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Supplemental Information

Intercellular Adhesion-Dependent Cell Survival and ROCK-Regulated Actomyosin-Driven Forces Mediate Self-Formation of a Retinal Organoid

Albert Lowe, Raven Harris, Punita Bhansali, Ales Cvekl, and Wei Liu

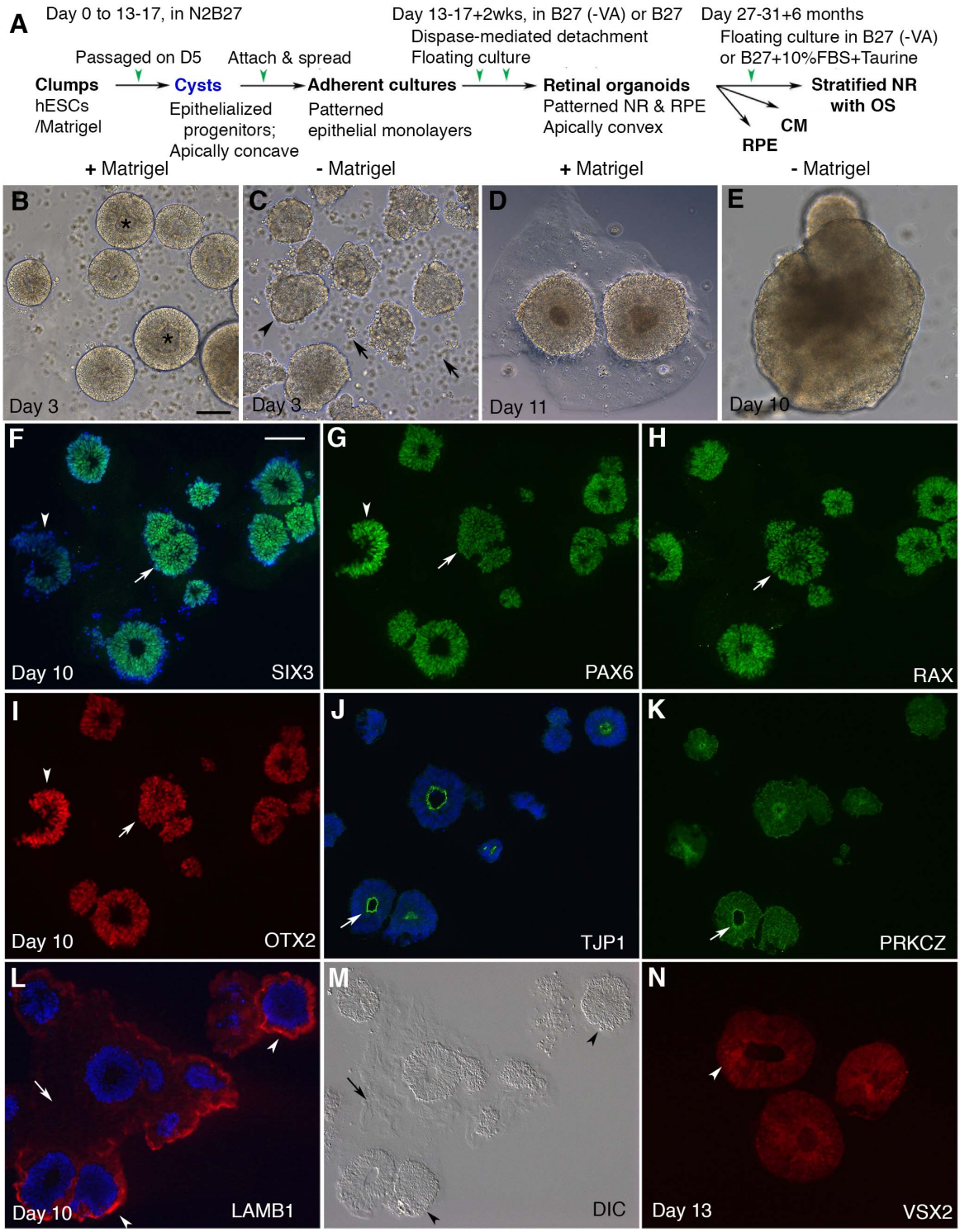

Figure S1. Related to Figure 1. (**A**) **A procedure for retinal differentiation from H1 hESCs via the cysts.** NR, neural retina; RPE, retinal pigment epithelium; CM, ciliary margin; OS, outer segment. (**B-N**) **Generation of polarized early retinal progenitor cells via Matrigel-mediated cyst formation.** Culturing hESCs that were directly embedded in Matrigel generated large quantities of cysts with a single lumen (**B**,**D,** asterisks in B). In the absence of Matrigel, most cells died (arrows in **C**); the surviving cells formed solid cell masses (C,**E**). The cysts on day 10 widely expressed the eye field markers SIX3, PAX6, RAX, and OTX2 (**F-I**). Tight junction protein TJP1 and apical protein PRKCZ were found at the lumen surface (arrows in **J**,**K**), and basal protein LAMB1 was present at the outer surface (arrowheads in **L**). The locations of LAMB1 immunostaining differed from the sites of Matrigel (as revealed by DIC images; compare arrows and arrowheads in L,**M**), indicating that the immunostaining detects the LAMB1 self-deposited by the polarized cells rather than the LAMB1 as a component of Matrigel. VSX2 was weakly expressed in some cells on day 13 (**N**). Scale bar, 100 μm.

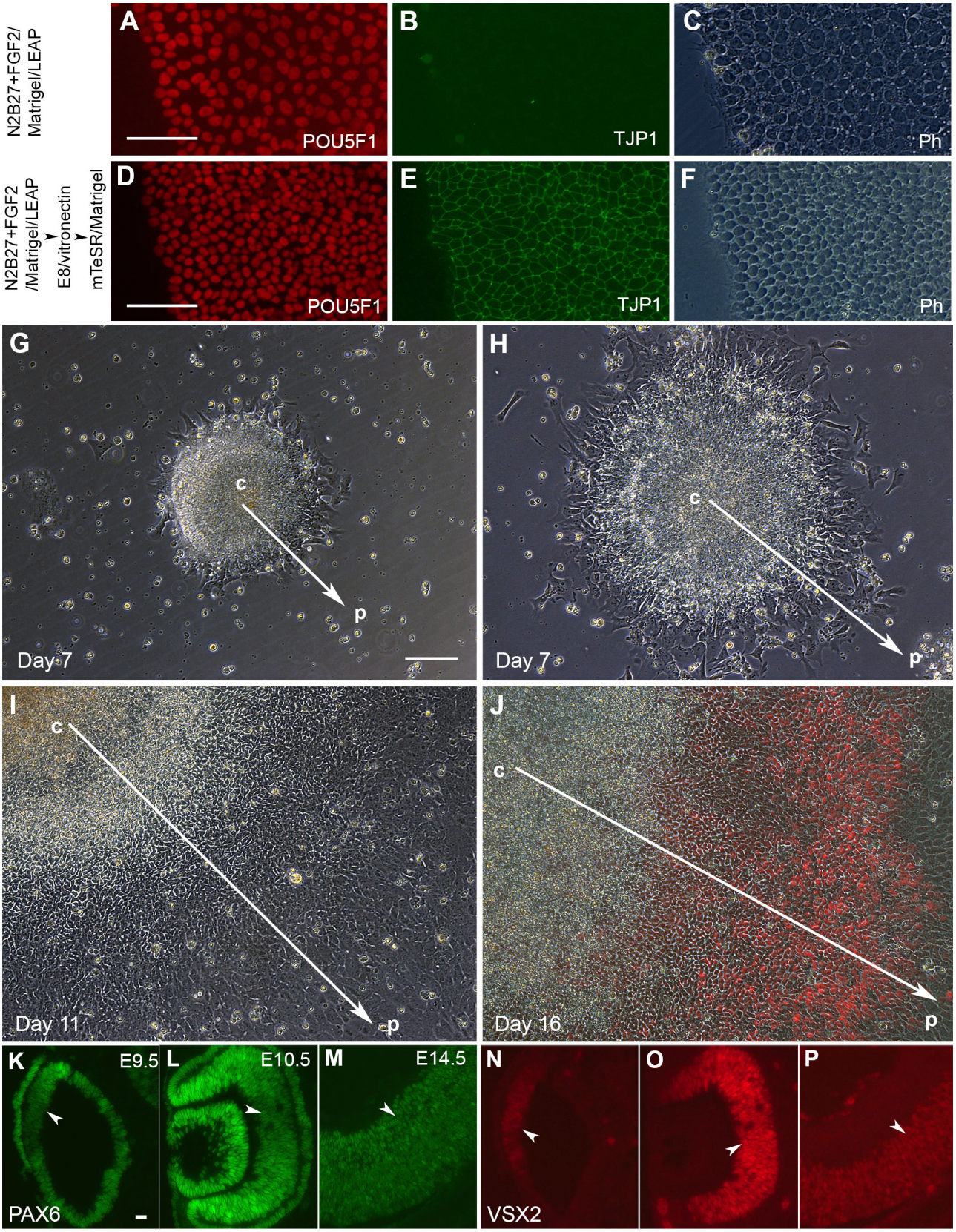

Figure S2. Related to Figure 1. (A-F) TJP1 expression in undifferentiated H1 hESCs depends on culture conditions. H1 hESCs cultured in two conditions were used for immunocytochemistry: 1) hESCs on Matrigel-coated plates in N2B27 medium containing FGF2 and passaged with laser-enabled analysis and processing (LEAP; Cyntellect) (condition 1, **A-C**); 2) hESCs initially in condition 1 changed to E8/vitronectin and then to mTeSR/Matrigel for 3 passages (condition 2, **D-F**).

In both conditions, hESCs highly expressed pluripotency marker POU5F1 (A,D; phase contrast images in C,F. Note the different morphology). However, TJP1 was expressed in hESCs in condition 2, but not in condition 1. **(G-J) Spontaneous attachment and spreading of the cysts.** The cysts spontaneously attached to culture surfaces starting at day 7 (**G,H,** two different colonies) and spread radially to form adherent cultures with a center-periphery axis on day 11 and day 16 (**I**,**J**). $VSX2^+$ cells formed a ring shape (J). C, center; p, periphery. (**K-P**) **Expression of PAX6 and VSX2 in mouse retinal development.** Sections adjacent to those in Figure 2K-P were used. PAX6 was expressed at the optic vesicle (arrowhead in **K**) and surface ectoderm at E9.5; at RPCs (arrowhead in **L**), RPE progenitors, and lens vesicle at E10.5; and at RPCs on E14.5 (arrowhead in **M**). VSX2 was expressed in RPCs at E9.5, 10.5, and 14.5 with its peak level at E10.5 (arrowheads in **N-P**). Scale bar, 100 μm.

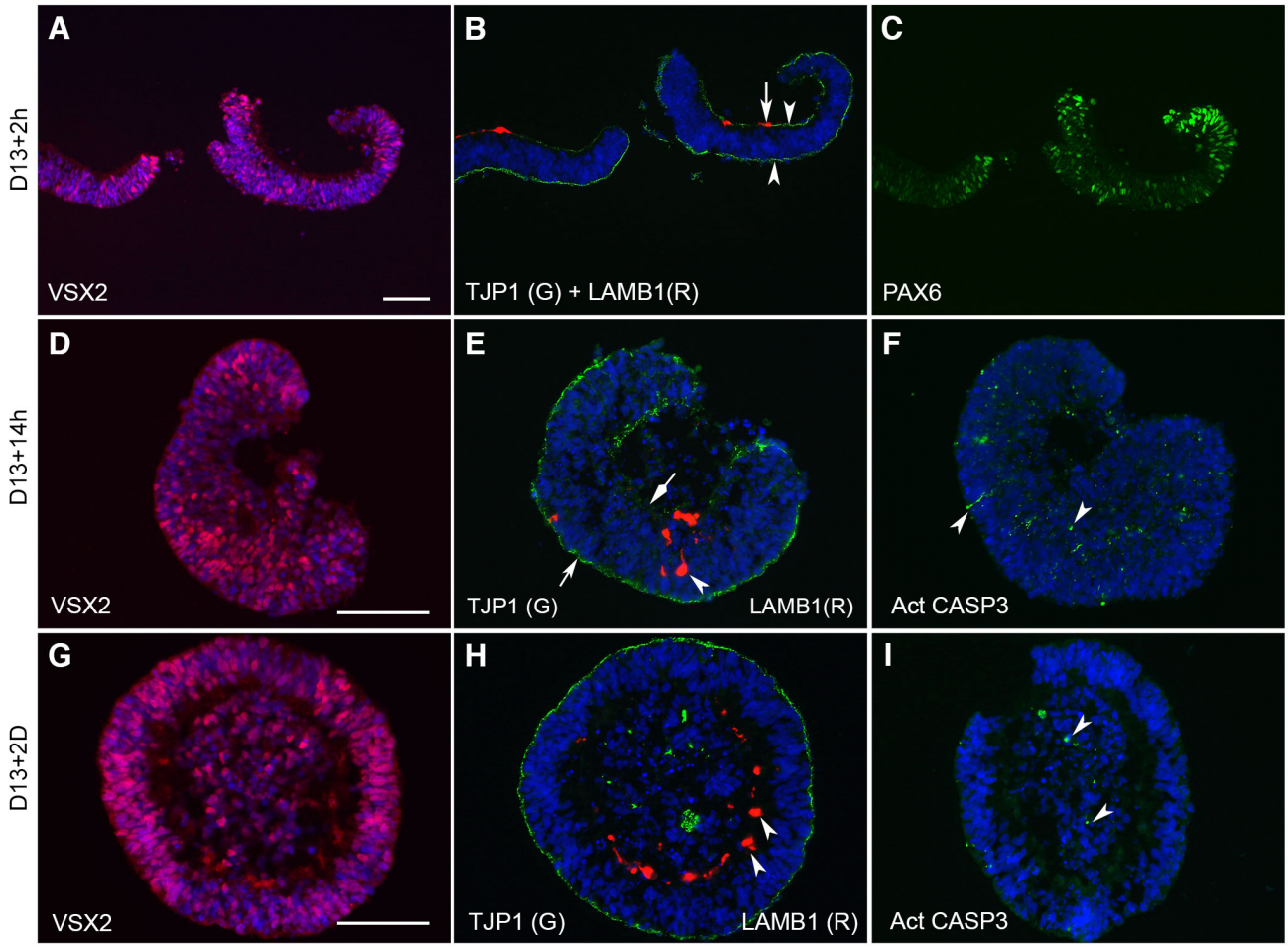

Figure S3. Related to Figure 3 and Figure 4C. In some cases, newly detached cell sheets displayed relatively fewer $VSX2^+$ RPCs and $PAX6^+$ cells (A, C, n=7/10, independent cell sheets), or expressed comparable amounts of TJP1 at the both surfaces (B, n=3/10, independent cell sheets). At later stages, however, $VSX2^+$ RPCs gradually formed apically convex epithelium with high abundance in all cases (D,E,G,H, n=5/5, independent aggregates). See also Figure 4C for the increase in VSX2 expression. Substantial levels of apoptosis occurred in the remodeling (F,I, n=4/4, independent aggregates). The retinal organoid in G-I is the same one as in Movie S2. Scale bars, 100 μm.

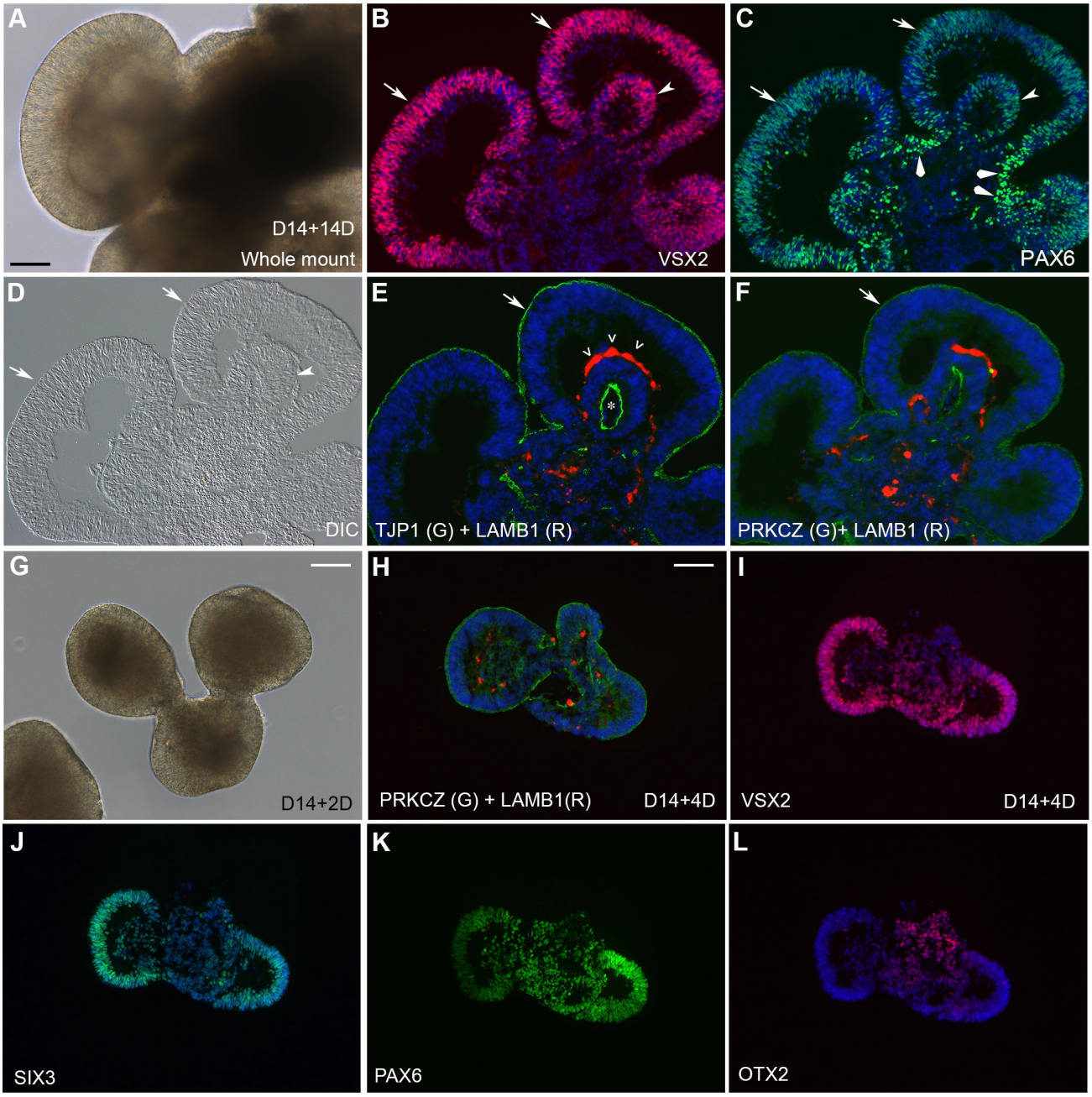

Figure S4. Related to Figure 4. (A-F) Morphology, higher-magnification views for Figure 4J-L, and the expression of PRKCZ in a fused retinal organoid at D14+14D. Morphology (whole-mount view in **A**, section view in **D**). VSX2 was specifically expressed in the epithelium (**B**). PAX6 was expressed at moderate levels in the $VSX2^{+}$ epithelium (arrows in **C**), and at high levels in the peripheral $VSX2^{-}$ cells (diamond arrowheads in C). $VSX2^{+}$ epithelium was apically convex, as TJP1 and PRKCZ were expressed at the convex outer surface of the retinal organoid (arrows in **E**,**F**). An internal $VSX2^{+}$ epithelium with the opposite cell polarity (asterisk in E) appeared to be fusing to the outer epithelium and flipping out (arrowheads in B-F). Abundant LAMB1 was present at the basal surface of the internal $VSX2^{+}$ epithelium (arrowheads in E). **(G-L) Validation of the retinal differentiation procedure in a line of hiPSCs.** Retinal organoids were generated from a line of hiPSCs using the same procedure that was employed for H1 hESCs. The retinal organoids at D14+2D showed very similar morphology (**G**) and expression pattern (**H-L**) compared those generated with H1 hESCs (Figure 2F, Figures S6A,C-E). On D14+4D, $VSX2^{+}$ RPCs formed apically convex epithelium (H,I) and co-expressed SIX3 and PAX6 but not OTX2 (I-L). Scale bars, 100 μm.

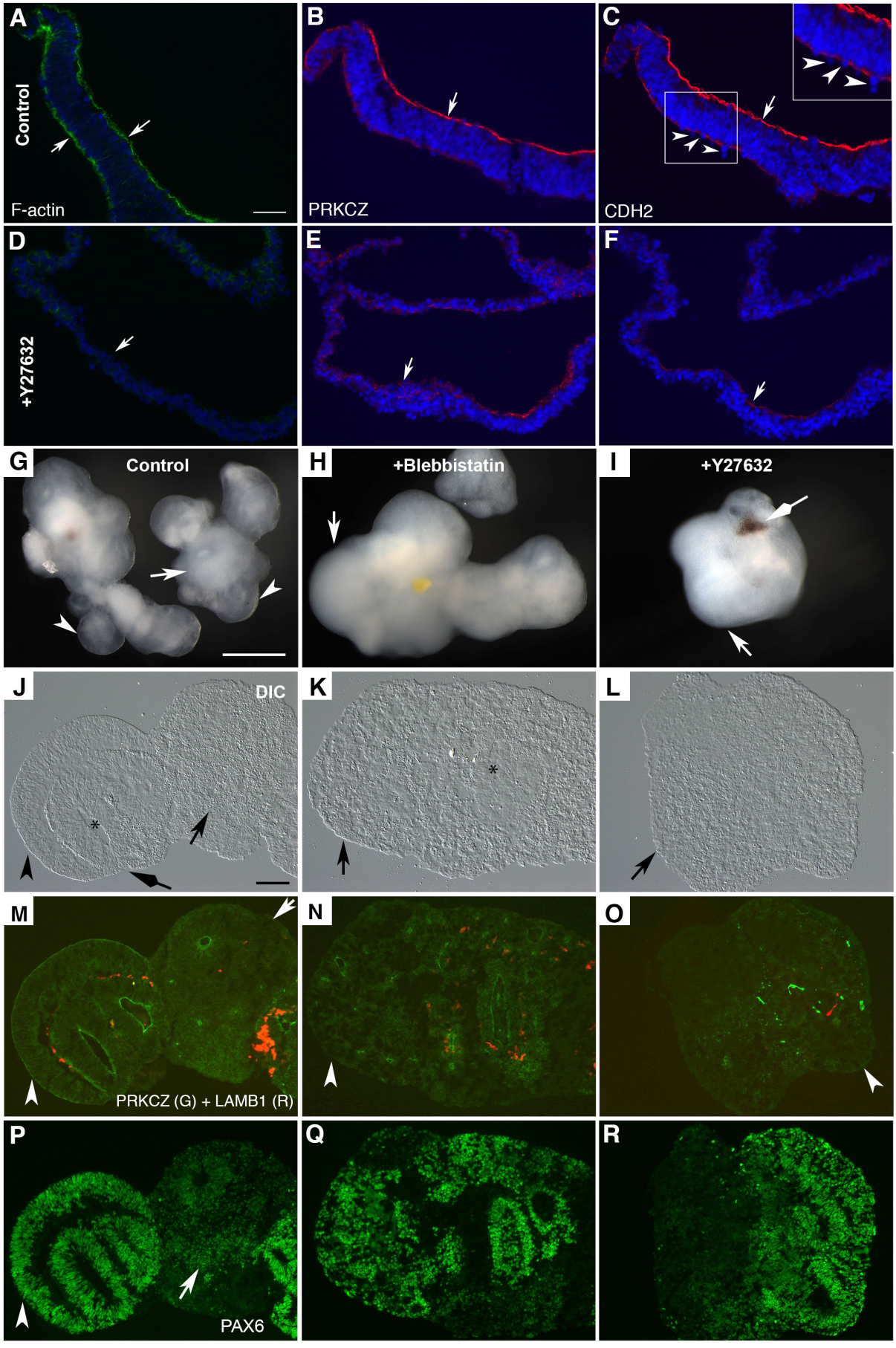
A
Control
F-actin
B
PRKCZ
C
CDH2
D
+Y27632
E
F
G
Control
H
+Blebbistatin
I
+Y27632
J
DIC
K
L
M
PRKCZ (G) + LAMB1 (R)
N
O
P
PAX6
Q
R

Figure S5. Related to Figure 5. (A-F) ROCK is required for the polarized expression of F-actin, PRKCZ, and CDH2 at the surfaces of the detached cell sheets. Sections adjacent to Figure 5A,C were used. ROCK inhibitor Y27632 was supplemented to the medium one hour before Dispase treatment. Then, Dispase was added to detach the adherent cultures. After two hours of floating culture, cell sheets were fixed and processed for immunostaining. In the controls, F-actin, PRKCZ, and CDH2 were present at the surfaces of the detached cell sheets (arrows in **A**,**B**,**C**), but they were undetectable or significantly downregulated after Y27632 treatment (arrows in **D**,**E**,**F**, n=4/4, independent cell sheets). The cells without proper CDH2 were disintegrating (arrowheads in C). (**G-R**) **Morphology of the aggregates in Figure 5M-R and the expression of PRKCZ, LAMB1, and PAX6.** On day 16, myosin inhibitor blebbistatin (50 μM) and ROCK inhibitor (10 μM) were supplemented to the medium one hour before, during, and the next 10 days after Dispase treatment. On D16+10D, dark field views revealed translucent areas in the controls (arrowheads in **G**), but these translucent areas were absent in the aggregates treated with either blebbistatin or Y27632 (**H,I;** n=4/4 for Y27632, n=3/4 for blebbistatin, independent aggregates). On sections, epithelium was evident in the control (arrowhead in **J**; an internal epithelium indicated by asterisk in J was fusing to the outer $VSX2^{+}$ epithelium and flipping out at the region indicated by diamond arrow in J), but no such structure was found in the treated aggregates (arrows in **K,L**). In the control, PRKCZ was expressed at the convex surface of the outer epithelium and the concave surface (the lumen) of the inner epithelium (**M**). In the one treated with blebbistatin, PRKCZ was either at the lumen of deeply embedded vesicles (asterisk in K) or at irregular positions (**N**). In the one treated with Y27632, PRKCZ expression was downregulated and displayed irregular pattern (**O**). LAMB1 expression was also irregular in the treated aggregates (N,O). Nevertheless, PAX6 was expressed in the control as well as the treated aggregates (**P-R**). Scale bars, 100 μm.

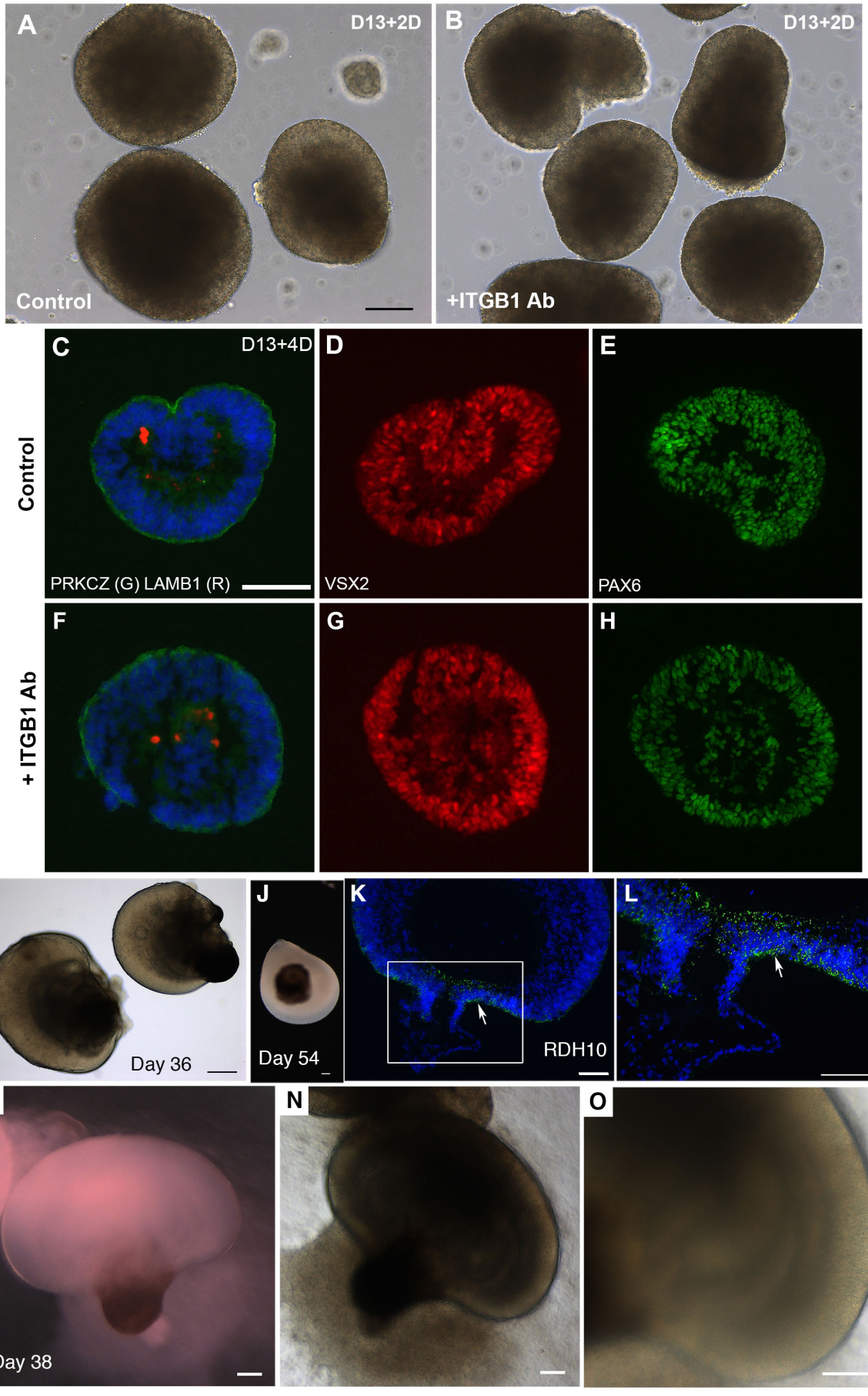
A
D13+2D
Control
B
D13+2D
+ITGB1 Ab
C
D13+4D
PRKCZ (G) LAMB1 (R)
D
VSX2
E
PAX6
Control
F
G
H
+ ITGB1 Ab
I
Day 36
J
Day 54
K
RDH10
L
M
Day 38
N
O

Figure S6. (**A**-**H**) **Related to Figure 5. Supplementing an antibody neutralizing ITGB1 to the medium does not affect self-formation of retinal organoids.** (**A**,**B**) Morphology. (**C**-**H**) Immunohistochemistry of the retinal organoids on D13+4D revealed a very similar pattern in the expression of PRKCZ, LAMB1, VSX2, and PAX6 for the control and experimental groups. (**I**-**O**) **Related to Figure 6. Morphology of the retinal organoids on day 36 (I) and day 54 (J), and the expression of ciliary margin marker RDH10 on day 54 (K,L;** sections adjacent to those in Figure 6L**).** Note the patterning of the dark and the translucent areas. (**M**-**O**) **Occasionally, self-formation of retinal organoids was found in adherent cultures.** Cyst-derived adherent cultures grown in N2B27 medium were changed to B27 (-VA) medium on day 17. On day 38, partially detached retinal organoids became visible. Micrographs in dark field (**A**) and in bright field (**B,** high power view in **C**). Scale bars, 100 μm.

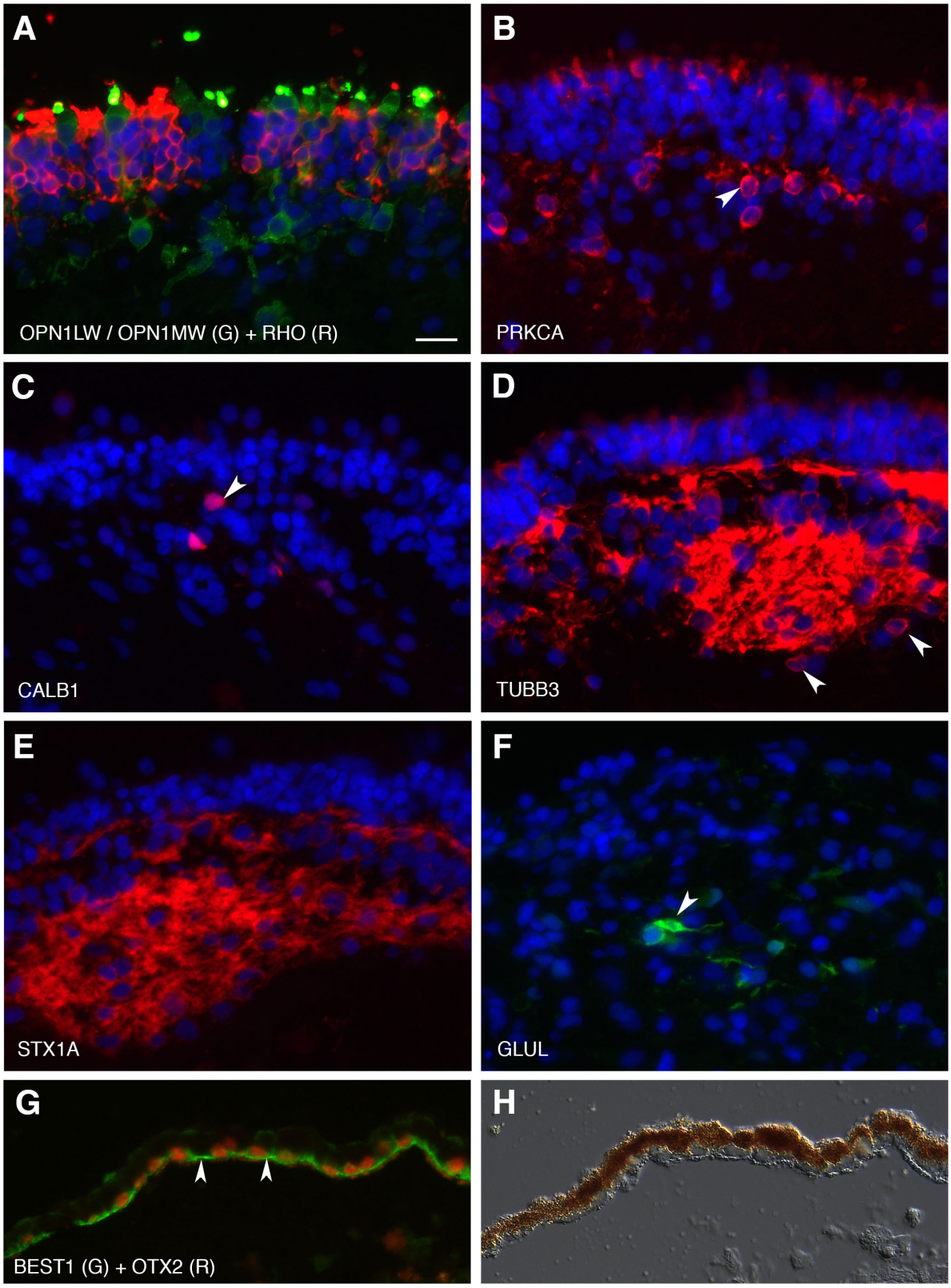

Figure S7. Related to Figure 7. Retinal cell types in retinal organoids on day 187 as revealed by immunohistochemistry. Cone photoreceptors were revealed by an antibody against OPN1LW and OPN1MW (green), and rod photoreceptors by an antibody against RHO (red) (**A**); bipolar cells by an antibody against PRKCA (**B**); horizontal cells by an antibody against CALB1 (**C**); ganglion cells and amacrine cells by an antibody against TUBB3 (**D**); amacrine cells by an antibody against STX1A (**E**); Müller cells by an antibody against GLUL (**F**); RPE cells by antibodies against BEST1 and OTX2 (**G**; bright field in **H**). Scale bar, 20 µm.

Movie S1. Related to Figure 2. Live imaging of Dispase treatment. Cyst-derived adherent cultures on day 13 were supplemented with Dispase (1 mg/ml in DMEM/F12) and then imaged on AxioObserver Z1 fully motorized microscope (EC Plan-Neofluar 10x/0.30 Ph1 WD=5.2 M27) every 2 minutes for 40 min. The cell sheet lifted at the edge, contracted, and folded up. Some cells disintegrated from the cell sheet during this process.

Movie S2. Related to Figure 3 and 5. Two-day live imaging of a retinal organoid. A detached cell sheet on day 13 was cultured in B27 culture medium for imaging on AxioObserver Z1 fully motorized microscope (EC Plan-Neofluar 10x/0.30 Ph1 WD=5.2 M27) every 15 minutes for 2 days. Some cells disintegrated from the sphere during this process. At the end of day 2, a bright ring was visible at the periphery of the sphere.

Movie S3. Related to Figure 5. Effect of ROCK inhibitor Y27632 on morphogenesis of a retinal organoid. A detached cell sheet in B27 culture medium supplemented with Y27632 one hour before, during, and after Dispase treatment on day 13 was imaged on AxioObserver Z1 fully motorized microscope (EC Plan-Neofluar 10x/0.30 Ph1 WD=5.2 M27) every 15 minutes for 2 days. Some cells disintegrated from the sphere as seen in the control condition. At the end of day 2, however, no bright ring was visible.

Supplemental Experimental Procedures

Maintenance of hESCs and hiPSCs

The project was approved by ESCRO and IRB committees at Albert Einstein College of Medicine. Undifferentiated H1 hESCs were maintained with the following feeder-free methods: 1) were grown on Matrigel-coated plates in DMEM/F12 medium supplemented with N2 and B27, 0.5% bovine serum albumin, 1 mM l-glutamine, 1% penicillin-streptomycin, 100 ng/ml of bFGF and were passaged every 5 to 7 days with laser-enabled analysis and processing (LEAP; Cyntellect) or Dispase (Yao et al., 2006, Chang and Bouhassira, 2012); 2) were grown on Matrigel-coated plates in mTeSR and passaged with Dispase or ReLeSR (STEMCELL technologies); 3) were grown on vitronectin-coated plates in Essential 8™ Medium (Gibco). Passages 48 to 75 were used. The hiPSCs were described previously (Chang and Bouhassira, 2012).

Retinal differentiation

Cysts were generated with a method modified from a previous one (Zhu et al., 2013). Briefly, undifferentiated H1 hESCs ready for passage on a 6-well microplate were partially lifted by Dispase, thoroughly rinsed with DMEM/F12 medium, cut into pieces (6-9 pieces per colony) with a glass needle, scraped off with a cell scraper in DMEM/F12 medium, and then harvested in a 15-ml conical falcon tube by centrifugation (300g, 5 minutes). Alternatively, undifferentiated H1 hESCs were treated with ReLeSR similar to cell passage and then were harvested in 15-ml conical falcon tube by centrifugation. Pellets of the cell sheets were suspended in 0.25 ml of ice-cold Matrigel (Corning) or Growth Factor Reduced Matrigel (Corning), which yielded similar results. After gelling at room temperature for 15-30 minutes, 9 ml of N2B27 Medium (DMEM/F12+GlutaMAX (GIBCO):Neurobasal medium (GIBCO) = 1:1, 0.5 x B27 supplement (GIBCO), 0.5 x N2 supplement (GIBCO), 0.1 mM b-mercaptoethanol, and 2 mM L-GlutaMax) was added gently to disperse the clump with a 5 ml pipet. Next, the suspension was dispensed into 18 wells on a 24-well plate, designated as day 0. Cysts with a single lumen were visible on day 1. On day 4 or 5, floating cysts were transferred to new 24-well plates, 20-40 per well, 4-5 plates in total. The cysts were further cultured in N2B27 Medium with medium changes every 2-3 days.

Starting at day 7, the cysts spontaneously attached to culture surfaces, spread, and became adherent cultures by day 13. At a time point during day 12 to 17 when the monolayer colonies were well spread out but before extensive interactions between neighboring colonies, the adherent cultures were detached with these methods: 1) 0.05% Trypsin/EDTA to remove the loosely attached cells, 2) 0.05% Trypsin/EDTA to remove the loosely attached cells and then Dispase (1 mg/ml) for 30 minutes, 3) Dispase (1 mg/ml) for about 30 min, or 4) Collagenase IV (2 mg/ml) for 40 minutes. Dispase treatment sufficiently detached the adherent cells as sheets and was used thereafter. After Dispase treatment, the cells without intercellular adhesions were suspended into single cells or small aggregates and were in suspension. In contrast, the cells with intercellular adhesions were lifted as cell sheets and settled at the bottom of culture wells. After 3-5 rinses with DMEM/F12 medium, the single cells and smaller aggregates were mostly removed from the system by aspiration with a Pasteur pipet. The detached cell sheets were then cultured as floating aggregates and remodeled into retinal spheres with bright rings (referred as retinal organoids). These media were tested for culture: 1) Medium 1, DMEM+GlutaMAX™, 20% KOSR (Gibco), 1xNEAA, 1x GlutaMax, and 0.1 mM β-mercaptoethanol; 2) Medium 2, DMEM/F12 (3:1), 2% B27 (-VA), 1xNEAA; 3) Medium 3, DMEM/F12 (3:1), 2% B27, 1xNEAA; 4) N2B27 Medium as described above. These media gave similar results. For consistency, Medium 2 was used at this step unless it is stated differently. In the first 7 days of floating culture, substantial amounts of cell death were found and the medium was changed every other day. When the cysts were seeded at 20-40 per well on day 5, single retinal organoids were abundant in the floating cultures, 400-600 retinal organoids were generated from a 6-well plate of undifferentiated H1 hESCs. Sometimes fused retinal organoids were trimmed out with scalpel (#11). After two weeks of floating cultures, retinal organoids were grown in the following media: 5) Medium 5, DMEM/F12 (3:1), 2% B27, 1xNEAA, 10% fetal bovine serum (Gibco), 100 μM Taurine (Sigma), and 2 mM GlutaMAX; or 6) Medium 6, DMEM/F12 (3:1), 2% B27 (-VA), 1xNEAA, 10% fetal bovine serum (Gibco), 100 μM Taurine (Sigma) and 2 mM GlutaMAX. Medium 5 and Medium 6 gave similar results. For consistency, Medium 5 was used for long-term culture unless stated differently. Medium was changed every 3 days. Pigmentation in the retinal organoids became visible from day 25-30. Agitation during the culture helps maintain floating cultures.

Assay for Ca^{2+}-dependent intercellular adhesions

EGTA (3 mM as a final concentration) was added during Dispase treatment.

Cell viability assay

Trypan Blue staining was used. 20 μl of the mixture of cell suspensions and Trypan Blue was loaded onto a slide and mounted with a coverslip for imaging. The cell suspensions were: 1) the disintegrated cells that shed off from retinal organoids in floating cultures; 2) the single cells from the cell sheets that were detached by Dispase and were then dissociated by 0.25% Trypsin/EDTA; 3) the single cells after 2-day suspension culture; or 4) the single cells after 2-day floating culture in Matrigel clumps. Matrigel-embedded single cells were prepared as follows. Dissociated single cells from

the detached cell sheets were mixed with cold Matrigel and kept at room temperature for 15-30 minutes for gelification to form a clump. Subsequently, the clump of Matrigel/single cells was dispersed into small clumps by gentle pipetting with a 5 ml pipet and then grown as floating cultures. After 2 days, the clumps of Matrigel/cells were harvested by centrifugation, and the cells in the clumps were recovered by dissolving Matrigel with Cell Recovery Solution (Corning) in a refrigerator for 40 minutes. The recovered cells were mixed with Trypan Blue for viability assay.

Immunohistochemistry and immunocytochemistry
At least three biological replicates were used for each assay. Cysts, retinal organoids, and aggregates were fixed in 4% PFA for 15-30 minutes at room temperature and processed for cryosectioning at 8 mm. For immunocytochemistry, the cultures were fixed in 4% PFA for 10-15 minutes at room temperature. The primary antibodies used were: POU4F2 (also known as BRN3, 1:100, Santa Cruz SC-6026), POU5F1 (also known as OCT4, 1:500, Santa Cruz, sc-5279), ISLET1/2 (1:100, DSHB 39.4D5-c), Ki67 (1:600, BD 556003), LAMB1 (also known as β1-laminin, 1:500, Millipore MAB1904), MITF (1:1000, Heinz A), OTX2 (1:1500, R&D AF1979), PAX6 (1:500, Covance PRB-278P), PRKCZ (also known as PKCζ, 1:500, Santa Cruz sc-216), pH3 (1:2000, Millipore 05-806), RAX (1:500, Abcam ab86210), RECOVERIN (1:2000, Millipore AB5585), SIX3 (1:500, Rockland), TUBB3 (also known as Tuj1 (1:500, Covance MMS-435P), VSX2 (1:200, Millipore AB9016), TJP1 (also known as ZO-1, 1:500, Invitrogen 40-2200), CDH2 (also known as N-cadherin (1:250, BD 610920), FUT4 (also known as SSEA-1, 1:100, Millipore MAB4301), COL4A3, (also known as Collagen IV, 1:500, American Research Products 03-10760), Phospho-myosin Light Chain 2 (Thr18/Ser19) (1:100, Cell Signaling 3674P), Peanut Agglutinin-568 (1:250, Invitrogen L-32458), L/M-opsin and S-opsin (1:2000, J Nathan), BEST1 (also known as Bestrophin, 1:200, ab155252), PRKCA (also know as RKCα, 1:800, Millipore 05-154), CALB1 (also known as Calbindin D 28k, 1:500, Sigma C9848 clone CB-955), STX1A (also known as Syntaxin, 1:1000, Sigma S0664), GLUT (also known as Glutamine Synthetase, 1:200, BD Biosciences 610517), RDH10 (1:200, Proteintech 14644-1-AP). Primary antibodies were visualized by Alexa Fluor 488- or 568-conjugated secondary antibodies.

Electron microscopy (EM)
EM was performed by Analytical Imaging Facility in Albert Einstein College of Medicine with a standard method. Retinal organoids were fixed in 0.1M Cacodylate buffer containing 2% paraformaldehyde and 2.5% gluteraldehyde for 60 minutes at room temperature, and then processed for EM.

Integrin blocking
Detached cell sheets were cultured in a medium supplemented with a neutralizing antibody against ITGB1 (also known as Integrin-b1) (5 mg/ml, Calbiochem) on day 13 for 2 days. The retinal organoids were analyzed on day 17.

Live imaging
AxioObserver Z1 fully motorized microscope (EC Plan-Neofluar 10x/0.30 Ph1 WD=5.2 M27) with environment control was used for live imaging. For imaging Dispase treatment, adherent cultures in a 24-well plate were supplemented with Dispase (1 mg/ml in DMEM/F12) and then photographed every 2 minutes for 40 min. For imaging the morphogenesis of the retinal organoids, a detached cell sheet cultured in 35 mm dish was photographed every 15 min for 2 days.

RT-qPCR
To quantify gene expression in retinal organoid morphogenesis, RNA from three individual wells of adherent cultures on D13 (the time before the cell detachment), four individual wells of adherent cultures on D13+13D, and four groups of retinal organoids on D13+13D (15 pooled retinal organoids in each group) were isolated for measurement using RT-qPCR. The adherent cultures were grown in N2B27 medium until D13 and then changed to Medium 2 for another 13 days of cultures. The retinal organoids were generated via Dispase-mediated detachment on D13 and subsequent floating culture in Medium 2 for another 13 days. Individual or trimmed retinal organoids gave indistinguishable expression profile. cDNA was synthesized with SuperScript III First-Strand Synthesis System using oligo d(T) primer (Invitrogen). qPCR was performed using Power SYBR Green (Applied Biosystems) with four technical replicates for each biological replicate. RNA quantity for each biological replicate was determined with $2^{-\Delta Ct}$ method with *ACTB* (also known as actin, beta) as an internal control as described previously (Schmittgen and Livak, 2008). The mean and standard deviation of biological replicates for each group were determined using Excel software, generating the quantity of gene expression for each group for each gene. Then, the quantity of gene expression for each group was normalized to that on D13 adherent cultures for each gene and was thus expressed as fold changes (mean ± SD of biological replicates). Sequences of PCR primers were from literature (Meyer et al., 2009, Lamba et al., 2006), PrimerBank (Wang et al., 2012), or designed using Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/): *VSX2*, forward, GGC GAC ACA GGA CAA TCT TTA, reverse, TTC CGG CAG CTC CGT TTT C; *SIX6*, forward, ACC CCT ACG CAG GTG GGC AA, reverse, TGA AGT GGC CGC CTT GCT GG; *TJP1*, forward, AGA CCG TGC TGA CTT CTG GAG ATT, reverse, ACT TTG TTT GAA CAG GCT GAG CGG; *PAX6*, forward, AGT GAA TCA GCT CGG TGG TGT CTT, reverse, TGC AGA ATT CGG GAA ATG TCG CAC; *CDH2* (also

known as N-cadherin, forward, AGC CAA CCT TAA CTG AGG AGT, reverse, GGC AAG TTG ATT GGA GG GATG; *OTX2*, forward, AGA GCA GCC CTC ACT CGC CA, reverse, AGT CGG CCC AAA TCG GGG GT; *SNAI2*, forward, CGA ACT GGA CAC ACA TAC AGT G, reverse, CTG AGG ATC TCT GGT TGT GGT; *ACTB*, forward, GCG AGA AGA TGA CCC AGA TC, reverse, CCA GTG GTA CGG CCA GAG G.

Supplemental References

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