EPITHELIAL GEOMETRY REGULATES SPINDLE ORIENTATION
AND PROGENITOR FATE DURING FORMATION
OF THE MAMMALIAN EPIDERMIS

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ABSTRACT

The ability to control cell fate through oriented division is imperative for the proper development of many organs, such as the stratified epidermis. Basal progenitor cells of the mammalian epidermis orient their divisions parallel or perpendicular to the basement membrane to expand the stem cell pool or produce differentiated stratified layers. Although any given basal cell is capable of either division orientation, the mechanisms that regulate the choice between division orientations are unknown. Here, using time-lapse imaging to follow divisions and fates of basal progenitors, we investigate the role of planar cell polarity (PCP) and tissue architecture during epidermal stratification. We find that embryos defective for the core PCP gene, Vangl2, exhibit increased perpendicular, asymmetric divisions at the expense of planar, symmetric divisions, but surprisingly, this is not due to defective Vangl2 function in the epidermis. We link this reduction in planar divisions to alterations in cell geometry and packing, which are indirectly caused by the neural tube closure defects characteristic of planar cell polarity mutants. We demonstrate that early in epidermal stratification, there is a close relationship between cell density, interphase cell height-to-width aspect ratio, and mitotic spindle orientation. These data show that basal epidermal cells utilize packing and shape, rather than cortical PCP cues, to inform division orientation. We propose a model in which local tissue architecture regulates the decision between symmetric and stratifying divisions, a mechanism that allows flexibility for basal stem cells to adapt to the needs of the developing tissue.
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CHAPTER 1: Introduction
Structure and Development of the Epidermis

The mammalian skin is a complex organ that serves as a protective barrier against an organism’s external environment. It constantly defends against infection and UV radiation, withstands mechanical stresses, and prevents trans-epidermal water loss. In conjunction with the underlying dermis, it also plays important roles in thermoregulation, touch perception, and wound healing. The skin is comprised of three distinct but interdependent compartments: the epidermis, dermis, and subcutaneous fat (Figure 1).

The epidermis is the outermost protective covering. The intermediate skin layer is the dermis, which contains many different cell types involved in a variety of biological processes. The deepest layer of skin is subcutaneous fat, connecting the dermis to skeletal muscle and providing insulation and fat storage (Gould, 2018). The dermis, in addition to providing elasticity to the skin, is home to a variety of cell types and appendages such as sweat glands and hair follicles. Sensory neurons innervate much of the skin, enabling the ability to sense stimuli such as heat, pressure, or pain (Gould, 2018; Hsu et al., 2014). A vast array of immune cells interact with both the dermis and epidermis, playing important roles in wound healing and protection from infectious microorganisms. Blood vessels and vasculature coursing through the dermis provide a supply of nutrients and hormones.
Figure 1. Mammalian Skin (adapted from Julie Gould, Nature Outlook 2018)

The skin is a large and complex organ, organized in three main compartments: the epidermis, dermis, and subcutaneous fat. Each compartment plays an essential role in the structure and function of the skin. The epidermis is the organism’s first line of defense against harmful microbes and mechanical stresses. The dermis provides elasticity, and houses sweat glands, blood vessels, and hair follicles. Subcutaneous fat allows insulation and energy storage. Most of the mammalian skin is hairy skin, while glabrous skin can be found on the palms of the hands and soles of the feet.
The epidermis is comprised of four main layers: the basal layer, spinous layer, granular layer, and stratum corneum. Keratinocytes begin in the basal layer as progenitor cells, which divide and/or differentiate to maintain the tissue. As these cells terminally differentiate, they move upward through the stratified layers until they are eventually sloughed off at the surface.
The outermost compartment of the skin, the epidermis, is itself organized into four distinct layers comprised mainly of keratinocytes. The basal layer, spinous layer, granular layer, and stratum corneum are defined by differences in cell shapes, adhesions, and gene expression profiles (Figure 2).

The basal layer of progenitor cells are stem cells specific to the skin, and are indispensable for its development, structure, and function. These cells express high levels of keratins K5 and K14, which form a network of filaments that allow them to withstand external mechanical forces. They also express high levels of integrins in order to connect to the basement membrane separating the epidermis from the dermis (Fuchs and Green, 1980). Basal cells give rise to the other layers of the epidermis through two mechanisms: oriented cell division (further discussed below) and delamination, a process by which cells detach from the basement membrane and migrate upward through the skin’s stratified layers (Watt and Green, 1982).

As keratinocytes enter the spinous and granular layers, they undergo a molecularly-defined process of terminal differentiation. This includes a switch to expression of keratins K1 and K10, forming an even more complex arrangement of filamentous cables (Fuchs and Green, 1980), as well as a decrease in proliferation. Previous work has shown transcriptional alterations of suprabasal cells is dependent on the Notch signaling pathway (Pan et al., 2004; Williams et al., 2011). The Notch intracellular domain (NICD), a transcriptional activator, exhibits nuclear localization in suprabasal cells, and induction of constitutive Notch signaling in the basal layer greatly increases the production of spinous layers (Blanpain et al., 2006). Additionally, experimental knockdown of the NICD binding partner results in impaired epidermal
differentiation (Williams et al., 2011). Differentiating keratinocytes also form strong intercellular adhesions called desmosomes, and adopt a more squamous morphology. Finally, as these cells entire the stratum corneum, they extrude their cellular organelles and form a dense layer of dead cornified sacs. This layer is continuously sloughed off and replaced by cells originating from the basal layer. The cycle of new cell production driven by basal progenitors coupled with terminal differentiation replenishes the skin during its natural turnover. In addition, basal cells must proliferate, migrate, and differentiate to restore tissue integrity after wounding (Fuchs, 2007). While sufficient proliferation and stratification are necessary to ensure proper barrier function, they must be kept in careful balance to prevent hyperproliferative skin disorders such as psoriasis and cancers (Fuchs, 2007; Schon, 1999).

Unlike cells in simple single-layered epithelia, which divide exclusively within the plane of the tissue, basal epidermal cells can divide in either a planar orientation, parallel to the underlying basement membrane, or they can divide perpendicularly (Figure 3). These two division orientations are tightly linked to a defining feature of stem cells: the ability to self-renew, and the potential to create differentiated cell types. Planar cell divisions are self-renewing and symmetric, in that they generate two basal daughter cells. Planar divisions are necessary to conserve and expand the population of basal progenitors. Perpendicular divisions generate one basal and one suprabasal daughter cell, which will differentiate and contribute to the stratified layers of the tissue. These divisions generate both a molecular and positional asymmetry in the two daughter cells. The basal daughter maintains contact with the basement membrane and signaling factors, and as a result remains a basal stem cell. The suprabasal daughter loses these contacts,
inherits apically localized factors, and begins differentiation (Lechler and Fuchs, 2005). The ability of basal epidermal cells to conduct both types of division orientations is imperative for the proper formation and maintenance of the tissue. For example, too many planar divisions prevents sufficient stratification, leading to defective barrier formation. Conversely, too many perpendicular divisions can deplete the progenitor pool (Niessen, et al., 2013; Williams et al., 2011). How these symmetric and asymmetric divisions are balanced during formation of the epidermis is not understood.

Figure 3. Basal epidermal cells proliferate to grow and maintain the epidermis

Basal stem cells of the epidermis can divide in two orientations: parallel to the epithelial plane, or perpendicular to it. Planar, symmetric divisions produce two basal daughter cells, which expands the stem cell pool and facilitates tissue growth. Perpendicular, asymmetric divisions produce a suprabasal daughter cell that will differentiate and contribute to tissue stratification. A/P mark the anterior/posterior axis.
Early in murine embryonic development, at approximately embryonic day (E) 8.5, a sheet of ectodermal progenitors becomes specified to adopt epidermal fate, and the skin remains a single layer of multipotent epithelial cells that primarily undergo planar division orientations until E12.5 (Lechler and Fuchs, 2005; Smart, 1970). As the epidermis continues to develop, it transforms into a stratified and differentiated epithelium with complete barrier function by E17.5 (Hardman et al., 1998; Kulukian and Fuchs, 2013; Muroyama and Lechler, 2012). Stratification initially begins between E13.5-E15.5, where oblique and perpendicular divisions produce the first few skin layers. While this balance of oriented cell division is imperative for proper epidermal formation, what regulates division orientations early in epidermal development remains undetermined.
**Oriented Cell Division in Organ Formation**

Oriented cell division is a fundamental mechanism through which multicellular organisms build complex tissue architecture (Bergstralh, et al., 2017; Gillies and Cabernard, 2011). By controlling the angle of the mitotic spindle a cell can position its daughters to facilitate tissue expansion, establish multiple cell layers, or generate asymmetric fates. The proper development of many different organs and tissues relies on the ability of their constituent cells to effectively orient their mitoses. One relatively simple type of tissue growth that depends on directed cell division is planar expansion of an epithelial sheet, often in an anisotropic manner (Panousopoulou and Green, 2014). For example, the proximal-distal growth of the Drosophila wing disc is facilitated by oriented cell divisions aligned with that axis (Baena-Lopez et al., 2005). During Xenopus epiboly, the surface ectoderm thins and spreads to cover the embryo. For this process to occur, cell divisions are oriented exclusively parallel to the embryo surface to promote the planar spread of the tissue (Woolner and Papalopulu, 2012).

Other types of oriented tissue growth include thickening through stratification, and lumen formation. As previously mentioned, the mammalian epidermis is one type of tissue whose stratification is largely driven by cell divisions oriented perpendicularly to the basement membrane (Lechler and Fuchs, 2005; Poulson and Lechler, 2010; Williams et al., 2011; Williams et al., 2014). As neurogenesis begins in the mammalian cortex, cell divisions switch from exclusively planar orientations to vertical, thus allowing radial thickening of the cortex (Gotz and Huttner, 2005). Oriented cell division is also important for the formation of epithelial tubes, one of the most common structures of epithelial
organs. As the kidney develops, epithelial cells must generate tubules with central lumens in order to function properly. These tubules are comprised of a single layer of cells, with their apical surfaces oriented toward the luminal space. To preserve lumen continuity, epithelial cells orient their mitotic divisions orthogonal to the apical-basal axis (Gao et al., 2017).

Two primary mechanisms are known to direct the orientation of cell division: intrinsic cues that couple the mitotic spindle to cell polarity, and extrinsic factors that guide spindle alignment relative to physical or chemical cues (di Pietro et al., 2016). The Drosophila ovarian stem cell niche is one well-studied example of asymmetric cell division influenced by cell-extrinsic factors. Short-range signaling molecules from the surrounding niche cells impact germline stem cell division and determine the fates of the resulting daughter cells – one cell maintains its contact with the niche, and therefore its stemness, while the other daughter cell exits the niche and beings a program of differentiation (Knoblich, 2008).

In the epidermis, intrinsic polarity cues play an integral role in determining spindle orientation of proliferating basal cells to impact tissue structure. Late in epidermal development, these polarity cues orient perpendicular divisions through a conserved set of proteins that anchors spindle astral microtubules to the cell cortex: mInsc (mammalian Inscuteable), LGN, Gαi3, and NuMA (Figure 4) (Lechler and Fuchs, 2005; Poulson and Lechler, 2010, Williams et al., 2014). This mechanism of spindle orientation begins with the apically-localized polarity factors Par3 and atypical protein kinase C (aPKC) that induce downstream recruitment of the other spindle orienting factors. mInsc interacts with Par3 and promotes apical enrichment of LGN. Through a C-
terminal binding site, LGN can directly interact with membrane-anchored \( G\alpha_{i3} \), and through its N-terminus form a complex with NuMA. LGN is thought to act as a conformational switch, where in its closed form its termini interface with each other, and binding to either \( G\alpha_{i3} \) or NuMA promotes the open state of the protein for complex formation (Du and Macara, 2004; Willard et al., 2004). NuMA, the most downstream component of this pathway, contains a microtubule binding domain, and has been shown to exert mechanical tension on astral microtubules \textit{in vitro}. However, previous work discovered that this domain overlaps with its LGN binding site, preventing NuMA from simultaneously interacting with both LGN and microtubules (Du et al., 2002). Instead, NuMA is able to generate forces on microtubules in conjunction with dynactin, a microtubule motor protein (Lechler and Fuchs, 2005; Siller and Doe, 2009; Knoblich, 2010; Prehoda, 2009).
Late in epidermal development, apical polarity factors Par3 and aPKC induce apical enrichment of a series of spindle orienting components in order to conduct perpendicular division orientations. This LGN/NuMA-dependent spindle orienting pathway promotes epidermal stratification and skin barrier formation.
Together, these components are capable of influencing mitotic spindle positioning to conduct perpendicular cell divisions in the basal layer of the epidermis. Removal of any of these proteins alters spindle orientation, and in some cases overall tissue integrity. Removing mInsc, Gαi3, or Par3 function through genetic perturbation or RNAi results in a randomization of spindle orientation in the developing epidermis. Knockdown of either LGN or NuMA causes a shift toward primarily planar division orientations, and the reduction of perpendicular cell divisions eventually impairs proper skin barrier formation. Combined loss of both mInsc and Gαi3 produces a phenotype similar to that observed with loss of LGN, demonstrating the cooperativity of these proteins during perpendicular divisions (Williams et al., 2014).

Surprisingly, these factors are not required during the early phase of stratification (Williams et al., 2014). LGN localization is rarely detectable before E14.5, and loss of LGN at E15.5 does not alter division planes. Additionally, early induction of mInsc expression has no effect on division orientations prior to E16.5. What regulates division orientations early in epidermal development remains unknown. Moreover, the cues that direct planar division orientations in the epidermis have not been identified. While hard-wired spindle orientating programs are necessary for proper tissue assembly and the reproducible production of functional organs, cells also need flexibility to divide according to changing needs of the tissue. The mechanisms that ensure flexibility in spindle orientation during development are poorly understood.
Planar Cell Polarity

Given that apical-basal polarity cues direct perpendicular divisions to drive stratification, we hypothesized that planar, symmetric divisions may be controlled by polarity oriented along the epithelial plane, or planar cell polarity. Planar cell polarity (PCP) refers to the collective cell polarization across the sheet of an epithelial tissue, such as the anterior-posterior epidermal plane (Butler and Wallingford, 2017; Devenport, 2016; Goodrich and Strutt, 2011; Segalen and Bellaiche, 2009). Several striking examples of planar cell polarity can be found throughout nature, as demonstrated by the alignment of feathers on a bird, or the scales that line the body of a fish. Planar cell polarity also coordinates the organization of vital internal structures, as seen in the stereocilia bundles of the inner ear, the establishment of left-right asymmetry, and the synchronized cell migrations that occur during embryonic development (Curtin et al., 2003; Keller, 2002; Montcouquiol et al., 2003; Song et al., 2010; Wallingford et al., 2000; Wang et al., 2005). Because of its important roles in development, there are often severe consequences when PCP goes awry. Misaligned stereocilia bundles in the inner ear result in loss of hearing, and defects in convergent extension prohibit proper formation of the body axes (Curtin et al., 2003; Kim et al., 2010; Wang et al., 2006a,b). In mammals, a characteristic phenotype of several PCP mutants is failure to complete neural tube closure, and neural tube defects (NTDs) such as these are a common birth defect in humans (Kibar et al., 2001; Murdoch et al., 2001; Wallingford et al., 2013).

One of the most extensively characterized instances of PCP signaling is along the proximal-distal axis of the wings of Drosophila, where PCP acts to orient the wing hairs, actin-rich protrusions at the distal side of wing epithelial cells. Extensive experiments in
this system have discovered the existence of two planar cell polarity systems: the Fat-Dachsous pathway, and the “core” PCP pathway. The core PCP pathway relies on the asymmetric localization of several transmembrane components including Flamingo (Fmi; Celsr in vertebrates), Van Gogh (Vang; Van Gogh-like in vertebrates), and Frizzled (Fz), as well as cytoplasmic components Prickle (Pk), Dishevelled (Dsh), and Diego (Dgo). These components are initially distributed uniformly around the membrane. Previous work in Drosophila has dissected a complex series of inter- and intracellular interactions by which Fz, Dsh, and Dgo become localized to the distal side of the cell, while Vang and Pk preferentially localize to the proximal side (Figure 5). In contrast, Fmi is localized to both sides of the cell (Axelrod, 2001; Strutt 2001; Feiguin et al., 2001; Tree et al., 2002; Strutt 2001; Das et al., 2004).
Core PCP proteins are asymmetrically localized across cell borders. Celsr1 localizes to junctions at both sides of the cell. Vangl2 and Fz6 are exclusively localized to the anterior and posterior cell membranes, respectively. Celsr1 interacts with itself across cell-cell junctions. Positive interactions between Celsr1/Fz6 complexes and Celsr1/Vangl2 complexes reinforce the asymmetric distribution of PCP to propagate the polarity across cell distances.
This asymmetry is established and reinforced through both positive and negative feedback of these components. Fmi, an atypical cadherin, forms homotypic interactions across cell junctions and promotes recruitment of Fz and Vang to their respective edges (Chen et al., 2008; Strutt and Strutt, 2008; Struhl et al., 2012). The core cytoplasmic components also help amplify PCP protein asymmetry. Dsh, Dgo, and Pk exhibit both negative and positive feedback mechanisms. Overexpression of Pk has been shown to prevent membrane localization of Dsh (Carreira-Barbosa et al., 2003; Tree et al., 2002). Additionally, these cytoplasmic proteins can promote stable clusters of the transmembrane complexes to further strengthen asymmetry (Axelrod et al., 1998; Axelrod, 2001; Butler and Wallingford, 2017; Devenport, 2014; Jenny et al., 2003; Jenny et al., 2005; Strutt and Strutt, 2007; Strutt et al., 2011). Functional planar cell polarity relies on the presence and asymmetric localization of each of the core pathway proteins. Disruption or deletion of any component results in the randomization or abolishment of PCP and disorder in cellular structures that rely on PCP signaling.

In the murine epidermis, the PCP components Celsr1, Vangl2, and Fz6 localize to basolateral cell junctions preferentially along anterior-posterior edges (Aw et al., 2016; Devenport and Fuchs, 2008; Devenport et al., 2011). Unlike the Drosophila wing blade, where PCP components determine the alignment of the single-cell wing hair, planar cell polarity signaling in the epidermis is propagated and coordinated across great cell distances to instruct the downward growth of multicellular hair follicles (Figure 6). These follicles originate from the epithelium where they bud into the underlying dermis, and continue their downward growth with an anteriorly-directed tilt, so that the emerging hair of the animal points toward its posterior (Devenport and Fuchs, 2008).
Figure 6. Example of planar cell polarity in mammalian skin

In mammalian skin, PCP signaling acts to orient the polarized growth of complex multicellular hair follicles. Hair follicle orientation can serve as a readout for PCP function. In the absence of PCP, hair follicles become randomly misoriented or point straight downward.
The establishment of PCP protein asymmetry in the epidermis coincides with the onset of stratification, and mutation or deletion of these components confers phenotypes of varying severity. They include, but are not limited to, a randomization of hair follicle polarization (Fz6) or hair follicles that point straight downward, accompanied by severe open neural tube defects (Celsr1 and Vangl2) (Cetera et al., 2017; Devenport and Fuchs, 2008; Guo et al., 2004). We had previously noted that embryos harboring a homozygous mutation in Vangl2 (Vangl2<sup>LP</sup>) display increased epidermal thickness, suggesting a role for PCP in planar division orientations. The goal of the research presented here was to investigate the mechanisms underlying the relationship between PCP and mitotic spindle orientation, and to understand the cellular dynamics that dictate the alternation between planar and perpendicular division orientation in the developing epidermis.
CHAPTER 2: Results
**Introduction**

Using live imaging to trace the orientation and positional fate of basal cell divisions, we investigated the function of PCP and tissue mechanics early in epidermal stratification. In agreement with the hypothesis that PCP promotes planar basal cell divisions, we found that Vangl2 mutants display an increased proportion of stratifying divisions at the expense of planar divisions. Unexpectedly, this is not a direct result of defective PCP; rather, we show that failure of the epidermis to close over the neural tube in these mutants leads to cell crowding within the basal layer, concomitant with changes in cell width and height and a higher frequency of asymmetric cell divisions. Combining our live imaging approach with tissue-specific genetic perturbations and mechanical tissue manipulations, we have found that early in epidermal development, interphase 3D cell geometry and packing, rather than cortical PCP cues, influence mitotic spindle orientation. Although it is well established that cell divisions align with the interphase long axis in two dimensions, our data show that this mechanism can also impact spindle alignment in the third, apical-basal, dimension in a stratifying epithelium. We propose that by responding to changes in cell density and shape, basal cell division orientations can adjust to satisfy the needs of the tissue.

**Basal progenitor cells in Vangl2^{Lp/Lp} embryos undergo fewer planar cell divisions**

Previously, the relationship between division orientation and epidermal stratification was primarily inferred from observations made in fixed tissue sections. This method comes with some key disadvantages: it excludes divisions oriented orthogonal to the plane of sectioning and does not allow one to directly correlate mitotic spindle
alignment with the final positions of daughter cells. To overcome these limitations, we performed time-lapse imaging of live, flat-mounted skin explants expressing a nuclear marker, K14-H2B-GFP (Tumblar et al, 2004), which allowed us to observe cell divisions dynamically in three-dimensional space (Figure 7A-C). Z-stacks were captured every 10 minutes over approximately 8 hours of epidermal development and division planes were quantified by measuring the angle between the centroids of the two daughter nuclei during telophase. Divisions were categorized as planar (\(\Theta \leq 20^\circ\)), oblique (20° < \(\Theta \leq 70^\circ\)) or perpendicular (70° < \(\Theta \leq 90^\circ\)) depending on their orientation relative to the plane of the basal layer. Interestingly, we observed that chromatids aligned along the metaphase plate continued to rotate prior to separating at anaphase/telophase. Because of this instability in spindle alignment during metaphase, division planes were measured only after chromatid separation.

In wildtype explants at E14.5, approximately half of all basal cell divisions were oriented parallel to the epithelial plane (51% ± 6%). However, this proportion was reduced in explants from Vangl2\(^{Lp/Lp}\) mutants (27% ± 4% planar divisions), which exhibited an increased frequency of oblique and perpendicular divisions (Figure 7D, E). Importantly, division orientations observed by live imaging mirrored the distribution in fixed whole mount epidermis, confirming that our explant imaging system reflects division behavior in vivo (Figure 7F). The altered distribution of division orientations at E14.5 suggested a potential role for PCP in promoting planar basal cell divisions in the epidermis.

To explore trends in cell division orientation over developmental time, we quantified division planes in fixed whole mount skins across multiple time points from
E13.5 – E16.5 (Figure 7F). Consistent with previous reports, we observed a strong bias toward planar division orientations in wildtype embryos at E13.5, when the epithelium is predominantly comprised of a single layer (Lechler and Fuchs, 2005; Williams et al., 2014). At E15.5 and E16.5, the proportion of oblique and perpendicular divisions increased, coinciding with the expansion of stratified epidermal layers. However, our data show a much higher proportion of planar and oblique divisions at E16.5 compared to prior reports, which we attribute to differences in observing divisions in whole mount versus sagittal tissue sections, as planar and oblique divisions that align out of the plane of sectioning are likely to go uncounted (Asare et al., 2017; Lechler and Fuchs, 2005; Luxenburg et al., 2011; Niessen et al., 2013; Poulson and Lechler, 2010; Williams et al., 2011; Williams et al., 2014). Interestingly, Vangl2 mutant embryos displayed elevated oblique and perpendicular divisions at E13.5 and E14.5 but not at later embryonic stages, suggesting the requirement for PCP function in promoting planar divisions is restricted to an early developmental window of time corresponding to the onset of epidermal stratification.
Figure 7. Basal cell division orientations in wildtype and Vangl2 mutant embryos
Figure 7. Basal cell division orientations in wildtype and Vangl2 mutant embryos

(A) Schematic of E14.5 skin depicting several planar cell polarity components and example division orientations. Dotted line represents focal plane for live imaging. (B, C) Stills from time-lapse movies of Vangl2<sup>WT</sup> E14.5 skin explants expressing K14-H2B-GFP, showing examples of planar (a) and perpendicular (b) division orientations. Top panels are the planar view of the basal layer of the epidermis, bottom panels are XZ dimension. Scale bar = 10µm. See also Movie S1 and Movie S2. (D) Example and quantification of division angles in live epidermal explants at E14.5. Vangl2<sup>WT</sup>, n = 310 divisions pooled from three embryos. Vangl2<sup>Lp/Lp</sup>, n = 271 divisions from three embryos. Modified Kuiper Test, p < 0.0001. (E) Distribution of division orientations in live epidermal explants at E14.5. Planar: Θ<20°, unpaired two-tailed t-test p = 0.027; oblique: 20°<Θ<70°, p = 0.030; perpendicular: 70°<Θ<90°, p = 0.427. n = 3 explants from each genotype. (F) Angular frequency of division angles quantified from fixed whole-mount skins dissected from embryos e13.5 – e16.5. E13.5: Vangl2<sup>WT</sup>, n = 208 divisions from three embryos; Vangl2<sup>Lp/Lp</sup>, n = 198 divisions from two embryos; p < 0.0001. E14.5: Vangl2<sup>WT</sup>, n = 164 divisions from three embryos; Vangl2<sup>Lp/Lp</sup>, n = 166 divisions from three embryos; p < 0.0001. E15.5: Vangl2<sup>WT</sup>, n = 152 divisions from three embryos; Vangl2<sup>Lp/Lp</sup>, n = 116 divisions from three embryos; p = 0.3482. E16.5: Vangl2<sup>WT</sup>, n = 132 divisions from three embryos; Vangl2<sup>Lp/Lp</sup>, n = 115 divisions from three embryos; p = 0.1785.
Distinct epidermal compartments exhibit markedly different distributions of division orientations

While the majority of our time-lapse imaging experiments aim to observe cellular dynamics and division in the interfollicular region of the basal layer of the epidermis, we also captured information from two other distinct compartments: the initial suprabasal layer, and early hair follicle placodes. Interestingly, at E14.5 both of these regions exhibit differences in division orientations that potentially contribute to their specific developmental needs. Suprabasal cells frequently divided during this time, in almost exclusively planar orientations (95% of 155 divisions, Figure 8). In the early placode, however, basal cells favored more oblique and perpendicular division orientations over planar ones (11% planar of 82 divisions). These data suggest that cells residing in different epidermal compartments are capable of adjusting their division orientations to promote the tissue architecture specific to that region. Suprabasal cells divide in planar orientations perhaps to encourage the rapid formation of early stratified layers, while cells in the placode contribute to bud invagination through perpendicularly oriented mitoses.
**Figure 8. Division orientations in different epidermal compartments**

Distribution of division orientations in live epidermal explants at E14.5. (A) n = 155 divisions from three explants. (B) n = 82 divisions from three explants.

**Division plane is a strong predictor of the positional fate of daughter cells**

After each cell division occurs in the epidermis, the final fates and positions of the two daughter cells influence the stratified architecture of the developing skin. Daughter cells with suprabasal positioning differentiate and contribute to the skin’s upper stratified layers. Daughters with basal positioning retain progenitor fate and continue to divide. Previously, the fates of daughter cells were inferred by their division planes as measured in fixed tissue sections. To directly assess how a basal cell’s division plane relates to its positional fate within the tissue, we employed our live imaging system to track recently divided cells and assign each cell a final positional fate. Fates were defined as symmetric when both daughter cells remained in the basal layer for at least 1.5 hours following division. Asymmetric fates were scored when one daughter cell remained basal while the other cell moved suprabasally (Figure 9A-C). We found that in wildtype embryos at
E14.5 division orientation and cell fate were tightly linked. For example, 94% of cells that divided within 20 degrees of the epithelial plane adopted symmetric fates (Figure 9A, blue points). Similarly, 89% of cells that divided within 70-90 degrees of the plane adopted asymmetric fates (Figure 9A, green points). Daughter cells resulting from oblique divisions, by contrast, had a roughly equal chance of becoming symmetric or asymmetric (41% symmetric versus 59% asymmetric) (Figure 9A, magenta points). In Vangl2Lp/Lp embryos, however, the relationship between division orientation and final position was shifted toward asymmetric. Only 78% of planar divisions, and 21% of oblique divisions resulted in symmetric positional fates (Figure 9B, C).

Notably, we did not observe delamination of basal cells into the suprabasal layer. Although we were not specifically seeking to quantify delamination events in this study, it is striking that we did not observe them in our time-lapse experiments. In contrast to previous studies suggesting early epidermal stratification is driven predominantly by basal cell delamination (Miroshnikova et al., 2018; Wickstrom and Niessen, 2018; Williams et al., 2014), our results suggest that early stratification results from oblique and perpendicular divisions that generate suprabasal daughters.

To investigate how altered division planes contribute to skin structure in PCP mutants, we quantified epithelial thickness over the course of epidermal development. The epithelium of Vangl2 mutants was measurably thicker than control embryos as early as E15.5, and this difference increased by E16.5. At E18.5, Vangl2 mutant embryos continue to display significantly thicker skin than their wildtype littermates, suggesting the early bias toward oblique and perpendicular divisions, together with a preference for
asymmetric cell fates, may contribute to longer-term effects on epidermal structure (Figure 9E-H).

Figure 9. Positional cell fates correlate with division orientation
**Figure 9. Positional cell fates correlate with division orientation**

(A, B) The relationship between division angle and final cell positions. Daughter cells were followed for 1.5 hours after cell division, and assigned a positional fate: symmetric or asymmetric. Each dot represents a single division event. (C) Representation of the same data in (a,b), showing the distribution of fates for each division orientation. For planar divisions: symmetric fates = 94% [Vangl2\(^{WT}\)], 78% [Vangl2\(^{Lp/Lp}\)]. Oblique divisions: symmetric fates = 41% [Vangl2\(^{WT}\)], 21% [Vangl2\(^{Lp/Lp}\)]. Perpendicular divisions: asymmetric fates = 89% [Vangl2\(^{WT}\)], 94% [Vangl2\(^{Lp/Lp}\)]. (D) Combining all division orientations, Vangl2\(^{Lp/Lp}\) embryos display an overall bias toward asymmetric final cell positions. Unpaired two-tailed t-test, \(p = 0.017\). For (a-d), \(n = 284\) divisions pooled across three Vangl2\(^{WT}\) embryos; \(n = 238\) divisions pooled from three Vangl2\(^{Lp/Lp}\) embryos. (E) Sagittal sections of E18.5 skin from Vangl2\(^{WT}\); K14-H2B-GFP and Vangl2\(^{Lp/Lp}\); K14-H2B-GFP embryos. Involucrin (red) labels the outer stratified layers and nuclei are labeled with Hoechst. Scale bar = 50\(\mu\)m. (F) Quantification of skin thickness at E18.5. \(n = 10\) images for each of three embryos per genotype. Bars represent means of the three embryos, error bars are SEM. Unpaired two-tailed t-test, \(p = 0.017\). (G) XZ panels from whole-mount images of Vangl2\(^{WT}\) and Vangl2 mutant skins at E14.5 – E16.5, expressing membrane-tdTomato or immunostained for E-Cadherin. Yellow dotted lines outline the outermost epidermal layer. Scale bars = 10\(\mu\)m. (H) Quantification of skin thickness at E14.5 – E16.5. \(n = 30\) measurements per genotype per stage. Bars represent means, error bars are SD. E14.5: unpaired two-tailed t-test, \(p = 0.9340\); E15.5: unpaired two-tailed t-test, \(p = 0.0003\); E16.5: unpaired two-tailed t-test, \(p < 0.0001\).
Early in epidermal development, LGN localization does not correlate with planar spindle positioning

Previous work exploring the mechanisms underlying oriented cell division in the murine epidermis has shown that late in epidermal development, apical-basal polarity factors localize several spindle-orienting components, such as LGN. These components serve to position the mitotic spindle to promote perpendicular, asymmetric basal cell divisions (Lechler and Fuchs, 2005; Williams et al., 2011; Williams et al., 2014). We reasoned that PCP factors might recruit these components to the lateral cortex to orient planar divisions, and that a failure of LGN to localize laterally might explain the increased oblique and perpendicular divisions observed in Vangl2 mutants. After confirming prior reports of apical LGN localization in cells with perpendicularly-oriented spindles at E16.5 (Figure 10A), we characterized the localization of this protein in dividing cells at E14.5. At this earlier stage, we observed apical LGN in a subset of cells undergoing oblique and perpendicular divisions. However, in cells undergoing planar divisions, LGN exhibited lateral localization in only 14% of cells, and was most frequently undetectable (61% of planar divisions; Figure 10B, C). These patterns of LGN localization are consistent with those previously observed in thin tissue sections by Williams et al. Together with the observation that LGN knockdown leads to an increase in planar divisions (Williams et al, 2014), these data suggest that this spindle-orienting pathway is not the primary mechanism promoting planar basal cell divisions.

We also explored the possibility that the observed increase in oblique/perpendicular divisions and asymmetric cell fates in Vangl2Lp/Lp mutants is caused by more frequent apical LGN localization. However, the occurrence of apical
LGN (47.7% of divisions) was not sufficiently increased compared to Vangl2\textsuperscript{WT} (41.1% of divisions) to explain the altered division orientations (Figure 10D, E). These data are consistent with the LGN-depended spindle orienting pathway acting predominantly at E16.5 and later stages, and that alternative mechanisms must influence division planes at earlier developmental stages.

Although LGN localization does not correlate with early planar spindle orientation or explain the altered division orientations in the Vangl2 mutant, we did observe a temporal trend in LGN localization, consistent with prior models that over time, the LGN-dependent spindle orienting pathway is increasingly responsible for informing perpendicular division orientations (Williams et al., 2014). In wildtype skin explants at E14.5, LGN was apically localized in only 60% of oblique/perpendicularly dividing cells. At E15.5, the percentage of oblique and perpendicular divisions with apical LGN increased to 79% of cells. By E16.5, 83% of cells dividing in these orientations displayed apical LGN (Figure 10F). In agreement with previous reports, this suggests that an alternative mechanism influences division plane early in epidermal growth until the LGN/NuMA molecular pathway becomes a more primary factor to promote tissue stratification.
Figure 10. LGN localization does not determine planar division orientation
Figure 10. LGN localization does not determine planar division orientation

(A) Example image of apical LGN localization in a Vangl2<sup>WT</sup> E16.5 embryo. Shown is a mitotic cell in metaphase, poised to divide perpendicularly. In all images, Hoechst labels the nuclei, cell membranes are marked with E-Cadherin (red) and LGN is white. Yellow dashed lines outline the dividing cell, and yellow arrowheads mark LGN localization. (B) Examples from Vangl2<sup>WT</sup> embryos of cells dividing in a planar orientation with unilateral (left) and absent (right) LGN localization. In all images, Hoechst labels the nuclei, cell membranes are marked with E-Cadherin (red) and LGN is white. Yellow dashed lines outline the dividing cell, and yellow arrowheads mark LGN localization. (C) Frequency of LGN localization patterns in planar cell divisions in Vangl2<sup>WT</sup> embryos at E14.5. n = 36 cells (metaphase-telophase). ‘Mislocalized’ LGN refers to a detectable signal that does not correlate with division plane (ie, basal in a perpendicularly dividing cell). (D) Examples from Vangl2<sup>ΔTm/Lp</sup> embryos of cells dividing in a perpendicular orientation with apical (left) and absent (right) LGN. (E) Frequency of LGN localization patterns in all dividing cells in Vangl2<sup>WT</sup> and Vangl2<sup>ΔTm/Lp</sup> embryos at E14.5. n = 99 and 107 cells, respectively (metaphase-telophase). All scale bars = 10µm. (F) LGN localization patterns in oblique and perpendicularly dividing cells from E14.5 – E16.5. E14.5: n = 63 divisions; E15.5: n = 36 divisions; E16.5: n = 41 divisions.
Cell division orientation is correlated with basal cell geometry

Since division planes at E14.5 did not correlate with localization of a known epidermal spindle-orienting cue, we hypothesized that basal cells might instead divide according to cell geometry. Hertwig’s Rule, also known as the ‘long axis rule,’ states that a cell is most likely to divide along its longest interphase axis, but this has been shown mainly in cells dividing within a two-dimensional plane (Bosveld et al., 2016; Thery and Bornens, 2006; Thery et al., 2005; Wyatt et al., 2015). In the epidermis, where basal cells divide in three-dimensional space, a cell’s longest axis may lie perpendicular to the epithelial plane, which could promote perpendicular spindle alignment and asymmetric division (Chalmers et al., 2003; Xiong et al., 2014). Indeed, we noted that basal cells appeared taller and narrower in Vangl2 mutant embryos (Figure 11A) which, according to Hertwig’s Rule, might account for the observed increase in asymmetric divisions. To test this idea we first quantified basal cell area and height in fixed whole mount explants from E14.5 wildtype and Vangl2Lp/Lp embryos. Compared to Vangl2WT embryos, basal cells in Vangl2Lp/Lp mutants were significantly smaller in cross-sectional area along the planar axis and taller along the apical-basal axis (Figure 11A-C). This increase in height:width ratio was not accompanied by changes in the number of neighbors, as Vangl2Lp/Lp and wildtype embryos displayed a similar distribution of cells with 4,5,6,7, and 8 edges (Figure 11D). Basal cells in Vangl2Lp/Lp embryos were also packed more densely into the basal layer compared to wildtype (258 cells vs 215 cells per 1200μm² field of view) (Figure 11E), but this increase in cell density was not due to elevated proliferation (Figure 11F). Thus, the geometrical differences observed in Vangl2Lp/Lp mutants are defined not by differences in cell sidedness, but rather by a change in height-
to-width aspect ratio. We observed similarly altered cell geometries in Vangl2 mutants at E13.5, when the proportion of oblique and perpendicular divisions is also increased. However, we did not observe such differences at E15.5, when division planes are similar to wildtype (Figure 12), perhaps because elevated asymmetric divisions at earlier stages relieves basal cell crowding. Together these data show that early in epidermal development, Vangl2\textsuperscript{Lp/Lp} cells are more crowded and elongated along their apical-basal axes and, according to Hertwig’s Rule, may be more likely to divide in an oblique or perpendicular orientation.

After observing this tissue-wide correlation between 3D cell geometry and cell division orientations, we wanted to more directly assess this relationship on a per-cell basis. We therefore performed live imaging of E14.5 skin explants expressing either a membrane-GFP or membrane-tdTomato marker (Muzumdar et al., 2007), which allowed us to measure cell geometry in interphase and observe that same cell’s division orientation. Confirming what has previously been shown for simple epithelia dividing in a two-dimensional plane, we found that basal cells dividing within the plane of the epidermis do so along their longest XY axis (Figure 11G). We also observed a modest but significant relationship between division plane and height:width aspect ratio along the apical basal axis, wherein cells that divided in a planar orientation tended to have smaller height:width ratios (average = 0.8307) compared to cells that divided perpendicularly (average height:width ratios = 1.275) (Figure 11I). This relationship was also observed in Vangl2 mutant embryos (Figure 11H, J), where perpendicular divisions were associated with larger height:width ratios. These results suggest that during the early phase of epidermal stratification, the decision to divide in a planar or perpendicular orientation is
influenced by a cell’s three-dimensional geometry. Furthermore, these results also show that basal cells can still divide according to their geometrical state in the absence of PCP function.

Figure 11. Cell division orientation correlates with basal cell geometry
(A) Representative images of basal layer labeled with E-Cadherin from E14.5 Vangl2WT and Vangl2Lp/Lp embryos. (B) Quantification of cross sectional areas of basal cells. Each dot represents average area of all cells in a single field of view. n = 10 total fields of view from three embryos per genotype. Bars are mean with SEM. Unpaired two-tailed t-test, p = 0.0057 (C) Quantification of cell height along apical-basal axis. 300 cells were measured from across 3 embryos per genotype. Whiskers indicate minimum and maximum values, + indicates the mean. Unpaired two-tailed t-test, p < 0.0001. (D) Quantification of distribution of polygon classes, as defined by the number of each cell’s neighbors. n = 10 total fields of view from three embryos per genotype. Error bars denote SD. (E) Quantification of cell density, as number of cells per field of view (1200μm²). Each dot represents a single field of view. n = 10 fields of view across three embryos per genotype. Bars are mean with SD. Unpaired two-tailed t-test, p = 0.0001. (F) Proliferation rates, quantified as the number of mitotic cells in a field of view as a percentage of total number of cells. n = 10 fields of view across three embryos. Whiskers denote min-max values, mean shown as ‘+.’ Unpaired two-tailed t-test, p = 0.9987. (G) Still images from a time-lapse movie of E14.5 Vangl2WT ; K14-Cre; mTmG explant showing an example of a basal cell dividing within the plane of the epidermis, along its longest interphase axis. Red line denotes orientation of the longest XY axis of the cell. (H) Still images from a time-lapse movie of an E14.5 skin explant from Vangl2ΔTm/Lp ; mTmG embryo showing a cell dividing in a perpendicular orientation. Daughter cells are marked with asterisks in the XZ view. (I, J) Relationship between height:width aspect ratio and division orientation. H:W ratios were measured from interphase cells at the time just before the onset of mitotic rounding. Width is defined as the longest planar axis of the cell. Each dot corresponds to a single division event, and divisions were binned according to the angle of the division plane in cytokinesis. Bars are mean with SD. (I) n = 72 divisions pooled from three E14.5 embryos. One-way ANOVA, p < 0.0001. Tukey’s Multiple Comparison Test: planar vs oblique, p < 0.05; planar vs perpendicular, p < 0.05; oblique vs perpendicular, p > 0.05. (J) n = 66 divisions from two E14.5 Vangl2ΔTm/Lp embryos. One-way ANOVA, p <0.0001. Tukey’s Multiple Comparison Test: planar vs oblique, p < 0.05; planar vs perpendicular, p < 0.05; oblique vs. perpendicular, p < 0.05. All scale bars = 10μm.
Figure 12. Cell shape quantifications at E13.5 and E15.5

(A) Representative images of basal layer labeled with membrane-tdTomato from E13.5 Vangl2\textsuperscript{WT} and Vangl2\textsuperscript{Lp/\Delta Tm} embryos. (B) Quantification of cross sectional areas of basal cells. Each dot represents average area of all cells in a single field of view. n = 12 total
fields of view from three Vangl2<sup>WT</sup> embryos and two Vangl2<sup>Lp/ΔTm</sup> embryos. Bars are mean with SEM. Unpaired two-tailed t-test, \( p < 0.0001 \). (C) Quantification of cell height along apical-basal axis. \( n = 300 \) cells from across three Vangl2<sup>WT</sup> embryos and two Vangl2<sup>Lp/ΔTm</sup> embryos. Whiskers indicate minimum and maximum values, + indicates the mean. Unpaired two-tailed t-test, \( p < 0.0001 \). (D) Quantification of distribution of polygon classes, as defined by the number of each cell’s neighbors. \( n = 12 \) fields of view per genotype. Error bars denote SD. (E) Quantification of cell density, as number of cells per field of view (700\( \mu \)m<sup>2</sup>). Each dot represents a single field of view. \( n = 12 \) fields of view per genotype. Bars are mean with SD. Unpaired two-tailed t-test, \( p < 0.0001 \). (F) Proliferation rates, quantified as the number of mitotic cells in a field of view as a percentage of total number of cells. \( n = 12 \) fields of view per genotype. Whiskers denote min-max values, mean shown as ‘+.’ Unpaired two-tailed t-test, \( p = 0.4416 \). (G) Representative images of basal layer labeled with membrane-tdTomato from E15.5 Vangl2<sup>WT</sup> and Vangl2<sup>ΔTm/ΔTm</sup> embryos. (H) Quantification of cross sectional areas of basal cells. Each dot represents average area of all cells in a single field of view. \( n = 15 \) total fields of view from three embryos per genotype. Bars are mean with SEM. Unpaired two-tailed t-test, \( p = 0.2314 \). (I) Quantification of cell height along apical-basal axis. \( n = 300 \) cells from across three embryos per genotype. Whiskers indicate minimum and maximum values, + indicates the mean. Unpaired two-tailed t-test, \( p < 0.0001 \). (J) Quantification of distribution of polygon classes, as defined by the number of each cell’s neighbors. \( n = 15 \) fields of view per genotype. Error bars denote SD. (K) Quantification of cell density, as number of cells per field of view (700\( \mu \)m<sup>2</sup>). Each dot represents a single field of view. \( n = 15 \) fields of view per genotype. Bars are mean with SD. Unpaired two-tailed t-test, \( p = 0.6472 \). (L) Proliferation rates, quantified as the number of mitotic cells in a field of view as a percentage of total number of cells. \( n = 15 \) fields of view per genotype. Whiskers denote min-max values, mean shown as ‘+.’ Unpaired two-tailed t-test, \( p = 0.7641 \). All scale bars = 10\( \mu \)m.
Cell shape and division orientation defects in Vangl2 mutants are indirect, and likely a consequence of improper neural tube closure

The altered cell geometries and bias toward oblique and perpendicular cell divisions observed in Vangl2<sup>Lp/Lp</sup> embryos could be directly or indirectly influenced by PCP function. As planar cell polarity components are highly expressed in the basal layer, they could have a direct role in regulating basal cell shape. Alternatively, changes in epidermal cell shape could arise as a secondary consequence of the open neural tube phenotype characteristic of several PCP mutants (Curtin et al., 2003; Hamblet et al., 2002; Kibar et al., 2001; Murdoch et al., 2001; Wang et al., 2006). The process of neural tube closure pulls the adjoining surface ectoderm over the dorsal midline to cover the spinal cord. When this process is disrupted, as in Vangl2 knockouts, the epidermis fails to enclose the embryo. To distinguish between direct and indirect functions for PCP in epidermal stratification, we conditionally deleted Vangl1 and Vangl2 in the epidermis using K14-Cre (Vangl1; Vangl2 dckO) (Vasioukhin et al., 1999). These embryos complete proper neural tube closure, but display severe planar cell polarity defects in the epidermis; PCP proteins fail to asymmetrically localize in the basal layer (Figure 13A) and hair follicles emerge with vertical instead of anterior-directed growth (Cetera et al., 2017; Chang et al., 2016). Nevertheless, in Vangl1; Vangl2 dKO embryos, the height and cross-sectional area of basal cells are comparable to control littermates (average surface area = 116.5μm<sup>2</sup> vs 116.8μm<sup>2</sup>; average height = 7.89μm vs 7.84μm for control and Vangl1,2 dKO, respectively), which is also reflected in the unchanged cell density (Figure 13B-D). Moreover, the distribution of division orientations in these mutants was similar to control embryos (Figure 13E, F), suggesting that the phenotypes observed in
the Vangl2<sup>Lp/Lp</sup> embryos are a result of the failure to perform proper neural tube closure, and not directly due to the absence of Vangl2 or planar cell polarity function in the epidermis.

We also quantified division planes and cell geometries in embryos lacking Frizzled-6, which display strong planar cell polarity defects in the skin (Cetera et al., 2017; Chang et al., 2016; Guo et al., 2004), but complete proper neural tube closure (Guo et al., 2004; Wang et al., 2006). We observed no changes in cell geometries or division orientations in these mutants, further suggesting open neural tube defects impact epidermal division planes independently of PCP function (Figure 14).
Figure 13. PCP mutants that undergo proper neural tube closure do not display altered cell shapes or division orientations

(A) Representative images of the basal layer of Vangl1<sup>fl/fl</sup>; Vangl2<sup>fl/fl</sup>; mTmG control and Vangl1<sup>fl/fl</sup>; Vangl2<sup>fl/fl</sup>; K14-Cre; mTmG embryos. Brightness of Vangl2 panel in Vangl1<sup>fl/fl</sup>; Vangl2<sup>fl/fl</sup>; K14-Cre; mTmG was increased to show lack of Vangl2 staining. Scale bars = 10 μm. (B) Quantification of basal cell cross-sectional areas. Each dot represents average area of all cells in a single field of view. n = 15 fields of view across three embryos per genotype. Bars are mean with SEM. Unpaired two-tailed t-test, p = 0.9209. (C) Quantification of cell height along apical-basal axis. n = 300 cells per genotype. Whiskers indicate minimum and maximum values, + indicates the mean. Unpaired two-tailed t-test, p = 0.4209. (D) Quantification of cell density, as number of cells per field of view (700μm<sup>2</sup>). Each dot represents a single field of view. n = 15 fields of view across three embryos per genotype. Bars are mean with SD. Unpaired two-tailed t-test, p = 0.7840. (E) Division orientations in Vangl1<sup>fl/fl</sup>; Vangl2<sup>fl/fl</sup>; mTmG control embryos. n = 211 divisions, from 45 images across three embryos. (F) Division orientations in Vangl1<sup>fl/fl</sup>; Vangl2<sup>fl/fl</sup>; K14-Cre; mTmG embryos. n = 181 divisions, from 45 images across three embryos. Modified Kuiper Test, p = 0.3743.
Figure 14. Embryos lacking Frizzled-6 do not display altered cell shapes or division orientations

(A) Representative images of the basal layer of Fz6WT and Fz6KO embryos expressing membrane-tdTomato. Scale bars = 10μm. (B) Quantification of basal cell cross-sectional areas. Each dot represents average area of all cells in a single field of view. n = 15 fields of view across three embryos per genotype. Bars are mean with SEM. Unpaired two-tailed t-test, p = 0.1357. (C) Quantification of cell height along apical-basal axis. n = 300 cells per genotype. Whiskers indicate minimum and maximum values, + indicates the mean. Unpaired two-tailed t-test, p = 0.4283. (D) Quantification of cell density, as number of cells per field of view (700μm²). Each dot represents a single field of view. n = 15 fields of view across three embryos per genotype. Bars are mean with SD. Unpaired two-tailed t-test, p = 0.1739. (E) Division orientations in Fz6WT embryos. n = 167 divisions, from three embryos. (F) Division orientations in Fz6KO embryos. n = 167 divisions, from three embryos. Modified Kuiper Test, p = 0.0644.
To further test this hypothesis, we asked whether epidermal-specific expression of Vangl2-GFP could rescue the cell geometry and division orientation defects of Vangl2 mutants. Expression of Vangl2-GFP under the K14-promoter rescued PCP protein asymmetry (Figure 15A) and hair follicle orientations in the epidermis (Devenport et al., 2011), but not the neural tube closure defect. Similar to Vangl2ΔTm/Lp mutants, basal epidermal cells of K14-Vangl2-GFP; Vangl2ΔTm/Lp embryos had decreased cross-sectional areas, were taller than K14-Vangl2-GFP; Vangl2WT control littermates, and packed more densely into the basal layer (Figure 15B-D). These embryos also displayed a greater proportion of oblique and perpendicular division orientations than control embryos (Figure 15E, F). The occurrence of these cell shape and division orientation defects, even in the presence of functioning epidermal PCP, again suggests that they are a secondary consequence of the open neural tube phenotype, and not directly influenced by planar cell polarity. We propose that failure of the neural folds to stretch the surface ectoderm over the midline in PCP mutants leads to crowding and narrowing of basal cells in the flanking epidermis, biasing division planes toward oblique and perpendicular orientations.
Figure 15. Cell shape and division orientation defects in Vangl2 mutants are indirect, and likely a consequence of failed neural tube closure.
Figure 15. Cell shape and division orientation defects in Vangl2 mutants are indirect, and likely a consequence of failed neural tube closure

(A) Representative images of the basal layer of Vangl2WT and Vangl2ΔTm/Lp embryos with or without K14-Vangl2-GFP. Green = 488 channel, Greyscale = Celsr1, Red = m-tdTomato. Rose plots quantify Celsr1 polarity in the basal layer. Red line indicates the average magnitude and direction of polarity. Rose plots were generated from 3 images per genotype. All scale bars = 10μm. (B) Quantification of cell surface areas. Each dot represents average surface area of all cells in a single field of view. n = 15 fields of view across three embryos per genotype. Bars are mean with SEM. Unpaired two-tailed t-test, p < 0.0001. (C) Quantification of cell height along apical-basal axis. n = 300 cells per genotype. Whiskers indicate minimum and maximum values, + indicates the mean. Unpaired two-tailed t-test, p < 0.0001. (D) Quantification of cell density, as number of cells per field of view (700μm²). Each dot represents a single field of view. n = 15 fields of view across three embryos per genotype. Bars are mean with SD. Unpaired two-tailed t-test, p < 0.0001. (E) Division orientations in Vangl2WT; K14-Vangl2-GFP embryos. n = 134 divisions, from 45 images across three embryos. (F) Division orientations in Vangl2ΔTm/Lp; K14-Vangl2-GFP embryos. n = 159 divisions, from 45 images across three embryos. Modified Kuiper Test, p < 0.0001.
Spatial differences in epithelial packing and shape correlate with cell division orientations and the timing of stratification

To investigate whether cell shape and density regulate division orientations in the context of normal skin development, we examined an earlier stage of epidermal development, E13.5, when basal cell geometries naturally vary in a spatially defined pattern (Aw et al., 2016). Along the midline, basal cells are stretched over the spinal cord whereas laterally, cell shapes are more isometric. We quantified cell morphologies in three distinct regions of the skin and observed large differences in cell density and geometry along the mediolateral axis (Figure 16A). Basal cells along the midline were highly flattened compared to cells at the intermediate and lateral positions (average cell height = 4.6µm at the midline vs 6.9µm laterally; Figure 16B). This reduction in cell height at the midline was accompanied by a large increase in cross-sectional area (average XY surface area = 363.5µm² in the midline vs 164.9µm² and 157.9µm² at intermediate and lateral zones, respectively) and decrease in cell density (Figure 16C, D). Interestingly, although cells at lateral and intermediate positions had similar heights and surface areas, those at intermediate positions were significantly more elongated (Figure 16E; (Aw et al., 2016)), suggesting the curvature of neighboring midline exerts mediolateral tension upon cells in this intermediate space.

We next examined cell division orientations in each region, and found that division planes correlated with spatial differences in cell geometry. Although oblique and perpendicular divisions are relatively rare at E13.5, the majority were observed within lateral regions of the embryo where cells are taller and more crowded compared to medial regions (Figure 16F-H). In the intermediate space, we observed an increased frequency of
planar divisions relative to the lateral region, likely as a result of the increased cell
elongation in this space. Along the midline, where cells are flatter and have greater planar
surface areas, divisions were almost exclusively planar (94% of divisions) (Figure 16I-L).
These spatial differences in cell geometry and division plane were not observed between
lateral and intermediate zones of Vangl2ΔTm/Lp mutants whose skin fails to close over the
midline (Figure 16). Thus, coverage of the epidermis over the spinal cord induces a
gradient of cell density and elongation along the mediolateral axis, which in turn
regulates cell division planes and the timing of stratification.
Figure 16. Spatial differences in cell geometries are associated with regional changes in division orientations
Figure 16. Spatial differences in cell geometries are associated with regional changes in division orientations

(A) Schematic of an E13.5 Vangl2WT embryo, depicting the lateral, intermediate, and midline regions. (B) Quantification of cell height along apical-basal axis. n = 300 cells per genotype. Whiskers indicate minimum and maximum values, + indicates the mean. Lateral vs Intermediate, unpaired two-tailed t-test, p < 0.0001; Lateral vs Midline, unpaired two-tailed t-test, p < 0.0001; Intermediate vs Midline, unpaired two-tailed t-test, p < 0.0001. (C) Quantification of basal cross-sectional areas. Each dot represents average surface area of all cells in a single field of view. Two images per region were analyzed, from each of three embryos, for a total of 6 measurements per region. Bars are mean with SEM. Lateral vs Intermediate, unpaired two-tailed t-test, p = 0.4872; Lateral vs Midline, unpaired two-tailed t-test, p < 0.0001; Intermediate vs Midline, unpaired two-tailed t-test, p < 0.0001. (D) Quantification of cell density, as number of cells per image (875μm²). Each dot represents a single field of view. Two images per region were analyzed, from each of three embryos, for a total of 6 measurements per region. Bars represent mean with SD. Lateral vs Intermediate, unpaired two-tailed t-test, p = 0.8567; Lateral vs Midline, unpaired two-tailed t-test, p < 0.0001; Intermediate vs Midline, unpaired two-tailed t-test, p < 0.0001. (E) Quantification of cell elongations. Each dot represents the average elongation value of all cells in a field of view. Two images per region were analyzed, from each of three embryos, for a total of 6 measurements per region. Bars represent mean with SEM. Lateral vs Intermediate, unpaired two-tailed t-test, p = 0.0125; Lateral vs Midline, unpaired two-tailed t-test, p = 0.2256; Intermediate vs Midline, unpaired two-tailed t-test, p = 0.0432. (F-H) Representative images of cell divisions in the lateral, intermediate, and midline regions in E13.5 Vangl2WT; K14-H2B-GFP embryos. Hoechst and membrane-tdTomato mark the nuclei and cell membranes, respectively. (I-K) Division orientations in the lateral, intermediate, and midline regions. Lateral: n = 99 divisions pooled from three embryos; Intermediate: n = 109 divisions pooled from three embryos; Midline: n = 50 divisions pooled from three embryos. (L) Distribution of planar vs oblique + perpendicular division orientations in the lateral, intermediate, and midline regions.
Figure 17. Vangl2 mutants lack regional changes in cell geometry and division orientation, as a result of failed neural tube closure.
Figure 17. Vangl2 mutants lack regional changes in cell geometry and division orientation, as a result of failed neural tube closure

(A) Quantification of cell height along apical-basal axis. Quantification of cell height along apical-basal axis. n = 300 cells per genotype. Whiskers indicate minimum and maximum values, + indicates the mean. Unpaired two-tailed t-test, p = 0.9412. (B) Quantification of basal cross-sectional areas. Each dot represents average surface area of all cells in a single field of view. Three images per region were analyzed, from each of two embryos, for a total of 6 measurements per region. Bars are mean with SEM. Unpaired two-tailed t-test, p = 0.3670. (C) Quantification of cell density, as number of cells per image (875µm²). Each dot represents a single field of view. Two images per region were analyzed, from each of three embryos, for a total of 6 measurements per region. Bars represent mean with SD. Unpaired two-tailed t-test, p = 0.3385. (D) Quantification of cell elongations. Each dot represents the average elongation value of all cells in a field of view. Three images per region were analyzed, from each of two embryos, for a total of 6 measurements per region. Bars represent mean with SEM. Unpaired two-tailed t-test, p = 0.2755. (E, F) Representative images of cell divisions in the lateral and intermediate regions in E13.5 Vangl2^{Lp/ΔTm}; K14-H2B-GFP embryos. Hoechst and membrane-tdTomato mark the nuclei and cell membranes, respectively. (G, H) Division orientations in the lateral and intermediate regions. Lateral: n = 87 divisions pooled from two embryos; Intermediate: n = 111 divisions pooled from two embryos. (I) Distribution of planar vs oblique + perpendicular division orientations in the lateral and intermediate regions.
To further explore the dynamics of division orientation at this early stage of epidermal development, we utilized our time-lapse imaging system to observe live E13.5 wildtype skin explants expressing nuclear marker K14-H2B-GFP. Surprisingly, we found that the distribution of division orientations did not mirror those observed in fixed whole mount explants. Instead, we observed division orientations similar to those in fixed E13.5 Vangl2<sup>Lp/Lp</sup> explants (Figure 18A). We attribute this reduction in planar cell divisions to the elimination of tissue-wide tension induced by the midline. When live explants are removed from the embryo, this tension is released, and basal cells are no longer highly biased toward planar division orientations. However, these cells still exhibit a tight correlation between division orientation and final cell positions (Figure 18B, C).

**Figure 18. Division orientation and cell fate at E13.5**

(A) Angular frequency of division angles quantified from live whole-mount skin dissected from a wildtype embryo at E13.5. n = 420 divisions from one flank skin explant. (B) Distribution of positional cell fates. Each dot represents a single division
event. Daughter cells were followed for 1.5 hours after cell division, and assigned a positional fate: symmetric or asymmetric. (C) Representation of the same data in (B), showing the distribution of fates by division orientation. For planar divisions: 94% symmetric, 8% asymmetric. For oblique divisions: 39% symmetric, 61% asymmetric. For perpendicular divisions: 3% symmetric, 97% asymmetric.

**Exogenous stretching of skin explants increases the frequency of planar cell divisions**

Our results thus far indicate that basal epidermal cells can orient their division planes to respond to changes in packing and geometry. The neural tube defect of PCP mutants offered a genetic tool with which to experimentally induce cell crowding and increase oblique and perpendicular division orientations. Therefore, we reasoned that mechanical manipulations that stretch the epidermis should bias cell divisions toward planar orientations. To test this, we cultured embryonic skin explants on flexible substrates and applied uniaxial stretch across the tissue (Aw et al., 2016). Initially, we tested the effect of stretch on wildtype E15.5 explants, where we previously observed an increase in oblique and perpendicular divisions relative to earlier developmental stages. When these explants were stretched over the course of 1 hour, basal cells became elongated along the axis of strain and divisions were shifted toward planar division orientations compared to unstretched controls (Figure 19).
Figure 19. Basal cell divisions shift toward planar orientations under exogenously applied stretch

(A) Representative images of the basal layer of E15.5 Vangl2WT control and stretched skin explants with vectors illustrating direction and magnitude of cell elongation. White, membrane-tdTomato. Scale bars = 10μm. (B) Quantification of cell elongation in control vs stretched skins. Each dot represents the average elongation value of all cells in an field of view. n = three images from each of two separate embryos/experiments. Bars are mean with SEM. Unpaired two-tailed t-test, p = 0.0001. (C) Quantification of cell height along apical-basal axis. n = 450 cells per genotype. Whiskers indicate minimum and maximum values, + indicates the mean. Unpaired two-tailed t-test, p < 0.0001. (D) Division orientations in control E15.5 Vangl2WT explants, n = 61 divisions pooled from two explants. (E) Division orientations in stretched E15.5 Vangl2WT skin explants. n = 50 divisions pooled from two explants. Modified Kuiper Test, p < 0.0001.
We then tested whether planar division orientations could be restored in E14.5 Vangl2ΔTm/Lp skin explants by stretching along the medial-lateral axis to mimic the tension experienced by the epidermis when the neural tube has closed. Stretching Vangl2ΔTm/Lp skin explants significantly increased the planar elongation of basal cells (average elongation = 30.79 a.u. in stretched explants, vs 20.30 a.u. in control explants) and reduced cell heights as compared to unstretched controls (Figure 20A-C).

Additionally, while basal cells of Vangl2ΔTm/Lp control explants underwent primarily oblique and perpendicular divisions, stretched Vangl2ΔTm/Lp explants exhibited an increased proportion of planar division orientations, similar to the distribution observed in E14.5 Vangl2WT embryos (62.5% planar divisions in stretched explants vs 27.1% in control) (Figure 20D, E). The ability to rescue the division orientation defect of Vangl2ΔTm/Lp embryos through uniaxial stretch demonstrates that basal cells are able to integrate tissue dynamics with cell division orientation to respond to and influence tissue architecture.
Figure 20. Exogenously applied stretch rescues the division orientation defect of Vangl2 mutant embryos

(A) Representative images of the basal layer of E14.5 Vangl2<sup>ΔTm/Lp</sup> control and stretched skin explants. White, membrane-tdTomato. Scale bars = 10μm. (B) Quantification of cell elongation in control vs stretched skins. Each dot represents the average elongation value of all cells in a field of view. n = three images from each of three separate experiments. Bars are mean with SEM. Unpaired two-tailed t-test, p<0.0001. (C) Quantification of cell height along apical-basal axis. n = 450 cells per genotype. Whiskers indicate minimum and maximum values, + indicates the mean. Unpaired two-tailed t-test, p < 0.0001. (D) Division orientations in control E14.5 Vangl2<sup>ΔTm/Lp</sup> explants. n = 48 divisions pooled from three explants. (E) Division orientations in stretched E14.5 Vangl2<sup>ΔTm/Lp</sup> embryos. n = 57 divisions pooled from three explants. Modified Kuiper Test, p < 0.0001.
Conclusions

By live imaging of the developing epidermis, we have shown that the orientation of cell divisions within the skin’s basal progenitor layer is closely linked to the positional fate of daughter cells. Basal cells dividing with telophase angles greater than 45 degrees of the basement membrane will have asymmetric fates, whereas division angles less than 45 degrees will generate daughters with symmetric fates. Although this has been assumed to be true based on division planes in fixed tissue sections, these data show directly that during early phases of epidermal stratification, the angle between telophase daughter cells is a strong indicator of the final positions and fates of the two daughters. We also found, through investigation of the hyperstratification defect of a well-studied PCP mutant, that during early stratification stages, the orientation of cell division is influenced by cell shape and packing rather than cortical PCP cues or LGN localization. Basal cells that are taller along the apical-basal axis are biased toward perpendicular divisions, while flatter and wider cells tended to divide parallel to the epithelial plane. Crowding within the epithelium, a consequence that is likely due to failure of the skin to stretch and cover the midline in PCP mutants, is associated with increased asymmetric divisions, while stretching the epithelium promotes planar divisions and expansion of the progenitor layer. These findings show that embryonic basal epidermal cells are flexible to adjust their divisions, at the level of spindle orientation, in response to changes in the tissue environment. We propose that under normal developmental conditions, this mechanism allows for expansion of the progenitor layer to accommodate embryo growth while also allowing for the generation of new layers when the basal layer has reached sufficient density.
CHAPTER 3: Discussion and Perspectives
Model for Early Epidermal Stratification

Based on our observations, we propose an updated model for epidermal stratification. The epidermis begins as a single layer of progenitor cells that divide almost exclusively within the plane of the tissue, until increases in cell density and height-to-width ratios promote stratification through oblique/perpendicular divisions. Epithelial stretch or tension, such as that exerted on the skin by embryo growth or morphogenetic changes like neural tube closure, delays stratification while crowding of the basal layer promotes its occurrence. These cues of stretch and crowding provide an effective way to balance basal cell division orientations and ensure proper tissue structure early in epidermal development. Notably, although other studies have suggested stratification at this early stage is driven primarily by delamination (Miroshnikova et al., 2018; Wickstrom and Niessen, 2018; Williams et al., 2014), we did not observe any delamination events through the analysis of our live imaging data. Late in embryonic development, there is a developmental shift toward generating more skin layers, as forming an effective barrier from the exterior environment becomes imperative. To facilitate this change in tissue architecture, apical mInsC polarization promotes perpendicular spindle orientations and formation of a stratified barrier (Lechler and Fuchs, 2005; Poulson and Lechler, 2010; Williams et al., 2011; Williams et al., 2014).

Oriented Cell Division in Epidermal Development and Homeostasis

During homeostasis of the adult epidermis, yet another shift occurs in the balance between division and stratification. Division angles are once again primarily planar and delamination, rather than asymmetric division, becomes the mechanism by which cells
are able to replenish the skin’s stratified layers (Clayton et al., 2007; Mesa et al., 2018; Rompolas et al., 2016; Smart, 1970). During adult homeostasis, differentiation triggers neighboring basal stem cells to divide. Thus, similar to what we’ve observed in early epidermal stages, homeostasis is influenced by the needs of the tissue, as sensed through local tissue architecture (Mesa et al., 2018).

The different strategies that basal cells employ to orient divisions as the epidermis develops raises many new questions that warrant further investigation. Firstly, it is unclear what regulates the developmental switches in division modes. For example, the switch to mInsc/LGN/NuMA-mediated perpendicular divisions coincides with an increase in mInsc polarization (Lechler and Fuchs, 2005; Poulson and Lechler, 2010; Williams et al., 2011; Williams et al., 2014), but how this change in polarization is regulated is unknown. Additionally, we do not know precisely when or how the switch back to planar divisions during homeostasis occurs. Secondly, the cortical cues that orient planar spindle alignment, at any developmental stage, remain to be identified. In single-layered epithelia, such as the Drosophila follicular epithelium and the chick neuroepithelium, planar spindle orientation depends on the LGN/Pins-NuMA/Mud complex, which anchors astral microtubules to the lateral cell cortex through the polarity factor Dlg (Bellaiche et al., 2001b; Berghstrahl et al., 2013; Nakajima et al., 2013; Saadaoui et al., 2014). Murine epidermal progenitors require LGN-NuMA for perpendicular spindle orientation during the latter phase of stratification, but these proteins are dispensable for planar spindle alignment (Williams et al., 2014). Moreover, although core PCP components orient parallel divisions in several other contexts (Bellaiche et al., 2004; Bellaiche et al., 2001a; Ciruna et al., 2006; Fischer et al., 2006;
Gong et al., 2004; Segalen et al., 2010), the core PCP component Vangl2 is not required for parallel, symmetric divisions in the skin. Conditional ablation of β1 integrin or α-catenin leads to randomization of division planes in the basal layer (Lechler and Fuchs, 2005) suggesting planar spindle alignment requires both basal and lateral adhesion proteins. However, cell polarity might be generally compromised in these mutants. Thus, the cortical cues that pull the spindle in a planar orientation still need to be identified, and it is unclear whether such factors would be localized to the lateral or basal cortex. Finally, the mechanisms that connect spindle orientation to cell geometry in the early stages of stratification remain unknown.

**Hertwig’s Rule**

Although the propensity for a cell to divide along its longest interphase axis, Hertwig’s Rule, has been known for over 100 years, it is not entirely intuitive how the mitotic spindle, which assembles and anchors only after the cell has rounded, orients relative to the cell’s geometry when it was in interphase. Evidence that cells retain a ‘cortical memory’ of their interphase shape has been shown in the pupal notum epithelium of Drosophila and single cells in culture. In planar divisions of the Drosophila notum, NuMA localizes to tricellular junctions, where it captures astral microtubules to dictate division orientation. Because tricellular junction position is preserved from interphase, their position serves as a memory of interphase geometry when the cell rounds up during mitosis (Bosveld et al., 2016). When HeLa cells are plated on fibronectin micropatterns, retraction fibers left behind during mitotic rounding present spatial cortical cues that can bias the localization of subcortical molecular factors, which
in turn influence mitotic spindle orientation (Fink et al., 2001; Thery et al., 2005). Ablation of these fibers or adjustment of their distribution through pattern variation affects spindle positioning (Fink et al., 2011).

In the murine epidermis, however, the mechanisms responsible for communicating cell shape and/or tension information to the mitotic spindle are unknown. Whether the same principles by which cell geometry orients divisions in two dimensions apply to the three-dimensional division planes of basal cells is also unclear. Because each cell is dividing in the context of the surrounding tissue, perhaps neighboring cells exert tension on the dividing cell as shared junctions are distorted due to cell shape changes that occur during mitosis. For example, cells that are elongated within the plane of the epidermis would have the greatest tension exerted upon them from lateral neighbors as they progress from an elongated to a rounded shape, and thus would be more likely to divide in a planar orientation. Conversely, cells that have more isometric cross-sectional areas and are elongated along their apical-basal axes may lack these planar forces. Alternatively, there may be a cell intrinsic system for interphase shape memory that guides the spindle to align along its interphase 3D long axis. Ultimately, deciphering the processes by which basal epidermal cells connect local tissue mechanics to cell division orientations will illuminate a new method of cell fate control.
MATERIALS AND METHODS

Mouse Lines and Breeding. All procedures involving animals were approved by Princeton University’s Institutional Animal Care and Use Committee (IACUC). Mice were housed in an AAALAC-accredited facility in accordance with the Guide for the Care and Use of Laboratory Animals. This study was compliant with all relevant ethical regulations regarding animal research. E13.5-E18.5 embryos from C57BL6 backgrounds were used unless otherwise indicated. Both sexes were used as sex was not determined in embryos. Vangl2Lp lines generated extremely high frequencies of hermaphrodites, severely limiting the availability of female animals. Therefore, both Vangl2Lp and Vangl2ΔTm females were used for experiments.

Whole-mount immunostaining. For immunostaining, embryos were dissected in PBS and fixed in 4% paraformaldehyde. E13.5 and E14.5 embryos and embryonic explants were fixed at room temperature for 1 hour, and E15.5 and E16.5 embryos and embryonic explants were fixed at room temperature for 1.5 hours. Skins stained with anti-LGN antibodies were processed as follows: skins were dissected from fixed embryos and blocked overnight at 4°C in 2% fish skin gelatin, 1% bovine serum albumin, 2% normal donkey serum, and/or 2% normal goat serum in PBT (1X PBS with 0.2% or 0.3% Triton X-100). Skins were incubated in primary antibodies diluted in blocking solution for 24 hours at 4°C. Skins were washed with 0.2%-0.3% PBT overnight, incubated with secondary antibodies for 24 hours at 4°C followed by Hoechst. All other skins were blocked for 1 hour at RT in 1% BSA, 2% NDS and/or 2% NGS then incubated in primary antibodies diluted in block overnight at 4°C. After washing with 0.2%-0.3% PBT, skins
were incubated in secondary antibodies followed by Hoechst. Samples were mounted in either glycerol-based anti-fade non-curing medium or Vectashield Antifade mounting medium. The following primary antibodies were used: rabbit anti-involucrin (1:500, D. Devenport), guinea pig anti-Celsr1 (1:1000, D. Devenport), anti-E-Cadherin (1:25, purified in lab), rat anti-E-Cadherin (1:2000, DECMA-1, Thermo Pierce, Cat: MA1-25160), guinea pig anti-LGN (1:500, gift from Scott Williams), rabbit anti-LGN (1:3000, gift from Scott Williams), rat anti-Vangl2 (1:100, Millipore, Cat: MABN750). Alexa Fluor-488, -555, -647 secondary antibodies were used at 1:2000. Hoechst (Invitrogen, Cat: H1399) was used at 1:1000 (1mg/mL). Images were acquired on a Nikon Ti-E Spinning Disc or A1 scanning confocal microscope controlled by NIS Elements software using a Plan Fluor 40X/1.3NA, Plan Apo 60X/1.4NA, or Plan Apo 100x/1.45 oil immersion objective. NIS Elements software and Photoshop were used for image processing.

**Live imaging.** A portion of live imaging for Figure 1 was done as follows: E14.5 flank skin explants were dissected in PBS and mounted on the bottom side of Sarstedt 35mm Lumox membrane dish (Cat# 94.6077.331) and secured in position using 13mm diameter, 8mm Nucleopore polycarbonate membrane (Fisher Scientific Cat# 09-300-57) overlaid with Matrigel (Fisher Cat# CB-40230A). This dish was then mounted inside a Sarstedt standard TC dish (Cat# 83.3900) filled with F-Media with 10% FBS. This setup was necessary to enable imaging from the epidermal side of the explant using an upright microscope. Time-lapse imaging was undertaken using an upright Prairie Ultima multiphoton microscope equipped with 40X Plan Apochromat, 1.0NA immersion.
objective with incubation chamber to maintain 37°C and 5% CO2. 1024 x 1024 pixel images were collected in Z-stacks space at 1.0μm apart. 3D Z-stacks were collected at 10-minute intervals for 24 hours. Volocity software was used for movie processing. For all other movies: E14.5 dorsal flank skin explants were dissected in PBS and transferred to a 1% agarose gel with F-Media containing 10% fetal bovine serum. Explants were sandwiched between the gel on the dermal side and a 35mm lummmox membrane dish (Sarstedt) on the epidermal side. Z-stacks with 0.5 or 1.0μm step sizes were acquired at 10 minute intervals for 8-18 hours. Images were acquired using a Nikon Ti-E Spinning Disc with a Plan Fluor 40X/1.3NA oil objective. Explants were cultured in a humid imaging chamber at 37°C with 5% CO2 during the course of imaging. In all live imaging experiments, cells close to the edge of the explants were not imaged to avoid differences in division orientations that could be induced by a wound healing response. NIS Elements software was used for movie processing.

**Stretching Experiments.** A custom-designed stretch chamber (Aw et al., 2016) was constructed to culture and uniaxially stretch skin explants on a flexible 7 x 3cm PDMS membrane (SSP-M823-005, Specialty Silicone Products, Inc). The PDMS membranes were exposed to UV light for 15-20 minutes then coated with fibronectin. E14.5 or E15.5 dorsal flank explants were dissected in PBS and positioned dermis side down onto the membranes and submerged in E-media containing 1.5mM calcium. E14.5 explants were cultured with 10% mouse serum, isolated in the lab. The explants were allowed to rest for 3 hours before stretching. One half of each explant was cultured in a similar chamber with no applied stretch, as a control. Experiments were performed by stretching the
membrane 4mm over the course of 1 hour. Skin explants were kept on the PDMS membranes for 15 minutes following stretching, then fixed at room temperature for 1 hour, then removed from the PDMS membrane and mounted in Vectashield mounting medium for imaging. Basal cell elongation was quantified using Packing Analyzer V2 software, which determines the direction and magnitude of elongation as previously described (Aw et al., 2016).

**Quantification of division angle, skin thickness, cell height, surface area.** Division angle in both live and fixed imaging was determined by drawing a line connecting the two daughter nuclei during telophase or cytokinesis (either using Volocity or NIS Elements software). Skin thickness was quantified using fixed sagittal tissue sections or whole-mount skins and by measuring the area of skin in an image, normalized to length. For whole-mount images, area and length were measured in YZ views. Cell heights were measured using membrane signal (either E-Cadherin or membrane-tdTomato, but held consistent within each experiment) and XZ views of images with either 0.2μm or 0.5μm Z-step sizes in NIS Elements software to determine the distance from the apical surface of the cell to the basement membrane. Cell surface area was calculated using Packing Analyzer V2 software. Images were imported into the software and cells were segmented using a membrane signal, with any necessary hand corrections. Surface areas were calculated using the software’s watershed formula (Aw et al., 2016).

**Statistics.** Statistical analyses were conducted as noted in each figure legend. Circular statistics: to compare rose plots of division angles, p values were calculated from the non-
parametric Kuiper’s test to compare distributions along a circle. This test was modified to account for data limited to 0-90° range, and p values were calculated using Matlab. All other statistical analyses were performed in GraphPad Prism.
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stress dissipation through divisions oriented along the long cell axis. Proc Natl Acad Sci USA 112, 5726-5731.