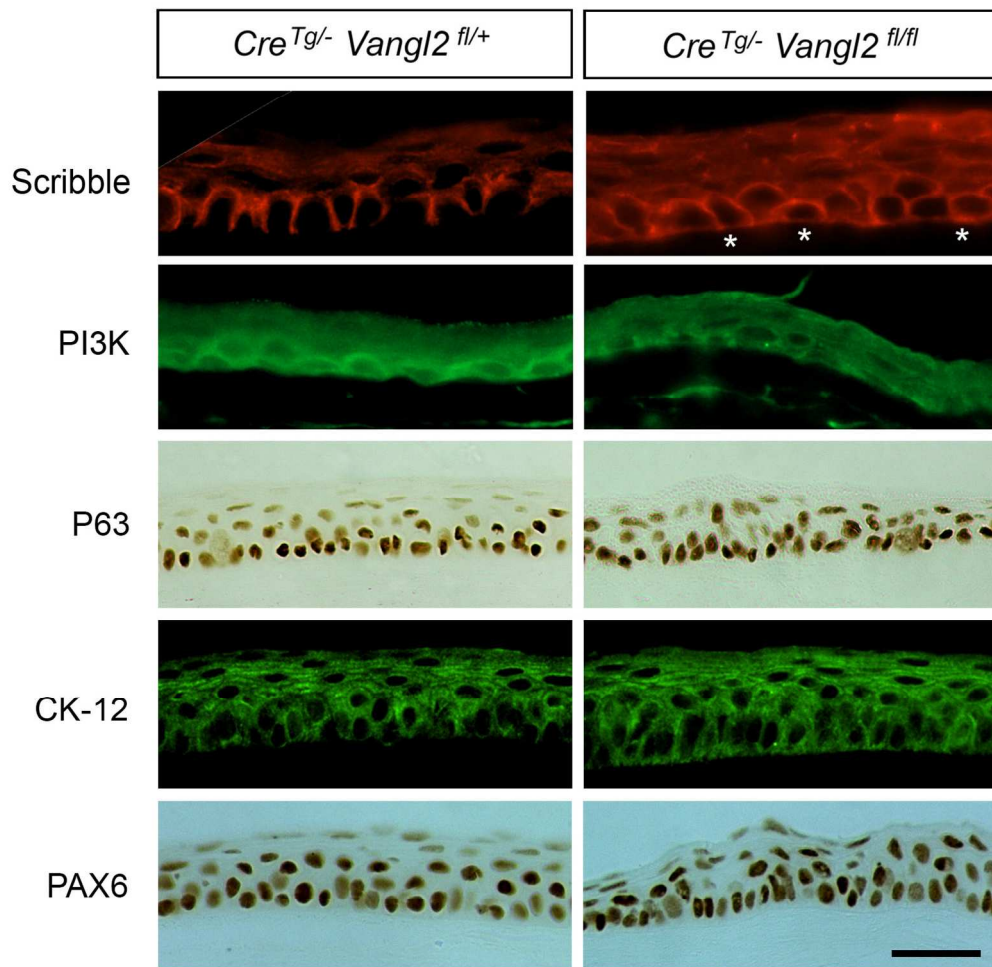
Morphometric analysis of Vangl2-mutant corneas. Corneal epithelial diameter (top) and thickness (bottom) were measured. Le-Cre-positive corneas were slightly but very significantly smaller than Le-Cre-negative corneas, irrespective of Vangl2 genotype. Conditional knockout of Vangl2 lead to increased mean thickness of the corneal epithelium, both centrally and peripherally, accompanying the cellular, morphological and molecular defects described in this paper.

161x232mm (300 x 300 DPI)



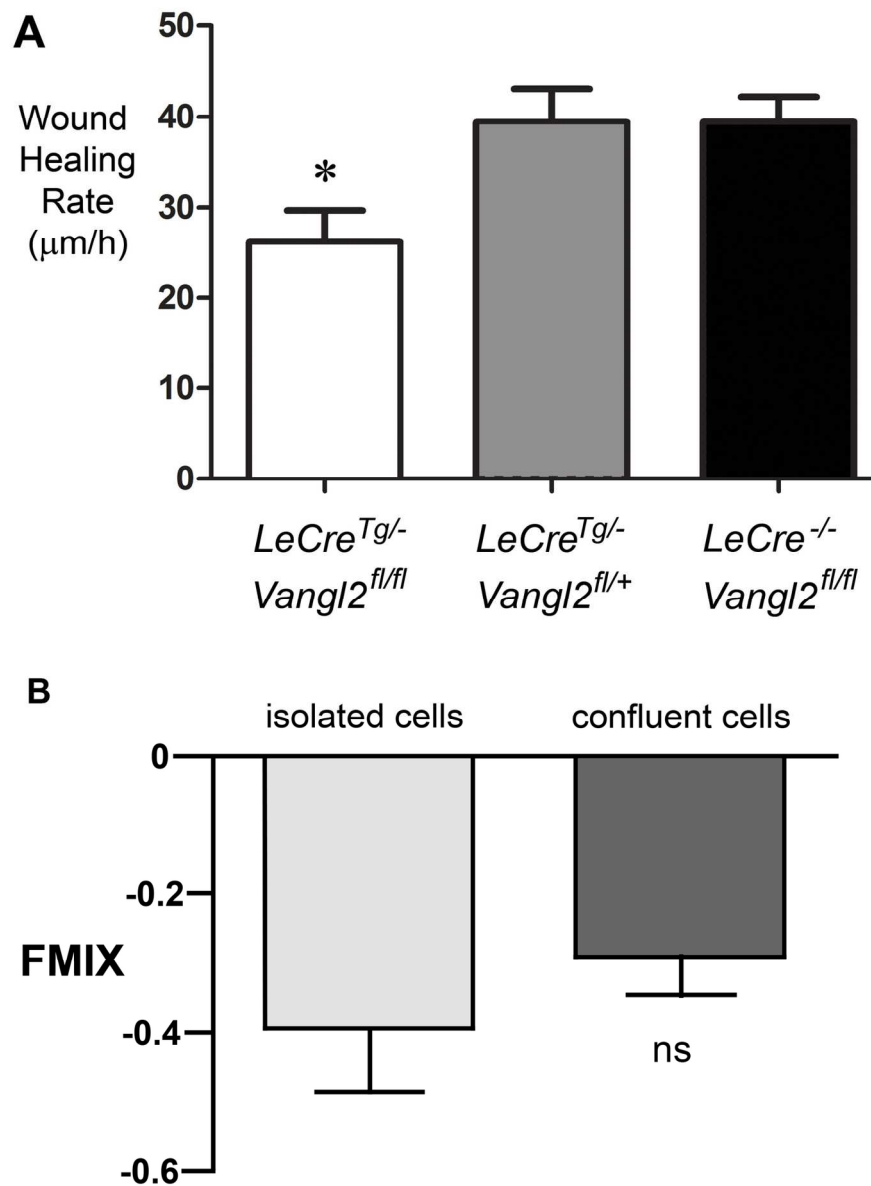
Molecular analysis of cell proliferation and apoptosis in *Vangl2*-mutant corneal epithelia. (A-C) TUNEL analysis of apoptotic cell death. In both *Le-CreTg<sup>-</sup> Vangl2<sup>fl/+</sup>* control corneas (A) and *Le-CreTg<sup>-</sup> Vangl2<sup>fl/fl</sup>* conditional knockouts (B) apoptosis (green fluorescent labelling) was restricted to the most superficial cell layer, with no apoptotic events noted in any other layer of the corneal epithelium ( $n = 3$  of both genotypes). (C) is a positive control - tissue section digested with DNase1 to create double-stranded breaks in DNA of all cells). (D, E). Immunohistochemical analysis of cell proliferation in corneal epithelium of mice after a single injection of BrdU. In both *Le-CreTg<sup>-</sup> Vangl2<sup>fl/+</sup>* control corneas (E) and *Le-CreTg<sup>-</sup> Vangl2<sup>fl/fl</sup>* conditional knockouts (F), DNA replication was restricted to the basal layer of the corneal epithelium, with no proliferation events more apically.

124x221mm (300 x 300 DPI)



Cell polarity and cell fate markers in corneal epithelia of *Vangl2*-mutants. Immunohistochemical labelling of Scribble, PI3K, P63, cytokeratin-12 and Pax6 in *Le-Cre<sup>Tg/-</sup> Vangl2<sup>fl/+</sup>* control corneas and *Le-Cre<sup>Tg/-</sup> Vangl2<sup>fl/fl</sup>* conditional knockouts. Asterisks denote selected *Vangl2*-mutant cells with atypical localisation of Scribble to the basal boundary, suggesting disrupted apical-basal polarity of the basal epithelial cells. PI3K is upregulated in the basal cells of control epithelia but not of *Vangl2* knockout cells. Other markers of cell fate and apical-basal identity are expressed normally in mutant epithelia.  $n = 3-9$  for all markers. Scale bar represents  $40 \mu\text{m}$ .

148x148mm (300 x 300 DPI)



Planar migration defects in *Vangl2*-deficient corneal epithelial cells. (A) Wound healing rate of in vitro cultured adult mouse corneal epithelial cells. The rate of migration of *Le-CreTg<sup>-/-</sup> Vangl2<sup>fl/fl</sup>* cells following scratch-wounding was significantly slower than that of *Le-CreTg<sup>-/-</sup> Vangl2<sup>fl/+</sup>* and *Le-Cre<sup>-/-</sup> Vangl2<sup>fl/fl</sup>* cells. (One-way ANOVA:  $F = 3.720$ ,  $P = 0.0309$ ). (B) Cathodal migration (expressed as forward migration index – FMIX – as described in Materials and Methods), for human corneal epithelial cells after *VANGL2* knockdown to ~30% of normal protein levels. The response of cell cultured at low density (individual cells) (light grey) was not significantly different from that of confluent cells (dark grey).

134x183mm (300 x 300 DPI)

**The core Planar Cell Polarity gene, *Vangl2*, maintains apical-basal organisation of the corneal epithelium.**

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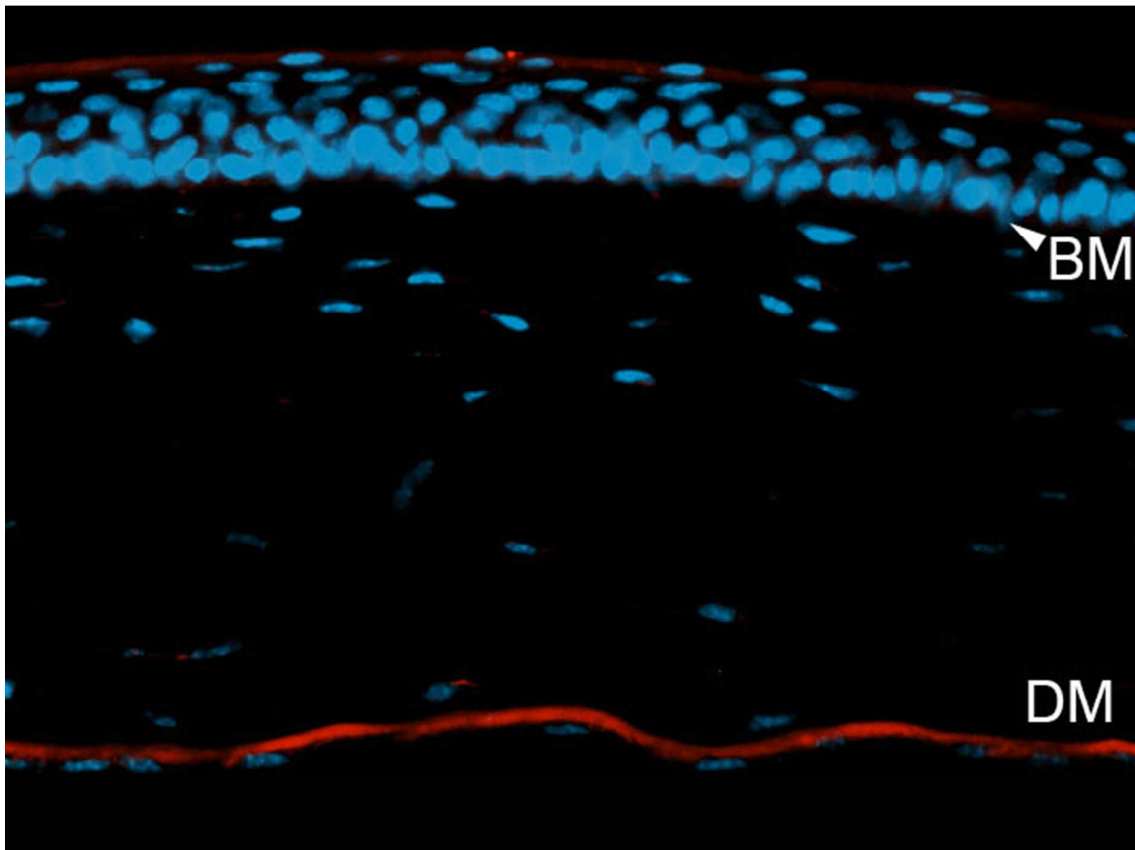
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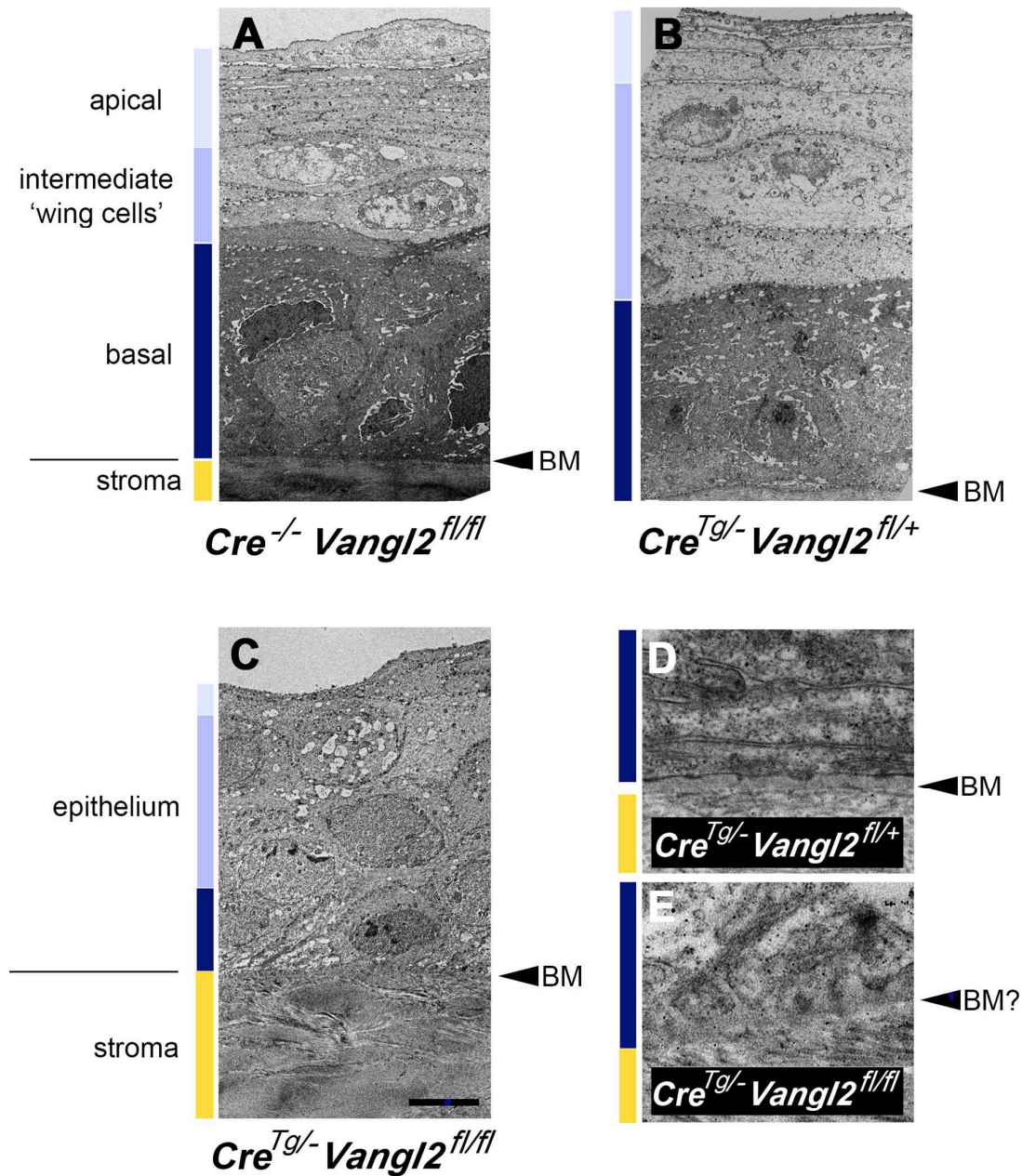
Running title: *Vangl2* in the corneal epithelium

Supplementary Figure 1



**Legend:** Collagen IV immunostaining of *Le-Cre<sup>Tg/-</sup> Vangl2<sup>fl/fl</sup>* conditional knockout cornea showing that although Collagen IV was absent from the epithelial basement membrane (BM, arrowhead) it was still detectable in Descemet's membrane (DM)

## Supplementary Figure 2



**Legend: Transmission electron microscopy of control and *Vangl2*-mutant corneas.** **A)** Transmission electron microscope image of the corneal epithelium of a *Le-Cre*-negative mouse. Corneal stroma, the basal, intermediate and apical epithelial cells are labelled at left. The junction between the basal epithelial cells and the underlying stroma is highlighted with an arrowhead (right). Cytoplasm of the basal layers of cells is electron dense. Suprabasal 'wing' epithelial cells and superficial flattened desquamating cells are visible. **(B)** Comparable image from *Le-Cre*<sup>Tg/-</sup> *Vangl2*<sup>fl/+</sup> control mouse showing apical-basal organisation similar to that of *Le-Cre*-negative mice (A). **(C)** Comparable image



from *Le-Cre<sup>Tg/-</sup> Vangl2<sup>fl/fl</sup>* conditional knockout mouse showing disrupted apical-basal organisation. The epithelium is thin and cells in all layers are of superficially similar shape. The proliferative basal cells are neither electron-dense nor overtly polarised, unlike the controls. **(D)** High magnification image of the basement membrane of a *Le-Cre<sup>Tg/-</sup> Vangl2<sup>fl/+</sup>* control corneal epithelium showing clear differentiation between the epithelial cells (top) and the underlying stromal collagen fibres (bottom). Basement membrane is denoted by an arrowhead. There is a thin, electron-dense basal lamina directly under the epithelial cells overlying a distinct basement membrane. **(E)** Comparable image to (D) from a *Le-Cre<sup>Tg/-</sup> Vangl2<sup>fl/fl</sup>* conditional knockout. The boundary between the epithelial cells and stromal collagen (at the level of the arrowhead) is indistinct, with no electron-dense lamina.

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