RESEARCH ARTICLE

REVISED Planar cell polarity proteins determine basal cell height in the later stage embryonic mouse epidermis'

[version 2; peer review: 2 approved]

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Abstract

Background: Complex organ formation requires the coordinated morphogenesis of adjacent tissue layers. Here, we report a role for the planar cell polarity (PCP) proteins Fz6 and Celsr1 in generating squamous basal cells in the later stage embryonic epidermis of the mouse is reported, which may impact upon the shape of overlying suprabasal cells.
Methods: The depth of the epidermis and basal layer as well as cell proliferation index was scored from immunostained wax sections taken from different mouse embryos mutant in planar cell polarity signalling and their wild-type littermates. Orientation of epidermal cell division in *Celsr1 Crash/Crash* mutants was determined from thick frozen immunostained sections. Immunostained wax sections of wild-type skin explants cultured using the Lumox method enabled any changes in epidermal and basal layer depth to be measured following the release of surface tension upon dissection of skin away from the whole embryo.
Results: Increased numbers of columnar and cuboidal basal epidermal cells were observed in *fz6-/-* mutant and *Celsr1* mouse mutant *Crash/Crash* which correlated with visibly more rounded suprabasal cells and a thicker epidermis.
Conclusions: Altogether these data support tissue intrinsic roles for PCP proteins in 'outside-in' (radial) skin architecture.

Keywords

mouse epidermis, PCP, cell shape, coordination across tissue layers

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REVISED **Amendments from Version 1**

Based on reviewer suggestions we made scatter plots of individual embryos and noted that one of the *Celsr1-/-* embryos exhibited a distinct phenotype to the others. To ensure that there had not been a mix-up with wild-type littermates during blind analyses we analysed further litters of the *Celsr1-/-* which revealed that there was variability between *Celsr1-/-* embryos. We show representative data for the different phenotypes observed. Further work is required to understand the underlying basis of this variability. The text has been revised accordingly. Based on reviewer comments further analysis was performed, including frequency plots, correlation and r squared, and new Figures generated. Measurements were repeated to exclude any distortion in tissue sections. Statistical analysis has also been improved with one way ANOVA and multiple comparisons on biological replicates.

Any further responses from the reviewers can be found at the end of the article

Introduction

Complex organs comprise multiple tissue layers. During organ formation, morphogenesis of the different tissue layers is coordinated to achieve the correct architecture for healthy organ function. A useful model for this is the mammalian embryonic epidermis which is a stratified epithelium comprising of a progenitor basal cell layer, a suprabasal layer and a surface periderm layer (Hu *et al.*, 2018). Recent studies have revealed two independent phases of suprabasal layer formation. A nascent suprabasal layer emerges via delamination of basal progenitors and is proliferative (Damen *et al.*, 2021; Miroshnikova *et al.*, 2018; Williams *et al.*, 2014). Subsequently, the suprabasal layer differentiates and become highly keratinised. Later stage suprabasal cells (E15.5 onwards) are generated via asymmetric cell division of basal progenitors (Damen *et al.*, 2021; Lechler & Fuchs, 2005; Williams *et al.*, 2014).

In addition to epidermal stratification, embryonic skin becomes globally and locally patterned along the tissue plane. The core planar cell polarity (PCP) pathway plays a major role in this process which manifests in the orientation and alignment of hair follicle down-growth along the head-to-tail axis of mouse back skin (Cetera *et al.*, 2018; Chang *et al.*, 2016; Devenport & Fuchs, 2008; Wang *et al.*, 2010). As hair placodes form, PCP proteins exhibit planar enrichment at opposing interfaces between basal progenitor cells along the head-to-tail axis (Cetera *et al.*, 2018). Two seven-pass transmembrane receptors *frizzled6* (*fz6*; Wang *et al.*, 2006) and *Celsr1* (homologue of Drosophila *flamingo*; Hadjantonakis *et al.*, 1998; Usui *et al.*, 1999) play important roles in planar hair follicle alignment as their loss of function (*fz6-/-*, *Celsr1-/-*) leads to randomisation of hair follicle orientation in adult back skin (Ravni *et al.*, 2009; Wang *et al.*, 2010). The same phenotype is reported for embryonic E16 and post-natal back skin of *fz6-/-* embryos (Chang *et al.*, 2016; Wang *et al.*, 2010), indicating on-going roles for PCP proteins in global alignment of epidermal appendages. Notably, live imaging of E15.5 *fz6-/-* embryonic explants demonstrated loss of head-to-tail alignment of local cell rearrangement in hair placode cells (Cetera *et al.*, 2018). Proposed dominant mutations in *Celsr1* (*Crash;Crsh,* Curtin *et al.*, 2003) and in the four-pass transmembrane PCP protein *Vangl2* (*Loop-tail:Lp*, Murdoch *et al.*, 2001) together with *Vangl1/Vangl2* double knockouts (Cetera *et al.*, 2018) however, lead to hair follicle growth vertically downwards into the underlying dermis rather than at an oblique angle, as in wild-type (Devenport & Fuchs, 2008), which implies that PCP proteins play a primary role at the local cell level. Live imaging of E15.5 *Vangl1/Vangl2* double knockouts reveals severe reduction/loss of hair placode cell rearrangements (Cetera *et al.*, 2018). Recent study of *Crsh* and *Lp* homozygote mutants has uncovered epidermal defects along an 'outside-in' tissue axis (Box *et al.*, 2019; Panousopoulou *et al.*, 2016), which can also be defined as 'superficial-basal' in terms of epidermal architecture or 'radial': effectively the 'radial' tissue axis is perpendicular to 'planar'. The study of Panousopoulou *et al.* (2016) proposes that radial cell rearrangements within the surface ectoderm at E13.25 fail in *Celsr1 Crsh/Crsh* mutants. Here it is reported that *Crsh* homozygotes together with *frizzled-6* (*fz6*) null mutants exhibit an abnormal thickening of the later stage epidermis (E17-E17.5), which correlates with a loss of squamous basal cells. Increased numbers of taller basal cells in each mutant correlate with more rounded suprabasal cells. Altogether the data presented is consistent with a model where PCP mouse mutants exhibit a late stage, tissue intrinsic failure to locally generate or maintain squamous basal cell shape which may directly impact on suprabasal cell shape. This is exciting because PCP proteins are expressed within the basal epidermal layer, raising interesting questions about how basal layer PCP signalling impacts upon adjacent, overlying suprabasal layers. These data also provide additional support for core-PCP protein function(s) in radial skin architecture.

Methods

Ethical statement

We expected mice litter sizes of 4–8 with at least one homozygote mutant per litter, thus we determined that for each condition six pregnant females would be required to guarantee sufficient control and mutant embryos for an experimental analysis with n=3 embryos. The same embryos were used for tissue height and proliferation analyses. Two fertile heterozygote males only for each genotype were maintained throughout the study. All mice were bred according to UK Home Office guidelines under Project Licence that was reviewed by the Animal Welfare and Ethical Review Body (AWERB) at King's College London before Home Office review, the Establishment Licence number for King's College London is X24D82DFF. Prior to individual studies taking place, a study plan was also reviewed within King's College London. Mice were held in individually ventilated cages at an average 21°C within a 12h light/dark cycle with food and water provided ad-lib. Mating trios (one male, two females) were set up and monitored morning and afternoon until all females had plugged. At desired gestational age, pregnant females were killed via schedule 1 (cervical dislocation) with all embryos killed by hypothermia and exsanguination.

Mice

The *fz6* and *Celsr1* mouse mutants have been described previously (Curtin *et al.*, 2003; *Crsh/Crsh*: Guo *et al.*, 2004; *fz6-/-*: Ravni *et al.*, 2009; *Celsr1-/-*). Genotypes are: *Crsh/Crsh*, inbred BALB/c strain; *fz6-/-*, C57BL6 × 129 background; *Celsr1-/-*,

floxed mice 129 x 1/Sv 129S1/Sv background (http://www.informatics.jax.org/allele/key/606422) were crossed with the germline deleter PGK-Cre and the heterozygous progenies (*Celsr1*+/-) were inter-crossed to generate homozygous mutants (*Celsr1*-/-) and controls (*Celsr1*+/+). All mice were between 8 weeks and 1 year of age. Mice were genotyped by polymerase chain reaction (PCR). Wild-type mice used for explant cultures were the outbred CD-1 strain (in-house). Timed mating was performed and 9am of the first day of plugging was taken as E0.5. Wild-type and homozygous mutant mice from the same litters were compared. No inclusion or exclusion criteria were set, no embryos were excluded from any of the analyses performed. Mutant embryos were not randomised but due to variability in *Celsr1-/-* embryo phenotypes data from 3 representative embryos are shown of n=8 *Celsr1-/-* embryos analysed. One wild-type embryo for each condition was randomly chosen for data analysis. Analyses were carried out as outlined in ARRIVE checklist (Formstone, 2022).

Risk assessments were generated prior to experimentation.

Histology and immunohistochemistry

Paraffin sections were generated and immunostained according to Oudin *et al.*, 2011. Haematoxylin and Eosin (*H&E) staining* was as described in Panousopoulou *et al.* (2016). *Suspension explants* were embedded into wax as described in Panousopoulou *et al.* (2016). Wax sections were photographed using a Zeiss HBO50 light microscope with an Axiocam 503 colour camera and Axiovision rel.4.7 software (Zeiss). Images were exported into Adobe Photoshop CS5.1 and manipulated using the *crop* function.

The primary antibodies used were cytokeratin1 (K1) antibody (rabbit polyclonal, 1:500; Covance AF109), Ki67 (mouse monoclonal, 1:1000; BD Pharmingen, clone 56, 556003) and PhosphoHistoneH3 (PHH3; rabbit polyclonal. 1:300, Millipore 06-570). Secondary antibodies (Life Technologies) were fluorescently labelled and used at 1:1000.

Measurement of total epidermal thickness and basal cell height

For whole embryos, epidermal thickness and basal cell height measurements were from longitudinal wax sections, every 10 basal cells across inter-follicular epidermis from mid-trunk back skin. Immunostained or H&E-stained wax sections of epidermis were viewed under 40X magnification, bright field illumination and measurements were taken using the *measure* tool of Axiovision rel.4.7 software (Zeiss). Measurements for total height were excluded if the epidermis in section appeared distorted. For each basal cell measured, total height of the overlying epidermis including the height of the basal cell was also scored. At least 75 measurements were made from three independent sections from each of n=3 embryos (from two litters) for each condition. Total adult animals used was n=6. Analysis was blinded, embryos embedded in wax for each gene knockout condition and wild-type were assigned a number by the histologist. One wild-type was randomly chosen for analysis from each mutant cohort. Mutant embryos (n=8) from more than two litters were analysed for *Celsr1-/-* to confirm the variability in phenotype observed in original litters for one embryo. Embryos exhibiting representative phenotypes are shown for *Celsr1-/-*. Immunostained sections were analysed using Axiovision rel 4.7 software, measurements were taken and then the genotype linked to each number was revealed. Histograms were generated using Graphpad Prism 7, but Microsoft Excel could also be used. Statistical analysis was one-way ANOVA with Bonferetti post-hoc test, using Prism software. Frequency graphs, correlation plots and their R-squared values were generated by Prism software.

For suspension explants, five measurements of epidermal thickness and basal cell height were taken from a wax section derived from the central portion of each explant, n=3 explants, total of 15 measurements, using the measure tool of Axiovision rel 4.7 software. For each basal cell measured, total height of the overlying epidermis including the height of the basal cell was again scored. A total or two measurements were taken from each mid-flank of n=3 transverse wax sections taken from each of n=3 mouse embryos, total of 18 measurements. All embryos were taken from the same two wild-type litters. Total adult animals used was n=2. Histograms were generated using Graphpad Prism 7. Statistical analysis was unpaired two-tailed Student's t-test, using Prism software. Analysis of explants was not blinded.

Determination of cell proliferation

Ki67 staining was used to assess any unusual patterns of cell proliferation. Mitotic index was scored manually using immunostaining for pHH3 and H&E staining of telophase divisions from three images analysed from three embryos (from two litters) for each condition, basal and suprabasal divisions were scored for each 1000 basal cells counted, n> 1000 basal cells for each condition. Total adult animals used was n=6, the same embryos were used to score for cell proliferation as were used to measure epidermal thickness. Histograms were generated using Graphpad Prism 7 and statistical analysis was unpaired two-tailed Student's t-test, using Prism software. Analysis was blinded, embryos embedded in wax for each condition were assigned a number by the histologist. Immunostained sections were analysed using Axiovision rel 4.7 software, measurements were taken and then the genotype linked to each number was revealed.

Quantification of oriented cell division

Thick longitudinal frozen sections (100µm) were immunostained with fibronectin to label the basal lamina, E-cadherin to label cell outlines and DAPI to label nuclei. Sequential images along a stretch of mid-trunk back skin were taken on a confocal microscope (Nikon A1R). Z-stack images were generated using 0.3µm steps. For each image, 3-D reconstructions of each Z-stack using Volocity software were generated (see Figure 2C). All telophase divisions within inter-follicular epidermis for which chromatids were fully visible within the Z-stack and their underlying basal lamina were individually cropped and the resulting images rotated (3D opacity mode) until the segregation of each chromatid pair could be measured relative to the basal lamina (marked by staining for fibronectin). One snapshot was taken from opposing sides of any given division. Angles of cell division relative to the basal lamina were measured using the *ruler* tool in Adobe Photoshop CS5.1. In each

case, a line was first drawn along the basal lamina and then through the centre of each chromatid, which appeared in side-view. The mean angle for each division (of the two angles derived) was calculated. A total of n=37 wild-type divisions and n=36 *Crsh/Crsh* divisions were analysed from skin sections taken from n=3 embryos (from four litters) for each condition. Total adult animals used was n=4. Statistical analysis using unpaired two-tailed Student's t-test compared wild-type and *Crash/Crash* divisions in bins shown, significant differences were not found. Histograms were generated using Graphpad Prism 7 and statistical analysis was unpaired two-tailed Student's t-test, using Prism software. Analysis was not blinded.

Results

A curious observation about E17-E17.5 *Crsh/Crsh* embryonic skin is that the suprabasal layer was thickened compared to wild-type, with visibly more rounded, cytokeratin-1 expressing suprabasal cells (nuclear shape was taken as a proxy for cell shape) overlying the basal layer in mutant skin compared to wild-type littermates (Figure 1A, B, E) (Formstone, 2022). *Crsh/Crsh* mutants exhibited an open neural tube (Curtin *et al.*, 2003), which may contribute to the skin thickening phenotype (Box *et al.*, 2019), however, study of *fz6* homozygote knockout (KO: -/-) mouse embryos, which exhibit closed neural tubes, revealed that the epidermis of *fz6-/-* embryos was significantly thickened compared to wild-type embryos (Figure 1A,C,E), suggesting an underlying tissue intrinsic defect.

Scatter plots of total epidermal height against basal cell height (total epidermal height was measured for each individual basal cell for which cell height was measured) were generated for individual embryos revealing strong similarities between *fz6-/-* and *Crsh/Crsh* embryos compared to wild-type (Figure 1G). Individual *Celsr1-/-* embryos however demonstrated variability in phenotypes (representative data is shown; Figure 1G) which was consistent across more than three litters (embryos shown are representative for the different phenotypes observed in n=8 embryos analysed). More than one *Celsr1-/-* embryo exhibited evidence of thickened epidermis (Figure 1G). Indeed, graphs showing the frequency distribution of total epidermal height revealed values greater than 59µm (shown by thin vertical line, Figure 2A) for all PCP mutants, which was the maximum height measured in wild-type embryos. Frequency data for *Celsr1-/-* embryos was generated using total and basal height measurements from each of the representative embryos shown in Figure 1G. Further study at earlier embryonic stages are required in this mutant to evaluate the underlying basis of the observed variable phenotype.

No significant changes in cell proliferation index in either basal or suprabasal layers of *fz6-/-* embryos were observed that might account for the epidermal thickening phenotype (Figure 3A,B). From E15.5 onwards, Inscuteable and LGN protein crescents on the apical side of epidermal basal cells are reported to define oblique and vertical epidermal basal cell divisions as asymmetric, giving rise to a basal cell daughter as well as a differentiating suprabasal cell daughter (Lechler & Fuchs, 2005; Williams *et al.*, 2011). Further support for this model was provided recently by Damen *et al.* (2021). The proportion of basal cell divisions exhibiting horizontal, oblique and vertical orientations in E16-E16.5 *Crsh/Crsh* skin compared to wild-type was similar (Figure 3C), consistent with a recent study of *fz6-/-* skin (see supplementary data, Box *et al.*, 2019). Thus, the ratio of symmetric to asymmetric divisions, which promote epidermal stratification in later stage skin, appears to be undisturbed in *fz6* and *Celsr1 Crsh/Crsh* PCP mutants.

A frequency distribution of values for basal cell height measurements from images of wax sections immunostained with cytokeratin-1, which labels suprabasal cells, demonstrated that the skin basal layer (nuclei of which were co-stained blue) in wild-type contained a mixture of tall (columnar), square (cuboidal) and flattened (squamous) epithelial cells with a bias towards squamous-type: most frequent height was found for bin centre 6µm (Figure 2B) Conversely, *fz6-/-* basal cells were predominantly columnar (most frequent heights were around 8-10µm; Figure 2B) whilst *Crsh/Crsh* basal cells exhibited bias towards columnar or cuboidal (most frequent height was found for bin centre 8µm; Figure 2B). Both *fz6-/-* and *Crsh/Crsh* basal cells were rarely less than 5µm in height (Figure 2B).

Notably, the bias towards taller basal cells in *fz6-/-* and *Crsh/Crsh* correlated with suprabasal cells which were more visibly rounded in shape (Figure 1B,C). In wild-type, squamous basal cells were generally overlain by a flattened suprabasal layer where suprabasal cells were squamous in appearance (black arrows, Figure 1A). Cuboidal/columnar wild-type basal cells however were overlain by a thicker suprabasal layer containing suprabasal cells that were rounded in appearance (white boxed area, Figure 1A). Correlation analysis on scatter plots where basal cell height was plotted against total epidermal height for wild-type embryos (Figure 1G) revealed mean r-squared values for wild-types as +0.56 (*P* value <0.0001) supporting a positive correlation between basal cell height and total epidermal height (Figure 1G). Mean r-squared values for *fz6-/-* and *Crsh/Crsh* however were significantly lower 0.18 (r squared *P* value range of 0.0002- <0.0001) and 0.17 (r squared *P* value range of 0.0031- <0.0001) respectively. Mean r-squared value for representative *Celsr1-/-* embryos was 0.33 (r squared *P* value range of 0.0002- <0.0001). One way ANOVA (Bonferonni post-hoc test) across all PCP mutants and wild-type controls gave a *P* value of 0.024 with multiple comparisons revealing *P* values of 0.0266 (*fz6*KO), 0.0223 (*Crsh/Crsh*) and 0.22 (*Celsr1-/-*) for individual mutants compared to wild-type controls. We found therefore a generally lower correlation between basal cell height and total epidermal height for all PCP mutants analysed.

Wax sections for *fz6-/-* and *Crsh/Crsh* mutants show visibly more rounded suprabasal cells, we were not able to robustly quantify suprabasal cell shape however in wax sections. One simple explanation for the presence of more rounded suprabasal cells directly above the more columnar height of basal cells observed in *fz6-/-* and *Crsh/Crsh* skin is direct mechanical contact. We have recently used suspension explant (*ex-vivo*) culture to investigate skin morphogenesis and found that

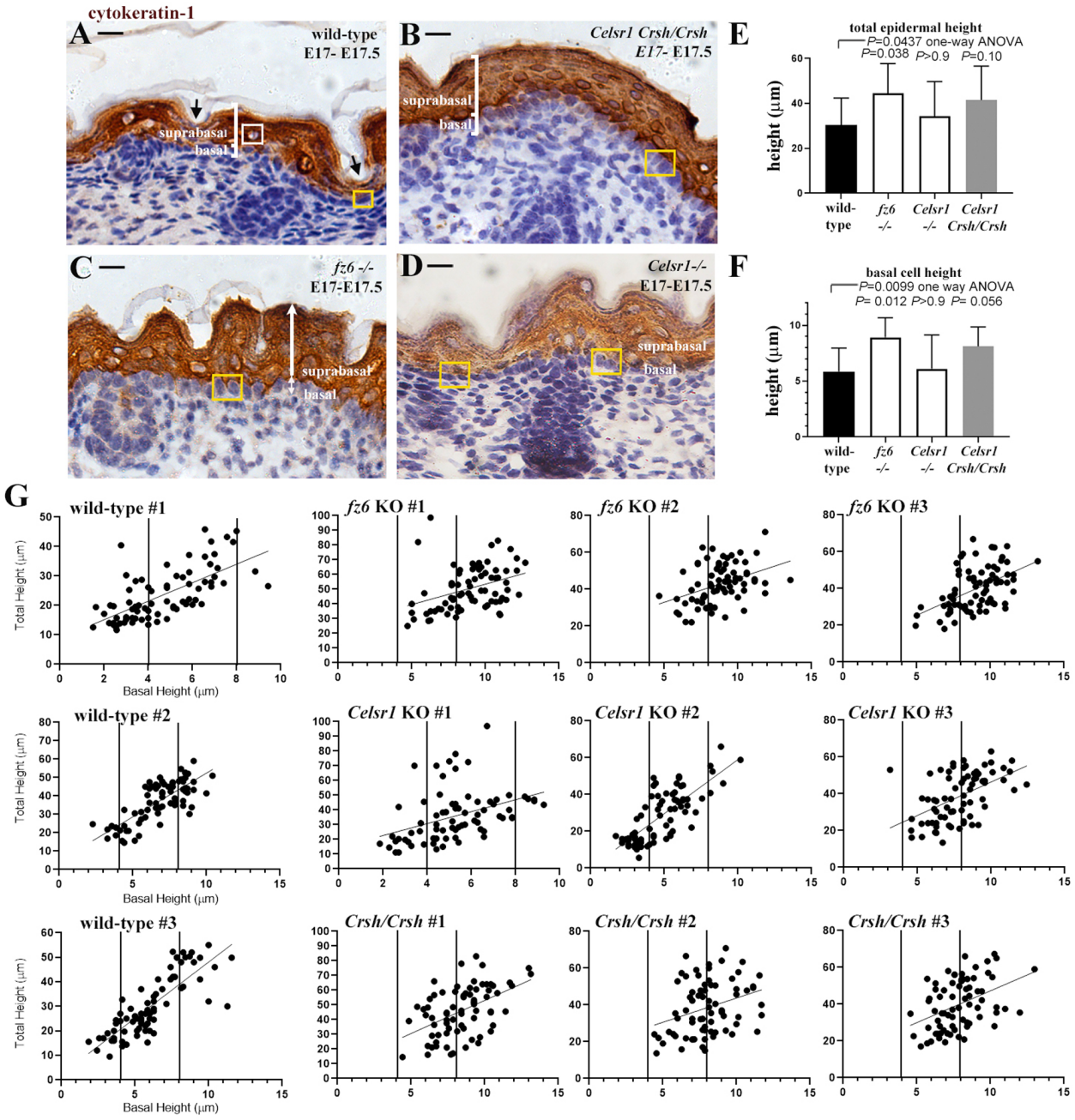

Figure 1. Planar Cell Polarity (PCP) pathway components expressed in the skin basal layer determine later stage basal cell height. (**A**–**D**) Representative longitudinal wax sections of E17-E17.5 mid-trunk back skin from (**A**) wild-type (**B**) *Crash* homozygotes (*Crsh/Crsh*), a mutant in Celsr1 (Curtin *et al.*, 2003) which expresses Celsr1 protein but lacks PCP protein planar asymmetry, (**C**) *frizzled6* (*fz6-/-;* Wang *et al.*, 2010) and (**D**) *Celsr1* (*Celsr1-/-*; Ravni *et al.*, 2009) knock-out embryos. Skin sections were immunostained with cytokeratin-1 to visualise suprabasal cells, counterstain for nuclei is blue. White brackets label basal and suprabasal layers in (**A**,**B**). Black arrows in (**A**) label thinner sections of the suprabasal layer correlating with squamous basal and suprabasal cells. White box in (**A**) labels a rounded nucleus of a suprabasal cell. Yellow box in (**A**) highlights a squamous basal cell. Yellow boxes in (**B**,**C**) label columnar basal cells. Yellow boxes in (**D**) label squamous (left hand box) and cuboidal (right hand box) basal cells. White arrows in (**C**) denote basal and suprabasal height measurements taken. Skin surface is to the top, scale bar is 20μm in all cases. (**E**,**F**) Histograms of total epidermal depth (**E**) and basal cell height (**F**), n=75 measurements from wax sections from individual embryos, n=3 for each condition from at least two litters. Measurements made were taken every 10 basal cells across the inter-follicular epidermis. Measurements for wild-type are combined measurements for one wild-type littermate taken from one litter for each PCP mutant. Mean and SD are shown. Statistical analysis was one-way ANOVA with Bonferroni post-hoc test between mean values for n=3 biological replicates for each condition, individual values show comparisons between individual mutants and wild-type. (**G**) Correlation plots of total epidermal height at location of individual basal cells against individual basal cell height for each embryo analysed. Measurements made are shown by white arrows in (**C**) and were taken every 10 basal cells across the inter-follicular basal layer of mid-trunk back skin. 75 measurements were taken from n=3 sections from each of n=3 embryos (from at least two litters) for each condition.

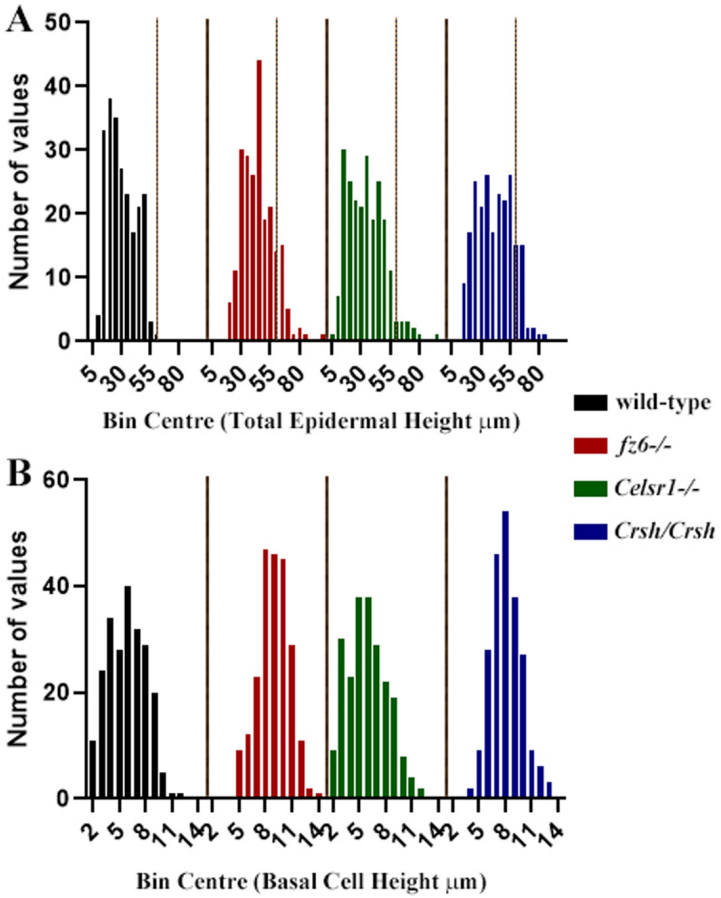

Figure 2. Each PCP mutant exhibits evidence of a thickened epidermis. Frequency plots for total epidermal (**A**) and basal cell (**B**) height made using GraphPad Prism. Prism allocated bin centres using the same measurements as for graphs and correlation plots shown in Figure 1.

explants of wild-type surface ectoderm deform when excised away from the intact embryo, demonstrating that the embryonic skin is under extrinsic mechanical tension (Lumox culture: Panousopoulou *et al.*, 2016). Explant of E15.5-E16 mid-flank wild-type skin (+8h, Lumox suspension culture) also resulted in epidermal deformation Figure 3D,E). Basal cells became significantly taller and suprabasal cells became more rounded *ex-vivo* compared to *in vivo,* leading to a significant increase in epidermal depth (Figure 3F). These data reveal that both basal and suprabasal cells respond to loss of extrinsic force by changing their height, indicating that coordination of tissue mechanics between epidermal layers could result in a thicker epidermis in PCP mutant embryos which exhibit a bias towards more columnar basal cells.

Discussion

Previous studies reveal a role for core-PCP proteins in determining epithelial cell shape (Nishimura *et al.*, 2012; Shi *et al.*, 2014). Here it is reported that two different mouse mutants in core-PCP genes exhibit loss of squamous basal cells at later stages of embryonic epidermal development. Both *fz6* and *Celsr1 Crsh/Crsh* mutants exhibit a bias towards taller (more columnar or cuboidal) epidermal basal cells. A similar later stage thickening of the epidermis has also been reported in the *Vangl2 Lp/Lp* mutant, which was linked to the presence of taller basal cells and an increased frequency of oblique/perpendicular basal cell divisions at the expense of planar orientations much earlier in skin development i.e., at around E14.5 (Box *et al.*, 2019). Damen *et al.* (2021) find that centriole loss in skin where p53-mediated cell death has been suppressed does not disrupt skin morphogenesis prior to E15.5. The authors proposed therefore a two-stage system (early/late) for epidermal development, with later stages (E15.5 onwards) relying on asymmetric basal cell divisions to supply new suprabasal cells Damen *et al.* (2021). In light of this model and the highly similar later stage epidermal thickening phenotype in *Crsh/Crsh* and *fz6-/-* mid-flank skins at E17-E17.5, it is proposed here that both *Crsh* homozygotes and *fz6-/-* exhibit a similar later stage tissue intrinsic defect in the generation of squamous basal cell shape.

R-squared values from scatter plots suggest that flattened (squamous) basal cell shape in later stage skin impacts on suprabasal cell shape. Suspension explants of E15.5-E16 wild-type skin provides evidence for a mechanical coordination in cell height between different epidermal layers. Significantly lower

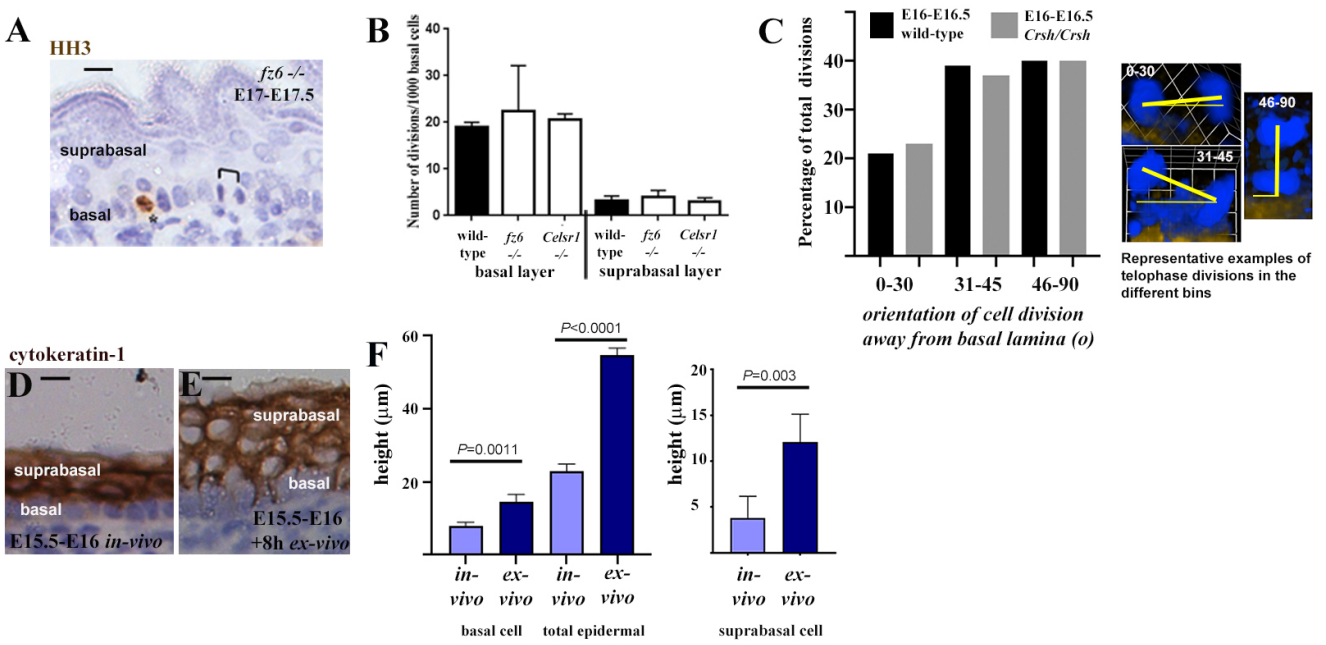

Figure 3. Cell proliferation index and orientation of cell division in PCP mutants are similar to wild-type. (**A**) Representative longitudinal wax section of mid-trunk back skin, asterisk labels a dividing cell in the basal layer stained with pHH3 (brown stain), black bracket labels a telophase division. Scale bar is 10µm. (**B**) Histogram showing number of cell divisions scored per 1000 basal cells in both basal and suprabasal layers of mid-trunk back skin from wild-type and PCP mouse mutants, n=3 embryos from 2 litters for each condition. Statistical analysis was Students t-test of biological replicates from wild-type and each mutant, no significant differences were found. (**C**) Percentage of total divisions which exhibit specific angles of orientation away from the basal lamina. Only telophase divisions were scored within inter-follicular epidermis. Angles are binned 0–30, 30–45, 46–60, 61–75 and 76–90 to represent horizontal (0–30), oblique (30–45, 46–60) and vertical (61–75) divisions (Lechler & Fuchs, 2005). Frozen longitudinal sections of mid-trunk back skin were analysed from n=3 wild-type and n=3 *Crsh/Crsh* skins from four litters in each case, a total of n=37 wild-type divisions and n=36 *Crsh/Crsh* divisions were scored. 3D images of Z-stacks for representative telophase divisions are shown. Nuclei are in blue, yellow lines label angle of cell division orientation relative to the basal lamina (gold staining). (**D**,**E**) Representative image of wax sections of E15.5-E16 wild-type epidermis in vivo and explants of E15.5-E16 wild-type epidermis following an 8 hour (8h) suspension culture (*ex-vivo*), n=3 whole embryos and n=3 explants, taken from the same two litters. Scale bar is 10µm. Counterstain of nuclei is in blue. (**F**) Histograms of basal cell height, suprabasal cell height and total epidermal height at E15.5-E16 *in vivo* and E15.5-E16 + 8h in suspension culture (*ex-vivo*). Mean and SD are shown, statistical analysis was Students t-test of biological replicates (n=3), n=15 measurements per biological replicate.

r-squared values for *fz6-/-* and *Crsh/Crsh* scatter plots compared to wild-type revealed however a low correlation between basal cell height and total epidermal height. This might be accounted for by there being a limit to the maximum height of individual suprabasal cells in the intact embryo. Given however that *fz3* becomes restricted to epidermal suprabasal layers at E17 (Hung *et al.*, 2001) and *fz3-/-* E17 skin is also thickened (Dong *et al.*, 2018, supplementary data) it is also entirely possible that PCP protein signalling between different epidermal layers also plays a role. Indeed, this may explain the variability in phenotype in *Celsr1-/-* mutants.

In wild-type, squamous basal cells make up just 30% of the total number of basal cells in the later stage epidermis. Thus, PCP proteins appear to act locally within specific cell communities to generate squamous basal cells. Local PCP protein signalling is well-documented in the skin e.g., hair placodes use local planar oriented cell-cell rearrangements within the basal layer of the hair placode to create a cellular asymmetry, which couples to the global alignment of their subsequent oblique oriented down-growth into the underlying dermis (Cetera *et al.*, 2018; Devenport & Fuchs, 2008). *Crash* and *Lp* homozygote hair placode/follicle phenotypes (Devenport & Fuchs, 2008) also infer local disruption to PCP signalling. Additionally, E16 basal cells are reported to rely upon local cues from neighbouring interphase cells to orient their planar axis of cell division (Oozeer *et al.*, 2017). PCP signalling processes in the skin are defined by the asymmetric enrichment of PCP proteins along the axis of planar polarity (Devenport & Fuchs, 2008). It is not certain, however, whether asymmetric PCP protein distribution is necessary to generate squamous basal cell shape in later stage skin. Fz6, for example, is not unique in the Frizzled family in playing a role in epithelial cell height. Cell height is reduced in the monolayer epithelium of developing mouse lung tubules of *fz2-/-* embryos (Kadzik *et al.*, 2014).

In conclusion, we find that the presence of squamous basal cells in the later stage mouse embryonic epidermis is dependent upon core-PCP protein function. Further study is necessary to understand whether squamous versus columnar basal cell height impacts overlying suprabasal cell shape and whether PCP proteins impact on their potential association. The findings presented here also support a role for core-PCP proteins in the 'outside-in' (radial) organisation of

the developing mammalian skin. Future work will focus on a more detailed analysis of the correlation between basal and suprabasal cell shape in wholemounted skin at earlier embryonic stages which will enable packing and cell shape in both layers to be fully investigated.

Data availability

Underlying data

OSF: Formstone 2022 WT Open Research. https://doi.org/10.17605/OSF.IO/G39WT (Formstone, 2022)

This project contains the following underlying data:

- Data files for Tables Figure 1 file
    - Data for epidermal height and orientation of cell division – E17-E17.5 epidermis
    - Data for orientation of cell division
    - Data for epidermal height – E15.5-E16 explants
- Photomicrographs for Formstone 2022 Wellcome Trust Open Research
    - Photomicrographs for wild-type epidermis
    - Photomicrographs for Celsr1 KO epidermis
    - Photomicrographs for fz6 KO epidermis
    - Photomicrographs for Celsr1 Cras/Crash epidermis
    - Photomicrographs for E15.5-E16 whole embryo and explants

Reporting guidelines

OSF: ARRIVE checklist for 'Planar Cell Polarity protein-dependent basal cell height in the later stage mouse epidermis impacts on the shape of overlying suprabasal cells'. https://osf.io/ts8fr/?view_only=16c87f5bf6ba4318a12d9a8c3bfde25a (Formstone, 2022)

Acknowledgements

Sincere thanks to Professor Anthony Graham for reading the manuscript and Dr Jenny Murdoch for providing the initial wax sections of *Crash/Crash* mutants. Also, to Dr Fadel Tissir for *Celsr1-/-* embryos and Professor Jeremy Nathans for *fz6-/-* mice.

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Open Peer Review

Current Peer Review Status: ✔ ✔

Version 2

Reviewer Report 16 March 2023

https://doi.org/10.21956/wellcomeopenres.20902.r54329

✔ **David Strutt**
School of Biosciences, University of Sheffield, Sheffield, UK
Helen Strutt
The University of Sheffield, Sheffield, UK

The authors have taken on board our suggestions and we have no further comments.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Cell and developmental biology of planar polarity

We confirm that we have read this submission and believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 26 January 2023

https://doi.org/10.21956/wellcomeopenres.20902.r54328

✔ **Hisham Bazzi**
Department of Dermatology and Venereology, CECAD Excellence Cluster, Medical Faculty, University of Cologne, Cologne, Germany

No further comments.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 26 July 2022

https://doi.org/

?

David Strutt
School of Biosciences, University of Sheffield, Sheffield, UK
Helen Strutt
The University of Sheffield, Sheffield, UK

This short manuscript follows up on previous observations of epidermal thickening in Vangl2 mutant mice. The authors report a similar thickening of the epidermis in Celsr1 and Fz6 mutant animals, and describe cell shape changes in both the basal cell layer and the suprabasal cell layer.

1. A diagram of the epidermal layers would be useful to help orient less expert readers. An alternative to a cartoon might be clear 'brackets' beside one of the figure panels indicating the cell layers. The white arrows in panel C partly address this point, but are rather thin and hard to see.

   Could the authors zoom in on one of the panels B-D and point out the basal cell layer, and also examples of squamous, cuboidal and columnar basal cells?

2. Panel E, F: Total epidermal depth and basal cell height are measured, n = 75 measurements from 3 embryos, measurements taken every 10 basal cells. Given the very low p-values, we wonder if the 'n' value used for the statistics was in fact n=75 rather than n=3. In our view, the true n is 3 (number of embryos), not 75. The 25 measurements within an embryo seem to fall into the category of technical replicates, not biological replicates. We would suggest taking an average per embryo and doing stats between embryos (if this wasn't what was done). Most likely the result will still be significant (or close to significant), but in either case reporting the actual p-values would provide useful information.

   Regarding the statistical test used, the legend indicates 'Students t-test', however multiple comparisons are shown and the Methods mention both t-tests and ANOVA. We think the appropriate test for multiple comparisons is ANOVA with post-hoc correction for multiple samples (e.g. Bonferroni – we think 'Bonferretti' in the Methods is a typo?). Could the authors clarify the test used?

   Panel H legend also states t-test, but as there are 3 genotypes, ANOVA looks more

appropriate. Although the authors state in the text that differences are not significant, giving the actual p-value would be more informative (e.g. p = 0.051 is conventionally regarded as 'not significant' while p =0.049 is regarded as 'significant', but both p-values are very similar and it's hard to justify regarding them as qualitatively different).

Panel M: We have similar questions regarding technical vs biological replicates and the appropriate n number.

3. It would be nice (but not essential) to have a sample image to accompany panel I, showing examples of different orientations of cell division.

4. Panel J: Celsr, Fz6 and Crsh mutants had more columnar and cuboidal cells relative to wild-type. The total epidermal depth for each of these classes was then plotted, with the conclusion that having columnar/cuboidal cells rather than flat/squamous cells correlates with overall epidermal thickness.

   As there will be a range of cell heights within each class, we wonder whether it might be also useful to show scatter plots of individual basal cell height versus total epidermal thickness? This might firm up the connection between basal cell height and epidermal thickness.

   We were again uncertain about the stats. Is the p-value given the overall ANOVA p-value for comparisons of epidermal depth for all classes, or for comparison of epidermal depth in columnar vs squamous (as the bars above the graphs might imply)? Clarifying this in the figure legend would be good. If the former, why not also show the pair-wise values on the graph?

   There is also the question of the correct n number for the ANOVA. Averaging within animals and then comparing between animals would again seem most appropriate? However, there may not be enough measurements in each animal for some classes (e.g. squamous basal cells) to do this. This is why scatter plots of individual cell height vs epidermal depth might be more valid, using either pooled data or plotting each animal individually, and looking at the r-squared values for these plots to show a correlation.

5. The authors say the bias towards taller basal cells in fz6-/-, Celsr1-/- and Crsh/Crsh correlated with suprabasal cells which were more rounded in shape. While not essential, can this be quantitated in some way?

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Partly

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Cell and developmental biology of planar polarity

We confirm that we have read this submission and believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however we have significant reservations, as outlined above.

Author Response 08 Jan 2023

Caroline Formstone

I would like to thank the reviewer for their most helpful comments which have significantly improved our manuscript. I apologise for the delay in submitting the revised manuscript. Based on your comments, whilst analysing the data for the individual embryos I noticed that one *Celsr1-/-* embryo was very different to the other two and was concerned about a mix up with wild-types and so I asked for more *Celsr1-/-* litters from my collaborator. I had then to perform the histology which has taken me some time. The new litters revealed further similar embryos demonstrating that there is variability between embryos in this mutant. The Figures and text have been altered accordingly. Based on comments from both reviewers we have made additions to Figures and added two further Figures.

Specific comments

1. Thank you for these improvements. We have added brackets to Fig.1A and Fig.1B and labelled the two layers. We have also made the line demarcating the suprabasal layer measurement thicker in Fig.1C to aid visualisation. We have also outlined examples of squamous, cuboidal and columnar basal cells on Fig.1A-D. We hope this improves ease of understanding of the photographs presented.
2. Thank you for this important comment on our statistical analysis. We previously performed an unpaired student's T-test for the basal and total height measurements making comparisons between mutants. You are correct that we compared technical replicates rather than averages for each embryo. As you suggest, we have now performed ANOVA on the three biological replicates for each condition and provided updated P values for the overall comparison as well as showing individual P values for Bonferroni's multiple comparisons test where each mutant is compared to wild-type.
3. We agree with the reviewer and have added in the images as requested which can now be found in Fig.3.
4. We agree with the reviewer and have generated scatter plots for individual embryos which replace the original scatter plot. New measurements were made for all embryos for each condition based on comment #2 of reviewer 1 and we have added

new measurements for the Celsr1 KO based on additional litters. We have now made 75 measurements for each embryo to improve data analysis. We have added the new scatter plots to Fig.1 and used the same data to generate frequency plots which are shown in a new Fig.2. We have also generated r-squared values and altered the text to reflect this new data. Remaining data is now in Fig.3.

5. We tried different strategies to quantify suprabasal cell shape in wax tissue sections but were not convinced by their robustness. We were however able to measure suprabasal cell height in wax sections of explants and this data is now included in Fig.3. We have improved our statistical analysis as suggested and compared biological replicates of all measurements for each condition. Thank you again for flagging this up.

Competing Interests: No competing interests were disclosed.

Reviewer Report 13 May 2022

https://doi.org/10.21956/wellcomeopenres.19624.r50043

?

Hisham Bazzi
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The manuscript by Hobbs and Formstone, “Planar cell polarity protein-dependent basal cell height in the later stage of embryonic mouse epidermis impacts on the shape of overlying suprabasal cells” reports a brief and targeted analyses of epidermal dynamics in the planar cell polarity (PCP) constitutive mutants for Fzd6 and Celsr1. The authors focus mainly on embryonic day (E) 17.5 of skin development and the data correlate epidermal thickness with basal cell height as well as suprabasal cell shape.

The data in the manuscript extend previous findings by Box *et al*, 2019 on Vangl2 mutants to other PCP mutants, namely Celsr1 and Fzd6. The authors conclude that “basal cell height...impacts on the shape of overlying suprabasal cells”; however, a causal relationship is difficult to establish from correlation in fixed samples and at a single timepoint during development. The authors acknowledge the correlation in the abstract and main text but the title asserts a causal link with the word “impacts on”. A more objective conclusion from the data maybe that the thickness of the epidermis correlates with the packing of the cells and their shapes in both the basal and suprabasal layers, which also applies to the data on the ex-vivo skin cultures in K-M, and perhaps the basal cell packing reported in Box et al., 2019. In my opinion, the outstanding questions are: why is the cellular packing different in PCP mutants? If it does not correlate with proliferation rates as shown in Fig. 1H (and Box *et al*., 2019), then what about cellular densities and cell sizes in both

the basal and suprabasal layers from E13.5 – E17.5? A more thorough analysis of PCP mutants is required to address these questions.

The authors raise an interesting point on a potential non-cell autonomous role for PCP proteins that are expressed in the basal layer and affect the suprabasal layers, which is an area for further investigation into the relationships between the different layers of the epidermis.

Below are more specific comments:

1. Fig. 1A: The examples of squamous suprabasal cells shown by the black arrows coincide with the troughs of epidermal ridges, which are hallmarks of the morphogenetic processes that occur in the skin after E15.5. The suprabasal cell shape was not quantified to support the author's conclusion regarding the correlation with the basal cell height.

2. Fig. 1D: The orientation of the section shown appears slanted and may impact on the thickness of the epidermis and cell shapes.

3. Damen *et al.* 2021 is not correctly cited in reference to the roles of asymmetric cell division in the later stages of epidermal development. The author's conclusion in this article is more focused on the roles of delamination than asymmetric cell division per se.

4. Perhaps a typo in reference to Box *et al*. 2019 analyzing Fzd6 mutants, when the authors likely mean Vangl2 mutants instead.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 08 Jan 2023

Caroline Formstone

I would like to thank the reviewer for their helpful comments which have significantly improved our manuscript. I apologise for the delay in submitting the revised manuscript. Based on reviewer 2 comments, whilst analysing the data for the individual embryos I noticed that one *Celsr1-/-* embryo was very different to the other two and was concerned about a mix up with wild-types and so I asked for more *Celsr1-/-* litters from my collaborator. I had then to perform the histology which has taken me some time. The new litters revealed further similar embryos demonstrating that there is variability between embryos in this mutant. The Figures and text have been altered accordingly. Based on comments from both reviewers we have made additions to Figures and added two further Figures.

General comments. We understand your concerns and provided a shorter title which we feel represents the overall message of the revised manuscript. We agree with the reviewer that a more thorough investigation of cell shape correlation between basal and suprabasal layers is required. These studies are underway at younger embryonic stages in wholemounted skins. We thank the reviewer for recognising the importance of the potential non-autonomous role for PCP proteins in suprabasal morphogenesis. *Specific comments*

1. Thank you for suggesting this improvement. Robust measurement of suprabasal cell height in late-stage wild-type skin from wax sections *in vivo* was found to be impossible however as cells are highly squamous in many images and we cannot distinguish individual cells. We could make these measurements at E15.5 *in vivo* and thus we have added in a quantification for height of suprabasal cells for explant of E15.5 skin into lumox dishes. These data have been included in the new Fig.3F.
2. We acknowledge the concerns of the reviewer and have remade all the measurements excluding any sections where there was any concern about tissue artefacts or tissue distortions. We have included a statement to this effect in the methods.
3. We apologise for any error in our interpretation of the data from Damen et al, 2021 and we have removed the statement from the revised version.
4. The data mentioned for *fz6* mutants is in the supplementary Figures of Box et al, 2019 and we have now clarified this in the text.

Competing Interests: No competing interests were disclosed.