Contraction of basal filopodia controls periodic feather branching via Notch and FGF signaling

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Branching morphogenesis is a general mechanism that increases the surface area of an organ. In chicken feathers, the flat epithelial sheath at the base of the follicle is transformed into periodic branches. How exactly the keratinocytes are organized into this pattern remains unclear. Here we show that in the feather follicle, the pre-branch basal keratinocytes have extensive filopodia, which contract and smooth out after branching. Manipulating the filopodia via small GTPases RhoA/Cdc42 also regulates branch formation. These basal filopodia help interpret the proximal-distal FGF gradient in the follicle. Furthermore, the topological arrangement of cell adhesion via E-Cadherin re-distribution controls the branching process. Periodic activation of Notch signaling drives the differential cell adhesion and contraction of basal filopodia, which occurs only below an FGF signaling threshold. Our results suggest a coordinated adjustment of cell shape and adhesion orchestrates feather branching, which is regulated by Notch and FGF signaling.
Biological systems utilize various principles to achieve periodic pattern formation\textsuperscript{1–4}. Periodic epithelial branching is a widely used mechanism to increase the surface area of an organ. Such a mechanism is exemplified in feather branching, which characterizes modern birds\textsuperscript{5,6}. In this process, the epithelial sheath at the base of the follicle organizes into periodic branches\textsuperscript{7–11} (Fig. 1). Recently, the regularly branched feather structure was utilized as a model to dissect the pathological principles of tissue damage due to chemo- and radiation therapy, because any perturbations of feather development are recorded in the final feather morphology\textsuperscript{12–15}. Thus, the formation of the exquisite feather branches is of both evolutionary and medical interest.

Feather branching has been considered as a classical example of how periodic structures result from the reaction-diffusion mechanism during pattern formation\textsuperscript{10}. The involvement of the antagonistic molecule pairs such as BMP4/Noggin and BMP2/Shh has been proposed\textsuperscript{16,17}. Furthermore, a set of core signaling molecules, including BMP, Shh, Wnt and FGF, has been shown to regulate this process\textsuperscript{9–11,16–18}. However, it remains unclear at the cell level how the keratinocytes are organized into the periodic branches.

Here we report how cells accommodate the rapid formation of feather branches through the rearrangement of cell adhesion and changes in cell shape, and how molecular signaling controls the patterning of the periodic feather branches. We find that extensive filopodia present on basal keratinocytes before branching, which disappear after branch formation. These filopodia are regulated by the Rho family small GTPases RhoA and Cdc42, and help interpret the FGF signaling gradient in the feather follicle. FGF and Notch signaling regulate the branching process and further control the formation of the filopodia. Calculating the surface area before and after branching reveals a scaling effect resembling the “coastline paradox”, which was proposed by Benoit Mandelbrot in the 1960s to describe the fractal nature of the coastline\textsuperscript{19}. Thus counter-intuitively, the surface area increase during feather branching morphogenesis is actually prepared in advance. These results provide mechanistic insight into the epithelial branching process.

**Results**

Filopodia in basal keratinocytes of the feather epithelium. We examined the ultrastructure of feather epithelium before and after branching (Fig. 2a, b; and Supplementary Fig. 1). Transmission electron microscopy (TEM) analysis revealed extensive filopodia in basal keratinocytes in the pre-branch feather epithelium (Fig. 2c). Higher magnification views showed clear basal lamina along the filopodia, including the lamina densa and lamina lucida (Fig. 2c). Depending on the specific location in the feather follicle, these filopodia vary in size and length. On average, each basal cell extends 3–5 filopodia about 2–10 μm long as counted/measured from the TEM images, with no single filopodium showing dominance over the others. Filopodia from two neighboring cells may fuse together, with the cell membranes running side by side to separate the cells (Supplementary Fig. 1a). Upon branching, the filopodia disappear and a smooth basal lamina is formed. Still, adjacent basal keratinocytes form tight junctions (TJs) in the apical/basolateral border, and zone of adherens junctions (AJs) at the sites of lateral cell-cell contact (Fig. 2d and insert). Therefore, even with the extensive filopodia, the basal keratinocytes retain these classical adhesion structures.

We characterize the filopodia by additional marker analysis (Fig. 2e). FITC-phalloidin showed strong staining in the filopodia, suggesting the presence of rich F-Actin bundles. E-Cadherin and β-Catenin are also expressed. When double-stained with a mesenchymal marker Tenascin C (Tn), we found inter-digitation of β-Catenin and Tn staining, suggesting the filopodia project into the mesenchyme and do not result from artificial contraction of the epithelium or mesenchyme during sample preparation. Finally, VASP and Fscn1 are markers normally associated with filopodia; they also stained positive in these structures (Fig. 2e and Supplementary Fig. 2).

Contraction of filopodia controls feather branching. Filopodia are cell projections that are subject to the regulation of Rho family small GTPases. We tested whether these molecules can regulate the filopodia in basal keratinocytes. We cloned both the constitutively active (CA) and dominant negative (DN) forms of RhoA, Rac1, and Cdc42 into lentivirus. The capability of these constructs to regulate filopodia was confirmed in vitro (Supplementary Fig. 3). In vivo, two independent methods were used to examine the roles of these genes in feather development (Fig. 3a, b; Supplementary Fig. 4): In the first method, we directly injected lentivirus into actively growing feather follicles and examined the impact of local gene perturbation\textsuperscript{14,15}; In the second method, we made transgenic feathers via lentiviral-mediated overexpression or RNAi knockdown\textsuperscript{18}. The CA forms did not disrupt feather formation, while the DN forms of RhoA and Cdc42 induced ectopic branches in the rachis, and loss of rachis in the feathers (Fig. 3c, d). DN-Rac1 produced normal feathers, consistent with its inability to disrupt filopodia in cell culture.

Because the DN forms of GTPases may elicit non-specific effects\textsuperscript{20,21}, and there are over 20 Rho family GTPases in the avian genome (Supplementary Table 1)\textsuperscript{22,23}, we further verified the impact of RNAi knockdown of these small GTPases. The knockdown efficiency and specificity of RNAi were each verified in vitro and in vivo (Supplementary Figs. 4 and 5). In particular, RNAi for RhoA or Cdc42 did not perturb the expression of other Rho family GTPases (Supplementary Fig. 5). Consistent with the results from overexpressing the DN forms, knockdown of RhoA or Cdc42 produced feathers with weaker or no rachis in the upper part, whereas knockdown of Rac1 resulted in normal feathers.
These results demonstrate that the regulation of basal filopodia is causally linked with feather branching morphogenesis.

**E-Cadherin regulates feather branching.** A few possibilities may explain, at the cell level, how exactly the feather epithelium is organized into branches: differential proliferation, differential cell death/apoptosis, and differential cell adhesion. No pre-patterned cell proliferation or apoptosis was found before or immediately after branch formation, as shown by TEM analysis, PCNA staining, or TUNEL staining (Fig. 4a, b and Supplementary Fig. 6). We reasoned that cells are directly re-arranged into the periodic pattern, possibly through a differential adhesion mechanism. Consistently, we found that there are higher levels of E-Cadherin and β-Catenin in each barb plate, while the marginal plate cells express lower levels of these molecules (Fig. 4c, d).

Because E-Cadherin-mediated cell adhesion depends on its organization at the nanoscale, we examined in detail its distribution pre- and post- feather branching. In the pre-branch basal keratinocytes, AJJs were formed between two adjacent cells; however, it appears the E-Cadherin molecules were more diffusively distributed (Fig. 4e, f). On the other hand, in the branched feather barbs, the outer layer cells (marginal plate) use more stable TJs and desmosomes to build cell connections, as these cells have reduced levels of E-Cadherin (Supplementary Fig. 1b). The inner barb cells, which have higher levels of E-Cadherin, showed distinct puncta of E-Cadherin distribution (Fig. 4e), and a continuous zone of AJJs under TEM (Fig. 4f). These structures resemble the previously described adhesion
Thus, E-Cadherin is down-regulated in the basal keratinocytes and re-distributed in the suprabasal cells during feather branching.

We tested the functional significance of E-Cadherin-mediated cell adhesion in feather branching morphogenesis. When E-Cadherin was ectopically overexpressed, the branching of feather epithelium was blocked; conversely, we obtained feathers with supernumerary branches and reduced rachis size when E-Cadherin expression was suppressed (Fig. 4g, h). Therefore, differential epithelial cell adhesion via redistribution of E-Cadherin is required for feather branching.

Activation of Notch signaling drives feather branching. We then explored the molecular pathways that control the feather branching process. Notch signaling is often harnessed to generate periodic spatial patterns and has been implicated in embryonic feather development. In our effort to profile gene expression in the adult feather follicle, we identified members of the Notch signaling pathway including Notch1, Notch2, Serrate1, Serrate2 (Supplementary Table 1). Here we mapped the expression of these genes during feather branching (Fig. 5a–d and Supplementary Fig. 7): Notch1 and Serrate1 are enriched in the pre-branch feather epithelium but are expressed at low levels in the basal keratinocytes. Notch2 expression is more ubiquitous, whereas Serrate2 is mainly expressed in the basal keratinocytes. After branching, Notch1 is enriched in the barb plate, Serrate2 is enriched in the complementary marginal plate, whereas Notch2 and Serrate1 are more ubiquitously expressed.

The role of Notch signaling in feather development is examined in vivo. RNAi-Notch1 (and Notch2) produced feathers with supernumerary branches (Fig. 5e–h). Similar results were obtained for RNAi-Serrate1 and RNAi-Serrate2 (Supplementary Fig. 8). In contrast, overexpression of Notch1 resulted in barb fusion and the formation of multiple rachises. The specificity and knockdown efficiency for each RNAi construct were examined in vitro and in vivo (Supplementary Figs. 4 and 5). Moreover, mis-expression of Delta1 in the feather follicle also disrupted the regular branched pattern (Supplementary Fig. 8), further supporting the involvement of Notch signaling in feather branching.
Given the complexity of Notch signaling activation\textsuperscript{4,30–32}, we examined the activity of this pathway in the feather follicle. Three independent criteria were explored. First, we examined the expression patterns of downstream Notch target genes including \(L\)-Fringe and Hey1\textsuperscript{33,34}. In situ hybridization showed that they both are expressed in the marginal plate (Fig. 5i, j; Supplementary Fig. 7). Second, we cloned a Notch reporter into lentivirus, where GFP expression was driven by a promoter containing \(6 \times \text{RBP-J}\) binding elements\textsuperscript{35,36}. In the developing feather follicle, GFP is only expressed in the marginal plate, indicating specific activation of Notch signaling in this region. For the control, a viral vector where GFP expression was driven by a CMV promoter showed widespread expression (Fig. 5k, l). Finally, we constructed a secretory form of Serrate2 (sSer2; Supplementary Fig. 9a), which is known to inhibit Notch signaling\textsuperscript{37,38}. We demonstrated sSer2 can reduce the Notch reporter activity in cell culture (Supplementary Fig. 9b). When overexpressed in vivo, sSer2 induced ectopic rachis formation (Fig. 5m). Altogether, these results suggest that activation of Notch signaling is required for the periodic feather branching.

Fig. 4 Topological arrangement of E-Cadherin-mediated cell adhesion is required for feather branching. \(a, b\) Neither pre-patterned cell proliferation nor apoptosis play a role in feather branching morphogenesis as shown by TEM analysis and PCNA staining. \(c, d\) Differential distribution of \(\beta\)-Catenin and E-Cadherin in feather branching. mp, marginal plate; bp, barb plate. \(e, f\) Higher magnification views of regions in \(d\) showing E-Cadherin was diffusely distributed in pre-branch feather epithelium, but as puncta in branched barbs (e). Arrow heads indicate unstable adherens junctions (AJ) in the filopodia, as compare to AJ clusters in branched barbs (f). Dashed green lines indicate the basal lamina. Representative images from five repeated experiments are shown. N, nucleus; nm, nuclear membrane. \(g, h\) Overexpression or knockdown of E-Cadherin disrupted feather branching. Histology and gross feather morphology are shown. The numbers indicate the occasions of feathers with the phenotype. Ra, rachis; eRa, ectopic rachis; eBr, ectopic branch. Bar = 2 \(\mu\)m in \(a\), 20 \(\mu\)m in \(b-d\) (shown in \(c\)), 5 \(\mu\)m in \(e\), 0.3 \(\mu\)m in \(f\), 100 \(\mu\)m in \(g\), 2 mm in \(h\).
it directly binds β-Catenin in Drosophila and in vertebrate cells (Supplementary Fig. 10a). In addition, activation of Notch signaling down-regulates E-Cadherin expression (Supplementary Fig. 10b), a regulatory module often found in cancer metastasis. Furthermore, activation of Notch signaling reduces cell filopodia (Supplementary Fig. 10c). Indeed, when Notch signaling was ectopically activated in the rachis in vivo, we found reduced E-Cadherin and contraction of filopodia in the ectopic branches (Fig. 5n). In summary, the periodic activation of Notch signaling may drive the differential cell adhesion and contraction of basal filopodia, leading to feather branching.

**Filopodia help interpret the proximal-distal FGF gradient.** The fact that the feather epithelium branches only at a distance from the most proximal anchoring site, the dermal papilla (dp), suggests a morphogen gradient is in control. We have shown previously a proximal-distal FGF signaling gradient regulates feather branching. Indeed, FGF2 and FGF10 showed a graded distribution pattern in the feather follicle (Fig. 6a–c). Perturbation of FGF signaling by local injection of RNAi-FGFRI or through a specific chemical inhibitor SU5402 induced ectopic branches in the rachis region (Fig. 6d). Furthermore, FGF signaling also regulates the filopodia in basal keratinocytes. SU5402 treatment resulted in ectopic branching and the filopodia disappeared (Fig. 7a). In contrast, implantation of FGF10-soaked beads blocked epithelial branching and induced ectopic rachis formation: in the ectopic rachis, filopodia were also induced (Fig. 7b). These data are consistent with a role of FGF signaling in the regulation of cell filopodia in vitro (Supplementary Fig. 11). Conversely, we found that the filopodia can sense FGF molecules as they stained positive for FGFR1 and can transport FGF10 molecules (Fig. 7c). TEM analysis further documented vesicle-like structures inside the filopodia, supporting its role in transportation (Fig. 7d). Thus, a positive feedback loop may exist between FGF signaling and the filopodia, which helps to interpret the proximal-distal FGF gradient in the feather follicle.
Filopodia and the surface area change in feather branching.
Since feather branching is coupled with the disappearance of basal filopodia, we wonder how the total surface area actually changes during this process. We designed an algorithm to delineate the epithelial-mesenchymal (E-M) border in the image, and calculated the ratio of surface area increase due to the filopodia or branch formation (simplified to 2D situation, the length of the E-M border $\lambda$ versus the linear distance $d$)\cite{39}. The $\lambda/d$ ratio is in the range of 5–16 and averaged 11 before branching. After branching, $\lambda/d$ is in the range of 4–8 and averaged 6 (Fig. 7c). Therefore, the total surface area is actually decreased by about 50% after feather branching. This situation resembles the coastline paradox\cite{19}, which claims that a given landmass may not have a fixed coastline length because of the fractal-like property of its coastline. Thus at the nanoscale, emerging features - in this case basal filopodia, increase the surface area of the pre-branch feather epithelium.

Discussion
We propose a model that integrates the molecular and cellular events in feather branching morphogenesis (Fig. 7f): A proximal-distal gradient of FGF signaling cooperates with periodic Notch activation to regulate feather branching. Notch signaling is activated only when FGF levels fall below a threshold. The periodically activated Notch signaling then drives the contraction of filopodia and differential cell adhesion, promoting branch formation.

The basement membrane of an epithelial tissue is often viewed as a flat sheet where basal keratinocytes attach. This is not true even for mammalian skin: in the mouse footpad, basal keratinocytes also have filopodia about 0.5–1 $\mu$m long, and the basal lamina follow the outline of the filopodia (Supplementary Fig. 1c). These structures are particularly distinct in the feather follicle, possibly due to the intensive epithelial-mesenchymal interactions in feather branching morphogenesis. Similar elongated cyto- projections have been implicated in several examples of organ...

Fig. 6 A proximal-distal FGF signaling gradient regulates feather branching. a Semi-quantitative RT-PCR analysis revealed graded expression of FGF10 and FGF2 in the feather follicle. The dermal papillae (dp) and the pulp represent the proximal and distal compartment of the follicle, respectively. b In situ hybridization and c immunofluorescence showing the graded expression of FGF2 and FGF10 in the feather follicle. d Whole mount view and HE sections of the feather rachis after manipulating the FGF signaling via RNAi-FGFR1 lentivirus local injection, or SU5402 (100 $\mu$M) chemical inhibitor treatment in vivo. Ectopic branches were induced by both treatments. Representative images from five repeated experiments are shown. Ra rachis, eBr ectopic branch. Bar = 100 $\mu$m
Filopodia sense and transport FGF10 mole-
ules, which may help interpret the proximal-distal FGF gradient.

The feather branching process is accompanied by a complete
contraction of the basal filopodia. We have shown that by reg-
ulating the filopodia via small GTPases, the feather branching
pattern was perturbed. Thus the contraction of filopodia is cau-
sally linked with branching morphogenesis. The positive feedback
loop between FGF signaling and filopodia may contribute to this
abrupt contraction. Additionally, Notch signaling also contributes
to the contraction of filopodia, as demonstrated by our in vivo
manipulation of this pathway. Filopodia contraction may facil-
itate Notch pathway activation and feather branching by reducing
FGF signaling.

Given the complex expression patterns of the various Notch
ligands and receptors in the feather follicle, and the potential cis
and trans-interactions of the ligands/receptors, the activation
of Notch signaling in the feather follicle is likely to be
complicated. We have examined the impact of overexpression
and knock-down of both the receptors (Notch1, Notch2) and
ligands (Ser1, Ser2). It appears Notch2 is the endogenous re-
erator that is responsible for the activation of Notch signaling in the
marginal plate keratinocytes. Ser1 serves as the ligand to drive its
activation, whereas Ser2 acts in cis to inhibit its activation. The
expression of L-Fringe may further modulate Notch activation.

Our results are consistent with the current understanding of
Notch signaling activation, overexpression of Notch receptors
inhibits branching, because limited amounts of endo-
genous ligands (Ser1, Ser2) will be sequestered in cis, thus reduc-
ing Notch signaling trans-activation. Conversely, suppression of
Notch receptors will render more available ligands for Notch
signaling trans-activation. Similarly, because Ser1/2 is inhibitory
in cis, down-regulation of these molecules will promote Notch
activation and branch formation.

The periodic branching of feather epithelium is an example of
the classical reaction-diffusion mechanism in pattern forma-
tion. Here we propose that a proximal-distal FGF gradient
cooperates with periodic activation of Notch signaling to control
this process. Both in vivo (Fig. 7b) and in vitro (Supplementary
Fig. 10d), FGF signaling inhibits Notch activation. At the cell
level, contraction of filopodia and rearrangement of E-Cadherin-
mediated cell adhesion is critical for feather branching. Since
filopodia can actively sense and transport FGF molecules, they
may alter the fate of the basal keratinocytes so they are competent
to branch. Toward the base of the follicle, FGF levels are high and
may alter the fate of the basal keratinocytes so they are competent
to branch. Toward the base of the follicle, FGF levels are high and
inhibit branching. In contrast, activation of Notch signaling in the
distal feather drives branching and reduces the filopodia, and the
keratinocytes are more tightly compacted/connected. In this
sense, a pair of “activator–inhibitor” molecules still work together
to control the cell status and feather branching, although not in
the classic manner of “reaction–diffusion”.

Branching morphogenesis is widely used in many organs to
increase the surface area. Counter-intuitively, we show here that
the tissue actually prepares the surface area in advance, via filo-
podia, to accommodate feather branching. The total surface area
is reduced immediately after feather branching, although cell
proliferation may further increase the surface area in later feather
growth. Our data illustrate how complex molecular activities and
cell behaviors are integrated to control periodic pattern formation
in feather development.

Methods
Feather follicle manipulation in vivo. Three to six months adult male chickens
(Gallus gallus domesticus) were purchased from a local farm and housed in Fuzhou

Fig. 7 The filopodia sense and transport FGF10 and increase the surface
area for feather branching. a) SU5402 induced ectopic branch (eBr) in the
rachis and inhibited filopodia. b) Locally implanted FGF10 beads inhibited
branching and induced filopodia in basal keratinocytes. c) Filopodia
expressed FGF10, and transported FGF10. Notice the FGF10 puncta (red)
were lined up along the filopodia (green). d) A TEM image showing the
transported vesicles inside the filopodia (marked by arrowheads). Dashed
green line indicated the basal lamina. e) The circumference (J) and distance
(d) of the epithelial—mesenchymal border were measured, and their ratio
was calculated. Ten follicles were analyzed and representative images are
shown. **, p < 0.01 by t-test. f) Schematics showing FGF signaling
cooperates with periodic Notch activation to regulate the filopodia and E-
Cadherin/β-Catenin-mediated cell adhesion, which then control the
periodic feather branching. Bar = 50 μm in a, b, c; 10 μm in c; 2 μm in the
TEM images.
University Animal Facility Center. All experiments were approved by the Animal Research Committee in Fuzhou University. The chickens were anesthetized using pentobarbital (intraperitoneal injection, 50 mg kg\(^{-1}\)) before surgery. For lentiviral-mediated gene overexpression and RNAi knockdown, fully grown primary wing feathers were plucked and 200 μl virus solutions were injected into the follicle cavity using a micropipette. Feathers were allowed to regenerate for one month before sample collection to document the gross morphology using a stereo dissecting microscope (Chongqing Optical Instrument, China). For localization of virus/protein/chemical reagents, contour feathers in the wing in their actively growing phase were used. Two to five microliter solutions were injected into the desired locations in the follicle using a homemade glass microneedle. FGFI0 (0.1 μg μl\(^{-1}\), Sangon, Shanghai, China) or SU5402 (100 μM, Santa Cruz, Dallas, USA) was mixed with Sepharose 4B beads in PBS before injection. Samples were collected 48 h later and fixed in 4% paraformaldehyde (PFA) before processing for documentation of the gross morphology or histology\(^{13}\).

Cell culture and transfection. All cells were purchased from the Cell Library of the Chinese Academy of Sciences, Shanghai (293 T cells, #GNHu17; DF-1 cells, #GN036; HeLa cells, #TCHu187; MCF7 cells, #TCHu24). Cells were cultured with 10% fetal calf serum (HyClone, XiAn, China) in DMEM (Life Technology, Guangzhou, China). Cells were maintained in a humid incubator at 37 °C with 5% CO\(_2\). Plasmids were transfected using calcium phosphate (for 293 T cells) or electroporation (for DF-1/HeLa/MCF7 cells). A home-made electroporator was used for electroporation (600 V/30 ms for 1 pulse, 10 μl plasmids mixed with 4 × 10\(^{6}\) cells in 120 μl total volume). Cells were lysed for total RNA extraction (DF-1 cells) or sonicated for protein collection (293T cells) using the standard protocols.

Lentiviral construction. We used the pL3.7 vector for short hairpin RNAi knockdown. Target sequences for RNAi were listed in Supplementary Table 2, and a scramble control was used\(^{13}\). The vector used for gene overexpression was pLVX-ZeGreen. Source of the genes: full-length human E-CADHERIN was purchased from Sinobioscicals, Beijing, China; human SERRATE on pCIG (a generous gift from Dr Fernando Giraldez, Universitat Pompeu Fabra, Spain) was digested with BamHI to achieve the secretory form (amino acid 1-994); full length mouse Tenascin C (Developmental Studies Hybridoma Bank; M1-B4, 1:40 dilution), FGF10 (Sangon, Shanghai, China; D163308, 1:200 dilution), FGFR1 (Sangon, Shanghai, China; D124097, 1:200 dilution), FGF2 (Sangon, Shanghai, China; D160122, 1:200 dilution), VASP (Sangon, Shanghai, China; C2206, 1:1000 dilution), Actin (Sangon, Shanghai, China; D110001, 1:1000 dilution), β-Catenin (Sigma, Shanghai, China; C2206, 1:1000 dilution), Actin (Sangon, Shanghai, China; D110001, 1:1000 dilution), Full-length gel images can be found in Supplementary Fig.12. Repre- sentative results from three repeats were shown.

Luciferase reporter assay. In total 293 T cells were subcultured in 24-well plate 24 h before transfection. Cells were transfected with a 6XCSL Notch reporter plasmid, with or without a secreted fluorescent reporter. Luciferase activity was measured 48 h post-transfection. For control, the small molecular inhibitor of Notch signaling N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) was added to the culture medium at a final concentration of 46 nM 24 h before sample collection. Each experiment was repeated at least three times and representative results were shown.

Image processing. We used a region-based active contour model (ACM) to perform image segmentations and locate the boundaries of objects\(^{39}\). This method transforms image segmentation to an energy minimization problem, where the solution is represented as the signed distance function of the curve\(^{39}\). The Heaviside function and Dirac function is approximated as

\[ H(x) = \begin{cases} 1 & \text{if } x > 0 \\ 0 & \text{if } x < 0 \end{cases} \]

where \(H(x)\) is the Heaviside function, and \(\delta(x)\) is the Dirac function.\(^{39}\) The Heaviside function and Dirac function is approximated as

\[ h_\theta(x) = \frac{1}{2} \left( 1 + \frac{2}{\pi} \arctan \left( \frac{x}{\theta} \right) \right) \text{ and } \delta(x) = \frac{1}{\pi \theta^2} e^{-x^2/\theta^2} \text{ for } 0 < \theta < 1 \]

Applying the gradient descent method to Eq. (1), the optimal values of \(c_1, c_2, f_2\) for minimizing the energy functional defined by Eq. (1) can be achieved as following:

\[ c_1 = \frac{\int |u(x)|^2 H(x) dx}{\int H(x) dx} \]

\[ c_2 = \frac{\int |u(x)|^2 H(x) dx}{\int (1-H(x)) dx} \]

\[ f_1(x) = \frac{K(x) - \left| u(x) \right|^2}{K(x) \left| u(x) \right|^2} \]

\[ f_2(x) = \frac{K(x) - \left| u(x) \right|^2}{K(x) \left| u(x) \right|^2 + (1-H(x))} \]

\(\phi, k, \ldots\) denotes the level set function at iteration \(k\).
Fixing $c_1,c_2,f_1,f_2$ and using the calculus of variation method, one can have:

$$\nabla F(\phi_i) = \delta_i(\phi_i) \nabla \left[ \eta_i \left( u - c_1 \right)^2 - \eta_i \left( u - c_2 \right)^2 - V \cdot \left( \frac{\sigma}{\nabla} \right) \right]$$

$$+ \lambda \left( \frac{c}{c_0} - 1 \right) \nabla \left( \frac{\sigma}{\nabla} \right) = 0$$

Thus the iterative formula of the gradient descent method has the form:

$$\phi_{i+1} = \phi_i - a_i \nabla F(\phi_i)$$

where $a_i$ is the time step length. $a_i := -\nabla F(\phi_i)$ is the gradient descent direction. Meeting the conditions of convergence, the optimal values of $\phi$ to minimize the energy function $(i)$ is the boundary of the image. Detailed coding information to execute the ACM algorithm is provided in the supplementary software file (Supplementary Note 1), which is implemented in MATLAB R2012a under the Windows XP system.

**Statistics.** For feather follicle manipulation in vivo, at least five follicles were used for each experimental condition and representative results were shown. Data are available from the corresponding author upon reasonable request. The RNA-seq data generated in the present study have been deposited in the GEO database under accession code GSE110591.

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Author contributions
Z.Y., R.B.W., and C.M.C. conceived the work. D.C., X.Y., G.Q., and J.Z. contributed equally to this work and performed most of the experiments. D.C. characterized the filopodia and analyzed the role of Rho GTPases. X.Y. and J.Z. performed the in situ hybridization analysis. X.Y., G.Q., and T.F. analyzed the role of Notch signaling and E-Cadherin. G.Q. and Y.T. analyzed the role of FGF signaling. J.Z. analyzed the RNAi knockdown efficiency. H.W. and W.H. performed the TEM analysis. H.X. and M.W. analyzed the image. P.W. analyzed the role of Delta. J.Z. helped draft the manuscript. Z.Y., R.B.W., and C.M.C. drafted the manuscript. All authors read and approved the final manuscript.

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