Radial intercalation of ciliated cells during *Xenopus* skin development

Jennifer L. Stubbs1,2, Lance Davidson3,*, Ray Keller3 and Chris Kintner1,†

Cells with motile cilia cover the skin of *Xenopus* tadpoles in a characteristic spacing pattern. This pattern arises during early development when cells within the inner layer of ectoderm are selected out by Notch to form ciliated cell precursors (CCPs) that then radially intercalate into the outer epithelial cell layer to form ciliated cells. When Notch is inhibited and CCPs are overproduced, radial intercalation becomes limiting and the spacing of ciliated cells is maintained. To determine why this is the case, we used confocal microscopy to image intercalating cells labeled using transplantation and a transgenic approach that labels CCPs with green fluorescent protein (GFP). Our results indicate that inner cells intercalate by first wedging between the basal surface of the outer epithelium but only insert apically at the vertices where multiple outer cells make contact. When overproduced, more CCPs are able to wedge basally, but apical insertion becomes limiting. We propose that limitations imposed by the outer layer, along with restrictions on the apical insertion of CCPs, determine their pattern of radial intercalation.

**KEY WORDS:** Ciliated cells, Intercalation, Epithelium

**INTRODUCTION**

Cells with beating cilia are a common feature of many organ systems that depend on a directed fluid flow to function (Afzelius, 1995). For example, ciliated cells produce fluid flow in tissues as diverse as: the respiratory tract of mammals, where they clear mucous and debris; the choroid plexus, where they circulate the cerebral spinal fluid into the ventricles of the brain; and the reproductive tract, where they transport the egg along the oviduct. Proper development and function of these organs, therefore, requires the formation of a specialized epithelium containing cells with motile cilia.

The skin of the amphibian embryo also produces a directed fluid flow generated by ciliated cells, thus serving as a model system for studying how such ciliated epithelia form during organogenesis. In *Xenopus*, the skin develops after gastrulation through the differentiation of two cell types that are derived from two distinct layers of the ectoderm (Fig. 1A) (Drysdale and Elinson, 1992). Cells in the outer layer of the ectoderm, also called the superficial layer, differentiate into mucus-producing epidermal cells, thus forming an occluding epithelial barrier on the embryo surface. Cells in the inner layer of the ectoderm, also called the sensory layer, spread out underneath the outer layer during epiboly (Keller, 1980) and a subset give rise to ciliated cell precursors (CCPs) during early neurulae stages (stages 12-14) (Deblandre et al., 1999; Drysdale and Elinson, 1992). These precursors then differentiate into ciliated cells by intercalating radially into the outer layer during mid neurulae stages (stages 16-20) and undergoing ciliogenesis, allowing them to produce a directed fluid flow by late neurulae stages (stages 22-26). Ciliated cell differentiation is precisely controlled, thus ensuring that the cells are distributed across the epidermal surface at high density in an evenly spaced pattern.

In many developing tissues, specific spacing patterns of differentiated cells are generated by lateral inhibition, an evolutionarily conserved process in which cells inhibit their neighbors from acquiring the same fate using the Notch signaling pathway (Kintner, 2003). Studies of Notch in the *Xenopus* skin indicate that lateral inhibition also operates during the formation of ciliated cells, whereby Notch negatively regulates the number of CCPs that form in the inner layer of the ectoderm (Deblandre et al., 1999). By determining CCP number, the process of lateral inhibition could conceivably act to distribute ciliated cells evenly across the skin surface. However, when Notch is inhibited and CCPs are overproduced, the density of ciliated cells detected at tadpole stages only increases about twofold; moreover, these cells remain spaced out (Deblandre et al., 1999). Thus, although Notch determines the number of CCPs that form in the inner layer, other factors determine the pattern of ciliated cells in the outer layer. As CCPs need to intercalate radially to become ciliated cells, one possibility is that this morphogenetic process is a crucial step in controlling the pattern of ciliated cell differentiation (Deblandre et al., 1999).

By marking inner and outer cells with lineage tracers, Drysdale and Elinson (Drysdale and Elinson, 1992) showed that inner cells contribute not only ciliated cells but also an equal population of intercalating non-ciliated cells (INCIs) to the outer layer (Fig. 1A). Thus, the pattern of CCPs in the outer layer may not only be determined by their ability to intercalate but also by interactions with the INCIs. To examine these issues, we used two assays to characterize inner cells during radial intercalation. We first show using a transplantation assay that inhibiting Notch leads to more CCs and INCIs in the outer layer, although their number and distribution differ significantly. We then develop a transgenic assay to distinguish CCPs from INCIs in order to describe the behavior of these two cell types during intercalation both normally and when they are overproduced after disabling Notch signaling. The results of these analyses reveal important morphological differences between CCPs and INCIs at the earliest stages of radial intercalation. We propose that these differences, along with limitations imposed on intercalation at the apical surface by the outer layer determine the pattern of ciliated cells found in the *Xenopus* skin.

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MATERIALS AND METHODS

Transgenic embryos and RNA injection

The promoter region of $\alpha$-tubulin was isolated by screening a Xenopus laevis genomic library (Stratagene) with 32P radiolabelled DNA fragment from the $\alpha$-tubulin cDNA (Deblandre et al., 1999) followed by plaque purification of positive clones. Templates for anchored PCR were generated by digesting purified phage DNA with either EcoRI, XhoI, HindIII or BamHI, followed by ligation to pBluescript digested with the same enzyme. DNA sequences lying upstream of the $\alpha$-tubulin gene were then amplified by PCR, using one primer corresponding to cDNA sequences around the start of translation of the $\alpha$-tubulin protein and the other corresponding to the T3 polymerase recognition sequence in pBluescript. The largest PCR fragment generated was cloned into the CS2 vector (Turner and Weinstaub, 1994) replacing the CMV promoter upstream of the membrane-localized form of GFP. Clones containing the correct region of the $\alpha$-tubulin gene were verified by sequencing. For transgenics, $\alpha$-tubulin-mGFP DNA was isolated away from vector sequences by digestion with SalI and Acc65I, mixed with sperm nuclei and injected into unfertilized eggs, as described by Amaya and Kroll (Amaya and Kroll, 1999) with modifications (Lamar and Kinney, 2005; Sparrow et al., 2000). Routinely, 50-70% of the embryos were transgenic. In some experiments, transgenic embryos were also injected at the two-cell stage with RNAs (1-5 ng) encoding a dominant-negative form of Su(H) (Wettstein et al., 1997), or the repressor form of Su(H) [Su(H)-H11008/H11008].

Transplant assays and explant cultures

Xenopus embryos were obtained by in vitro fertilization using standard protocols (Sive et al., 1998). To introduce lineage tracers, embryos were injected four times at the two- to four-cell stages with capped, synthetic RNA (Sive et al., 1998) encoding membrane-localized forms of GFP or RFP. At stage 10, a fine needle or hair was used to peel off the outer layer from a region of the ectoderm from a donor embryo, which was transferred onto the host embryos after removing a similar patch of outer cells. Although the transplanted tissue healed onto the host embryo, it was kept in place by pressurizing with a small piece of a glass coverslip, held in place with silicone grease. In some transplants, host embryos were not only injected with RNA encoding a tracer but also with RNA that either activates [JCD (Wettstein et al., 1997)] or inhibits the Notch pathway [dnHMM (Fryer et al., 2002)]. Transplants were performed in Danilichki’s buffer + 0.1% BSA (Davidson et al., 2002). After healing of the transplanted tissue, embryos were returned to 0.1% Mar’s Modified Ringers (MRR) (Sive et al., 1998). Ectoderm was also explanted onto coverslips coated with fibronectin as described (Davidson et al., 2002).

Immunofluorescence and confocal microscopy

Fixation of embryos for confocal microscopy was performed for 1 hour on ice with 4% paraformaldehyde in PBS, followed by dehydration in 100% ethanol. Fixed embryos were rehydrated, washed with PBS/0.1% Triton-X-100 (PBT), and blocked with PBT containing 10% heat-inactivated normal serum (PBT/HIGS) for at least 1 hour. Embryos were incubated with primary antibody in PBT/HIGS overnight as follows: rabbit anti-ZO-1 (Zymed 1:200), mouse monoclonal anti-acetylated tubulin (Sigma, 1:1000), mouse monoclonal anti-Xenopus E-cadherin (SD3, Developmental Studies Hybridoma Bank, 1:500) or rabbit anti-GFP (Molecular Probes, 1:100). After washing, embryos were incubated overnight in Cy2-, Cy3- or Cy5-labeled goat anti-IgG of the appropriate species (all used at 1:500, Jackson ImmunoResearch), washed in PBT and then mounted in PVA/DABCO. Mounted embryos were imaged on a BioRad Radiance 2100 confocal mounted to a Zeiss inverted microscope using a 40x or 63x objective.

Low light epifluorescence time-lapse sequences at two wavelengths were collected at multiple positions from a cooled CCD camera (Hamamatsu; Bridgewater, NJ) mounted on an inverted compound microscope (Olympus; Melville NY). Camera settings, XYZ-position, shutter and filters were computer controlled by image acquisition software (Metamorph; Molecular Devices, Downingtown PA).

RESULTS

Inner and outer cells during radial intercalation

In order to distinguish inner cells and outer cells during radial intercalation, we modified an assay in which outer layer ectoderm from a donor embryo was grafted onto the inner layer ectoderm of a host prior to gastrulation (Drysdale and Elinson, 1992). In this modification, host and donor cells were marked by injecting embryos at the two-cell stage with RNA encoding membrane-localized forms of GFP (mGFP) or RFP (mRFP), respectively, allowing inner cells that have intercalated into the outer layer to be imaged at high resolution with confocal microscopy (Fig. 1B). At stage 28, when the larval skin has fully differentiated and the embryo has undergone axial extension, approximately half of the inner cells that had intercalated into the grafted outer layer were ciliated cells, while the other half were not (Drysdale and Elinson, 1992). Intercalating non-ciliated cells (INCs) differ morphologically from ciliated cells (CCs) by more than just the lack of cilia (Fig. 1B). For example, although CCs rarely lie adjacent to each other, INCs can often be found close to or in contact with a CC. In addition, INCs are typically smaller than ciliated cells, perhaps accounting for the fact that INCs on average make contact with three cells in the outer layer while the CCs make contact with four to five cells (data not shown). Finally, INCs are columnar in shape, while CCs have a small round apical domain and broad basally (see Fig. S1 in the supplementary material). Thus, the inner cell layer contributes two morphologically distinct cell types to the outer layer in approximately equal numbers (Fig. 1F).

To examine how cells in the outer layer respond to radial intercalation, we imaged live grafts using low-magnification, time-lapse fluorescent microscopy (Fig. 1C). Prior to the onset of intercalation, the outer layer epithelium is organized in a typical honeycomb pattern, as predicated by the optimal packing of epithelial cells into a hexagonal array. During intercalation, cell-cell contacts between two neighboring outer cells remain intact, with little or no change in their dimensions (Fig. 1C). Outer cell division was rarely observed during imaging (data not shown), suggesting that division of outer cells is not necessarily associated with, and presumably not required for, most intercalation events. The most prominent change in the outer cells during intercalation was a local rearrangement of cell borders where vertices retract between outer cells in the immediate area where an inner cell intercalates (Fig. 1C, see circled vertex). Thus, these results indicate that the outer epithelium is a relatively static structure that rearranges locally to accommodate the insertion of new elements.

INCs and CCs respond differently to Notch inhibition

The static nature of the outer layer raises the possibility that it effectively limits the total number of intercalating inner cells. If this were the case, then one possibility is that when Notch is inactive, the total number of intercalating cells remains the same, but INCs are replaced by CCs, thus explaining the modest twofold increase in the density of ciliated cells found at tadpole stages (Deblandre et al., 1999). To test this possibility, we used the same transplantation assay but transplanted outer layer cells onto host embryos that express an inhibitor of Notch signaling (dnHMM) (Fryer et al., 2002) and then scored the number of CCs and INCs at stage 28. As expected, inhibiting Notch in the inner layer resulted in a small increase in the density of ciliated cells (Fig. 1E,F) compared with controls (Fig. 1D). However, inhibiting Notch did not produce a loss of INCs but rather a dramatic increase in their number (Fig. 1F). To accommodate this increase in the total number of intercalating cells,
many of the INCs were located adjacent to each other while the CCs remain evenly spaced (Fig. 1E). Conversely, when Notch signaling was activated by expression of the intracellular domain of Notch (ICD), both CCs and INCs were lost (see Fig. S2 in the supplementary material). Thus, these results indicate that CCs and INCs do not represent a reciprocal population, but are instead regulated in tandem by Notch signaling. Additionally, these results show that CCs and INCs behave differently during radial intercalation in both the number and spacing of cells observed in the outer layer.

Transgenic analysis of ciliated cell precursors
As the transplantation assay cannot distinguish between CCPs and INCs during intercalation, we developed a second assay to label CCPs using a transgenic approach, based on an isoform of \( \alpha\)-tubulin that marks ciliated cells (Deblandre et al., 1999). Accordingly, a 2.5 kb genomic fragment lying upstream of the \( \alpha\)-tubulin gene was cloned upstream of mGFP (called \( \alpha\)-tubulin-mGFP, see Materials and methods) and used to generate transgenic embryos (Amaya and Kroll, 1999). Embryos transgenic for \( \alpha\)-tubulin-mGFP first expressed mGFP soon after gastrulation (Fig. 2A), within a subset of inner cells (Fig. 2D) that resemble the pattern of cells expressing \( \alpha\)-tubulin RNA (Fig. 2E). At tadpole stages, mGFP was strongly expressed in the skin of transgenic embryos but only in cells with cilia (Fig. 2C,F). Given the perdurance of GFP, we conclude that the cells expressing the \( \alpha\)-tubulin-mGFP transgene during intercalation give rise to ciliated cells but not to INCs.

Using the \( \alpha\)-tubulin-mGFP transgenic assay, we imaged CCPs during radial intercalation using confocal microscopy both in embryos as well as in ectoderm explanted from transgenic embryos onto fibronectin-coated coverslips (Davidson et al., 2002). In these explants, the ectoderm spreads out onto the fibronectin matrix deposited on the glass, much as it normally does during epiboly and gastrulation. This preparation provides the added advantage of allowing one to image intercalating cells from both the inner and outer surfaces.

In both embryos and explants at early-neurula stages (stages 13-16), CCPs were visualized based on \( \alpha\)-tubulin-mGFP expression as a subset of the inner layer cells (Fig. 3A,B,G,H). At this stage, the cell bodies of the CCPs were already wedged between the basolateral surfaces of the outer layer cells, extending processes that go up to, but not through, the apical tight junctions that seal the outer layer of cells together, as marked by staining with antibodies to ZO-1 (Merzdorf and Goodenough, 1997) (Fig. 3A,G). Thus, inner cells initiate ciliated cell differentiation, at least as marked by \( \alpha\)-tubulin-mGFP expression, prior to integrating fully into the outer epithelium. When imaged in explants from the basal surface, the CCPs were separated from the fibronectin substrate by a layer of inner cells (Fig. 3L), suggesting that CCPs are not an integral part of the inner layer and initially intercalate basally into the outer layer. Thus, CCPs appear to initiate intercalation by establishing extensive contact with epidermal cells by wedging between them basally, prior to apical insertion.

During mid-neurula stages (st17/18), intercalating CCPs penetrate and join the outer epithelium as assessed by the interdigitation of GFP\(^*\) labeled membrane between the ZO-1-labeled, apical junctions (Fig. 3C,D). Significantly, CCPs did not penetrate the apical junctions randomly, but are restricted to inserting between outer cells at vertices where multiple outer cells make contact (Fig. 3C). In addition, by this stage, the intercalating CCPs that were embedded into the outer layer began to take on a regular spacing pattern, even those that had not yet inserted apically into the
outer layer (Fig. 3D, arrow). Perhaps as a consequence, intercalating CCPs were rarely if ever detected at the same apical insertion site, and thus, avoid cell-cell contact with each other at the apical surface. Around this stage, ZO-1 staining also revealed small apical domains that were GFP-negative, indicating that at least some of the INCs insert apically around the same time as CCPs (data not shown).

By late neurula stages (stages 20-24), most CCPs have inserted apically, typically making contact with four or five outer cells (Fig. 3E,F). As the vertices between outer cells involve three to four cells prior to intercalation (Fig. 3A,G, see also Fig. 1C), this observation implies that alterations of vertices to accommodate the intercalating CCPs result in a rearrangement of outer cells, as indicated by the time-lapse imaging described above. In regions of the developing skin where the density of CCPs was relatively high, the intercalating CCPs took on a lattice-like pattern (Fig. 3E,F). In explants where relatively little growth takes place, CCPs also only inserted into vertices, and observed a spacing pattern where two CCPs rarely shared the same apical insertion site, while associating with four or five outer cells (Fig. 3K,L). Thus, the radial intercalation of CCPs into the outer layer in both embryos and explant culture does not occur randomly but only at vertices, follows a spacing rule that precludes apical insertion of adjacent CCPs, and culminates in the association of four or five outer cells with each CCP.

**Overproduced ciliated cell precursors are precluded from intercalation**

Blocking Notch increases substantially the number of cells expressing $\alpha$-tubulin RNA (Deblandre et al., 1999) but only produces a small increase in density of ciliated cells (Fig. 1E,F). The previous interpretation of these observations is that only a fraction of the CCPs can intercalate while the ‘excess’ remained trapped internally. To confirm this interpretation, we followed CCPs using confocal microscopy after blocking Notch signaling in $\alpha$-tubulin-mGFP transgenic embryos by injecting dnHMM RNA (Fryer et al., 2002).

When imaged from the apical surface of the outer layer at stage 16, control and dnHMM injected transgenic embryos contained approximately the same density of intercalated CCPs (Fig. 4I). As we are limited in our ability to detect CCPs that are located deeper than about 10 µm from the apical surface, we peeled the skin from transgenic embryos and imaged the basal side of the CCPs through the inner layer (Fig. 4E,F). The density of CCPs detected basally in control regions was similar to that detected apically (Fig. 4I), indicating that most if not all of the CCPs gained access and intercalated into the outer layer by stage 16 (compare Fig. 4A with 4E). By contrast, in dnHMM regions, CCP density detected basally increased at least twofold relative to the control, with many of the excess CCPs clustered and overlapping each other making accurate quantification difficult (Fig. 4F,I). Thus, the total number of CCPs generated is regulated by Notch signaling but those associated with the outer layer are regulated by their ability to intercalate.

To determine whether any of the ‘excess’ CCPs produced in Notch-deficient embryos were ultimately able to intercalate, we next examined late neurulae stages. In control embryos, the density of CCPs found in the outer layer was actually lower at later stages than at earlier stages. As a fixed number of CCPs seem to be generated early and all of these intercalate, we assume that these are diluted out as the outer layer increases in size during embryo growth (Fig. 4C,I). By contrast, in regions expressing dnHMM, the density of CCPs intercalated into the outer layer remained high, while maintaining a lattice-like packing pattern (Fig. 4D,I). These were localized to cell clumps that were poorly attached to the inner layer (Fig. 4H), making the size of this population difficult to measure. Thus, these observations indicate that intercalation of CCPs is limited but that additional CCPs can intercalate as the area of the outer layer grows, thus leading to an increased density of ciliated cells. Even at late stages, however, spatial limits on intercalation continue to restrict the number of ciliated cells as at least some of the overproduced CCPs remain trapped in the inner layer.

**Intercalating CCPs differ morphologically from INCs**

The results above indicate that the insertion of CCPs into the outer layer is restricted during radial intercalation, thus limiting the number of ciliated cells. By contrast, the intercalation of INCs seems to be less restricted based on the transplantation assay. To explore the difference between the INCs and CCPs further, we took advantage of the transgenic assay using sperm nuclei prepared from an F1 transgenic male. When injected into eggs, these nuclei produced transgenic $\alpha$-tubulin-mGFP expression in one half of the offspring based on the expected Mendelian distribution of a single insertion site. In addition, transgenic sperm nuclei were injected into albino eggs, thus eliminating the surface pigment and allowing for deeper imaging of intercalating cells. Finally, transgenic embryos were injected with mRFP RNA to label cell surfaces, thus allowing one to visualize outer cells and intercalating inner cells, while the transgenic mGFP expression was used to distinguish CCPs from INCs (Fig. 5). Outer cells, CCPs and INCs were imaged in live
embryos beginning at stage 16 and proceeding to stage 22 when
ciliogenesis begins, and data were collected from several different
regions of the developing skin at hourly intervals.

To determine when INCs and CCPs intercalate, we scored those
located near the apical surface as well those inserted basally (Fig.
5A-D, Table 1). Under normal conditions, CCPs were found to
outnumber INCs, even as late as stage 22 when most CCPs had
intercalated (Table 1). As INCs and CCPs are present in equal
numbers at later stages (Fig. 1F), INCs apparently intercalate over a
more protracted period, including after CCP intercalation is
normally complete. When Notch was inhibited, INCs represented a
proportionally larger fraction of the intercalating cells at early stages,
although their numbers were still modest compared with the large
increase of INCs seen at later stages in the transplantation assay (Fig.
1F, Table 1). Perhaps more striking is the difference between the
number of INCs and CCPs located apically versus basally when
Notch is inhibited (Table 1, Fig. 5). Inhibiting Notch markedly
increased the number of INCs and CCPs located basally at early
stages, correspondingly increased the number of INCs located
apically at late stages, but had little effect on the number of apically
located CCPs. These findings indicate that the ability of CCPs to
intercalate becomes limiting between basal and apical insertion.
Furthermore, as apical insertion becomes limiting, INCs may
compete with CCPs for intercalation space.

The difference in the rate of basal versus apical insertion of
intercalating cells is also evident in the behavior of the outer cells.
Under normal conditions, only a small fraction of the space present
around the basolateral circumference of an outer cell is taken up by
intercalating cells (Fig. 5B, broken lines). When Notch is blocked,
most of this space becomes occupied early on by both INCs and
CCPs, although two neighboring outer cells were never separated by
more than one intercalating cell, except at tricellular corners (Fig.
5D, broken lines). To accommodate the increase in intercalating
cells, therefore, outer cells respond basally by losing contact with
each other and narrowing around their circumference (Table 2; 6
μm column). By contrast, outer cells decrease their cell-cell contacts
more slowly at the apical surface (Table 2, apical column) in the face
of increased numbers of intercalating cells (Fig. 5D, broken lines),
perhaps reflecting the static nature of apical contacts between
neighboring outer cells (Fig. 1C, see Discussion). Thus, outer cells
may limit the insertion and expansion of intercalating cells at the
apical vertex, thus restricting the amount of space available for
intercalation.

As space for intercalation becomes limiting, CCPs may be
impacted more than INCs based on morphological differences that
mirror those evident at later stages (Fig. 1). CCPs take up twice as
much space as INCs when they wedge between the basolateral
surfaces of the outer cells (Fig. 5, data not shown). As a
consequence, CCPs are much more bulb-like in shape compared with the INCs, which tend to be more triangular and columnar (see Fig. S1 in the supplementary material). Significantly, the shape and sizes of intercalating CCPs and INCs look similar in control and dnHMM conditions (data not shown), indicating that these cells do not change their morphology to increase the number of intercalating cells.

Finally, the ability of CCPs to insert apically may also be limited by self-exclusion at the apical vertex. During intercalation, CCPs make contact with each other basally, but are rarely observed sharing a vertex when they insert apically (Table 3, Fig. 5). By contrast, CCPs can share a vertex with one or more INCs (Table 3, Fig. 5). Finally, at the late phases of intercalation, when Notch is blocked and a large fraction of CCPs remain trapped below the apical surface, many of these are positioned beneath a vertex that already contains a ciliated cell and one to several INCs (Fig. 5E,F arrowheads). Thus, CCP intercalation may be limited by self-exclusion at the vertices, by competition with INCs for intercalation space, and by a limitation that the outer layer imposes on the amount of intercalation space available at the apical surface.

**DISCUSSION**

The *Xenopus* larval skin is a ciliated epithelium, evenly decorated with ciliated cells at relatively high density. A key step in the formation of this tissue architecture is a radial intercalation event, in which CCPs generated in the inner cell layer move into the outer layer. By the time the skin is removed and imaged from the internal surface, the number of mGFP-expressing cells located in the inner layer increases significantly in regions expressing dnHMM. At stage 18, the mGFP-expressing cells located internally spread out and extend protrusions (F), but at stage 24 have rounded up and appear poorly attached to surrounding cells (H).

**Table 1. Density of INCs and CCPs during radial intercalation**

<table>
<thead>
<tr>
<th>Time</th>
<th>CCP-apical</th>
<th>CCP-6 µm</th>
<th>CCP-10 µm</th>
<th>INC-apical</th>
<th>INC-6 µm</th>
</tr>
</thead>
<tbody>
<tr>
<td>t=1</td>
<td>Control</td>
<td>28±7</td>
<td>39±9</td>
<td>41±9</td>
<td>23±7</td>
</tr>
<tr>
<td></td>
<td>dnHMM</td>
<td>26±10</td>
<td>56±15</td>
<td>71±20</td>
<td>31±13</td>
</tr>
<tr>
<td>t=2</td>
<td>Control</td>
<td>32±9</td>
<td>40±10</td>
<td>42±10</td>
<td>28±11</td>
</tr>
<tr>
<td></td>
<td>dnHMM</td>
<td>45±18</td>
<td>74±14</td>
<td>88±17</td>
<td>49±23</td>
</tr>
<tr>
<td>t=3</td>
<td>Control</td>
<td>37±4</td>
<td>45±5</td>
<td>47±6</td>
<td>26±9</td>
</tr>
<tr>
<td></td>
<td>dnHMM</td>
<td>43±11</td>
<td>75±18</td>
<td>87±18</td>
<td>53±19</td>
</tr>
<tr>
<td>t=4</td>
<td>Control</td>
<td>35±7</td>
<td>41±6</td>
<td>43±6</td>
<td>26±9</td>
</tr>
<tr>
<td></td>
<td>dnHMM</td>
<td>42±20</td>
<td>66±25</td>
<td>76±21</td>
<td>54±10</td>
</tr>
<tr>
<td>t=5</td>
<td>Control</td>
<td>32±5</td>
<td>37±6</td>
<td>37±6</td>
<td>19±9</td>
</tr>
<tr>
<td></td>
<td>dnHMM</td>
<td>44±16</td>
<td>65±19</td>
<td>73±14</td>
<td>55±15</td>
</tr>
</tbody>
</table>

Table shows average number of cells per 100 outer cells. The number of each cell type (CCP, INC) was determined per field based on confocal images (Fig. 5) of control, mRFP injected and dnHMM + mRFP-injected embryos. Cells were counted at the indicated time points (t=1 to t=5), representing stages 18-22. The number of each cell type was standardized to 100 outer cells, and the average of data taken from three embryos (two to four fields per embryo). A field is defined as an area 191 µm by 191 µm containing between 55 and 70 outer cells.

**Table 2. Average length (µm) of contacts between adjacent outer cells**

<table>
<thead>
<tr>
<th>Time</th>
<th>Apical</th>
<th>6 µm</th>
</tr>
</thead>
<tbody>
<tr>
<td>t=1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>88.0±22.1</td>
</tr>
<tr>
<td></td>
<td>dnHMM</td>
<td>112.3±17.0</td>
</tr>
<tr>
<td>t=3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>76.3±14.4</td>
</tr>
<tr>
<td></td>
<td>dnHMM</td>
<td>80.6±24.9</td>
</tr>
<tr>
<td>t=5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>78.6±18.0</td>
</tr>
<tr>
<td></td>
<td>dnHMM</td>
<td>69.0±23.9</td>
</tr>
</tbody>
</table>

Table shows average length in µm of OC-OC contacts. Length of contacts between adjacent OCs at t=1 (stage 18), t=3 (stage 20) and t=5 (stage 22) based on confocal images (see broken white line Fig. 5A-D). Each number is the average of data based on five cells from three fields taken from three embryos. A field is defined as an area 191 µm by 191 µm containing between 55 and 70 outer cells.
Fig. 5. Morphology of INCs, CCPs and outer cells during radial intercalation. Albino embryos transgenic for \( \text{\(\tau\)}-\text{tubulin-mGFP} \) were injected at the two-cell stage with mRFP RNA alone (A,B) or a mixture of mRFP and dnHMM RNA (C-F). (A-D) Transgenic embryos at an intermediate stage in intercalation (stage \( \approx 18 \)) were imaged live in the confocal microscope to score the morphology and number of ciliated cells (mGFP), outer cells or INCs (asterisks). (A,C) A confocal slice viewed apically; (B,D) a slice \( \approx 6 \) \( \mu \text{m} \) below the apical surface. broken lines indicate cell-cell contacts between outer cells; white lines indicate outer cell area. (E,F) Confocal images of transgenic embryos at stage 22, showing trapped CCPs (arrowheads) located below the apical surface. Images are a composite of apical mRFP expression and a \( \approx 6 \) \( \mu \text{m} \) stack of mGFP expression. Scale bars: 10 \( \mu \text{m} \).

outer occluding epithelium. Although the trans-epithelial movement of cells has been studied in such models as germ cell migration (Kunwar et al., 2003) or the trans-endothelial migration of leukocytes (Luscinskas et al., 2002), comparatively little analysis has been carried out to determine how specialized cells join an epithelial cell layer during development (Carthew, 2005). We analyze this process in the developing larval skin by determining the morphogenetic rules that govern radial intercalation under normal conditions, as well as when intercalating cells are overproduced.

Notch regulation of intercalation

Previous lineage studies showed that INCs and CCs are normally present in equal proportions and are often located adjacent to each other in the outer layer (Drysdale and Elinson, 1992). These findings suggested that INCs and ciliated cells might arise in pairs following the asymmetric division of a common intercalating precursor and led us to ask whether Notch signaling mediates this binary fate decision. However, our lineage analysis using \( \text{\(\tau\)}-\text{tubulin-mGFP} \) expression as a tracer indicates that ciliated cell precursors and INCs are already distinct prior to intercalation. Furthermore, inhibiting Notch not only increases the number of ciliated cells but also, dramatically, the number of INCs. Thus, Notch may have an additional, more-general role in regulating the intercalation of inner cells in parallel with its function in inhibiting ciliated cell differentiation. In line with this possibility, we have also found that ectopic expression of ICD, a constitutively activated form of the Notch receptor, suppresses the appearance of INCs in addition to CCPs, at least through early neurula stages (see Fig. S2E in the supplementary material). Similarly, INCs and CCs are also eliminated when inner cells express ESR6e, a member of the family of HHLH repressors that acts as a Notch target gene in the skin (Deblonde et al., 1999) (see Fig. S2C,F in the supplementary material). These results suggest a model in which Notch activity in the inner layer induces targets such as ESR6e, which, in turn, repress the expression of genes required for radial intercalation.

Morphogenetic changes during intercalation

During the early phases of radial intercalation, time-lapse images reveal dynamic protrusive activity in which inner cells extend and retract processes between the basolateral surfaces of the outer layer cells, just below the apical junctional complexes. This behavior is similar to that which occurs at earlier developmental stages during epiboly when inner cells radially intercalate between each other to thin the sensorial ectoderm (Longo et al., 2004). During epiboly, however, inner cells migrate basally, making contact with a matrix of fibronectin that lines the blastocoel, and later on, the basal surface of the developing skin. By contrast, CCPs and INCs migrate in the opposite direction, thereby pushing up between the outer cells (wedging) and ultimately to the apical surface. Thus, inner cells may initiate intercalation behavior during epiboly but a switch must then occur that directs their migration apically rather than basally.

As inner cells move into the outer layer, they first intercalate by wedging between the lateral surfaces of the outer cells, prior to interdigitating between the apical junctions to join the epithelium. During wedging, intercalating cells can be located at any point around the circumference of an outer cell (Fig. 5), but when they insert apically, they do so exclusively at vertices: the points within an epithelium where at least three outer cells make contact. The preference for these points presumably reflects that an apical vertex is where the apical junctions between outer cells are interrupted as they pass from one cell to the next, and thus the place where the apical junctures can be disassembled to provide room for an intercalating cell to join the outer epithelium. Conversely, the vertex may also be the only place for an intercalating cell to establish new apical contacts in a manner that maintains a seal, while still allowing new tight junctions to form. The implication of this finding is that the apical vertex represents a key site for the disassembly or reassembly of junctional contacts that need to occur as cells join an epithelial layer. Similar arguments have been made in terms of how assembly of the junctional complex is regulated when cells form an epithelial sheet de novo in vitro (Adams et al., 1998; Adams et al., 1996; Vasioukhin et al., 2000) or when an epithelial sheet rearranges (Fristrom, 1988).

Maximal packing pattern of CCs

Under conditions where Notch signaling is normally active, \( \approx 30-40 \) CCPs form per 100 outer cells, and all of these, assuming no loss to cell death, gain access to and intercalate into the outer layer. When Notch is inhibited, the number of CCPs in a given area increases at least twofold, although we suspect that this is an underestimation as we can count only CCPs located within \( \approx 10 \mu \text{m} \) of the outer or inner surface. Despite this increase in CCPs, the number of ciliated cells that have inserted apically by early neurula stages is similar in dnHMM and control embryos (Table 1). As these embryos grow and the number of cells in the outer layer expands, the density of CCPs remains high in regions where Notch has been inactivated, suggesting a model where ‘trapped’ CCPs can intercalate when a space opens up. Nonetheless, we can still detect CCPs ‘trapped’ in the inner layer even at late stages, suggesting that a certain proportion of the CCPs never make it into the outer layer (Fig. 4). Thus, these observations suggest strongly that limitations on CCP intercalation largely determine the density and spacing of ciliated cells.
To determine why intercalation is limiting, we used confocal microscopy to analyze the three principle players (INC, CCPs and outer cells) in terms of their shape and number, both normally as well as when Notch is inhibited. One finding that emerges from this analysis is that intercalation is potentially limited at the apical surface by restrictions imposed by the outer layer. Thus, outer cells initially allow more intercalating cells to wedge between their basolateral surfaces, which they accommodate by narrowing to take up less space, and by making contact with intercalating cells around their circumference. At the same time, however, outer cells seem to restrict intercalating cells apically, particularly if that cell is a CCP (Table 1). One possible reason for this difference is the difficulty of establishing apical junctions with outer cells, which occurs only at the apical vertex. Moreover, once an intercalating cells inserts apically, the size of its apical domain grows slowly, remaining small relative to the space it occupies basally (Fig. 5). Again, this may reflect the difficulty of forming apical junctions with outer cells, but also the rate at which these junctions can form at the expense of those between outer cells, which appear static during intercalation (Fig. 1C). The picture that emerges from these observations, therefore, is one where the outer cells restrict junction formation apically by acting topologically as a bottleneck. As long the outer cells resist moving farther apart apically, they limit the space available for intercalating cells, both apically and basally (Fig. 5).

If the outer layer acts as a bottleneck, then the shape and size of inner cells is likely to influence the pattern of their intercalation. CCPs and INCs have a characteristic size, regardless of whether their density is low as in the normal case, or when they pack into the outer layer as when Notch is inhibited. However, a CCP takes up about twice as much area as an INC because they are bulb-shaped during intercalation, while INCs are more columnar. These differences in shape and size may impact the intercalation of CCPs more than INCs, as intercalating space becomes restricted.

The behavior of INCs and CCPs during intercalation raises the possibility that several inhibitory interactions may influence their patterns of intercalation, particularly as their numbers increase. Under normal conditions, intercalating CCPs initially outnumber INCs (see Fig. 3), suggesting that the former intercalates more readily than the latter. However, when Notch is blocked, the proportion of INCs to CCPs increases substantially, raising the possibility that INCs fill in the intercalating space available, and thereby inhibit the intercalation of CCPs. Until we find a means of eliminating INCs, we are currently unable to assess their role in limiting CCP intercalation. Nonetheless, competition between these two intercalating cells types may be significant factor, particularly when the number of intercalating cells surpasses the space in the outer layer that is available for new cells.

A second, potentially significant inhibitory interaction is one that occurs between CCPs. CCPs rarely if ever insert at the same apical vertex even when they lie adjacent to each other basally (Table 3, Fig. 5). By contrast, an apical vertex often contains both a CCP and an INC, or even two INCs, indicating that multiple cells can intercalate along each other apically as long as they are not both CCPs. These observations suggest that when CCPs insert apically they cannot overlap. This restriction may reflect the tendency of CCPs to occupy a large basal space coupled with the requirement that cells only insert apically at a vertex. In this model, as INCs are smaller, they are able to insert adjacent to each other or to CCPs. Alternatively, another possible mechanism is that during apical insertion, CCPs favor cell-cell contacts with outer cells or INCs, but not with themselves. In this model, when CCPs are specified, they express adhesion molecules that enable apical junctions to form more readily with outer cells or INCs, but not with each other. Evidence for both possibilities comes from the finding that when CCPs are overproduced many of the trapped cells are found at the basolateral membrane of the outer layer and lie adjacent to other CCPs that have already established an apical domain.

In summary, these results indicate that during the complex process of radial intercalation, the spacing pattern of intercalating cells is likely to be influenced by several factors. Many of these factors, however, seem to relate to the pivotal role that the apical vertex plays in the process of intercalation. Intercalating cells use the vertex as the entry point for establishing apical contacts with outer cells. Modification of apical contacts occurs at the vertices, thus allowing outer cells to move apart. This separation is potentially the rate-limiting step in providing space for the insertion of new cells into an epithelium both apically and basally.

### Table 3. Number of adjacent CCPs and INCs

<table>
<thead>
<tr>
<th>Time</th>
<th>CCP+CCP apical</th>
<th>CCP+CCP 6 μm</th>
<th>INC+INC apical</th>
<th>INC+INC 6 μm</th>
<th>CCP+1 INC</th>
<th>CCP+2+ INC</th>
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<td><strong>t=1</strong></td>
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<td></td>
<td></td>
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<tr>
<td>Control</td>
<td>0.91±1.14</td>
<td>0.45±0.52</td>
<td>2.55±1.37</td>
<td>6.82±2.23</td>
<td>4.82±2.56</td>
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<tr>
<td>dnHMM</td>
<td>0.91±1.14</td>
<td>0.45±0.52</td>
<td>2.55±1.37</td>
<td>6.82±2.23</td>
<td>4.82±2.56</td>
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<td></td>
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<td></td>
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<tr>
<td>Control</td>
<td>0.36±0.67</td>
<td>1.18±1.37</td>
<td>0.55±0.82</td>
<td>2.50±3.06</td>
<td>7.00±3.30</td>
<td>3.30±2.41</td>
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<td>dnHMM</td>
<td>0.25±0.62</td>
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<td>3.30±2.41</td>
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<td>dnHMM</td>
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</table>

Table shows number of adjacent cells per field. At each time point (t=1-5), corresponding to stages 18-22, the number of cell-cell contacts between CCPs and INCs were counted. Each number is the average of data based on two or three embryos. A field is defined as an area 191 μm by 191 μm containing between 55 and 70 outer cells.
and basally. Finally, the vertex is where CCPs may exclude each other during apical insertion, thus generating the spacing pattern where CCPs are only surrounded by outer cells or INCs. These observations suggest that the regulatory events that occur at the apical vertex are likely to be key in understanding the process of radial intercalation and how this process controls tissue morphology.

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Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/133/13/2507/DC1

References