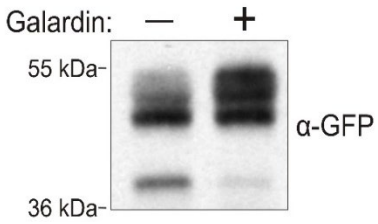


Fig. S1. Inhibition of EREG cleavage by the metalloprotease inhibitor Galardin. Three hundred thousand EREG-EGFP cells/well of 6-well dishes were seeded and incubated the following day with galardin (10 μM) for 16 h. Cells were then lysed, immunoprecipitated for GFP, blotted, and probed for GFP. Note reduction of the EREG 36 kDa membrane remnant form with a concomitant increase in the 55 kDa surface form after galardin treatment.

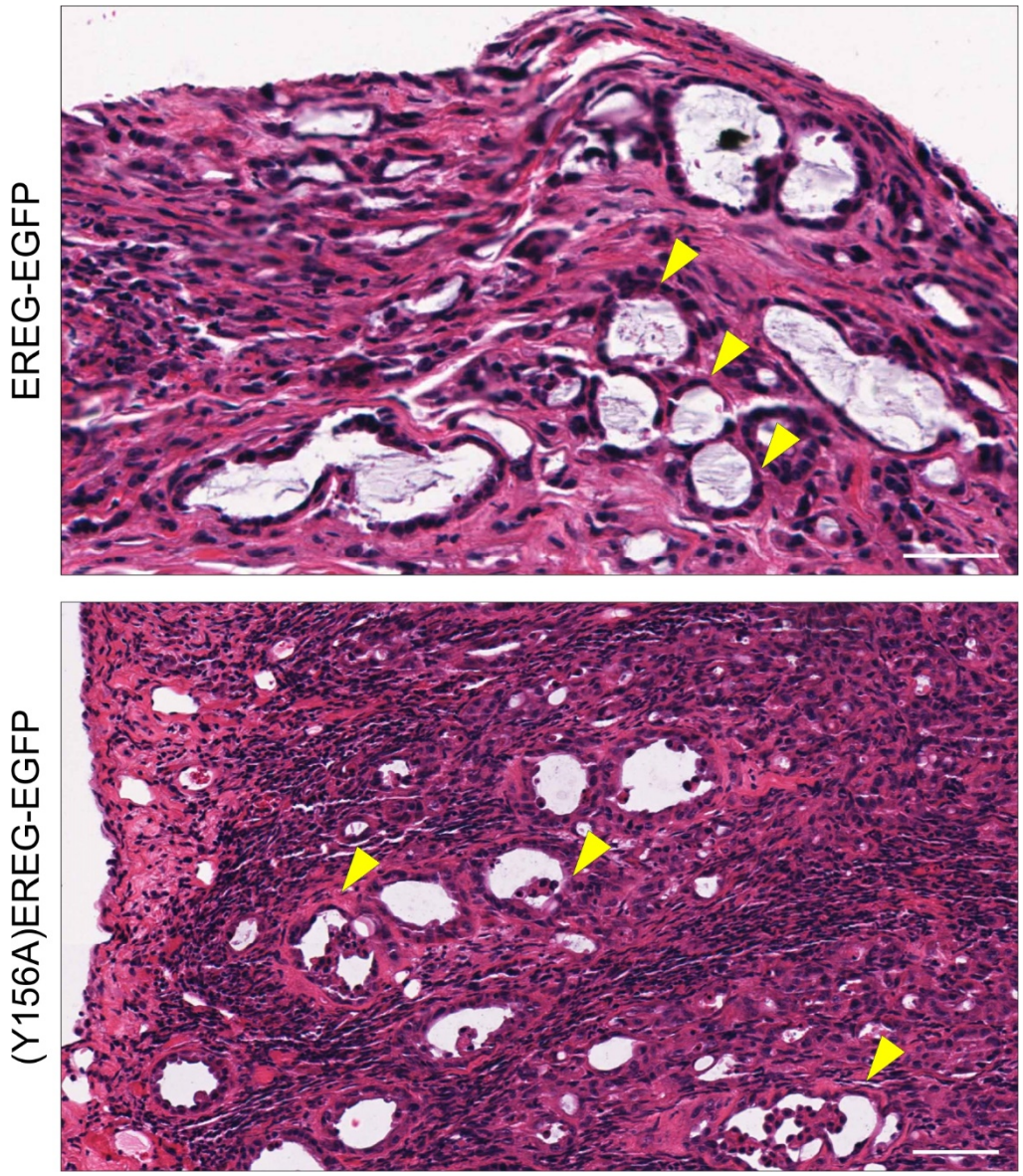


Fig. S2. (Y156A)EREG-EGFP expressing MDCK xenografts exhibit tubular structures with inward growth and multiple lumens. Four month old MDCK subcutaneous xenografts stably expressing EREG-EGFP (top panel) or (Y156A)EREG-EGFP (bottom panel) were formalin-fixed and paraffin-embedded followed by H&E staining. Please note that the tubular cystic structures in EREG-EGFP tumors (yellow arrowheads in top panel) are hollow in comparison to the inward growth and multiple lumens in the cystic structures within (Y156A)EREG-EGFP tumors (yellow arrowheads in bottom panel). Scale bar: 50 μm.

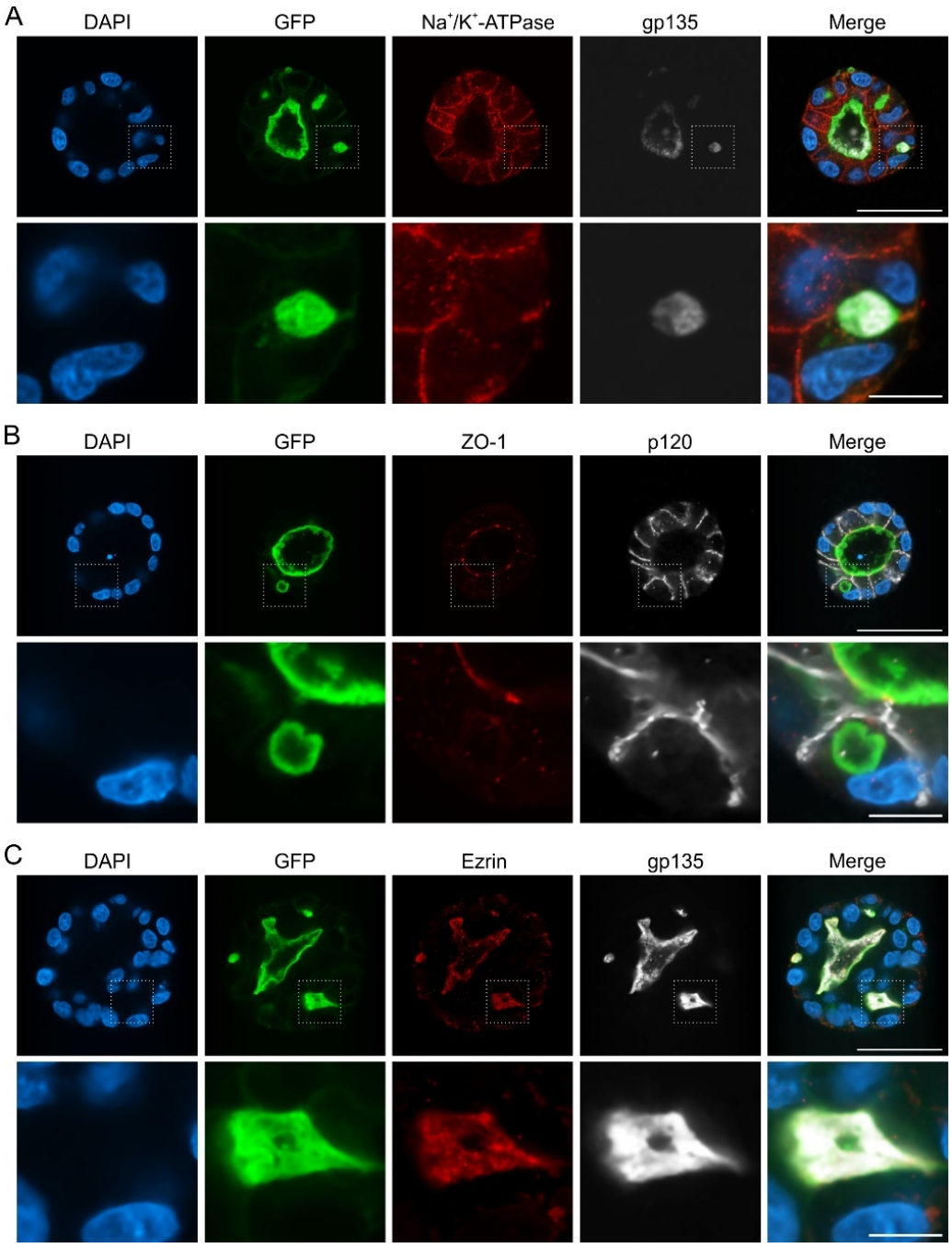


Fig. S3. EREG-induced ectopic lumens retain polarized trafficking of apical and basolateral proteins. (Y156A)EREG-EGFP-expressing MDCK cysts were fixed and stained for **(A)** Na^+/K^+-transporting ATPase α1 subunit (red) and gp135 (white), **(B)** ZO-1 (red) and p120 catenin (white), and **(C)** ezrin (red) and gp135 (white). Images from cyst equatorial planes are displayed on top and highlighted lumenal regions are displayed underneath at higher magnification. Scale bars: top panels, 50 µm; magnified lower panels, 10 µm.

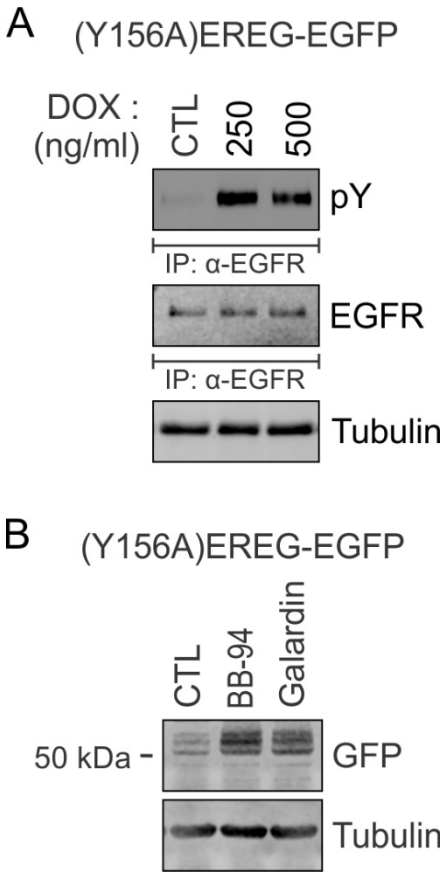


Fig. S4. Induction of (Y156A)EREG-EGFP expression in polarized MDCK cells leads to EGFR phosphorylation and (Y156A)EREG-EGFP cleavage is inhibited by addition of metalloprotease inhibitors (A) *EGFR phosphorylation by (Y156A)EREG-EGFP induction*: MDCK cells expressing an doxycycline-inducible (Y156A)EREG-EGFP were seeded on Transwell filters and allowed to polarize (~5 day in culture). Indicated concentrations of doxycycline (DOX) were added to induce EREG expression; untreated cells (lane 1) served as control (CTL). Cells were lysed 24 hr after induction and were subjected to immunoblot analysis as indicated. Induction of mutant apical EREG expression led to increased EGFR phosphorylation. **(B)** *Inhibition of (Y156A)EREG-EGFP cleavage by addition of metalloprotease inhibitors*: MDCK cells expressing an doxycycline-inducible (Y156A)EREG-EGFP were seeded on Transwell filters and allowed to polarize (~5 day in culture). Doxycycline (DOX, 1 mg/ml) was added to all wells along with either BB-94 (10 μM, lane 2) and galardin (10 μM, lane 3); doxycycline alone served as control (CTL). After 24 hr, cells were lysed and subjected to immunoblot analysis as indicated. Addition of metalloprotease inhibitors leads to increased full-length EREG.

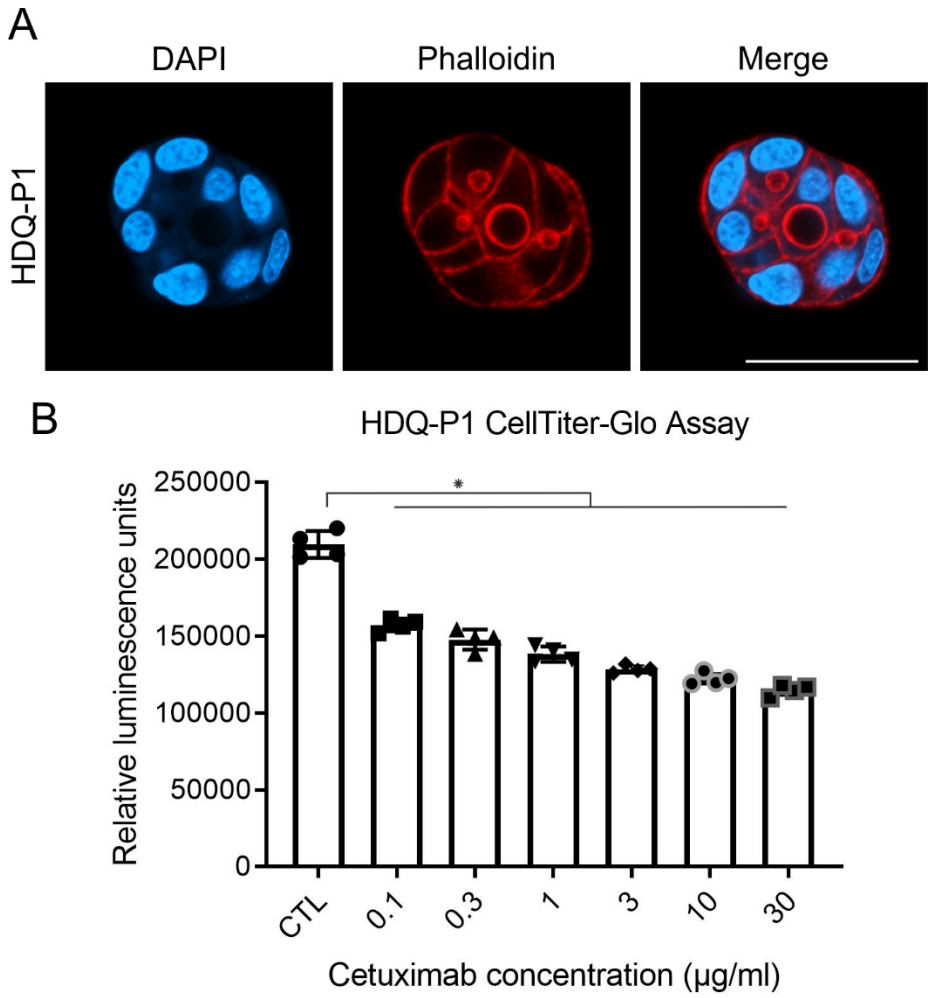


Fig. S5. Human breast cancer line, HDQ-P1, which harbors the EREG R147stop mutation, is sensitive to EGFR inhibition and forms ectopic lumens in 3D Matrigel cultures. (A) HDQ-P1 cells were seeded as a single cell suspension in Matrigel. After five days of culture, resulting cysts were fixed and stained for DNA (DAPI, blue) and F-actin (Phalloidin, red). Images from cyst equatorial planes are displayed here. Scale bars: 50 μm. **(B)** HDQ-P1 cells were incubated with indicated concentrations of the EGFR neutralizing antibody, cetuximab, and subjected to CellTiter-Glo assay after three days of drug incubation (mean ± s.d.). * = statistically significant difference, $p<0.05$ (two-tailed unpaired t-test).

Table S1. Average MDCK cyst diameters. Companion table for Fig. 4B with average cyst diameters and standard deviations (sd). CTL = parental MDCK cells; WT = EREG-EGFP; Y156A = (Y156A)EREG-EGFP cultured in the presence (induced) or absence (uninduced) of doxycycline.

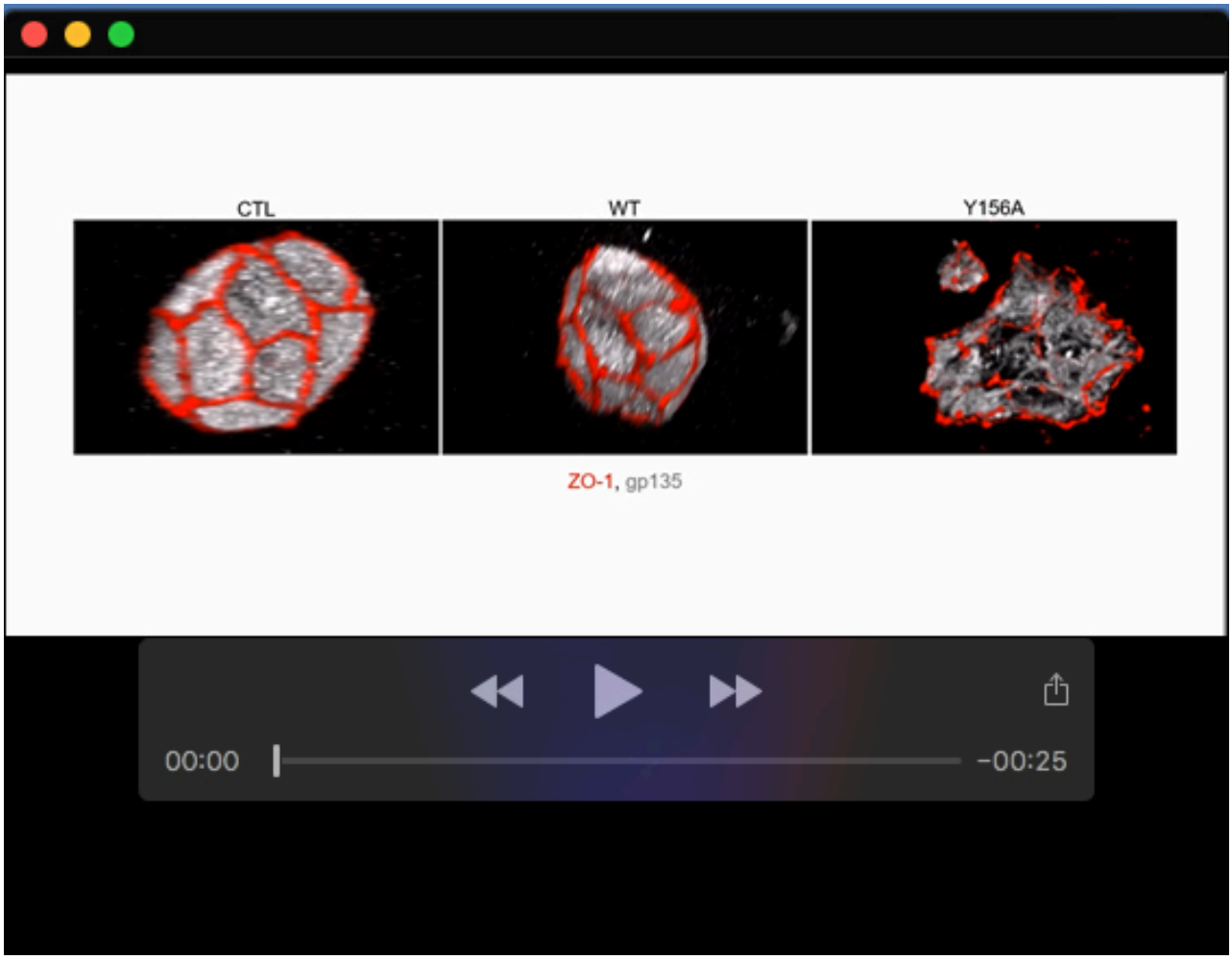
line	dox	mean	sd
CTL	Uninduced	64.49789	23.49156
CTL	Induced	66.87395	24.94377
WT	Uninduced	53.75644	16.88865
WT	Induced	64.20360	20.57492
Y156A	Uninduced	49.57858	20.02745
Y156A	Induced	62.71190	24.41907

Table S2. Ectopic lumen counts in MDCK cysts. Companion table for Fig. 4E with number of cysts containing ectopic lumens per low-magnification (10X) field of view (FOV). CTL = parental MDCK cells; WT = EREG-EGFP; Y156A = (Y156A)EREG-EGFP cultured in the presence of doxycycline. N = number of FOV imaged. EL = ectopic lumens counted per FOV.

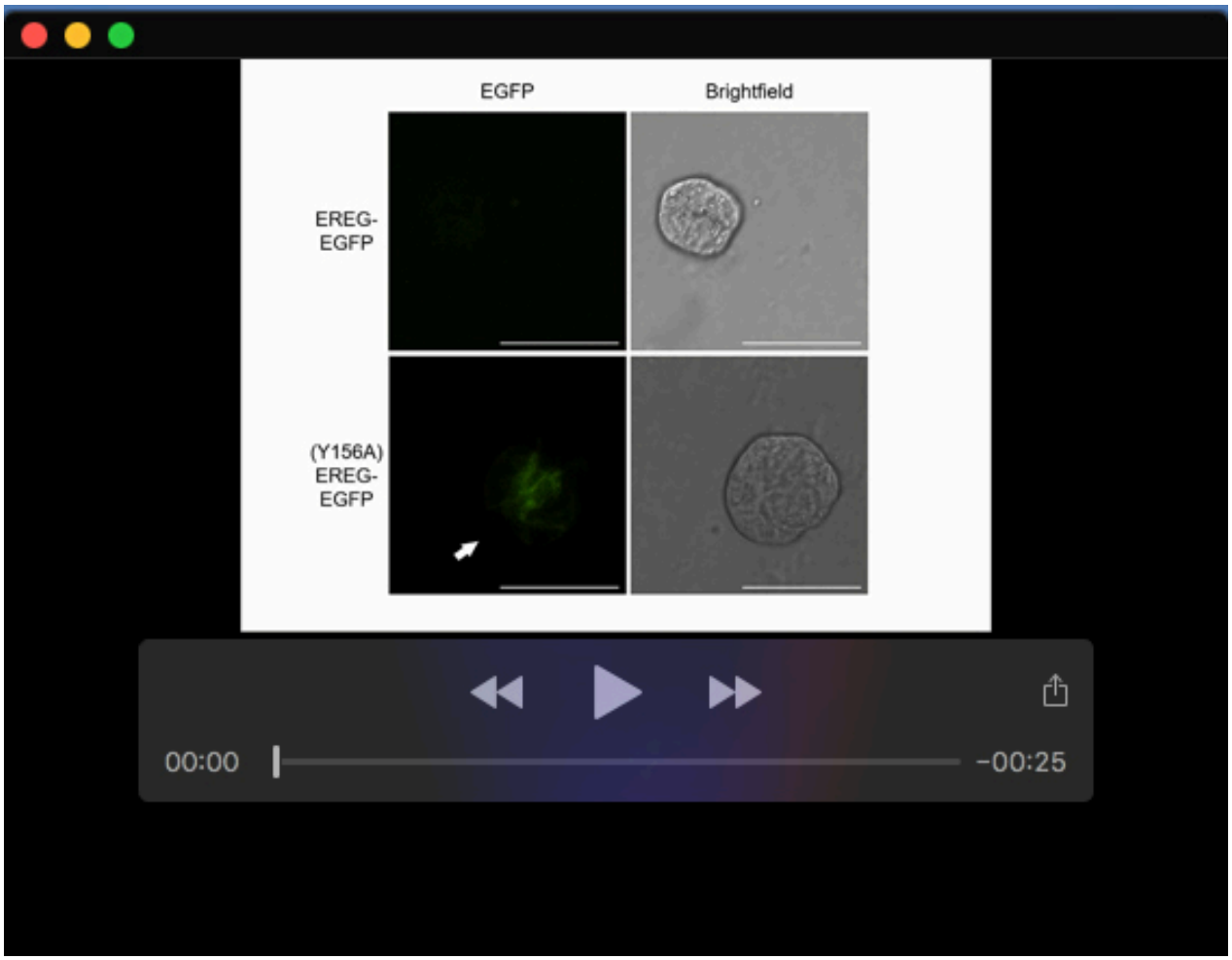
Characteristic	N	CTL, N = 9[1]	WT, N = 9[1]	Y156A, N = 9[1]	p-value[2]
EL	27	1.4 (0.7)	2.2 (1.2)	13.8 (5.8)	<0.001

[1] Statistics presented: mean (SD)

[2] Statistical tests performed: Kruskal-Wallis test



Movie 1. 3D reconstruction of apical surfaces of MDCK cysts. Six-day old MDCK cysts were induced with doxycycline at day 3 were fixed and stained with ZO-1 (red) and gp135 (white) and whole cysts were imaged with confocal microscope. 3D projection was constructed from all the confocal slices and depicted as 360° view. GFP fluorescence associated with EREG is excluded from the reconstructed images to highlight the underlying apical surfaces.



Movie 2. Ectopic lumens form *de novo.* MDCK cysts were induced at day 3 to express EREG-EGFP or (Y156A)EREG-EGFP as indicated and followed over the next 3 days by live cell imaging. Confocal images at the equatorial plane were taken every 40 min for EGFP (green) and Brightfield channels. All images were taken over 3 days were stitched into a single video and displayed here. Scale bars: 50 µm.