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Asymmetric Cell Divisions in the Epidermis

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Abstract

Generation of three-dimensional tissue with distinct cell types is required for the development of all organs. On its own, mitotic spindle orientation allows tissues to change in length or shape. In combination with intrinsic or extrinsic cues this can also be coupled to the generation of diverse cell fates - a process known as asymmetric cell division (ACD). Understanding ACD's has been greatly aided by studies in invertebrate model systems, where genetics and live imaging have provided the basis for much of what we know. ACD's also drive the development and differentiation of the epidermis in mammals. While similar to the invertebrate models, the epidermis is distinct in balancing symmetric and asymmetric divisions to yield a tissue of the correct surface area and thickness. Here we review the roles of spindle orientation in driving both morphogenesis and cell fate decisions. We highlight the epidermis as a unique model system to study not only basic mechanisms of ACD, but also to study their regulation during development.

Keywords

Inscuteable; epidermis; Asymmetric Cell Division; Spindle Orientation; NuMA

1. Introduction

During development, oriented cell divisions are crucial for correctly organizing and shaping a tissue. Mitotic spindle orientation can be coupled with cell fate decisions to provide cellular diversity through asymmetric cell divisions (ACDs), in which the division of a progenitor cell results in two daughters with different cell fates. Proper tissue morphogenesis relies on the coupling of these two phenomena being highly regulated. Studies in both *D. melanogaster* and *C. elegans* have provided great insight into the conserved molecular machinery required for ACDs. In mammals, the development of the mouse epidermis serves as a model in which to study the many levels that regulate ACDs. Basal cells within the epidermis can undergo both symmetric and asymmetric cell divisions. While symmetric divisions allow for an increase in surface area and progenitor cell number, asymmetric divisions drive the stratification of the epidermis, directly contributing additional cell layers (Lechler, Fuchs, 2005; Poulson, Lechler, 2010; Williams *et al.*, 2011). Recent work in the mouse epidermis suggests crucial roles for the protein Inscuteable (Insc) as both an instructive and regulatory cue for proper spindle orientation (Poulson, Lechler, 2010).

In this review, we begin by describing some of the myriad uses of spindle orientation in driving morphogenesis and ACD in diverse cell types. We then describe the machinery and the mechanisms that underlie spindle orientation and cell fate decisions. Finally, we expand on the epidermis as a physiological model for studying the regulation of ACD in mammals.

2. Oriented Cell Divisions in Morphogenesis

During development, the formation of tissues must be highly regulated to ensure proper shape, size and cell fate specifications. Oriented cell divisions are a highly conserved mechanism used to properly pattern and shape a tissue. Below we present various developmental and homeostatic functions for oriented spindles and give examples of each of these in diverse species and cell types.

2.1. Shaping a Tissue

Oriented cell divisions can function to drive morphogenesis by shaping tissues. A diversity of tissue architectures are generated and/or maintained by oriented spindles. These include the elongation of tissues, branching morphogenesis and maintenance of single lumens within epithelial tubes. In fact, evidence for oriented cell divisions directing these processes can be seen throughout development and evolution.

When cell divisions are oriented along a preferred axis, tissue elongation results (Fig. 1A). This simple mode of morphogenesis is seen throughout evolution. Even unicellular organisms such as some yeasts exploit spindle orientation to grow as hyphae (Finley *et al.*, 2008; Li *et al.*, 2005). One of the best studied examples of this occurs during *Drosophila* germband extension. Just after gastrulation, the germ-band elongates along the anterior-posterior axis of the embryo. This process requires both cell intercalations and oriented cell divisions. While cell intercalation occurs primarily in the anterior of the embryo, oriented divisions in the posterior drive the “fast phase” of elongation. Blocking cell division leads to a reduction in the amount of extension. Furthermore, mutants in patterning genes that result in a randomized spindle orientation and cell intercalation defects exhibit a reduction in germband extension (da Silva, Vincent, 2007). Thus, while oriented divisions contribute to elongation, the relative contribution of these divisions and cell intercalation during germband extension remain unclear.

Later in development, oriented spindles play a role in the proximal-distal lengthening of the fly wing. In this case, tension within the wing causes cells to align their long axis along the proximal-distal axis. The mitotic spindles also align along this axis (Aigouy *et al.*, 2010). Though not discussed in detail in this review, cell shape is often correlated with division orientation (Luxenburg *et al.*, 2011; Wildwater *et al.*, 2011). Additional evidence for the correlation between tissue lengthening and spindle orientation came from the analysis of labeled cell clones within the eye and wing imaginal disc (Baena-Lopez *et al.*, 2005; Mao *et al.*, 2011).

During gastrulation in zebrafish, the embryo elongates along the animal-vegetal axis. *In vivo* imaging of mitotic divisions during zebrafish gastrulation revealed that a majority of cells orient their spindle along the animal-vegetal axis. Inhibition of the planar cell polarity pathway led to altered spindle orientation as well as defects in elongation indicating a requirement for oriented cell divisions in zebrafish axis elongation (Gong *et al.*, 2004). Additionally, cell rearrangement studies in marine shrimp demonstrate that mesoderm cells direct spindle orientation in adjacent cells driving the process of elongation (Wang *et al.*, 2008).

Control of division orientation is also required to maintain existing structures. For example, in the adult hydra, the body column exists in a dynamic state in which the ectodermal cells are in a constant mitotic cycle. Cells are continuously displaced along the longitudinal axis but never in the circumferential direction (Shimizu *et al.*, 1995). These divisions do not lengthen the body column as cells are displaced at the top and bottom of the column, but they do appear to maintain the tissue architecture.

Chirality of some species is also determined by division orientations. In snails, for example, the division orientations at the 4 cell stage determine whether the final shell will be in the sinistral or dextral chiral form (Kuroda *et al.*, 2009).

In simple epithelia, mitotic spindles are oriented within the plane of the epithelia (parallel to the underlying substratum) (Fleming *et al.*, 2007). Their divisions therefore promote tissue expansion in two-dimensions. The importance of this organization has recently been demonstrated in cultured cells, though the *in vivo* equivalent has not yet been reported. When epithelial cells (often Caco2 or MDCK cells) are grown in matrigel matrices, they form three-dimensional structures in which a simple epithelium surrounds a central lumen. Disruption of normal lateral divisions in these cells results in the formation of multiple lumens (Jaffe *et al.*, 2008; Qin *et al.*, 2010). This suggests that both the developmental generation and homeostatic maintenance of single-lumened tubes (which includes the digestive tract, the airways and the urinary system, to name a few) requires control of cell division orientation.

Branching of tubes is also a common phenomenon – occurring in tissues as diverse as the vasculature, the mammary gland and the lung. While different mechanisms may drive branching, recent work has highlighted a role for spindle orientation in morphogenesis of the mouse lung (Fig. 1B). Early in development, the lung consists of two primary buds with an inner epithelial cell layer surrounded by mesenchyme. Subsequently, a highly regulated process of outgrowth and branching gives rise to the final lung structure (Metzger *et al.*, 2008). During outgrowth there is an enrichment for spindles oriented along the airway longitudinal axis, i.e. ones that promote outgrowth. If this population is lost, as is found in Sprouty loss of function and Kras gain of function mutant lung, the normal architecture and branching of the lung is disrupted (Tang *et al.*, 2011). This demonstrates the importance of oriented cell divisions in establishing the precise structure of the lung.

In addition to the role of oriented cell divisions in lung outgrowth, they may also play a role later in development. Studies looking at the spindle orientation in the distal epithelium of the lung revealed that a majority of cells divide perpendicular to the basement membrane. The protein Eya1 was shown to be required for cell polarity and proper spindle orientation (El-Hashash *et al.*, 2011). Whether these divisions are functionally asymmetric and their role in tissue patterning is not yet clear. Taken together these two types of oriented cell divisions in the lung demonstrate the ability of the tissue to regulate division orientation in diverse ways to control its final structure.

Another example of oriented cell divisions in morphogenesis occurs during neurulation in zebrafish. During zebrafish neurulation, 90% of neural plate cells undergo midline-crossing divisions (Lyons *et al.*, 2003). These result in mirror symmetric cells as polarity proteins accumulate at the site of cell division. This oriented cell division across the midline is achieved through a 90° rotation of the mitotic spindle (Geldmacher-Voss *et al.*, 2003). Cell division is required for these midline crossing events, as blocking cell division prevents most cells from crossing the midline (Ciruna *et al.*, 2006; Tawk *et al.*, 2007). The proper orientation of these divisions is required, as perturbation of oriented cell divisions in the neuroepithelium results in severe defects in the neural rod midline (Quesada-Hernandez *et al.*, 2010; Zigman *et al.*, 2011).

Oriented cell divisions can also be seen in the developing neuroepithelium of *Xenopus laevis*. *In vivo* four dimensional confocal microscopy revealed planar oriented mitotic spindles. Additionally, it was shown that similar to the zebrafish neural plate, cells must undergo spindle rotation in order to reach their final axis of division (Kieserman, Wallingford, 2009). Unlike oriented cell divisions in the zebrafish, these divisions appear to

be independent of the planar cell polarity (PCP) pathway. Despite defects in neural tube closure in PCP mutants, cells were still able to properly orient their mitotic spindles. Thus, while oriented cell divisions play a key role in development, there are multiple mechanisms by which proper spindle orientation can be achieved.

2.2. Generating Cellular Diversity

In addition to driving changes in morphology, oriented cell divisions function to generate cellular diversity in a tissue. Two non-mutually exclusion mechanisms can drive cell fate differences in asymmetric cell divisions. These are defined by whether the asymmetry is present during mitosis in the dividing cell (intrinsic), or whether it is provided by an asymmetry in the environment of the daughters (extrinsic) (Fig. 2A,B).

2.2.1. Extrinsic ACD's—In extrinsic ACDs cells divide away from an environmental niche so that one of the two daughter cells leaves the niche and can take on a new fate (Fig. 2B) (Betschinger, Knoblich, 2004). An excellent example of an extrinsic ACD is found in the *Drosophila* germline stem cell (GSC) niche. A group of ~10-15 closely packed non-dividing stromal cells referred to as the hub are located at the apical tip of the testes surrounded by 6-9 GSCs (Hardy *et al.*, 1979). Local secretion of Unpaired (Upp) from the hub cells activates the JAK-STAT pathway in adjacent GSCs. GSCs orient their mitotic spindle so that one of the spindle poles is closely apposed to the hub cell contact site. Thus, when the GSC divides, one daughter remains in contact with the hub and continues to receive its signals, while the other daughter is unable to receive sufficient levels of Upp, and thus, the JAK-STAT pathway is turned off (Kiger *et al.*, 2001; Tulina, Matunis, 2001). In addition to the Upp signal, the hub cells secrete bone morphogenic proteins (BMPs) (Kawase *et al.*, 2004). Activation of the BMP pathway in the GSCs is required to repress the transcription of the differentiation factor *bag of marbles* (*bam*) (Schulz *et al.*, 2004). As a result, GSCs must remain in close contact with the hub to retain their self-renewing capacity and to inhibit differentiation.

Recent live imaging studies of the *Drosophila* testis have revealed that GSCs can undergo direct differentiation by detaching from the hub (Sheng, Matunis, 2011). While this supports the interaction with the hub as a signal for maintaining self-renewing ability, it also indicates that oriented cell divisions are not the only mechanism by which GSCs can differentiate. In addition, by dividing parallel to the niche, progenitor cells can generate two self-renewing daughter cells, thereby increasing the progenitor cell number.

2.2.2. Intrinsic ACD's—In intrinsic ACDs cell-fate determinants are localized asymmetrically so that they are inherited by only one of the two daughter cells (Fig. 2A) (Betschinger, Knoblich, 2004). The *Drosophila* sensory organ precursor and neuroblasts provide two of the most well studied examples of intrinsic ACDs (Strain *et al.*). The SOP cells ultimately give rise to the external sensory organs of the fly. SOPs divide asymmetrically to generate an anterior pIIa and posterior pIIb cell. After this first division, the pIIb and pIIa cell divide once more to produce the four terminally differentiated cells of the organ (the socket, hair, sheath and neuron cells). The asymmetrically localized cell fate determinant Numb was first identified in SOP cells. Normally, Numb is inherited by the posterior pIIb cell. In Numb mutants, both SOP daughter cells take on the pIIa cell fate, which normally does not inherit Numb. Conversely, when Numb is overexpressed, both SOP daughter cells take on the opposite cell fate (Rhyu *et al.*, 1994). Numb ultimately exerts its effects through repressing the Notch pathway within the cell in which it is inherited (Berdnik *et al.*, 2002). Thus, by orienting the spindle along the anterior-posterior, axis only one resulting daughter cell will inherit Numb and repress Notch signaling.

Unlike the SOP cells that only undergo two rounds of division, *Drosophila* neuroblasts undergo multiple ACDs. The neuroblast cells align their mitotic spindle along the apical/basal axis of the cell, with the apical cortex being marked by the Par3 homolog Bazooka. Accompanying this alignment, cell fate determinants such as Brat, Prospero, and Numb localize to the basal cortex of the neuroblast. The resulting division produces an apical neuroblast progenitor and a basal differentiating ganglion mother cell (GMC)(Doe, 2008; Knoblich, 2010). Prospero is a transcription factor that acts to both inhibit neuroblast self-renewal and activate differentiation genes. Neuroblasts lacking Prospero produce GMCs that continue to proliferate and express neuroblast markers (Choksi *et al.*, 2006). In contrast to Numb and Prospero, the effect of Brat on cell fate specification is currently unknown.

Genetic manipulations that altered spindle orientation of the neuroblasts without altering the cells' intrinsic polarity were used to address the importance of spindle orientation in the inheritance of these cell fate determinants. Live imaging following cell divisions and, ultimately, analysis of markers for cell fate in both wildtype and mutant neuroblasts indicated that apical-basal spindle orientation was required for the inheritance of the proper ratio of cell fate determinants. In neuroblasts where the spindle aligned perpendicular to the apical-basal axis, both daughter cells took on a neuroblast fate (Cabernard, Doe, 2009). These studies revealed the importance of spindle orientation in correctly partitioning the basal cell fate determinants and properly specifying both neuroblast and GMC identity.

Studies of the first cell division of the *C. elegans* embryo have also revealed insights into the link between spindle orientation and ACDs. In response to a cue resulting from its fertilization, the *C. elegans* embryo becomes polarized (Goldstein, Hird, 1996). Polarity proteins become polarized to the anterior or posterior and the mitotic spindle aligns along this axis (Gonczy, Rose, 2005). This is accompanied by the localization of the cell fate determinants MEX-5/MEX-6 to the anterior and MEX-1, PIE-1, and POS-1 posteriorly (Guedes, Priess, 1997; Mello *et al.*, 1996; Schubert *et al.*, 2000; Tabara *et al.*, 1999). In addition P-granules serve as markers for the posterior, although they do not appear to act as cell fate determinants themselves (Gallo *et al.*, 2010). The resulting division gives rise to an anterior AB-cell and a posterior P1-cell. The AB-cell will give rise to the ectoderm, while the P1-cell will give rise to the germline, endoderm, and mesoderm. These cells have different sizes due to displacement of the mitotic spindle as well as different fates because of inheritance of different determinants.

Another example of extrinsic cues directing intrinsic asymmetric cell divisions occurs during the activation of T-cells. T-cells have the potential to develop into both effectors and memory T-cells. Under normal conditions T-cells have no intrinsic polarity cues to specify an axis of division. However, interactions between antigen presenting cells (APCs) and T-cells result in a polarized patch forming between these cells. The mitotic spindle aligns with this patch so that one daughter cells remains associated while the other one does not. Thus, in response to the APC, an axis of division is established so that an oriented cell division can generate two distinct daughter cells with different potentials to produce either effectors or memory T-cells(Oliaro *et al.*, 2010). The transcription factor T-bet is asymmetrically inherited because of its polarization proximal to the APC and may drive, at least in part, functional differences between daughters (Chang *et al.*, 2011). Thus oriented cell divisions that generate diversity are not limited to polarized cell types, but can also be established through induced cell polarity via external cues.

The key aspect of both intrinsic and extrinsic ACDs is the importance of a properly oriented mitotic spindle. The spindle must correctly orient and either divide in such a way to ensure the segregation of the cell-fate determinants or so that one of the daughter cells leaves the niche. Thus, the tight regulation of oriented cell divisions is crucial for proper ACD.

In addition to generating various cell types within a tissue, oriented cell divisions are common within stem cell populations to give rise to differentiated cell types as well as to renew a stem cell population. The *Drosophila* neuroblast is a good example of this. The apical neuroblast resulting from the division will maintain its proliferative capacity. Conversely, the basal GMC will divide only once more to give rise to two neurons. In this way, the neuroblasts can continuously divide and contribute to neuron development without depleting their overall number (Doe, 2008). These types of oriented stem cell divisions are seen in a variety of tissues including the *Drosophila* germ line, mammalian neuronal development, hematopoiesis, and stratification of the epidermis (Chenn, McConnell, 1995; Kiger et al., 2001; Lechler, Fuchs, 2005; Wu et al., 2007). However, it is important to note that for many adult stem cells, there is no evidence for asymmetric cell divisions.

2.2.3. Spindle Orientation and Loss of Basement Membrane Attachment—One environmental (extrinsic) factor that is frequently asymmetric in oriented cell divisions is connection to an underlying basement membrane. In divisions in which the mitotic spindle is oriented perpendicular to the basement membrane, one of the resulting daughter cells loses its attachment to the ECM. Conversely, divisions parallel to a basement membrane can serve to renew and increase the population of cells. As a result, precise coordination of each division orientation is required for proper development and/or homeostasis of a tissue.

The mouse epicardium is composed of a single layer of cells that attach to the basement membrane overlying the myocardium. During heart morphogenesis, some of these cells undergo an epithelial to mesenchymal transition (EMT) and migrate into the subepicardial space. This phenomenon is correlated with the epicardial cells dividing perpendicular to their basement membrane giving rise to one cell that remains in the epicardial layer and another that enters the myocardium. In addition to divisions perpendicular to the basement membrane, there are a subset of epicardial cells that divide parallel to the basement membrane (Wu et al., 2010). While it is clear that the perpendicular divisions contribute to epicardial EMT, it is not known how the balance of each division orientation is regulated.

The development of the mouse epidermis also involves divisions perpendicular and parallel to the underlying basement membrane (discussed in detail below). Like in the epicardium, the regulation of these divisions direct the morphologic process of shaping the tissue as well as generating cellular diversity.

2.2.4. Development and Stratification of the Epidermis—The development of the epidermis serves as a model to study how oriented cell divisions function to both shape a tissue and generate cellular diversity. The skin is one of the largest organs of the body and provides a protective outer covering in mammals. Primarily, the skin functions to control evaporation of water by creating a semi-impermeable barrier to water loss. Additionally, this tissue acts to protect us from external pathogens, regulate heat, and provide the sensation of touch. The epidermis, the outermost layer of our bodies, is essential for providing the waterproofing and barrier aspects of the skin. This protective function is established during development through the process of stratification in which the epidermis becomes a multilayered squamous epithelium.

In the mouse, the epidermis develops over a period of approximately 10 days, starting at E8.5 and completing around E18.5, just prior to birth. At E8.5, unspecified surface ectoderm cells overlying a basement membrane commit to an epidermal cell fate. While little is known about the mechanism driving this commitment, recombination experiments resulting in the transdifferentiation of one type of epithelium into another suggest that the underlying dermis provides the initial cue for epidermal specification (Ferraris et al., 2000; Pearton et al., 2005). This change in cell fate is accompanied by a change in keratin differentiation

markers. Surface ectoderm cells express keratins 8 and 18, while committed epidermal cells express keratin 5 and 14 (Byrne *et al.*, 1994; Moll *et al.*, 1982). This transition requires the transcription factor p63, which is expressed in the ectoderm/epidermis. In the absence of p63 stratification and the switch to keratin 5/14 does not occur (Mills *et al.*, 1999; Yang *et al.*, 1999). Committed epidermal cells comprise the first layer of the epidermis known as the basal layer. The basal cell layer contains progenitors for the terminally differentiated post-mitotic outer layers of the epidermis and thus promote the process of stratification. Additionally, basal cells must maintain their proliferative capacity and self-renew to replenish the loss of outer skin cells.

The process of stratification begins around E13.5 with the appearance of the first suprabasal cells. Similar to basal cells, these first suprabasal cells maintain the ability to divide. However, this proliferative ability is lost when these cells mature into the spinous layer (Smart, 1970). Stratification and the appearance of suprabasal cells are accompanied by a change in keratin expression. The suprabasal cells express keratin 1 and 10 (Fuchs, Green, 1980). Now destined towards a terminal differentiation pathway, spinous cells differentiate into the granular layer of the epidermis. Finally, the epidermal barrier is formed by an increase in cell adhesion through formation of tight junctions (Furuse *et al.*, 2002). Additionally, the formation of cornified envelopes, composed of lipids and cross-linked proteins, function to provide the final seal from the outside environment (Fig. 3A) (Steven, Steinert, 1994).

While the formation and characteristics of each of the epidermal layers has been well described, little is known about what drives/initiates the process of stratification. Recently, a link between division orientation and stratification of the epidermis has been established. Prior to stratification (between E9.5 and E13.5) when the epidermis is a single monolayer of basal cells, most mitotic basal cells divide symmetrically, or parallel to the basement membrane (Fig. 3A) (Lechler, Fuchs, 2005; Smart, 1970). However, at E14.5 when stratification begins to occur, there is a dramatic shift in spindle orientation where 70% of mitotic basal cells now divide asymmetrically, or perpendicular to the basement membrane (Lechler, Fuchs, 2005). By coupling the proliferative capacity of the basal cells with spindle orientation, these progenitors can directly contribute to stratification. Symmetric divisions increase the basal progenitor cell number and increase surface area. Asymmetric divisions promote stratification and produce cells of the differentiated layers (Fig. 1C). This is in contrast to alternative models in which delamination from the basement membrane would result in stratification. These oriented cell divisions have been shown to be required for proper stratification of the epidermis. Studies that have perturbed division orientation resulted in the inability to properly stratify the epidermis (Williams *et al.*, 2011). Taken together, oriented cell divisions contribute not only to the cellular diversity of the epidermis, but also drive the morphogenic process of stratification that is required for the epidermis to act as a barrier. That said, disruption of ACD's in epidermis did not lead to a complete loss of stratification. This is likely due both to the incomplete nature of the disruption of ACD's (knockdown versus null), as well as additional mechanisms to drive stratification, which may include both alternative ways to orient the mitotic spindle and also delamination of cells. The importance of spindle orientation in the adult mouse skin is also unclear. While many fewer divisions are clearly asymmetric, the cells are more squamous and many divisions are oblique with respect to the basement membrane (Clayton *et al.*, 2007). Whether these result in functionally asymmetric divisions is not known. During homeostasis, delamination may drive a significant portion of the turnover of cells, though this has never been directly imaged.

2.2.5. Cell Fate and ACD in the Epidermis—Unlike the invertebrate model systems described above, we do not yet have a molecular handle on the connection between ACD

and cell fate specification in the epidermis. However, studies suggest that both intrinsic cues and extrinsic cues are responsible for generating cellular diversity in the tissue (Fig. 2C). An excellent candidate for an extrinsic factor that controls cell proliferation and fate is the basement membrane. In culture, keratinocytes grown in suspension withdraw from the cell cycle and undergo terminal differentiation (Green, 1977). This phenomenon is due to the negative regulation of differentiation that results from the adherence of the keratinocytes to the extracellular matrix (ECM) (Watt *et al.*, 1993). *In vivo*, integrin expression within the basal cells facilitates their attachment to the underlying ECM (Watt, 2002). Conditional knockout of $\beta 1$ integrin within basal cells resulted in reduced proliferation of the epidermis (Brakebusch *et al.*, 2000). In contrast, misexpression of $\beta 1$ integrin in the differentiated cells of the epidermis caused them to proliferate inappropriately (Carroll *et al.*, 1995). In addition, other integrins and ECM ligands may also contribute to the proliferation/differentiation decision of keratinocytes (Owens *et al.*, 2005; Owens *et al.*, 2003). Finally, basement membranes can also serve as reservoirs for growth factors and their accessory molecules (such as HSPGs) and thus may mediate effects on basal cells other than through direct adhesion. Whether specific factors generated by dermal fibroblasts or other skin resident cells are required for this specification is also still an active area of investigation. Taken together, current data is consistent with the hypothesis that the basement membrane serves as a niche that supports the self-renewing capacity of basal cell progenitors. Thus, ACDs, which result in the detachment of a daughter cell from the basement membrane, would promote the cell's differentiation.

In addition to extrinsic signaling, intrinsic factors such as EGFR and Notch have been implicated in controlling cell fate in the epidermis. EGFR is asymmetrically distributed in dividing human keratinocytes. The EGFR⁻ population remained mainly quiescent while the EGFR⁺ population demonstrated a greater cycling activity (Le Roy *et al.*, 2010). The EGFR⁺ population is hypothesized to be transit amplifying cells which could be equivalent to basal cells undergoing ACDs in the mouse epidermis. Consistent with these findings, mice overexpressing rat EGFR in the basal layer of the epidermis exhibit hyperproliferation and a possible delay in differentiation (Kiguchi *et al.*, 2000). Loss of EGFR results in a hypoplastic epidermis (Hansen *et al.*, 1997). While these studies primarily illustrate the ability of EGFR to maintain proliferation, it has also been shown to inhibit differentiation. Inhibition of the EGFR pathway in human keratinocytes led to the induction of the differentiation markers K1 and K10 (Peus *et al.*, 1997). We have also noted an asymmetric accumulation of EGFR in both developing mouse epidermis and cultured mouse keratinocytes (unpublished data).

Unlike EGFR, Notch acts to repress proliferation and induce terminal differentiation. Notch loss-of-function studies resulted in the complete loss of the spinous layer and hyperplasia. Conversely, Notch gain-of-function expanded the spinous layer and induces the expression of early differentiation markers (Blanpain *et al.*, 2006; Rangarajan *et al.*, 2001). Interestingly, EGFR has been shown to be a negative regulator of Notch signaling. The induction of differentiation markers in response to EGFR inhibition in human keratinocytes requires Notch activity (Kolev *et al.*, 2008). Taken together, these data indicate that EGFR acts in the basal layer to promote proliferation and repress Notch activity, and thus, differentiation. In the absence of EGFR, suprabasal cells are then able to activate Notch, which in turn, can promote the first stages of terminal differentiation. However, this is likely to be only part of the story as there appears to be significant redundancy in the important choice of differentiation in the epidermis.

Loss of function studies by shRNA of genes required for ACD in the epidermis has also suggested a link to Notch. When ACD's were disrupted, normal Notch signaling did not occur in the differentiated cells of the epidermis. Neither a Notch reporter construct, nor the

Notch-target gene *Hes1* were expressed. However, it remains unknown whether this is a relatively direct or indirect effect. The most well-studied mechanism to control Notch activation – through inhibition by Numb – does not appear to be required for correct epidermal differentiation (Williams *et al.*, 2011).

3. How to Establish an Axis of Division

To ensure the segregation of cell fate determinants, the spindle must correctly align itself to correspond with their asymmetric localization. Similarly, to ensure the removal of one daughter cell from a niche environment, the spindle must align to divide away from the niche (Fig. 2). The mechanisms that mediate proper spindle alignment are conserved pathways utilized between various species and cell types.

3.1. Spindle Rotation vs Centrosome Migration

As mentioned previously, neural plate cells in zebrafish and *Xenopus* undergo spindle rotation to reach the final axis of division (Geldmacher-Voss *et al.*, 2003; Kieserman, Wallingford, 2009). While spindle rotation is a common mechanism to orient the mitotic spindle, another mechanism involves the migration of one of the centrosomes (Rebollo *et al.*, 2009; Rebollo *et al.*, 2007; Rusan, Peifer, 2007; Yamashita *et al.*, 2003). While these result in the same final state they have important differences in the timing of when the axis of division is established (Fig. 4). Interestingly, in at least some cases, the molecular machinery required for proper alignment can direct either spindle rotation or centrosome migration to control the final spindle orientation. Some divisions that are asymmetric by cell fate are also asymmetric by daughter cell size. In addition to their roles in directing spindle alignment, some of the same proteins are also used to generate asymmetries in cell size. The mechanisms of generating size asymmetry are also diverse and will not be covered here in detail, as there is no evidence that epidermal ACDs involve size asymmetry.

Both the large size as well as the advantages in genetics and imaging in the *C. elegans* zygote have made it an important model for understanding how spindle alignment and displacement occurs. As discussed, the first cell division of the *C. elegans* zygote produces an AB-cell and a P1-cell. In addition to their different fates, the anterior AB-cell is larger in size. This is achieved through proper positioning of the mitotic spindle which undergoes a two step process to reach its final placement. First, as the pronuclei migrate towards the center of the embryo, the centrosome-pronuclear complex undergoes a 90° rotation to align the spindle along the anterior-posterior axis. Subsequently, during anaphase, the mitotic spindle becomes displaced from the center of the cell and moves towards the posterior (Albertson, 1984). The displacement of the spindle results in the unequal size of the resulting daughter cells. Spindle and centrosome ablation/severing experiments have demonstrated that, during displacement, there are asymmetric forces pulling on astral microtubules at the anterior and posterior ends of the spindle (Grill *et al.*, 2001). The asymmetry of forces and spindle repositioning depend on correct embryo polarity which is defined by the Par proteins (Cheng *et al.*, 1995; Kemphues *et al.*, 1988). Thus, the cortical polarity of the embryo is an important cue that directs the displacement of the spindle. The polarity cue is relayed to the spindle through contact of astral microtubules with the cortex. Mutations that perturb astral microtubules so that they are unable to contact the cortex also prevent spindle positioning (Albertson, 1984; Bellanger, Gonczy, 2003; Le Bot *et al.*, 2003; Srayko *et al.*, 2003). To date, the observation of spindle forces by laser ablation approaches has only been performed during spindle displacement, but has not been examined during spindle rotation in any system. It will be important to determine in smaller cells and in cells undergoing active spindle rotation whether forces are generated in the same positions and in the same way. For example, in small cells the ability of microtubules to generate force by

pushing rather than pulling, is increased (Tolic-Norrelykke *et al.*, 2004). That said, the conservation of machinery suggests conservation of mechanism.

In the initial division of the *Drosophila* neuroblast, just subsequent to its delamination from the neuroepithelium, rotation of the spindle allows its alignment with the apical/basal axis of the cell. *In vivo* imaging of neuroblast spindles using the microtubule-binding protein tau-GFP demonstrated that the spindle initially forms parallel to the epithelial layer. However, during metaphase, the spindle undergoes a 90° rotation to align itself along the apical-basal axis (Kaltschmidt *et al.*, 2000). Like in the *C. elegans* embryo, this rotation requires cortical cues (Schober *et al.*, 1999). In addition to the proper alignment of the spindle, the neuroblast must produce daughter cells of unequal size. The division results in an apical neuroblast that is larger than the basal GMC. While in *C. elegans* this is achieved through spindle displacement, *Drosophila* neuroblasts rely on both an asymmetric spindle and a spindle-independent mechanism to basally displace the cleavage furrow. Tau-GFP visualization of spindles has revealed that, during anaphase, apical microtubules began to elongate, while basal microtubules began to shorten. The asymmetric spindle moves the midbody closer to the resulting GMC (Kaltschmidt *et al.*, 2000). In addition, some components of the asymmetric division machinery (including Pins, discussed below) acts to displace myosin basally resulting in unequal partitioning of the cytoplasm (Cabernard *et al.*, 2010).

Recent studies have shown that spindle rotation only occurs during the first cell cycle in the neuroblast. Subsequently, spindle alignment is achieved without rotation but rather by centrosome migration. Two-photo microscopy of embryos expressing YFP-Asl and GFP- α -Tub84B were used to visualize the centrosomes and their ability to organize microtubules as the spindle assembled (Rebollo *et al.*, 2009). One centrosome remained apical throughout interphase and maintained its ability to organize microtubules. The other centrosome had little microtubule-organizing ability and moved extensively throughout the cytoplasm during interphase. By prophase, this centrosome had settled at the basal side of the cell and began to regain its ability to generate microtubules (Rebollo *et al.*, 2009). As a result, an apical-basal axis of division is established prior to the onset of mitosis (Fig. 4A). Spindle orientation in the *Drosophila* testis utilizes the same process, but the mechanism has yet to be elucidated (Yamashita *et al.*, 2007). These studies confirmed previous results that described centrosome migration in *Drosophila* larval neuroblasts (Rebollo *et al.*, 2007; Rusan, Peifer, 2007). It still remains unclear why the neuroblast uses two different mechanisms to establish its axis of division.

Use of spindle rotation versus centrosome migration results in different cell cycle timing for alignment of the spindle (Fig. 4). In centrosome migration, the axis of division is predetermined prior to nuclear envelope break-down and the entry into mitosis (Fig 4A) (Rebollo *et al.*, 2009; Rebollo *et al.*, 2007; Rusan, Peifer, 2007). Conversely, during spindle rotation, the axis of division is established after nuclear envelope breakdown and as late as anaphase (Fig 4B) (Kaltschmidt *et al.*, 2000; Poulson, Lechler, 2010). Spindle orientation driven by reorientation might allow cells to respond to environmental cues to quickly alter the final axis of division. This could be particularly beneficial in a tissue like the mouse epidermis, which must maintain a proper balance between two distinct orientations of division. It may also be a consequence of a cell having two pathways for orienting the mitotic spindle. For example, epithelial cells have both lateral membranes and apical-basal polarity that can be used to orient a spindle. Spindle rotation in the neuroblast only occurs immediately after delamination where the neuroblast retains some of its epithelial character. After this, it relies on a positional cue – the apical centrosome as a marker.

3.1.1. Spindle Alignment in the Epidermis—Given that the basal cells in the epidermis can divide in two distinct orientations, the timing mechanism by which the final

axis of division is chosen could be crucial. Using centrin-GFP transgenic mice to visualize centrosomes, it was seen that they remain apically anchored during interphase, including after duplication in S-phase. During prophase some cells still maintained apically localized centrosomes, while others began to set up bipolar spindles. Additionally, the cells that had established mitotic spindles did so in random orientations with no clear indication whether they would divide asymmetrically or symmetrically. Given these results, it is clear that basal cells in the epidermis do not establish their axis of division prior to mitosis through centrosome migration, as in *Drosophila* larval neuroblasts (Poulson, Lechler, 2010).

To further address this question, NuMA-GFP expressing mice were used to visualize spindle poles and to measure orientation of division during the cell cycle. While the spindles were randomly oriented during prophase, by metaphase approximately 70 percent of the cells had spindles oriented perpendicular to the basement membrane and the remaining cells had spindles parallel to the basement membrane. Thus, between prometaphase and late metaphase, the spindle undergoes rotation to reach its final axis. Visualization of this rotation was accomplished in cultured keratinocytes from both transgenic mouse lines, but has not yet been directly documented in intact epidermis (Poulson, Lechler, 2010).

Though the epidermis achieves spindle orientation in a manner distinct from most neuroblast cells, much of the underlying machinery for spindle orientation is conserved. The identification and mechanism of this machinery is discussed below.

4. Molecular Mechanisms Controlling Spindle Orientation

Whether a cell undergoes spindle rotation or centrosome migration to properly align its spindle, it is clear that polarity cues at the cortex play a key role. This is the case in the *C. elegans* zygote, as discussed previously. It is also true in both the *Drosophila* neuroblast and in the epidermis, though the requirements for cell polarization are distinct. Like the factors controlling cell fate, cell polarity can be acquired through extrinsic and/or intrinsic means.

4.1. Par/Inscuteable-Dependent Pathway

Drosophila neuroblasts have the ability to orient their mitotic spindle even in the absence of extracellular cues. When cultured and removed from their normal environment, mitotic neuroblasts were still able to localize the key components required for spindle orientation. Furthermore, the resulting divisions were asymmetric, producing one neuroblast and one GMC (Broadus, Doe, 1997). This process is achieved through the intrinsic polarization of the neuroblast. However, extrinsic cues are required to refine this process. Only when in contact with epithelial cells are the neuroblasts able to maintain proper positioning of the centrosome and polarize the Par complex at prophase (Siegrist, Doe, 2006). The initial polarity of these cells comes from the polarized neuroectoderm from which the neuroblasts are derived. In the neuroectoderm, the polarity proteins Par-3/6 are localized apically just above the adherens junction. Neuroblasts then delaminate from the epithelium and, in the process, inherit their polarity cues. Par-3/6 can be found in the stalk that extends into the epithelium as delamination occurs (Betschinger, Knoblich, 2004). The result is a specified neuroblast cell with a clear apical-basal axis along which the spindle can align.

How does a mitotic spindle align along a predetermined polarity axis? A core component that connects the polarity cues to the mitotic spindle in the neuroblast is the protein Inscuteable (Insc). Insc is not expressed in the neuroectoderm, but during neuroblast delamination, Insc colocalizes with Par3/6 in the stalk. When the stalk is retracted and delamination is complete, Insc forms an apical crescent at the cortex (Knoblich, 2001). This localization is achieved through Insc directly binding to Par3. In neuroblasts lacking Par3,

Inscuteable remains cytoplasmic and is not recruited to the apical cortex (Schober *et al.*, 1999).

Once at the cortex, Inscuteable recruits the adaptor protein Partner of Inscuteable (Pins). Pins is characterized by the presence of N-terminal TPR repeats and C-terminal Goloco domains (Yu *et al.*, 2000). The Goloco domains facilitate the binding of Pins to the heterotrimeric G protein subunit G α i. This interaction targets Pins to the plasma membrane, while its interaction with Insc through its TPR repeats allows for its apical accumulation (Yu *et al.*, 2002). During interphase, Pins is found in an inactive conformation in which the Goloco domains interact with the TPR repeats. During mitosis, binding to both G α i releases this inhibition and lead to its recruitment to the membrane (Du, Macara, 2004). At the apical cortex where G α i levels are above threshold, Pins is able to efficiently recruit Mud (Smith, Prehoda, 2011). Interestingly, after their initial recruitment, both Insc and Pins are required to maintain each others localization at the apical cortex (Yu *et al.*, 2000). While this range of interactions is clear, and the crystal structures of some of the interactions have been solved, we still do not have a complete accounting of all the molecular players nor their dynamic interactions throughout the spindle orientation process.

The recruitment of Mud by Pins provides a link between the polarity cues and the mitotic spindle. This interaction is achieved through the Pins TPR repeats and a Pins binding domain in the C-terminal tail of Mud (Bowman *et al.*, 2006; Izumi *et al.*, 2006; Siller *et al.*, 2006). This resulted in a very simple model of linear recruitment (Bazooka- Insc – G α /Pins – Mud) to recruit Mud to the apical cortex. By homology, this would give a Par3-Insc-LGN-NuMA complex in mammals (Fig. 3B). However, recent structural and biochemical data have clearly demonstrated that isolated LGN-binding domains of Insc and NuMA compete with one another for binding to LGN (Zhu *et al.*, 2011). This suggests that they exist in two distinct complexes – perhaps an Insc-LGN complex is required for recruitment to the apical cortex while a LGN-NuMA complex mediates effects on the mitotic spindle. However, it is also important to note that either in the context of the full-length proteins or in the presence of additional factors, these proteins could exist in a single complex. In this case, the regulation of binding partners wouldn't effect the integrity/localization of the complex, but could affect its activity.

NuMA (Mud in *Drosophila*) is a large coiled-coiled protein that, in addition to binding LGN, can also directly bind microtubules through a region in its C-terminal tail. Interestingly, there is a small region of overlap between the microtubule- and LGN-binding domains (Du *et al.*, 2001; Du *et al.*, 2002). This again results in mutually-exclusive binding. While the functional relevance of this overlap is still not understood, one possibility is that LGN acts to recruit NuMA to the cortex where it is then offloaded so it can interact with the astral microtubules of the mitotic spindle. However, because NuMA can exist as a dodecamer, it is also possible that in the context of the native proteins multiple binding domains allow simultaneous binding of both microtubules and LGN (Harborth *et al.*, 1999). The relevance of the NuMA-microtubule interaction and how it directs the orientation of the mitotic spindle has yet to be tested. In mice, specific genetic loss of the microtubule binding domain causes early embryonic lethality (Silk *et al.*, 2009). This suggests that either this domain has an essential role in mitosis (as work in cultured cells has strongly demonstrated) or that essential spindle orientation functions of the domain are required in early development (Heald *et al.*, 1996; Merdes *et al.*, 2000).

Nevertheless, it remains clear that the Insc/G α /LGN/NuMA proteins are at least partially required for proper spindle alignment. Neuroblasts mutant for components of the complex show partial randomization of spindle orientation (Bowman *et al.*, 2006; Izumi *et al.*, 2006; Siller *et al.*, 2006). The partial requirement of the Insc/G α /Pins/Mud complex could likely

be explained by a second pathway that contributes to spindle orientation. In an S2 *Drosophila* cell culture system, studies revealed that a domain within Pins can recruit Dlg to the cortex. Dlg can bind to the kinesin Khc-73, which can ultimately interact with astral microtubules and direct spindle orientation (Johnston *et al.*, 2009). Both the relative contributions of each of these pathways to spindle orientation as well as the conservation of the Dlg/Khc-73 pathway in other cell types remains unclear.

As previously discussed, polarity in the *C. elegans* zygote is established by the site of sperm entry. In response to this cue, intrinsic polarity is established within the cell through the localization of the Par proteins. Par3 and Par6 localize to the anterior cortex, while Par2 and Par1 localize to the posterior cortex (Cuenca *et al.*, 2003). This intrinsic polarity allows for the asymmetric localization of a ternary complex consisting of Gα, GPR-1/2 (LGN), and Lin-5 (NuMA) to the posterior cortex. This complex is homologous to the Gα/Pins/Mud complex described in *Drosophila*, differing only in the absence of Insc. Like the Par proteins, the Gα/GPR-1/2/Lin-5 complex is required for proper spindle displacement (Cheng *et al.*, 1995; Kempthues *et al.*, 1988; Lorson *et al.*, 2000; Srinivasan *et al.*, 2003).

The large size of the *C. elegans* zygote and the robust displacement of the spindle towards the posterior during the first cell division has provided insight into how the Gα/GPR-1/2/Lin-5 complex interacts with and controls the mitotic spindle. The laser severing experiments described previously have demonstrated that there are greater pulling forces on astral microtubules in the posterior region of the cell (Grill *et al.*, 2001). While it still remains uncertain how the Gα/GPR-1/2/Lin-5 complex works to direct force on the mitotic spindle, experiments have implicated the minus-end motor protein dynein in the process. Co-immunoprecipitation experiments with various components of the Gα/GPR-1/2/Lin-5 complex showed that they are able to interact with members of the dynein complex. Furthermore, evidence indicates that this interaction occurs through Lin-5. The cortical localization of dynein requires the Gα/GPR-1/2/Lin-5 complex in *C. elegans*. This would result in the cortical anchoring of dynein, where it could generate pulling forces on astral microtubules (Fig. 3B). Consistent with this, various mutations resulting in an inactive dynein complex show reduced pulling forces (Nguyen-Ngoc *et al.*, 2007).

While dynein is strongly implicated as a force generator in spindle displacement, there is still much to be learned about how the force is generated and transduced. In addition to molecular motors, microtubule dynamics themselves can generate forces that can be harnessed by the cell. These include both pushing forces (for relatively short microtubules) and coupling the forces of depolymerization to movement. In the *C. elegans* zygote, microtubule dynamics have been directly quantitated. On the anterior end, the residence time of microtubule plus ends is higher than on the posterior end (where forces are higher) (Labbe *et al.*, 2003). Thus microtubule dynamics are positively correlated with force production. In support of this, local application of taxol at the anterior or posterior end of the embryo caused a significant drop in force production at that site (Nguyen-Ngoc *et al.*, 2007). Thus a combination of forces from dynein motors and microtubule depolymerization may control spindle positioning. It will be important to determine how these work together and/or are coordinated with each other.

4.2. PCP Dependent Pathway

In contrast to neuroblasts, *Drosophila* SOP cells use extracellular signals to establish their axis of division. Rather than setting up an apical-basal division, SOP cells divide along their anterior-posterior axis. The Planar Cell Polarity (PCP) pathway is used to define this axis within the plane of the epithelium. The transmembrane receptor Frizzled (Fz) and its cortical effector Dishevelled (Dsh) localize to the posterior cortex of the SOP cell. This localization restricts Fz signaling to the posterior of the cell, which ultimately translates into planar

polarization. Another PCP protein, Strabismus (Stbm), localizes to the anterior cortex and recruits Pins, where they both work to restrict Par-3/6 to the posterior cortex (Bellaiche *et al.*, 2004; Bellaiche *et al.*, 2001b). Like the *C. elegans* zygote, SOP cells lack Inscuteable expression, and thus, Pins and the Par proteins localize to opposite sides of the cell. Interestingly, spindle orientation along the A-P axis does not require Pins or the Par complex. Instead, both Fz and Dsh have been shown to be required (Bellaiche *et al.*, 2001a; Bellaiche *et al.*, 2001b). This is possibly due to the binding of Dsh to Mud, linking extracellular signaling through the Fz pathway to regulation of the mitotic spindle (Segalen *et al.*, 2010). Oriented cell divisions during zebrafish gastrulation have been shown to use this pathway as well (Gong *et al.*, 2004).

In addition to the PCP pathway that relies on Fz and Dsh, Fat and Daschous can also regulate the PCP pathway. Proper oriented cell divisions in the *Drosophila* wing require Fat and Daschous (Baena-Lopez *et al.*, 2005). Fat and Daschous are atypical cadherins that interact at cell junctions. A gradient of Daschous is expressed along the proximal-distal axis of the wing (Simon, 2004). Daschous can bind to Fat and transduce a signal to the atypical myosin Dachs. In response to this signal, Dachs becomes localized distally (Rogulja *et al.*, 2008). Mutant clones of Fat, Daschous, or Dachs show defects in spindle orientation (Baena-Lopez *et al.*, 2005). Additionally, Daschous is sufficient to induce oriented cell divisions in the wing, as altering the direction of its gradient also alters the direction of cell division (Mao *et al.*, 2011).

In conclusion, while there is conserved machinery that drives spindle reorientation in many cell types, there are many variations on the theme of how and when they are localized and functioning. There are also additional ways to place spindles, such as the actin and formin-dependent localization of meiotic spindles observed in oocytes (Pfender *et al.*, 2011; Yi *et al.*, 2011). While the mechanisms controlling each of the ACDs differs somewhat between species and cell type, it is clear that the proper orientation of the mitotic spindle is a key aspect in forming and shaping tissues as well as generating cellular diversity. The development of the mouse epidermis provides an attractive system for studying how oriented cell divisions couple with ACDs to drive the morphogenic process of stratification.

4.3. ACD machinery in the epidermis

Asymmetrically dividing basal cells of the mouse epidermis express many of the genes required for asymmetric cell division in neuroblasts (Lechler, Fuchs, 2005). The epidermal Insc/LGN/NuMA proteins are homologous to the Insc/Pins/Mud proteins described previously (Fig. 3B). Knock-down of LGN and NuMA in the developing epidermis result in spindle orientation defects, as well as stratification defects, providing the clearest evidence that ACDs in the epidermis drive stratification (Williams *et al.*, 2011). Additionally, short-term lineage tracing experiments have provided direct evidence that these oriented cell divisions are accompanied by cell fate changes. Labeled cells from a perpendicular (asymmetric) division gave rise to one basal cell expressing keratin-14 and one suprabasal cell expressing keratin-10 (Poulson, Lechler, 2010). In combination, these studies provided a functional link between asymmetric cell divisions and the development of the epidermis.

As discussed above, it is interesting that both basal cells and neuroblasts require the apical Insc/LGN/NuMA complex to orient ACDs, yet they use completely different mechanisms to establish the axis of division. This is most likely due to the cell cycle regulation of the complex. In basal cells, NuMA remains nuclear during interphase, while in neuroblasts NuMA is not nuclear and can accumulate at the apical cortex prior to nuclear envelope breakdown (Izumi *et al.*, 2006; Lechler, Fuchs, 2005). As a result, NuMA could act as an apical anchor for one of the centrosomes while the other centrosome migrates basally in neuroblasts. Conversely, in basal cells, by recruiting NuMA to the cortex during mitosis,

there is an additional opportunity to respond to environmental cues and alter the orientation of division.

Recent studies have implicated the transcription factor Srf and the actomyosin cytoskeleton as crucial for the proper alignment of the mitotic spindle. Conditional knock-out of Srf resulted in the inability of mitotic cells to round up, likely due to defects in the cortical actomyosin network. These mice also had mislocalized NuMA and LGN, and defects in spindle orientation (Luxenburg et al., 2011). These studies implicate the acto-myosin system and the regulation of cell shape in the proper orientation of the mitotic spindle.

5. Regulation of Division Orientation in the Epidermis

Another important difference between the epidermis and neuroblasts is that while neuroblasts obligately divide asymmetrically, both symmetric and asymmetric cell divisions occur within the basal layer of the epidermis. It has been proposed that a balance of symmetric:asymmetric cell divisions is required to give a tissue of the right size (to cover the body) and thickness (to generate the required differentiated layers). However, little is known about how the ratio of SCD/ACD is regulated in the developing epidermis.

5.1. Basal cells can divide along two axes

Two extreme models make predictions on how the SCD/ACD ratio could be balanced. First, basal cells could be pre-programmed to divide in a given orientation (either symmetric or asymmetric). This would suggest the existence of a stem-like cell that divides symmetrically and a transit-amplifying cell that divides asymmetrically. The different proliferation rates of each cell type could then be regulated as various cell types were needed. An alternative model suggests that every basal cell is equivalent and has the ability to divide in either orientation. Environmental cues would then be responsible for informing individual cells which way to divide. As a result, the ratio of SCD/ACD would be the sum of individual cell choices. While these two models present the extremes, it is possible that the reality lies somewhere in between.

To address this question, short term lineage tracing was done following basal cells through one or two divisions. Analysis of two cell clones reaffirmed previous results that during the process of stratification, approximately 70% of basal cells divide perpendicular to the basement membrane. Analysis of three cell clones revealed that the majority of basal cells could divide both asymmetrically and symmetrically. In addition to this main population of basal cells, there also appeared to be a small population that was biased towards dividing symmetrically. It is possible that this represents a small population of basal cells that is preprogrammed to undergo symmetric divisions, but it is impossible to conclude from the given data (Poulson, Lechler, 2010). As we learn more about the basal layer and its level of homo/heterogeneity, these experiments may need to be revisited and reinterpreted.

While these studies indicate that basal cells have a choice in which way to divide, long term imaging through multiple cell cycles *in vivo* would be ideal. With lineage tracing experiments, it is always possible that adjacent recombination events could alter the result as could selectivity of the cells in which recombination occurs. However, given the prevalence of single cells that underwent asymmetric and symmetric divisions, it is unlikely that many cells are hardwired in terms of division orientation.

The above studies suggest that during stratification, individual cells must respond to environmental cues to decide upon a division orientation. This is unique compared to the invertebrate models described above which obligately undergo ACDs. This makes the

epidermis a unique model to study the mechanisms that direct a cell to undergo an asymmetric division (discussed below).

5.2. Inscuteable Drives ACDs in the Epidermis

Because basal cells have a choice between two division orientations and because spindle rotation is used to achieve both alignments, the epidermis is an interesting tissue to study how ACDs are regulated. While knock-down studies have shown that both LGN and NuMA are required for ACDs, little is known about how the proper balance between symmetric and asymmetric divisions is controlled (Williams et al., 2011). One interesting candidate is the protein Insc, which links the cortical polarity complex with the ACD machinery. Insc expression is restricted to those cells undergoing asymmetric cell divisions. In *Drosophila*, Insc is specifically expressed in the asymmetrically dividing neuroblasts, but not in the symmetrically dividing neuroectoderm. In the absence of Insc, the neuroblasts fail to properly orient their spindles. Second, when Insc is ectopically expressed in the neuroectoderm, it can induce reorientation of the spindle (Kraut et al., 1996). Similar studies in the *Drosophila* SOP, where Insc is not normally present, reveal that Insc is sufficient to reverse the polarity of the cell (Bellaiche et al., 2001b). These data reveal that Insc is both necessary and sufficient to drive ACDs. Finally, Insc is an ideal candidate for promoting the processes of stratification by recruiting the general spindle orientation machinery to the apical cortex. Consistent with this idea, in the epidermis Insc levels are low at E11.5 but increase around E13.5, coinciding with the onset of the stratification process (Poulson, Lechler, 2010).

Transgenic mice allowing for epidermal-specific and doxycycline-inducible overexpression of Insc were used to highly regulate spatial and temporal control of its expression. After eight hours of induction at E14.5, when the process of stratification has begun, the induced Insc localized to the apical cortex where it colocalized with its downstream effectors LGN and NuMA. Additionally, an increase in the amount of asymmetric divisions (those perpendicular to the basement membrane) was observed. These were confirmed to be functionally asymmetric through lineage tracing experiments (Poulson, Lechler, 2010). In addition to transgenic Inscuteable inductions, lentiviral EYFP-mInsc mice demonstrated an increase in ACDs (Williams et al., 2011). Thus, like in *Drosophila*, Insc expression is sufficient to drive the apical localization of the ACD machinery and thereby promote asymmetric divisions. While these studies reveal that Insc is sufficient to drive ACDs in the epidermis, it is still unclear whether Insc is necessary. Recent work in neural stem cells has demonstrated a requirement for Insc in spindle orientation in those cells (Postiglione et al., 2011). Nevertheless, the sufficiency of Insc to drive ACDs in the epidermis implicates Insc expression as a key regulatory point that can drive the morphogenic process of stratification.

Interestingly, when Insc expression was induced in the epidermis over a three day period, the increase in ACDs that was seen in the short term inductions was not maintained. Under these conditions, the percentage of ACDs showed a slight decrease. Further analysis of the ACD machinery in these mice revealed the cause of this change. Apical crescents of both Insc and LGN were still observed. However while they are normally markers for ACDs, these apical crescents were observed in symmetrically dividing cells. Thus, division orientation can become uncoupled from the apical Insc/LGN complex. Even though Insc/LGN remained apical under these conditions, NuMA was noticeably absent from the cortex in symmetrically dividing cells.

One interpretation of these results is that the epidermis was able to sense an imbalance in the proper ratio and correct for it. Another possibility is that excess Insc competes with NuMA for binding to LGN. Without apical NuMA, the cells cannot divide asymmetrically. Neither of these answers are fully satisfactory with our expectations. For example, it has been

reported that LGN is required for planar spindle orientation in epithelial cells in culture (Peyre *et al.*, 2011). This appears not to be the case in the epidermis as: a) cells that have apical LGN in the Insc-induced mice undergo symmetric divisions, and b) knockdown of LGN in epidermis resulted in loss of asymmetric divisions, not a complete randomization of division orientation (Williams *et al.*, 2011). In addition, the inability of Insc/LGN complexes to recruit NuMA has also been observed in wild-type epidermis, where Insc is not being misexpressed. At a low rate, there is a disconnect between apical Insc/LGN and NuMA. In these cells, symmetric divisions occur. Therefore, NuMA can be functionally uncoupled from LGN in natural conditions (Poulson, Lechler, 2010). Taken together, these Insc studies have been important in revealing two key regulatory points in controlling ACDs. The level of Inscutable expression controls the onset of ACDs, but this signal can be overridden by regulating NuMA targeting to the cortex.

6. Concluding Remarks

Oriented cell divisions are crucial for a variety of developmental and homeostatic processes ranging from elongation to stratification. In addition to driving changes in shape, oriented divisions are used by many cell types to undergo ACDs, generating cellular diversity. Importantly, some stem cells undergo ACDs to promote differentiation while also maintaining the progenitor cell population. While invertebrate model systems have been important for understanding the machinery underlying ACD's, the epidermis is an important model for how ACDs are regulated to control proper tissue development.

Because the epidermis must maintain a balance of asymmetric:symmetric divisions, it must also regulate the ACD machinery to ensure proper development. Short term lineage tracing experiments have demonstrated that this regulation is not simply due to two unique populations within the basal cell layer. Rather, basal cells have the ability to divide in both orientations. As a result, regulation of the asymmetric cell division machinery is one way in which the ACD:SCD ratio could be controlled in the epidermis. Supporting this hypothesis, misexpression of Insc in the stratifying epidermis has both an instructive and a regulatory role. Given that Insc is sufficient to drive ACDs, its regulated expression at the onset of stratification is an excellent candidate for the trigger for this morphogenesis. The uncoupling of spindle orientation from Insc/LGN complexes suggests another level of control on ACDs. Mechanistically this was due to the loss of NuMA from the apical cortex resulting in symmetric divisions even though Insc/LGN were still apical.

The study of ACD, and especially ACD in the epidermis, is a relatively young field and many exciting questions remain to be answered. We highlight some of these below.

How is the machinery required for ACD localized to specific regions in the cell? While there have been significant advances in uncovering proteins involved in spindle orientation, we still do not have the full complement of players. In addition, large holes in our understanding of how these proteins are recruited to specific sites at the cell cortex still exist. New information is coming out quickly about additional binding partners for spindle orientation proteins and/or new requirements for their localization. While there is a lot of cell type specificity in the upstream players, in most cases, these appear to converge on LGN/Pins, NuMA and dynactin as major players in controlling spindle orientation.

Related to the above question is the issue of how the spindle orientation machinery generates productive force on the mitotic spindle. The specific roles of microtubule dynamics and dynein motor activity still need to be better understood. In addition, we have no clear molecular understanding of what stabilizes the force-generating machinery at the cell cortex. While the actin cytoskeleton is implicated in this, the connections between the force generators and the cortex are not clear.

All the issues discussed above also hold for symmetric divisions within the epidermis and in simple epithelia. Surprisingly, we know much less about how spindles align during symmetric divisions. As discussed above, there is conflicting evidence on the role of LGN in symmetric divisions. Therefore identifying this machinery and its regulation will also be paramount to understanding how tissue development occurs.

Developmental questions are also outstanding. How does the epidermis commit to stratification? What are the developmental cues, and what are the targets in addition to Inscuteable? This information may also shed light on how the balance of symmetric and asymmetric divisions is regulated to yield a normal sized and shaped skin.

One of the largest holes in our understanding of ACD's in the epidermis is the identification and unraveling of the intrinsic and extrinsic cues that connect these divisions to cell fate decisions. While we proposed some candidates, much work will be needed to test these and to decipher their relative contributions.

Loss of asymmetric cell divisions can lead to the formation of tumors in some experimental contexts. It has not yet been conclusively demonstrated that loss of ACDs in mammals can promote tumorigenesis, but the epidermis will be a valuable model system to test the possible pathological consequences.

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Figure 1.

(A) Tissue elongation is driven through oriented cell divisions along the axis of elongation. The overall affect of dividing in the same direction results in an overall elongation of the tissue. (B) Branching may occur through oriented cell divisions dividing perpendicular to the plane of a tissue. (C). Stratification of a tissue is driven through oriented cell divisions perpendicular to an underlying basement membrane. The resulting apical daughters cells will go on to form the stratified layers of the tissue.

Figure 2.

(A) Intrinsic signals rely on cell fate determinants being localized to one side of a cell. The mitotic spindle can then align the division so that there is an unequal inheritance of these factors. The unequal inheritance can drive different cell fates in each daughter cell. (B) Extrinsic cells rely on either attachment or proximity to a niche. The niche provides signals to nearby cells to maintain a particular cell fate. By dividing away from the niche, a daughter cell can enter a new environment and take on a different cell fate. (C) Both intrinsic cues and extrinsic cues determine cell fate in the epidermis. Basal cells rely on attachment to the underlying basement membrane through beta1-integrin. This attachment is required to maintain the proliferative capacity of the basal cell. Asymmetrically dividing basal cells also localize EGFR basally. Asymmetric inheritance of EGFR is thought to specify the basal cell fate. Conversely, apically inherited factors could specify the spinous cell fate. It is currently unknown if there are any apical cell fate determinants.

Figure 3.

(A) The epidermis is a stratified squamous epithelium. The basal layer is attached to an underlying basement membrane and makes up the proliferative compartment of the tissue. Divisions parallel to the basement membrane are symmetric and give rise to two basal daughter cells. Divisions perpendicular to the basement membrane are asymmetric and give rise to one basal cell and one differentiated suprabasal. Once a cell enters the suprabasal layer it begins a terminal differentiation program first becoming part of the spinous layer and then proceeding to the granular cell fate. Finally, a cornified layer is formed that ultimately provides the barrier function of the tissue. Each layer can be distinguished through various markers such as those listed. (B) Molecular machinery at the apical cortex of an asymmetrically dividing basal cell function to properly orient the mitotic spindle. Par3 localizes to and specifies the apical membrane. Par3 binds to Inscuteable which in turn recruits LGN to the apical cortex through binding its N-terminal TPR domains. LGN is also anchored to the membrane through the binding of Gai to its C-terminal GoLoco Domains. Once at the cortex, LGN recruits NuMA apically. NuMA, a microtubule binding protein, is thought to function through dynein to direct force on the mitotic spindle. Through minus ended directed movement along astral microtubules, dynein could create net pulling forces on the spindle towards the apical cortex. (C) This molecular mechanism is conserved across many species including *Drosophila* and *C. elegans*.

Figure 4.

(A) *Centrosome Migration* After centrosome duplication in S phase, one of the centrosomes remains apically anchored while the other migrates towards the basal end of the cell. This results in an axis of division being established prior to the onset of mitosis and nuclear envelope breakdown (NEB). (B) *Spindle Rotation* After centrosome duplication, both centrosomes remain apically anchored. Upon NEB, the spindle begins to elongate and rotate until an final axis of division is established.