SUPPLEMENTARY FIGURES

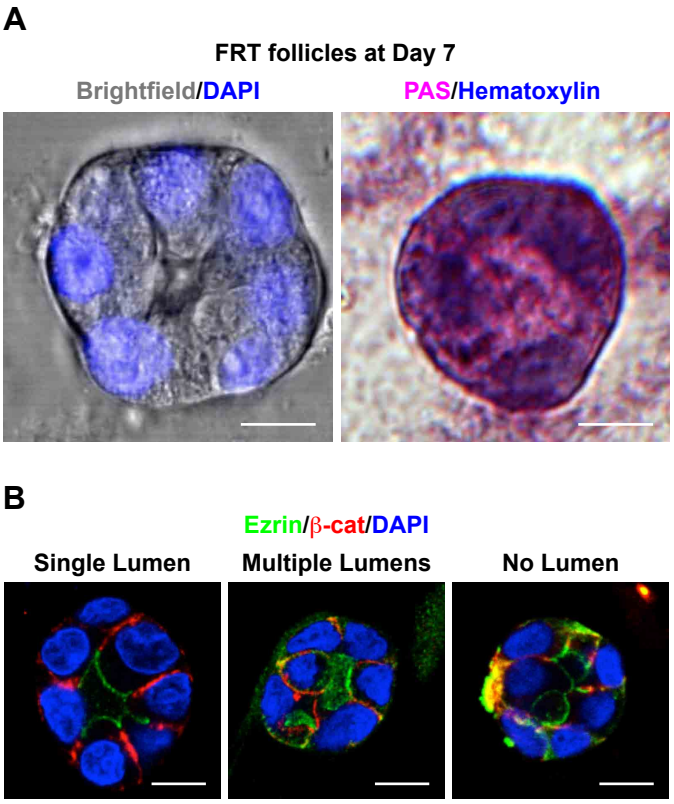


Fig. S1. Lumen formation during 3D FRT follicle organization (A) Brightfield images of 3D spherical, follicular structures derived from FRT cells after 7 days of 3D growth in Matrigel. Left panel: Representative brightfield and confocal microscopy merged image in which FRT follicle-like structures are characterized by a single layer of cells (nuclei stained with DAPI in blue) that surround a central lumen free from cells. Right panel: Representative brightfield image showing the follicular lumen filled with glycoproteins visualized by periodic acid-Schiff (PAS) staining (magenta). Nuclei in PAS samples are stained with hematoxylin (blue). Scale bars are 10 µm. (B) Different phenotypes of FRT structures regarding lumen formation were identified by examining them through the z-axes at the fifth day of growth. Follicle-like structures with single, cleared internal lumen were characterized as normal (left panel). Follicle-like structures with multiple lumens were identified by the presence of several small, apically lined lumens (middle panel). Some structures were cell aggregates without visible lumen due to loss of apical-basal polarity (right panel). Structures were fixed and stained for Ezrin (green) and β-catenin (red). Representative confocal middle z-sections are shown. All nuclei were stained with DAPI (blue) and scale bars are 10 µm.

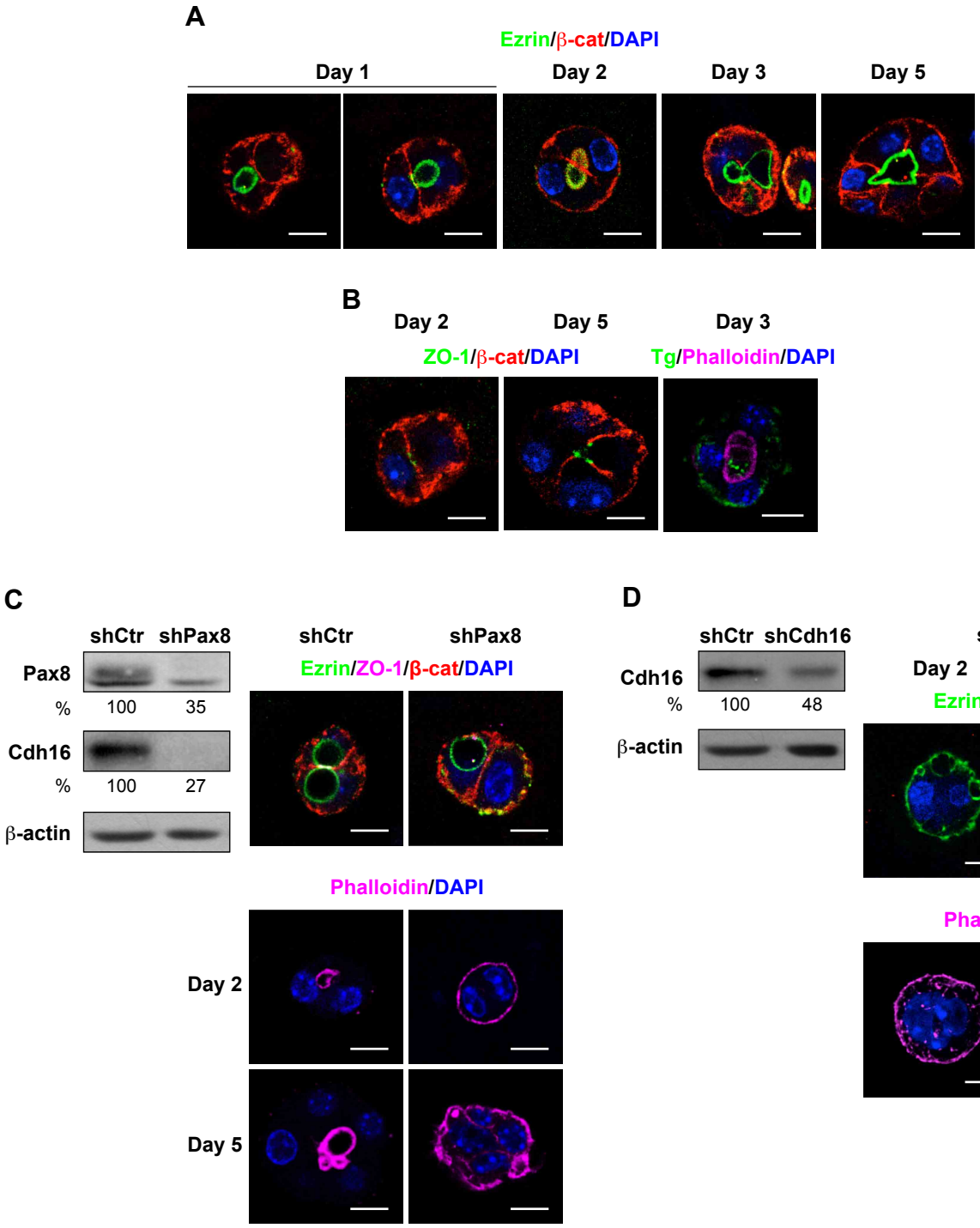
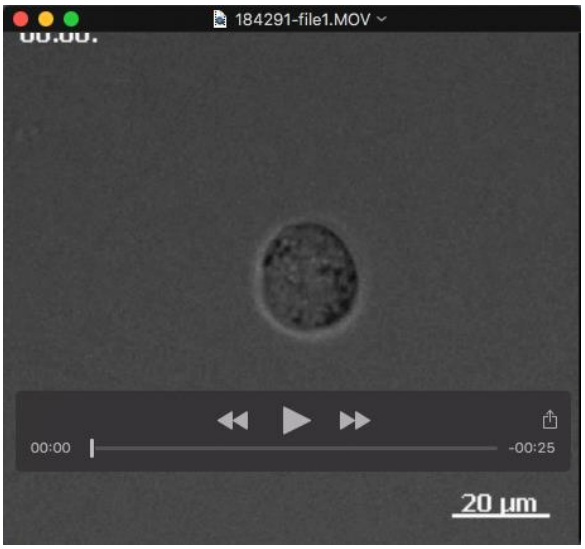


Fig. S2. Three-dimensional follicle reorganization of mouse primary thyrocytes requires apical-basal polarization and *de novo* lumen formation that are under the Pax8-Cdh16 regulation pathway. (A) Apical-basal polarization and lumen formation were observed in developing thyrocyte follicle-like structures. Structures were fixed at the indicated number of days and stained for Ezrin (green) and β-catenin (red). Representative confocal middle z-sections are shown. (B) Membrane polarization and thyroid differentiation in representative thyrocyte follicle-like structures at the indicated time points of culture. Structures were stained for ZO-1 (magenta) and β-catenin (red) (Day 2 and 5), thyroglobulin (Tg, green) and phalloidin (magenta) (Day 3). (C) Pax8 silencing disrupts apical membrane orientation, lumen formation and polarized actin organization in thyrocyte 3D structures. Left panels: Protein expression levels of Pax8 and Cdh16 in control primary thyrocytes (shCtr) and primary thyrocytes silenced for Pax8 (shPax8). Lysates were also immunoblotted for β-actin to ensure equal loading. Protein levels were quantified as percentages relative to shCtr expression levels. Right panels: (upper) Apical-basal polarization in shCtr and shPax8 thyrocytes grown for 2 days in 3D Matrigel culture. Representative single confocal sections through the middle of follicles stained for polarity markers: Ezrin (green), ZO-1 (magenta) and β-catenin (red); (lower) Actin filaments distribution in shCtr and shPax8 thyrocytes grown for 2 and 5 days in 3D Matrigel culture. Representative single confocal sections through the middle of structures stained for phalloidin (magenta). (D) Left panels: Protein expression of Cdh16 in primary thyrocytes after silencing of Cdh16 (shCdh16). Lysates were also immunoblotted for β-actin to ensure equal loading. Protein levels were quantified as percentages relative to control (shCtr) expression levels. Right panels: Representative single confocal sections of shCtr and shCdh16 structures at 2 and 5 days of growth in Matrigel stained for Ezrin (green), Cdh16 (red), and phalloidin (magenta). All nuclei were stained with DAPI (blue) and scale bars are 10 µm.

SUPPLEMENTARY DATA



Movie 1. Three-dimensional follicle morphogenesis. A time-lapse movie of follicle formation was created from single FRT thyroid follicular cells grown in 3D Matrigel culture for seven days. Time frame, 40 min. Scale Bar is 20 µm.

Movie 2. Three-dimensional follicle morphogenesis. A time-lapse movie of follicle formation was created from single mouse primary thyrocytes grown in 3D Matrigel culture for seven days. Time frame, 40 min. Scale Bar is 10 µm.

Table S1. Differentially expressed 3D *vs.* 2D genes. Excel file containing the upregulated (red) and downregulated genes (green) in alphabetical order.

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Table S2. KEGG gene set enrichment analysis. Excel file containing the biological processes overrepresented among upregulated (positive) and downregulated (negative) probes for 3D *vs.* 2D comparison.

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Table S3. Differentially expressed 3D *vs.* 2D genes related to epithelial tubulogenesis. List of probes over-represented among a gene set of epithelial tubulogenesis. FDR=0.008

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Table S4. Differentially expressed 3D *vs*. 2D genes involved in 3D MDCK cyst formation. List of probes over-represented among a gene set of 3D MDCK cyst morphogenesis. (FDR=0.002)

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Table S5. KEGG functional analysis. Excel file containing genes upregulated in 3D *vs*. 2D condition distributed in different biological processes according to KEGG annotations.

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Table S6. List of antibodies and dyes used in immunofluorescence (IF) and western blotting (WB)

Primary Ab	Cat. Number	Commercial source	Dilution
Ezrin	MS-661-P0	Thermo Fisher Scientific, Waltham, MA, USA	1:500 (IF)
β-catenin	1496-R	Santa Cruz Biotechnology, Santa Cruz, CA, USA	1:500 (IF)
ZO-1	33725	Santa Cruz Biotechnology, Santa Cruz, CA, USA	1:200 (IF)
GM130	610822	BD Biosciences, Bedford, MA, USA	1:100 (IF)
Pax8	PA0300	Biopat, Milan, Italy	1:2000 (WB)
Cdh16	15107	Protein Tech Group Inc., Chicago, IL, USA	1:100 (IF), 1:1000 (WB)
GFP	6455	Invitrogen, Carlsbad, CA, USA	1:200 (IF)
β-actin	1616-R	Santa Cruz Biotechnology	1:1000 (WB)
Acetylated tubulin	T6793	Sigma-Aldrich, St Louis, MO, USA	1:500 (IF)
Ki67	NCL-Ki67-MM1	Novocastra Laboratories Ltd., Newcastle, UK	1:100 (IF)
Cleaved caspase-3	9661	Cell Signaling Technology, Danvers, MA, USA	1:500 (IF)
Laminin-332	14509	Abcam PLC, Cambridge, UK	1:200 (IF)
Itgb1 (N20)	6622	Santa Cruz Biotechnology, Santa Cruz, CA, USA	1:500 (WB)
Tg	A0251	Dako-Agilent, Santa Clara, California, USA	1:1000 (IF)
Secondary Ab	**Cat. Number**	**Commercial source**	**Dilution**
Alexa Fluor anti-rabbit 488	A11034	Invitrogen, Molecular Probes	1:500
Alexa Fluor anti-mouse 546	A11030	Invitrogen, Molecular Probes	1:500
Alexa Fluor anti-rat 647	A21247	Invitrogen, Molecular Probes	1:500
Goat anti-mouse IgG-HRP	2005	Santa Cruz Biotechnology	1:2000
Goat anti-rabbit IgG-HRP	2004	Santa Cruz Biotechnology	1:2000
Dyes	**Cat. Number**	**Commercial source**	**Dilution**
Phalloidin 647	A22287	Invitrogen, Molecular Probes	1:40
DAPI	D1306	Invitrogen, Molecular Probes	1:10,000
PAS-Hematoxylin KIT	395	Sigma-Aldrich, St Louis, MO, USA	Standard procedure

Table S7. List of rat primers, oligonucleotide sequences and primer efficiency (%) used in q-PCR

Gene	Oligonucleotide sequence	Efficiency (%)
Pax8	F: 5´-GGACAGTTGTCGACTGAGCA-3´ R: 5´-GAATGAGGATCTGCCACCAC-3´	99.7
Cdh16	F: 5´-TTCCTGGAGGTTTCTGATGG-3´ R: 5´ AAAGGCTGAACTGGGAACTG-3´	97
Myo5b	F: 5´-AGTGGGGATGAGGGTTTCAT-3´ R: 5´-TTCCAGCATGGCAGAAACTA-3´	99.1
Rab17	F: 5´-AACACCATTGCCCAGGAG-3´ R: 5´-AGGCTGAGCTATCCTCCAC-3´	98.2
Wnt4	F: 5´-GCTGTACCTGGCCAAGCTGT-3´ R: 5´-GAACTGGTACTGGCACTCCTC-3´	97.4
Duox2	F: 5´-CATGCTGCGCGACCACGACA-3´ R: 5´-TACAGCCGGGGAGAGGGAGC-3´	100.4
Polr2g	F: 5´-GCAGGACGATGAGATACGCT-3´ R: 5´-ATCAAAGCAAGCTGGAGGCT-3´	99.1
Lamb1	F: 5´-GTGGAAGGAATGGTTCATGG-3´ R: 5´-AATCCATGCACAGCTCACAG-3´	98.5
Lamb2	F: 5´-GCAAATTGTGTGCCACTGTC-3´ R: 5´-TCAATGTTCCCACTGCACTC-3´	99.1
Lamb3	F: 5´-CCAAAGGTGTGACTGCAATG-3´ R: 5´-TTGGCTAGCAGCAAACACAG-3´	97.8
Lamc1	F: 5´-AATCTCGGACTTTGCTGTGG-3´ R: 5´-GAAAGGCAGGCACTTTTCAC-3´	99.2