# Supplementary Materials for

## Emergent Cellular Self-Organization and Mechanosensation Initiate Follicle Pattern in the Avian Skin

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## Materials and Methods

Embryos and Dissections

Fertilized chicken eggs were obtained from commercial sources, incubated at 37.5°C, and staged according to Hamburger and Hamilton. 2mm by 4mm dorsal skin pieces were dissected from E6 embryos using forceps and were either fixed within 10 minutes (in the case of the contraction experiments) or cultured. Skin layers were separated through gentle dissection or dissection after loosening in calcium/magnesium-free HBSS media.

To investigate the effect of acute compression on β-catenin localization, skin samples were removed from the E6 embryo, placed in media, and incubated at 37.5°C for 1, 2, or 4 hours. To control for the effects of media/culture, results were compared to skin that was cultured while remaining intact on the body (therefore in an uncontracted state).

Skin Culture

*Ex vivo* culture: dorsal skin pieces were dissected from E6 embryos and were gently placed dermal-side down atop culture well insert membranes (Fisher #353090). Culture well inserts were placed within wells containing DMEM with 2% chick serum and 10% FBS along with pharmacological agents including calyculin A (Sigma), blebbistatin (Abcam), SKI-1 (Abcam), collagenase (Worthington), and BIO (Sigma), XAV939 (Sigma) if applicable. Skin pieces were cultured for 48 hours at 37°C before being fixed in 4% paraformaldehyde in PBS. Reconstitution culture was performed as previously described in Jiang et al. 1999. All culture experiments were performed at least three times with three skin samples (each from a different embryo) per condition.

Polyacrylamide hydrogels were prepared as described previously (*26*) in glass bottom 6-well plates (MatTek Corporation). To conjugate fibronectin on the gel surface, gels were immersed in 1mL of 0.5 mg/mL sulfo-SANPAH (Pierce) in PBS and activated with 8 min UV exposure. After activation, gels were washed in PBS and incubated in 25μg/ml fibronectin for 1 hour at 37˚C. To mechanically characterize the polyacrylamide gels, gels were polymerized on an Anton Paar Physica MCR 301 rheometer using an 8-mm parallel plate and kinetics of polymerization were observed at 1 Hz and 5% amplitude. The storage modulus at 30 minutes after initiation of polymerization was recorded for at least 4 independent samples per condition. The storage modulus for each gel formulation was 0.025, 0.19, 0.42, 6.2 and 45kPa. Note that the stiffness reported here is the storage modulus instead of the Young's modulus.

Proliferation was quantified *ex vivo* by adding 10μM EdU to *ex vivo* culture of skin samples after exposure to the relevant drugs for 16 hours. Samples were exposed to EdU for 3 hours and then fixed, sectioned, and stained for DAPI and Edu using the Click-iT EdU kit Alexa Fluor 488 (ThermoFisher). The number of Edu-positive dermal cells per total dermal cells were compared across conditions. Percentage data was pooled from three biological replicates from three separate experiments.

Immunohistochemistry and fluorescent *in situ* hybridization

Full embryos between E6 and E8 stages, dissected skin pieces, and cultured dissected skin pieces were fixed in 4% paraformaldehyde in PBS and embedded in OCT. 14 μm thick longitudinal and transverse frozen sections were obtained for immunohistochemistry. Immunochemistry was performed with the following primary antibodies: laminin (1:200, ab11575 Abcam), E-cadherin (1: 200, ab76055 Abcam), β-catenin (1:100, C7207 Sigma), β-catenin Y654 (1:50, ab59430 Abcam). For β-catenin and β-catenin Y654 staining, antigen retrieval was performed prior to primary antibody administration. Incubation in primary antibodies occurred at 4°C overnight in CAS Block (Invitrogen). Sections were then incubated in Alexa Fluor 488 (1:300, Invitrogen) or Alexa Fluor 555 (1:300, Invitrogen) secondary antibodies in PBS with 0.1% Triton X-100 for two hours at room temperature. DAPI (1:1000, Molecular Probes) was used as a nuclear counter stain. Fluorescent *in situ* hybridization (FISH) experiments were performed using a protocol modified from Brent et al. 2003. Anti-DIG-POD (Roche) and Cy3 TSA (Perkin Elmer) were used to detect the probe signal. FISH assays were performed three times on whole embryo sections and performed twice (with three experimental replicates per trial) on culture samples.

Tissue-level traction force microscopy

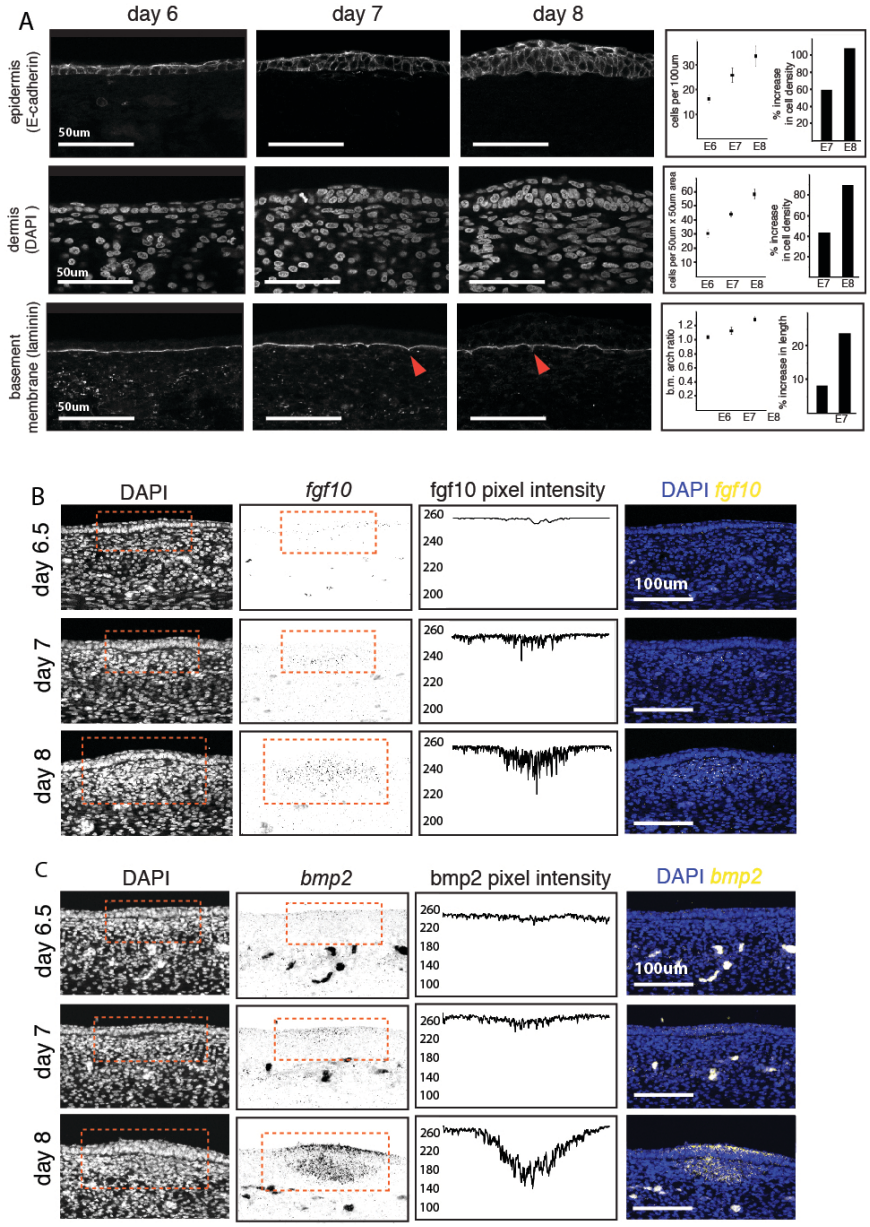
E6 unpatterned tissue was cultured on a 2 mg/ml collagen gel (ThermoFisher) seeded with 0.5μM fluorescently labeled beads (ThermoFisher) for 48 hours. Quantification of the brightness across the tissue perimeter was done using the plot profile function in Fiji. Images of cross-sections through the collagen/tissue were used to quantitatively compare the number of beads per area in the regions of the collagen outside of the tissue and under the tissue. The data represents three biological replicates for each of three separate experiments.

Quantification:

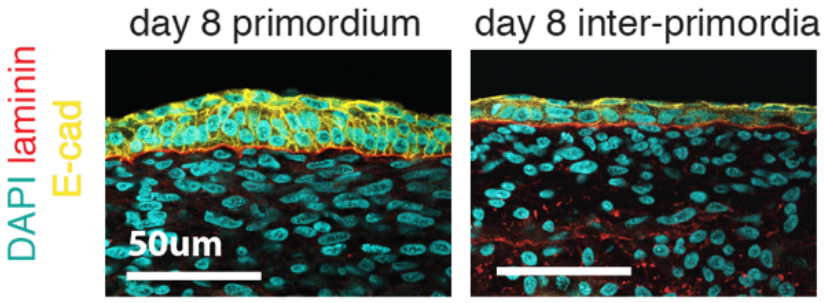
Density of epidermal cells was calculated as number of epidermal cell nuclei (DAPI stain) per 100μm length of the tissue section. Density of dermal cells was calculated as number of dermal cell nuclei per 50μm by 50μm square. Areas were determined and cells were counted manually in Fiji. The arching of the basement membrane was calculated by dividing the length of the membrane covering 100μm of the tissue section by 100μm. This membrane length was obtained using the freehand line drawing function in Fiji. Quantifications were done on three images, using three regions of the image.

A 50 μm wide box covering the length of the *in situ* image and encompassing the most superficial part of the tissue was selected and the plot profile function in Fiji was used to calculate the average pixel intensity for every point across the boxed length of the tissue. β-catenin antibody stain was quantified by using the Fiji plot profile function to analyze a free-hand line drawn through the contour of the epidermis. β-catenin Y654 antibody staining was quantified by using the Fiji plot profile function to analyze a free-hand line drawn through the contour of the basal surface of the epidermis where the staining is present. All graphs are quantifications of the single adjacent representative image. These staining were performed on two samples from each condition, from three separated experiments.

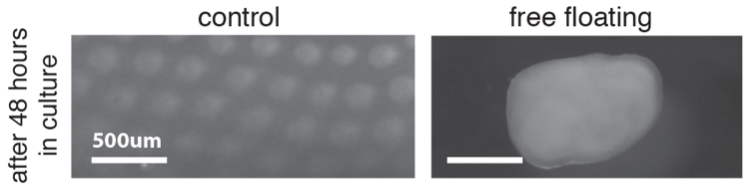
Dissected tissue surface area was calculated by using Fiji to measure the outlined tissue shape visible in images from a standard dissecting scope. Bud pattern geometry was calculated using Fiji to measure the relevant lengths. Geometry was measured on three samples, using three regions of the image.



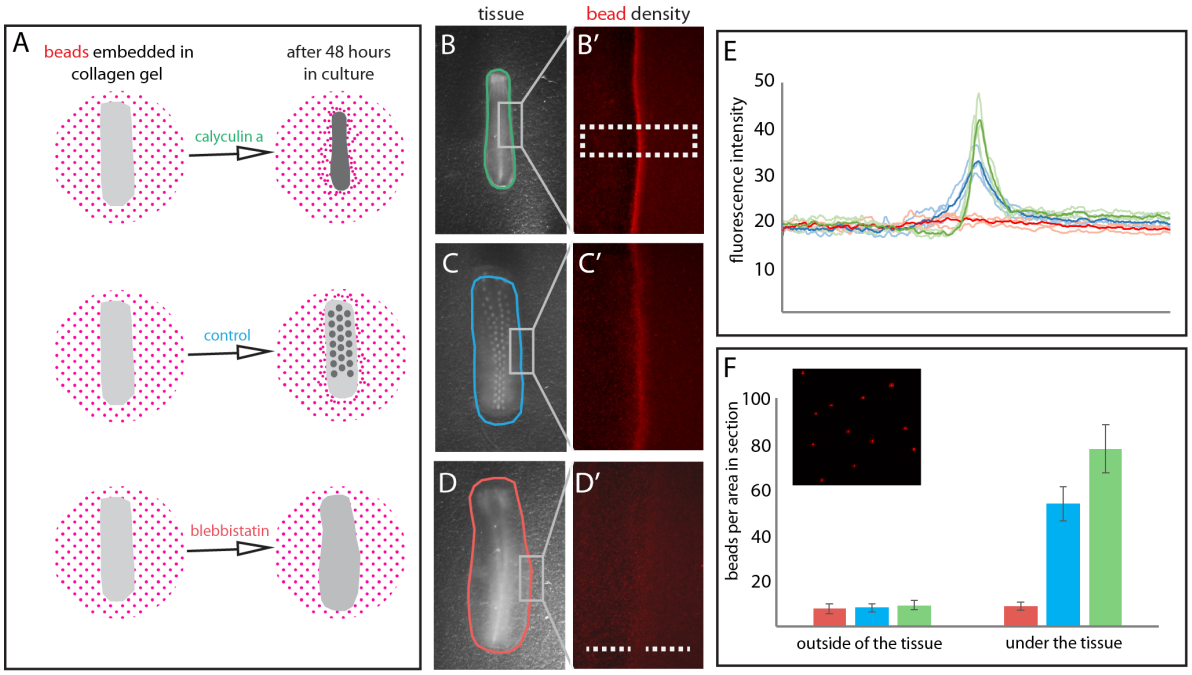

**Supplemental Figure 1. (A)** Cross-section of embryonic chick skin showing in detail architectural changes as a feather primordium forms from day 6 to day 8. Cells of the epidermis bunch, stack, and heighten; quantification of epidermal density (cells per 100um length) on the right. Cells of the dermis aggregate; quantification of dermal cell density (cells per 50µm by 50µm area) on the right. The basement membrane forms arches (arrowheads); quantification of the basement membrane arching ratio (membrane length per 100µm of the tissue) on the right (n>3 samples, at least three measurements per sample for all architecture measurements). Fluorescent *in situ* hybridization for *fgf10* **(B)**, *bmp2* **(C)** starting from the earliest signs of the primordium structure (E6.5 top) to the formed primordium (E8 bottom). Quantification of in situ pixel intensity is shown to the right of each expression pattern. Emerging primordium architecture is boxed. Bright spots in dermis are blood vessels. Error bars in the figure are +/- one SD.



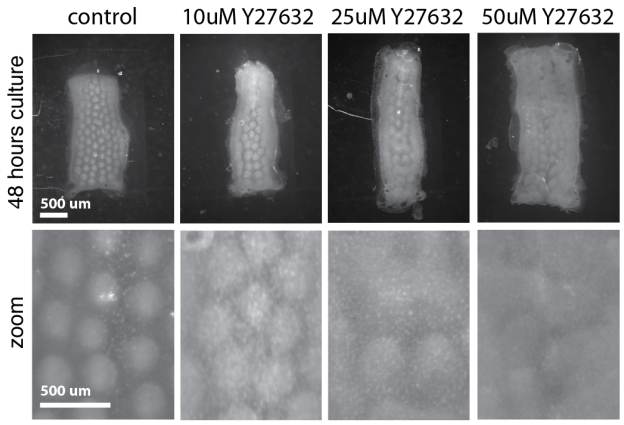

**Supplemental Figure 2. Co-aggregation of epidermal and dermal cells occurs only within the forming primordium and not in the inter-primordia space.** Cross-section of embryonic chick skin showing architectural changes as a feather primordium forms at E8 (left, panel from Fig. 1B). Architectural changes are not seen between primordia (right).



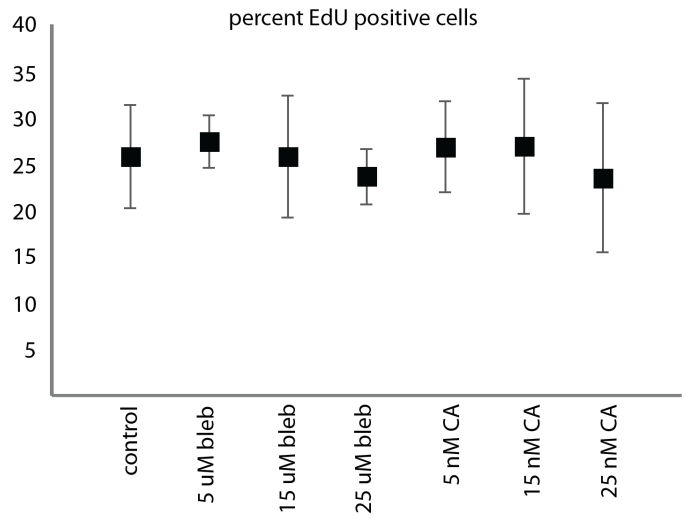

**Supplemental Figure 3. Pattern formation requires a rigid substrate.** Aerial view of day 6 skin samples cultured for 48 hours. The primordia pattern forms in control samples cultured on a tissue culture filter insert (left); however, no pattern forms when the sample is grown freely floating in media (right). (n=3, for each of three experiments)



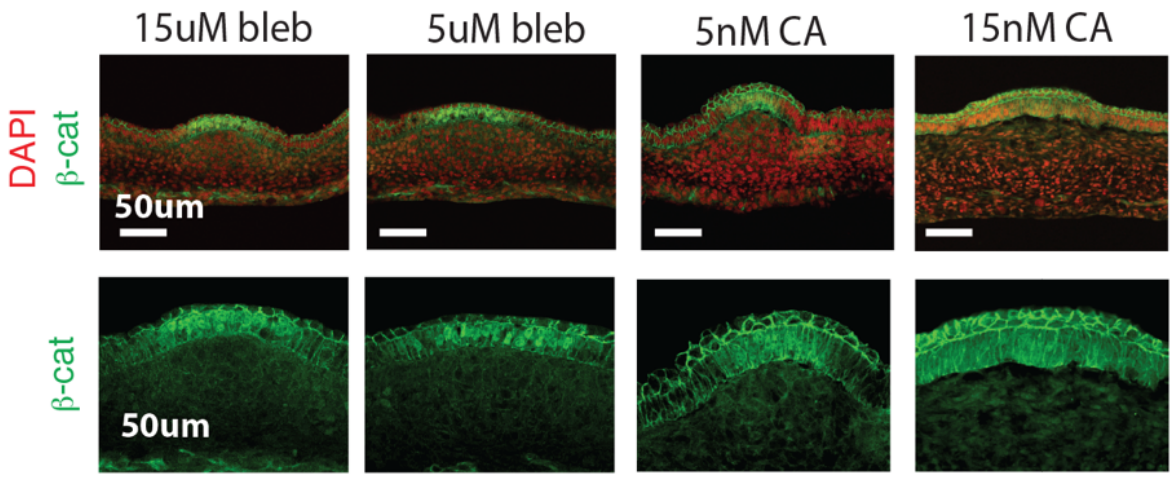

**Supplemental Figure 4. Pattern forming skin is contractile and perturbation of myosin II activity alters contractility. (A)** Schematic of tissue-level traction force microscopy, conducted on control samples **(C, C')** or with increased (5nM calyculin A, **B, B'**) or decreased myosin II activity (30μM blebbistatin, **C, C'**). E6 unpatterned tissue is cultured on a collagen gel seeded with fluorescently labeled beads for 48 hours. **(B, C, D)** Brightfield of tissue after 48 hours in culture. **(B', C', D')** View of fluorescence from labeled beads at the tissue perimeter. Brighter regions represent higher bead density and greater deformation of the substrate. **(E)** Quantification of the brightness across the perimeter using the plot profile function in Fiji (as in dotted box in **B'**). The light lines represent three separate biological replicates; the dark line is the average of these three samples. The data depicts one experiment that is representative of the findings from three total experimental replicates. The tissue (on the left side of the bright perimeter) obstructs the view of beads directly beneath it so cross-sections through the collagen/tissue (dotted lines in **D'**) were used to quantitatively compare the density of beads in the regions of the collagen underneath the tissue with density in regions not underneath the tissue **(F)**. The data represents three biological replicates for each of three separate experiments.



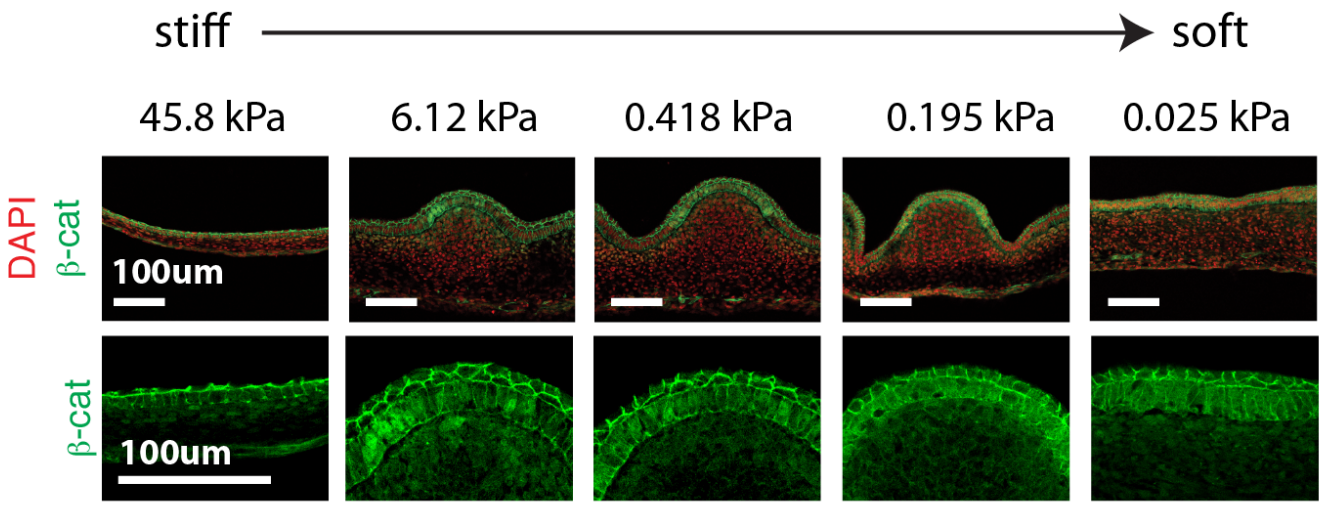

**Supplemental Figure 5. Cell contractility is required for proper pattern formation.** Aerial view of skin samples grown across a spectrum of traction through pharmacological inhibition of ROCK with Y27632 as compared to control; higher magnification below (n>3 samples for each of three experiments).



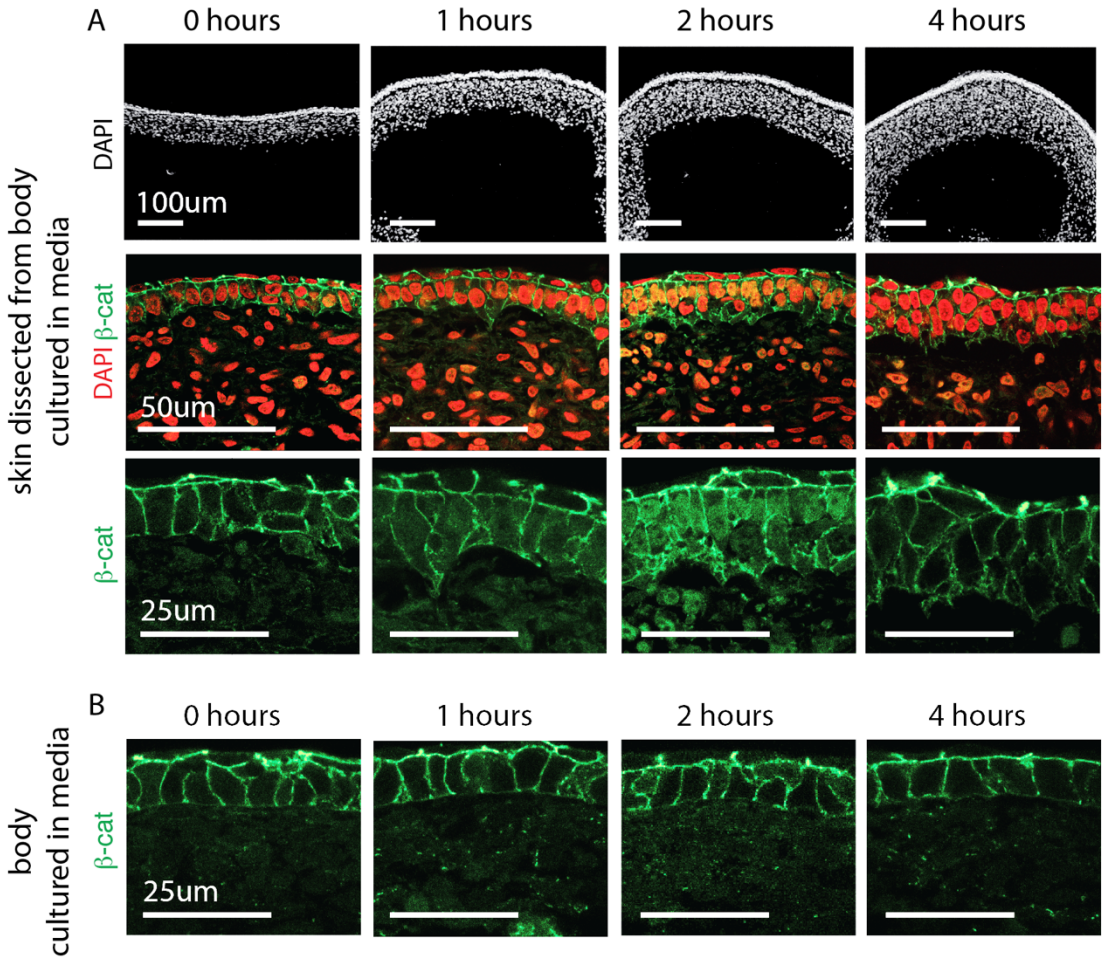

**Supplemental Figure 6. Proliferation is not altered in the presence of drugs that affect myosin II activity.** The number of Edu-positive cells per total cells was quantified for each culture condition. Samples were exposed to blebbistatin or calyculin A for 16 hours and then incubated with 10μM Edu for 3 hours. Tissues were then fixed, sectioned, and stained for Edu and DAPI. The data represents three biological replicates for each of three separate experiments.



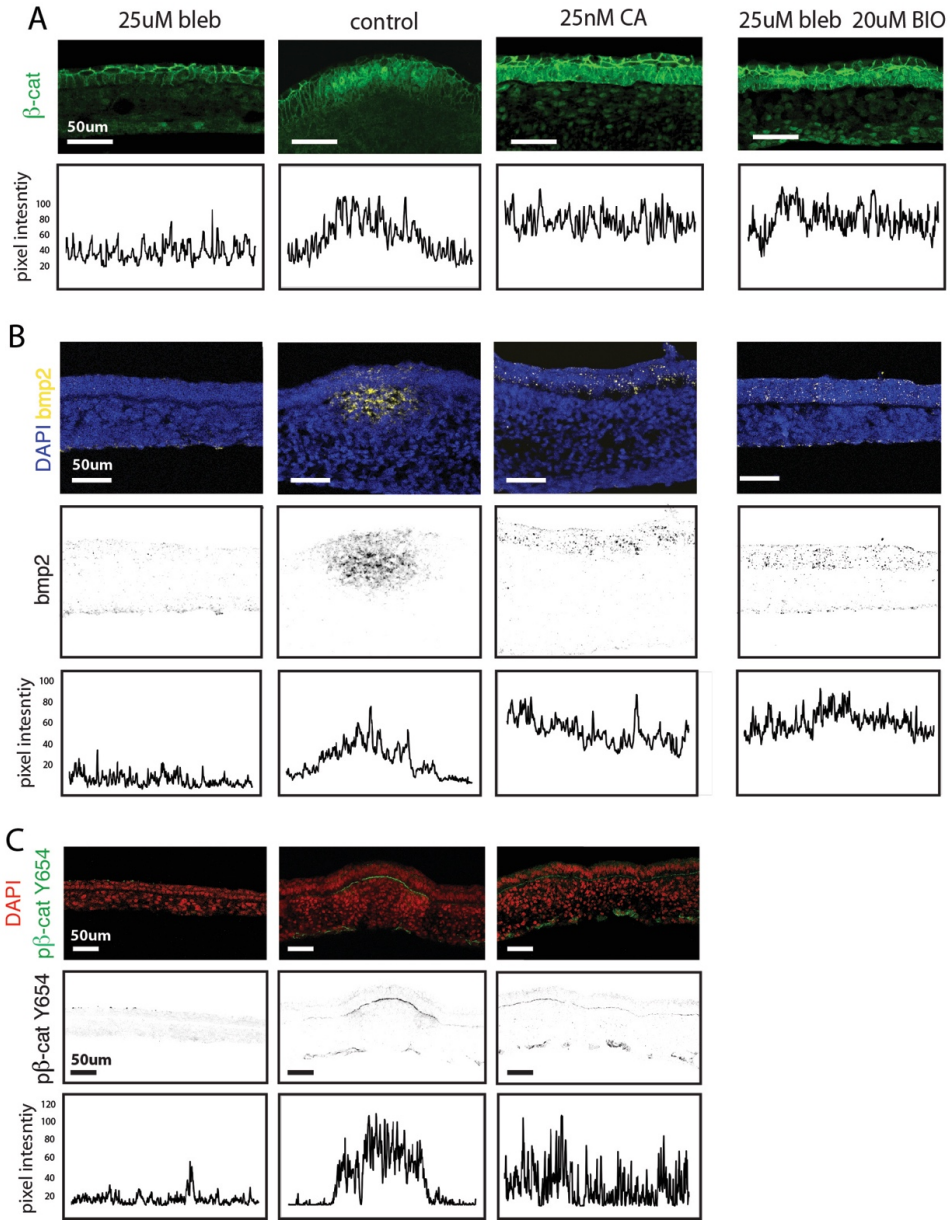

**Supplemental Figure 7. Movement of β-catenin to the nucleus is restricted to the forming primordia even as bud spacing changes.** β-catenin localization in samples in low (left) and high (right) traction; higher magnification shown below. Note in the presence of 15nM calyculin A, compression outside of the primordium reaches the threshold needed to move β-catenin to the nucleus, approaching the broad, pan-epidermal nuclear localization seen in the 25nM condition (Fig. 4A). (n=3, for each of three experiments)



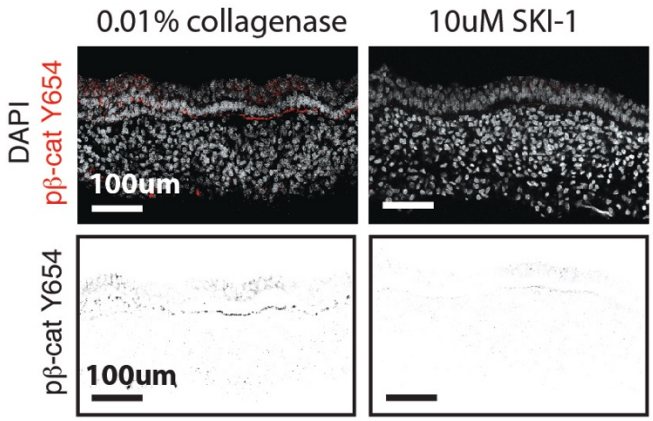

**Supplemental Figure 8. Movement of β-catenin to the nucleus in forming primordia is mechanically triggered.** β-catenin localization in samples cultured on substrates of varying stiffnesses. Higher magnification shown below. Note the lack of β-catenin in the nuclei of the uncompressed samples grown on the stiff substrate, where traction is strongly resisted (compare to low traction conditions [25μM blebbistatin] in Fig. 4A). Conversely, the samples grown on the soft substrates which weakly resist traction show nuclear localization across the highly contracted sample where the epidermis is compressed (compare to high traction conditions [25nM calyculin A] shown in Fig. 4A). At intermediate levels, nuclear β-catenin is restricted to forming primordia. (n=3, for each of three experiments)




**Supplemental Figure 9. Acute compression of the epidermis induces β-catenin translocation two hours. (A)** Top: cross sectional view of tissue shape after the skin is removed from the body and either immediately fixed (left) or cultured for 1, 2, or 4 hours freely floating in media. Freely floating pieces contract - note the thicker and increasingly curled tissue cross-section. Middle: β-catenin is found in the nuclei of the epidermal cells across the contracted samples after just 2 hours in culture. Also note the compaction of the epidermal cells of the contracted samples. The nuclear signal is lost by 4 hours. **(B)** Control samples that were cultured while attached to the body, and therefore not contracted, do not show this same nuclear translocation of β-catenin. Representative images are shown. The experiment was performed in triplicate with three biological replicates each.




**Supplemental Figure 10. β-catenin activation in the avian skin is mechanically triggered.** β-catenin protein localization **(A)**, FISH for *bmp2* **(B)** and Phosphorylated β-catenin Y654 staining **(C)** in samples with low (left) and high (right) contractility as compared to the control sample (center). Quantification of antibody staining and FISH signal intensity is shown below each expression pattern. Y654 staining is found adjacent to the basal membrane of epidermal cells, as has been observed in previous reports. (n=3)




**Supplemental Figure 11. β-catenin activation in the avian skin is Src-dependent and mechanically triggered.** Phosphorylated β-catenin Y654 staining in E6 skin samples cultured in the presence of collagenase to allow for increased compaction of the tissue and SKI-1 to inhibit Src activity. (n=3)