SUPPLEMENTAL INFORMATION

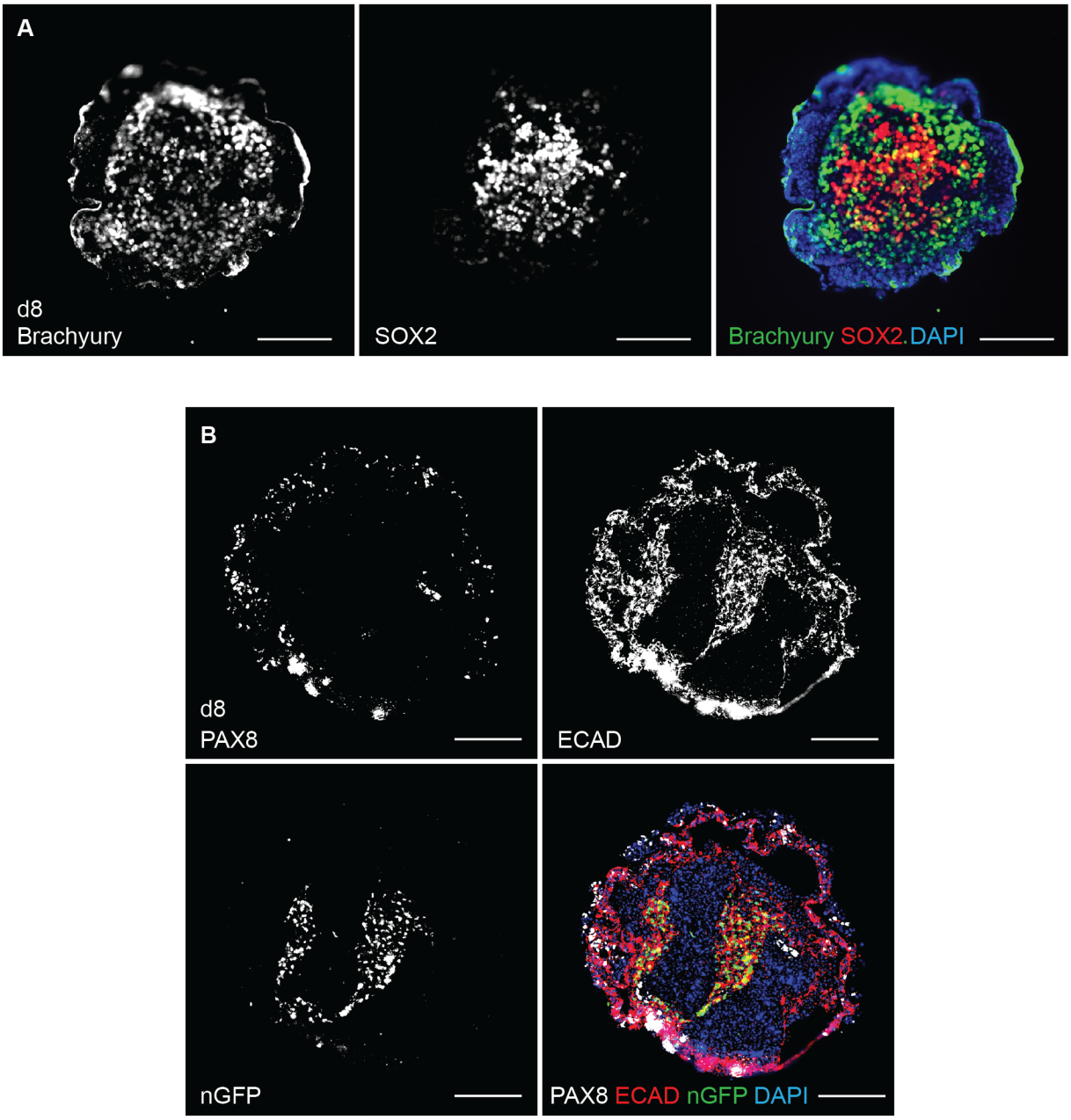


Figure S1: Mesoderm and epithelium induction in Atoh1/nGFP cell aggregates. Related to Figure 1A. A, By day 8 of differentiation, Brachyury^{+} mesodermal cells are induced in the intermediate layer, and undifferentiated SOX2^{+} pluripotent stem cells remains in the inner core of the aggregate. **B,** ECAD^{+} PAX8^{+} epithelium is detectable by day 8, suggesting the induction of cells in the cranial otic-epibranchial region. Undifferentiated Atoh1/nGFP^{+} pluripotent stem cells are still detectable in the core of the aggregate. Scale bars, 100 µm.

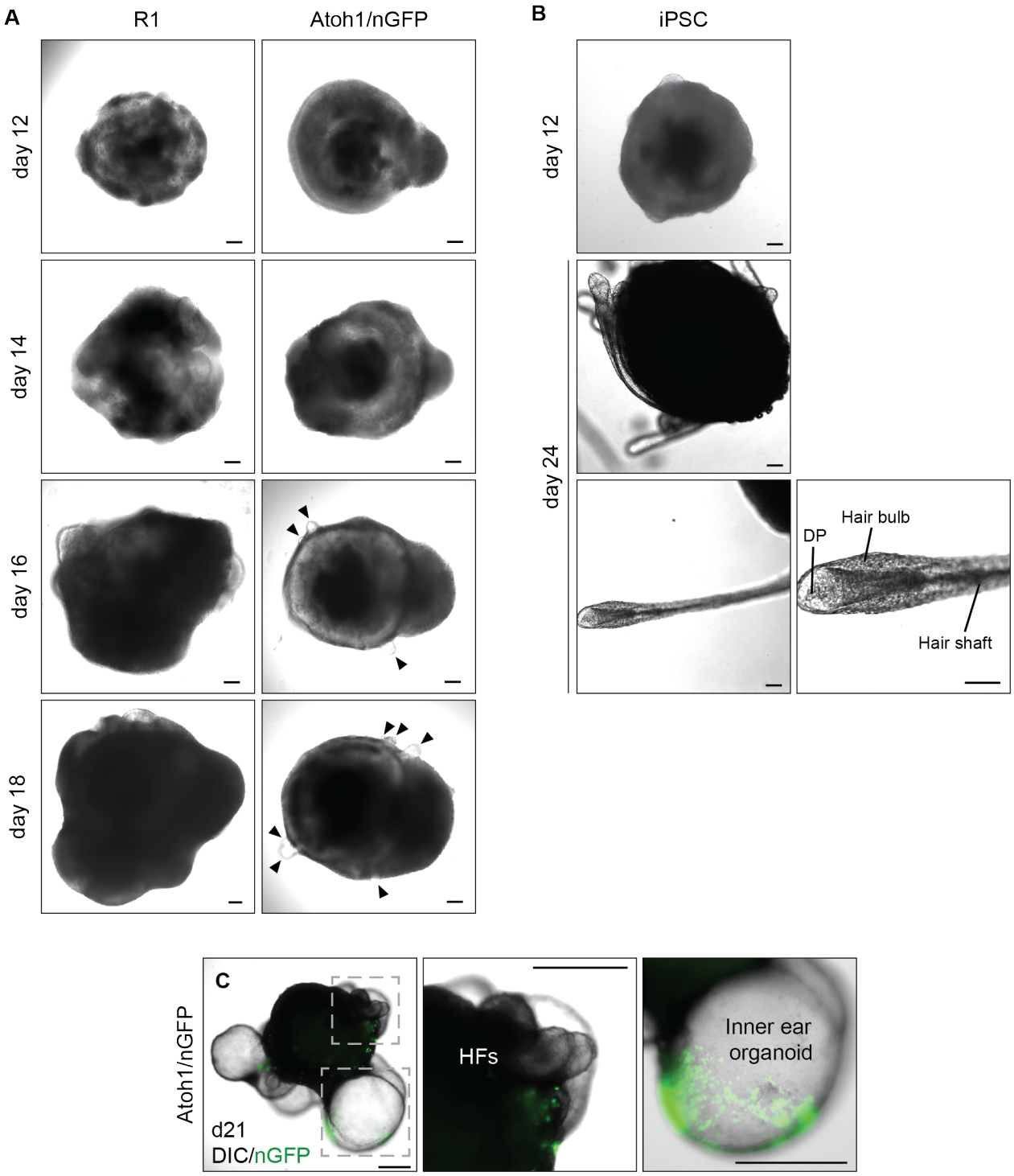
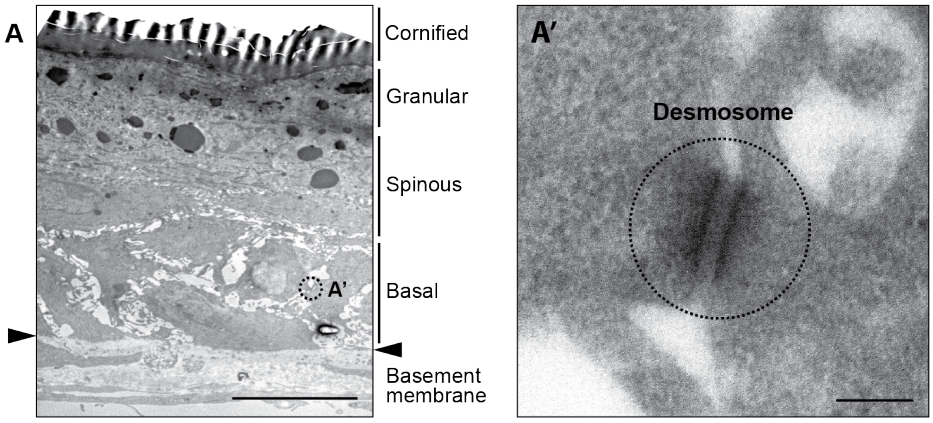


Figure S2: Comparison of developmental morphologies in different cell lines. Related to Figure 2. A, Representative DIC images of R1 and Atoh1/nGFP mESC-derived aggregates during days 12-18 of differentiation. Note that in R1 aggregates, non-epithelial tissue fully covers the epithelium, whereas in Atoh1/nGFP aggregates, the epithelium is only partially covered by non-epithelial tissue. By day 16, in Atoh1/nGFP aggregates, hair germs (arrowheads) visibly protrude through the thin layer of dermal tissue, covering the epithelium. **B,** Representative C57BL/BJ iPSC-derived aggregates on days 12 and 24. Like the Atoh1/nGFP cell line, iPSC aggregates typically lacked the large mass of non-epithelial tissue observed in R1 mESCs. High magnification images show development of hair bulb, dermal papilla, and hair shaft. **C,** Representative Atoh1/nGFP aggregate bearing HFs alongside inner ear organoids containing $nGFP^{+}$ hair cells on day 21 after CHIR treatment from days 8-10. Dash-lined boxes indicate the area of magnification. HFs; Hair Follicles. Scale bars, 100 μm.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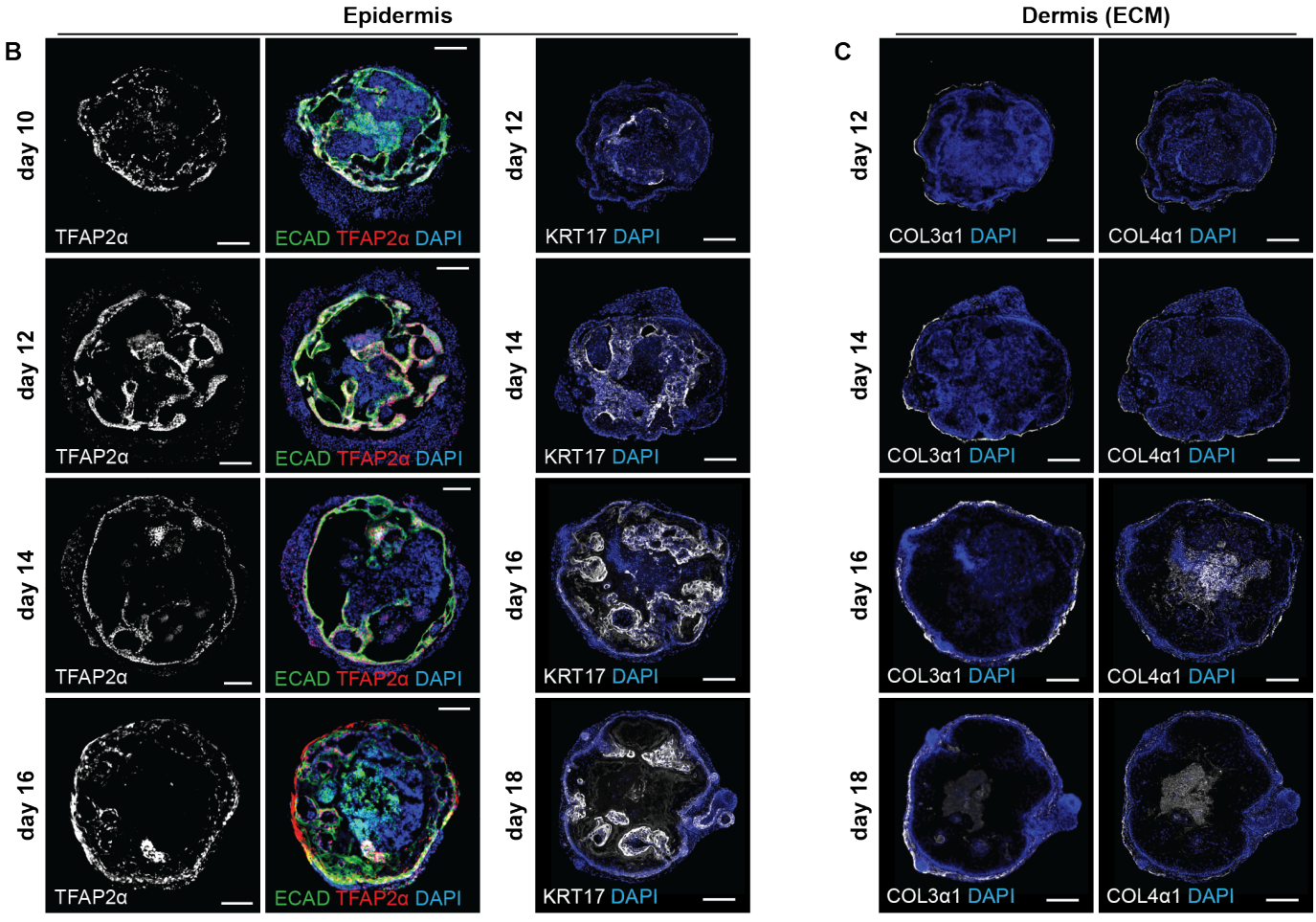
Cornified
Granular
Spinous
Basal
Basement membrane
A'
A'
Desmosome


Epidermis
Dermis (ECM)
B
C
day 10
day 12
day 14
day 16
day 18
TFAP2α
ECAD TFAP2α DAPI
KRT17 DAPI
COL3α1 DAPI
COL4α1 DAPI

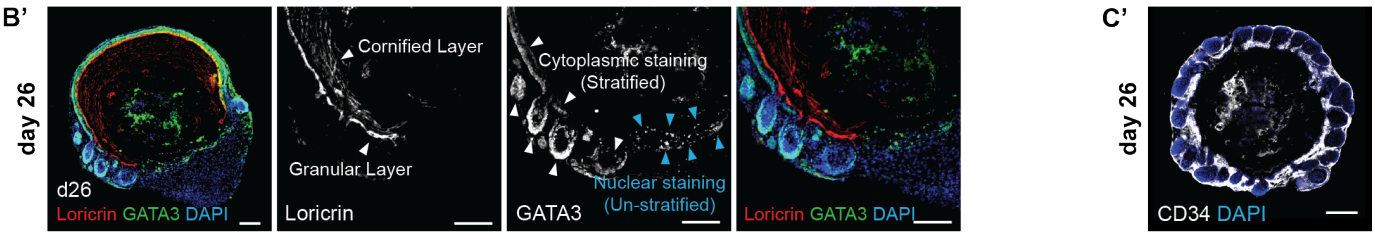
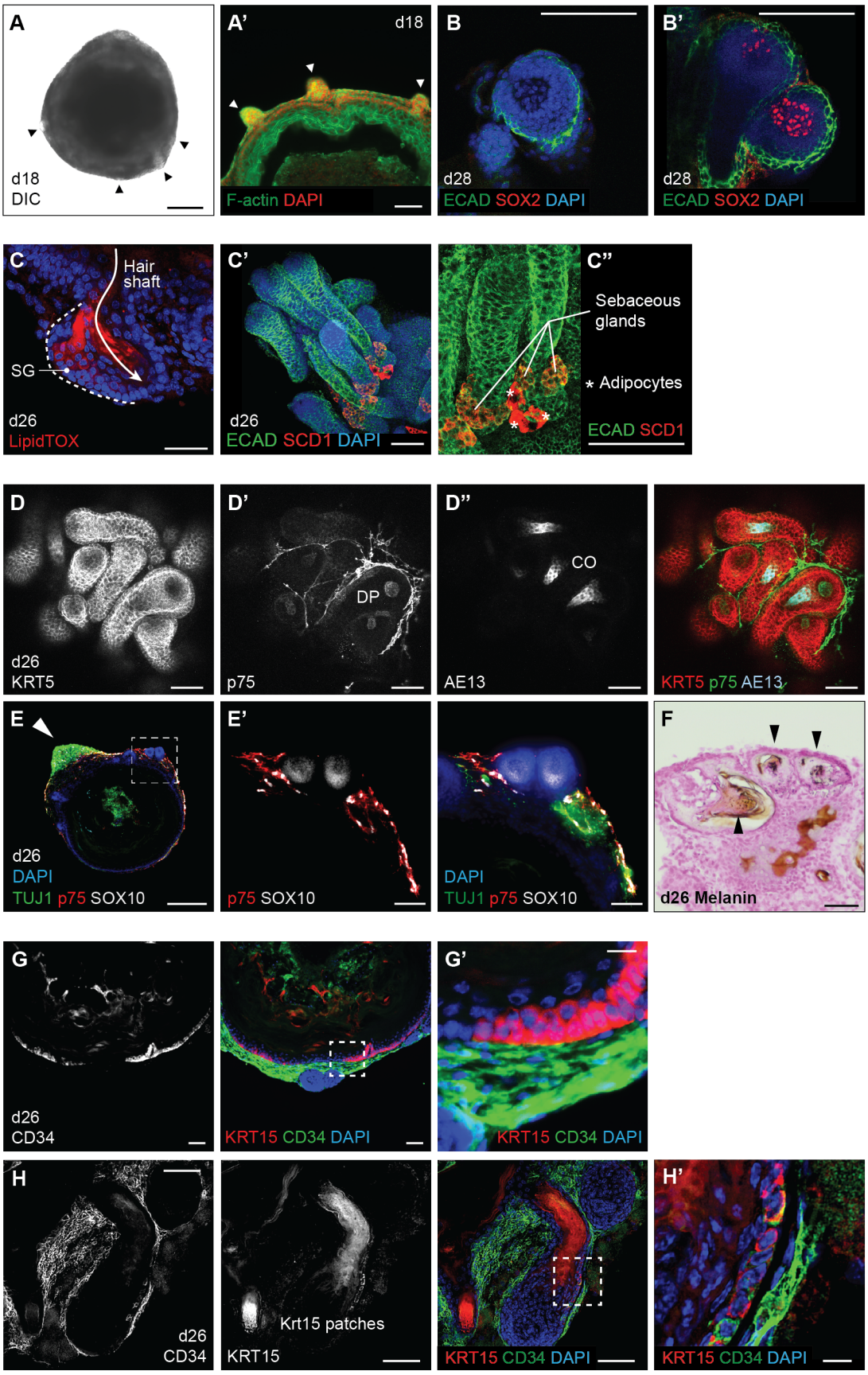

B'
day 26
d26
Loricrin GATA3 DAPI
Cornified Layer
Granular Layer
Loricrin
Cytoplasmic staining (Stratified)
Nuclear staining (Un-stratified)
GATA3
Loricrin GATA3 DAPI
C'
day 26
CD34 DAPI

Figure S3: Self-assembled skin layers in skin organoids. Related to Figure 3. A, A', Representative day 26 Transmission Electron Microscopy (TEM) images of stratified epidermis developed in Atoh1/nGFP mESC-derived skin organoids. (***A***) The epidermis self-assembled into cornified, granular, spinous, and basal layers (in an order from top to bottom). (***A'***) Desmosomes were present between cells in the basal layer. **B,** Early developmental stage of Atoh1/nGFP mESC aggregate, co-expressing ECAD and TFAP2α in the epidermis. The epidermal cells were visible from day 10 and persisted until day 32 (end of experiment). Interestingly, expression of KRT17 was first visible from the center of the aggregate, and spread out through the epidermis in the later days of differentiation. **B',** Late developmental stage of epidermis in R1 mESC aggregate. By day 26 of differentiation, Loricrin^{+} granular and cornified layers are visible (indicated with arrowheads). The cytoplasmic staining for GATA3 was noted in the basal layer of stratified epidermis (white arrowheads), while nuclear staining was detected in the basal layer of un-stratified epidermis (blue arrowheads). Note that the un-stratified epidermis is located adjacent to a mass of non-epithelial tissue. **C,** Expression of collagens at the epidermal-dermal interface. In Atoh1/nGFP aggregate, COL3α1 and COL4α1 were visible on day 12 and persisted throughout differentiation. **C'**, Late developmental stage of dermis in Atoh1/nGFP aggregate. CD34^{+} dermal fibroblast-like cells surround HF germs and pegs in a d26 organoid. Scale bars, 100 μm (***B-C'***), 10 μm (***A***), 100 nm (***A')***.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d18
DIC
A'
d18
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d28
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B'
d28
ECAD SOX2 DAPI
C
Hair shaft
SG
d26
LipidTOX
C'
d26
ECAD SCD1 DAPI
C''
Sebaceous glands
* Adipocytes
ECAD SCD1
D
d26
KRT5
D'
DP
p75
D''
CO
AE13
KRT5 p75 AE13
E
d26
DAPI
TUJ1 p75 SOX10
E'
p75 SOX10
DAPI
TUJ1 p75 SOX10
F
d26 Melanin
G
d26
CD34
KRT15 CD34 DAPI
G'
KRT15 CD34 DAPI
H
d26
CD34
Krt15 patches
KRT15
KRT15 CD34 DAPI
H'
KRT15 CD34 DAPI

Figure S4: Development of HFs and specialized cellular compartments in skin organoids. Related to Figure 4, 5 and 6. A, A', Hair germ induction on day 18 of differentiation. Representative (***A***) DIC image of d18 Atoh1/nGFP mESC-derived skin organoid with induced hair germs and (***A'***) IHC image of DAPI and Phalloidin (F-actin) reveal induction of hair germs (arrowheads). **B, B'**, Representative images of hair bulbs formed on skin organoid HFs on day 28 (***B***) with SOX2-negative hair bulb, indicative of a zigzag-like HF and (***B'***) with SOX2-positive dermal papilla, indicative of a nascent guard-, awl-, or achene-like HF. **C-C",** Representative images of sebaceous glands developing from HFs, visualized by (***C***) LipidTOX staining and (***C, C"***) whole-mount staining (double positive with SCD1 and ECAD). Asterisks represent $SCD1^{+}$ $ECAD^{-}$ adipocytes. **D-F,** Neural crest components in skin organoids. (***D-E'***) Skin organoids contain $p75^{+}$ $SOX10^{+}$ neural crest-like cells. TUJ1 expression was observed (***E***) in a neuroectoderm-like compartment (arrowhead) and (***E'***) in a subset of $p75^{+}$ $SOX10^{+}$ cells, suggesting the presence of sensory neurons. DP; Dermal Papilla, CO; Cortex. (***F***) Representative cryosection of day 26 Atoh1/nGFP aggregate that contains pigmented HFs. The pigmented HFs are visualized by Fontana-Masson staining, which stains melanin in the hair shafts. Arrowheads indicate melanocytes in the pigmented HFs. **G-H',** On day 26, $Krt15^{+}$ $CD34^{-}$ epithelium stem cell-like cell population reside in the basal layer of epithelium, surrounded by $CD34^{+}$ dermis (***G, G'***). $KRT15^{+}$ $CD34^{+}$ cells were rarely present in the outer root sheath of the HF (***H, H'***), suggesting that the organoids are at an early postnatal stage of maturity prior to development of $Krt15^{+}$ $CD34^{+}$ HF bulge stem cells. Scale bars, 250 μm (***E***), 200 μm (***A***), 100 μm (***B, B', C', C"***), 50 μm (***A', C, D-D", E', F, G, H***), 10 μm (***G', H'***).

SUPPLEMENTAL TABLES

Table S1. Comparison of hair follicle formation frequencies between cell lines and treatments. Related to Figure 2.

a. Comparison of hair follicle formation frequencies between R1 and Atoh1/nGFP mESC lines

Experiment ID	% of HF Formation per Experiment	Experiment ID	% of HF Formation per Experiment
R1 #1	25	Atoh1 #1	94
R1 #2	0	Atoh1 #2	100
R1 #3	40	Atoh1 #3	100
R1 #4	40	Atoh1 #4	63
R1 #5	0	Atoh1 #5	67
R1 #6	0	Atoh1 #6	100
-	-	Atoh1 #7	74
-	-	Atoh1 #8	77
-	-	Atoh1 #9	76
-	-	Atoh1 #10	67
Average (± SEM)%	**18 (± 8)%**	**Average (± SEM)%**	**83 (± 5)%**

b. Comparison of >15 hair follicle formation frequencies between SB/BMP-LDN and SB/BMP-FGF/LDN treatments in Atoh1/nGFP mESC-derived skin organoids

SB/BMP-LDN		SB/BMP-FGF/LDN	
Experiment ID	**% of >15 HF (***) Formation per Experiment**	**Experiment ID**	**% of >15 HF (***) Formation per Experiment**
S/B-L #1	50	S/B-F/L #1	100
S/B-L #2	33	S/B-F/L #2	19
S/B-L #3	20	S/B-F/L #3	67
S/B-L #4	0	S/B-F/L #4	60
S/B-L #5	0	S/B-F/L #5	38
S/B-L #6	0	S/B-F/L #6	24
S/B-L #7	0	S/B-F/L #7	33
S/B-L #8	0	S/B-F/L #8	31
-	-	S/B-F/L #9	32
-	-	S/B-F/L #10	45
-	-	S/B-F/L #11	50
Average (± SEM)%	**13 (± 7)%**	**Average (± SEM)%**	**45 (± 7)%**

Table S2. Media compositions. Related to Experimental Procedures.

a. Maintenance medium (LIF-2i)

Component	Supplier	Cat. No.	Stock Concentration	Final Concentration	Volume (50 ml)
Advanced DMEM/F12	Gibco	12634010	-	49% (v/v)	24.5 ml
Neurobasal Medium	Gibco	21103049	-	49% (v/v)	24.5 ml
N2 Supplement	Gibco	17502048	100X	0.5X	250 µl
B-27 Supplement, Minus Vitamin A	Gibco	12587010	50X	0.5X	500 µl
GlutaMAX Supplement	Gibco	35050061	100X	1X	500 µl
Leukemia Inhibitory Factor	PeproTech	250-02	10^7 U/ml	10^3 U/ml	5 µl
PD0325901	Stemgent	04-0006-02	10 mM	1 µM	5 µl
CHIR99021	Stemgent	04-0004-02	10 mM	3 µM	15 µl
Normocin	Invivogen	Ant-nr-1	50 mg/ml	100 µg/ml	100 µl

b. Ectodermal differentiation medium

Component	Supplier	Cat. No.	Stock Concentration	Final Concentration	Volume (50 ml)
GMEM	Gibco	11710035	-	96% (v/v)	48 ml
Knockout Serum Replacement	Gibco	10828010	-	1.5% (v/v)	750 µl
MEM Non-Essential Amino Acids Solution	Gibco	11140050	100X	1X	500 µl
Sodium Pyruvate	Gibco	11360070	100 mM	1 mM	500 µl
2-Mercaptoethanol	Gibco	21985023	55 mM	0.1 mM	91 µl
Normocin	Invivogen	Ant-nr-1	50 mg/ml	100 µg/ml	100 µl

c. Maturation medium

Component	Supplier	Cat. No.	Stock Concentration	Final Concentration	Volume (50 ml)
Advanced DMEM/F12	Gibco	12634010	-	98% (v/v)	49 ml
N2 Supplement	Gibco	17502048	100X	1X	500 µl
GlutaMAX Supplement	Gibco	35050061	100X	1X	500 µl
Normocin	Invivogen	Ant-nr-1	50 mg/ml	100 µg/ml	100 µl

Table S3. Antibodies list. Related to Experimental Procedures.

Protein	Host	Supplier	Catalog No.	Dilution
α-Smooth Muscle Actin (αSMA)	Mouse	Abcam	ab7817	1:50
βlll-Tubulin (TUJ1)	Mouse	BioLegend	801202	1:100
Brachyury	Goat	Santa Cruz	sc-17745	1:20
CD34	Rat	BD Biosciences	553731	1:50
Cytokeratin 5 (KRT5)	Rabbit	Abcam	ab53121	1:100
Cytokeratin 10 (KRT10)	Mouse	Santa Cruz	sc-23877	1:50
Cytokeratin 15 (KRT 15)	Mouse	Santa Cruz	sc-47697	1:50
Cytokeratin 17 (KRT17)	Mouse	Santa Cruz	sc-393091	1:50
E-Cadherin (ECAD)	Mouse	BD Biosciences	610181	1:50
Filaggrin (FLG)	Mouse	Abcam	ab3137	1:50
GATA3	Mouse	Invitrogen	MA1-028	1:100
Integrin-8α (ITGα8)	Goat	R&D Systems	AF4076-SP	1:100
Islet-1 (ISL1)	Mouse	DSHB	39.4D5	1:5
Ki67	Mouse	BD Biosciences	550609	1:100
Loricrin	Rabbit	Abcam	ab85679	1:50
NFATC1	Mouse	Thermo		1:100
p63	Mouse	Santa Cruz	sc-8431	1:50
p75NTR (p75)	Rabbit	Cell Signaling	8238	1:50
pan-Cytokeratin (AE13)	Mouse	Santa Cruz	sc-57012	1:50
PAX8	Rabbit	Abcam	ab97477	1:100
SCD1	Rabbit	Cell Signaling	2794	1:100
SOX2	Mouse	BD Biosciences	561469	1:100
SOX9	Rabbit	Millipore	AB5535	1:100
SOX10	Mouse	Invitrogen	14-5925-80	1:50
SSEA-1	Mouse	Stemgent	09-0067	1:100
TFAP2α	Mouse	DSHB	3B5	1:5

Note: All antibodies used for IHC were previously validated in IHC experiments. Citations can be found on the manufacture's website.

SUPPLEMENTAL VIDEO LEGEND

Video S1. 3D reconstruction of two conjoined hair follicle bearing skin organoids with neural crest-like cells. Related to Figure 5B and G. Wholemount aggregates were immunostained with antibodies for KRT5 (red), p75 (green) and AE13 (cyan). The sample was cleared using the Sca*l*eS protocol (see **Experimental Procedures** and **Supplemental Experimental Procedures**) and imaged using confocal microscopy. KRT5 labels epidermal keratinocytes in the skin organoid epidermis as well as outer root sheaths of the HFs. AE13 labels the cortex of the HFs. $p75^{+}$ neural crest-like cells reside in the dermal layer and surround the HFs. Dermal papilla cells are also $p75^{+}$. Refer to **Figure 5G and S4D-D"** for additional images of these specimens.

Video S2. 3D reconstruction of EdU labeled skin organoid hair follicles. Related to Figure 6A. Day 22 aggregates were incubated with 10 μM EdU for 24 hours. On day 23 (after 24 hours of 10 μM EdU incubation), the aggregates were fixed and immunostained with ECAD antibody. The samples were cleared using the Sca*l*eS4 solution and imaged using confocal microscopy. ECAD (white) labels entire epithelium in the skin organoid and reveals the morphology of HFs, including SGs. EdU (red) labeling represents proliferating cells as EdU incorporates into newly synthesizing DNA. EdU incorporation occurred throughout the HF matrix, in a subpopulation of cells in the putative bulge region, and in SG cells. Refer to **Figure 6A** for additional images and details.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Generation of induced pluripotent stem cells.

Induced pluripotent stem cells (C57BL/BJ) were generated from C57BL/6 mouse embryonic fibroblasts (MEF). Passage 3 MEFs were plated onto a 0.1% gelatin-coated 10 cm plastic dish at $5x10^5$ cells per plate, two days before infection. For lentivirus production, 293T HEK cells were transfected with FUW-rtTA and FUW-TetO-4F, a polycistronic vector containing *Oct3/4-Klf4-Sox2-c-Myc* separated by 2A sequences (Carey et al., 2009). 293T HEK cells were split onto 10 cm dishes at $2x10^6$ cells per plate, one day before transfection. For lentivirus production, lentiviral vectors and lentiviral packaging vectors were incubated in GeneJammer (Agilent Technologies) for 45 min and pipetted onto 293T HEK cell cultures. Medium was replaced 12 hrs after transfection, and supernatants were collected at 48 hr. Supernatants were filtered through a 0.45 μM filter, and FUW-TetO-4F and FUW-rtTA mixed at a 3.5:1 ratio. MEFs were infected by adding the supernatants to MEF medium supplemented with Polybrene (5 μg/ml, Millipore). One day after infection (day 0), medium was switched to mESC medium (DMEM, 15% FBS, LIF (Millipore), 2-Mercaptoethanol, penicillin/streptomycin, Non-Essential Amino Acids and Sodium Pyruvate supplemented with 2 μM Doxycycline (Sigma). Medium was changed every other day. On day 14, the culture medium was switched to mESC medium without doxycycline. On day 16, cell cultures were live-stained for SSEA-1 (Stemgent). SSEA-1-positive colonies were picked and transferred into a 24-well plate containing a feeder layer with radiation-inactivated MEFs. Medium changes were performed every other day. When clonal colonies were 80% confluent, cell cultures were split using Accutase and further expanded in feeder- and serum-free LIF-2i medium (DMEM/F12, Neurobasal Medium, N2 Supplement, B-27 Supplement (minus Vitamin A), Glutamine, 2-Mercaptoethanol, Penicillin/Streptomycin) (Ying et al., 2008).

Pluripotent stem cell maintenance culture.

ESCs (R1, kindly provided by Andras Nagy, and Atoh1/nGFP) and iPSCs (C57BL/BJ) were maintained in feeder-free conditions using LIF-2i medium as previously described (Koehler et al., 2013; Koehler and Hashino, 2014). PSCs were maintained on 0.1% (w/v) gelatin-coated plates with medium changes every other day and passages at 70-80% confluency. Passage 30 or lower PSCs were used for experiments to avoid over-passaging. LIF-2i medium was composed of a 1:1 mixture of Advanced DMEM/F12 (Gibco) and Neurobasal Medium (Gibco) supplemented with 0.5X N2 Supplement (Gibco), 0.5X B-27 Supplement (minus Vitamin A; Gibco), 1X GlutaMAX Supplement (Gibco), 10^3 U/ml Leukemia Inhibitory Factor (LIF; PeproTech), 1 μM PD0325901 (Stemgent), 3 μM CHIR99021 (Stemgent), and 100 μg/ml Normocin (Invivogen). See **Table S2** for media formulations.

Immunostaining.

Sample preparation - Collected aggregates were fixed with 4% Paraformaldehyde (PFA, Electron Microscopy Sciences) diluted in 1X PBS, for more than 20 min at room temperature (RT) up to overnight at 4 °C depending on aggregate sizes. The fixed samples were washed three times with 1X PBS (10 min per wash). For Immunohistochemistry (IHC), fixed and washed samples were cryoprotected with a graded treatment of 15 and 30% sucrose (Sigma-Aldrich) prior to embedding in tissue freezing medium (General Data Healthcare) on cryomolds (Endwin Scientific). The embedded samples were frozen and stored in -80 °C until usage. Frozen tissue blocks were equilibrated to the temperature in the cryostat prior to sectioning, then sectioned into 12 μm thickness cryosections. Cryosection slides were dried in a desiccator for 1 hr and stored in -80 °C until staining. For whole-mount staining, fixed and washed samples were used directly or stored in 1X PBS at 4°C until usage.

IHC – Frozen cryosections on slides were thawed and hydrated in 1X PBS for 15 min at RT. The sections were blocked with 10% Normal Goat (NGS)- or Horse (NHS)- serum (Vector Laboratories) in 1X PBS containing 0.1% Triton X-100 solution (1X PBS-T) for 1 hr at RT. Then, the sections were incubated with primary antibodies diluted in 3% NGS or NHS (Vector Laboratories) in 1X PBS-T for 1 hr at RT, followed by three-time washes with 1X PBS (10 min per wash) on an orbital shaker at 65 rpm. Secondary antibodies - Alexa Fluor 488-, 568-, and 647- conjugated anti-mouse IgG (Invitrogen), Alexa Fluor 647-conjugated anti-rat IgG (Invitrogen), or Alexa Fluor 568-conjugated anti-rabbit IgG (Invitrogen) - were diluted at a ratio of 1:2,000 in 3% NGS or NHS in 1X PBS-T, added to the sections, and incubated for 1 hr at RT, followed by three-time washes with 1X PBS. Lastly, the slides with sections were mounted with ProLong Gold Antifade Reagent with DAPI (Molecular Probes).

Whole-Mount Staining – Previously described Sca*l*eS protocol was used for whole-mount staining with major adjustments in incubation times to account for reduced tissue size (Hama et al., 2015). The fixed and washed samples went through **adaptation** step in Sca*l*eS0 solution for 12 hrs at 37 °C on a rotator. The composition of Sca*l*eS0 (pH 7.2) solution is: 20% (w/v) D-sorbitol (Sigma), 5% (v/v) Glycerol (Sigma), 1 mM Methyl-β-cyclodextrin (Santa Cruz), 1 mM γ-Cyclodextrin (Sigma), 1% (w/v) N-acetyl-L-hydroxyproline (Sigma), and 3% (v/v) Dimethylsulfoxide (DMSO; Sigma) dissolved in 1X PBS. The adapted aggregates were **permeabilized** sequentially as follows: incubated in Sca*l*eA2 solution for 24 hrs, in Sca*l*eB4(0) solution for 16 hrs, and in Sca*l*eA2 solution for 8 hrs, every incubation at 37 °C on a rotator. The composition of Sca*l*eA2 (pH 7.7) solution is: 10% (v/v) Glycerol (Sigma), 4 M Urea (Sigma), and 0.1% (v/v) Triton X-100 (Sigma), and Sca*l*eB4(0) (pH 8.4) solution is: 8 M Urea (Sigma), both dissolved in distilled water. Then, the samples were **descaled** in 1X PBS for 6 hrs at RT, and **blocked** in 10% (v/v) NGS or NHS (Vector Laboratories) in AbSca*l*e solution for 12 hrs at 37 °C on a rotator. The composition of AbSca*l*e solution is: 0.33 M Urea (Sigma) and 0.25% (v/v) Triton X-100 (Sigma) dissolved in 1X PBS. Then, the samples were incubated with **primary antibodies** diluted in 3% (v/v) NGS or NHS (Vector Laboratories) containing AbSca*l*e solution for 36 hrs at 37 °C on a rotator, followed by a **washing** step with AbSca*l*e solution at RT on a rotator sequentially for 15 min, 30 min, 1 hr, and 2 hrs. Washed samples were incubated with fluorescently-labeled **secondary antibodies** diluted in 3% (v/v) NGS or NHS (Vector Laboratories) for 36 hrs at 37 °C on a rotator followed by three **washes** in AbSca*l*e solution (2 hrs per wash) at RT on rotator. Then, the aggregates were **rinsed** with AbSca*l*e Rinse solution for twice (2 hrs per rinse) at RT on a rotator. The component of AbSca*l*e Rinse solution is: 2.5% (w/v) Bovine

Serum Albumin (Sigma) and 0.05% (v/v) Tween-20 (Sigma) dissolved in 0.1X PBS. Rinsed samples were **re-fixed** in 4% (v/v) PFA (Electron Microscopy Sciences) diluted in 1X PBS for 1 hr at RT on a rotator. Twice**-wash** (1 hr per wash) in 1X PBS at RT on a rotator was followed. Finally, the samples were **cleared** in Sca*l*eS4 solution for 12 hrs, and **mounted** by another 12 hr-incubation in fresh Sca*l*eS4 solution containing several drops of ProLong Gold Antifade Reagent with DAPI (Molecular Probes), both at 37 °C on a rotator. The composition of Sca*l*eS4 is: 40% (w/v) D-sorbitol (Sigma), 10% (v/v) Glycerol (Sigma), 4 M Urea (Sigma), 0.2% (v/v) Triton X-100 (Sigma), and 20% (v/v) Dimethylsulfoxide (Sigma) prepared in distilled water (Hama et al., 2015).

Microscopy was performed on a Leica DMi8 Inverted Microscope or an Olympus FV1000-MPE Confocal/Multiphoton Microscopes. Three-dimensional reconstruction was performed using the Imaris 8 software package (Bitplane) on computers housed at the Indiana Center for Biological Microscopy. See **Table S3** for a list of antibodies.

Fontana-Masson Staining.

A Fontana-Mason Staining Kit (Abcam) was used to visualize melanocytes. Prior to staining, dried cryosections were hydrated in distilled water. Then, the sections were incubated in Ammoniacal Silver Solution that was pre-warmed in water bath to 58 °C, until the sections turn into brownish color (~45 min), followed by three-time rinses in distilled water. The samples were incubated in 0.2% (v/v) Gold Chloride Solution for 30 sec, followed by three-time rinses in distilled water. Next, the sections were incubated in 5% (v/v) Sodium Thiosulfate Solution for 2 min, rinsed once in running tap water, and rinsed twice more in distilled water. Then, the samples were dehydrated by dipping 5 times in fresh Absolute Alcohol for three times. Lastly, the samples were cleared in Histochoice Clearing Agent (Sigma) for 2 min (1min per clearing) and mounted in Organo/Limonene Mount solution (Sigma).

SUPPLEMENTAL REFERENCES

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