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CELLULAR AND MOLECULAR GASTROENTEROLOGY AND HEPATOLOGY

RESEARCH LETTER

Protein Kinase D1 (PKD1) Signaling Induces Growth-Promoting Effects in Murine Enteroids

The mammalian intestine, covered by a single layer of epithelial cells, renews every 4–6 days throughout adult life. This high rate of turnover plays an essential role in the organization, maintenance, function, and restoration of intestinal tissue integrity. The ultimate source of cells are multipotent intestinal stem cells (ISCs) that reside in the lower regions of intestinal crypts.[1] Despite major advances in the identification of rapidly dividing ISCs and their regulation through growth factors and developmental cues from the microenvironment,[1–4] the intracellular signal transduction mechanisms remain incompletely understood.

Protein kinase D1 (PKD1), an evolutionarily conserved protein kinase family, has emerged as a key node in cellular signaling.[5,6] By using transgenic mice that overexpress PKD1 in the intestine (PKD1-Tg), we showed that PKD1 induces crypt cell hyperproliferation and leads to a change in tissue architecture, manifested by an increase in the size and total number of epithelial cells in intestinal crypts.[7,8] These findings prompted us to hypothesize that PKD1 controls the number and function of ISCs, a proposition that remained untested. Here, we determined the impact of PKD1 activity on the function of ISCs by assessing the capacity of isolated intestinal crypts to form 3-dimensional enteroids.[2,3]

We isolated crypts from PKD1-Tg and non-Tg littermates and plated them in Matrigel (Corning, Tewksbury, MA) containing R-spondin, noggin, and epidermal growth factor.[3] Initially, we verified that PKD1 expression and activity were markedly more pronounced in lysates of PKD1-Tg enteroids, as shown by Western blotting with antibodies that detect total PKD1 and catalytically active PKD1[5,6] phosphorylated at the activation loop $Ser^{744/748}$ or at the C-terminal autophosphorylation site Ser^{916} (Figure 1*A*). Enteroids generated from PKD1-Tg mice show a significant increase in area compared with enteroids generated from crypts isolated from non-Tg mice (Figure 1*B* and Supplementary Figure 1). After 7 days, the area of enteroids formed from PKD1-Tg mice was $58 \pm 2.3 \times 10^3$ μm^2 compared with $42 \pm 3.5 \times 10^3$ μm^2 in enteroids from non-Tg mice (Figure 1*C*). Similarly, the number of crypt-like buds, which contain the stem cell compartment,[3] was 8.1 ± 0.49 in enteroids from PKD1-Tg and 5.5 ± 0.31 in those from non-Tg mice (Figure 1*D*). Importantly, we obtained similar results with enteroids passaged 4 times in culture (Figure 1*B–D*), or with enteroids prepared from primary crypts from PKD1-Tg and non-Tg mice (Figure 1*E–G*). Given that the impact of PKD1 overexpression on enteroid morphology persists after serial subcultures, it is likely to be intrinsic to the epithelial cells.

Because PKD1 overexpression increases enteroid size and complexity, we next determined whether acute PKD inhibition produces the opposite effect. The structurally unrelated PKD family inhibitors CRT0066101 (Supplementary Figure 2) and kb NB 142-70 (Supplementary Figure 3) markedly inhibited the increase in enteroid area and budding of crypt-like structures in enteroids derived from either PKD1-Tg or non-Tg mice in a dose-dependent manner.

Next, we investigated the effect of PKD1 activity on the proliferation of enteroid cells, as scored by 5-ethynyl-2′-deoxyuridine incorporation. Consistent with crypt hyperproliferation in vivo,[7,8] enteroids grown from crypts isolated from PKD1-Tg mice showed a 54% and 50% increase in the proportion of cells in DNA synthesis on days 3 and 6 of culture, respectively (Figure 2*A* and *B*). Conversely, PKD inhibition with CRT0066101 blunted DNA synthesis (Figure 2*A* and *B*) in a reversible manner (Supplementary Figure 4). Thus, the increase in cell proliferation likely explains the increase in size of enteroids derived from PKD1-Tg mice.

We hypothesized that PKD1 hyperactivity increases the number and function of ISCs in the crypt-like structures, thereby enhancing enteroid size and complexity. Immunofluorescence analysis for olfactomedin 4, a marker of rapidly dividing ISCs,[9,10] showed an increase in ISCs in the crypt-like domains of enteroids generated from PKD1-Tg mice, as compared with those derived from non-Tg mice (Figure 2*C* and Supplementary Figure 1). Paneth cells, interspersed among ISCs in the crypts of the small intestine, produce and secrete growth-promoting factors that contribute to form a microenvironment propitious for ISC proliferation.[4] Therefore, PKD1 could increase the number of ISCs in the crypt-like domains of enteroids through an increase in Paneth abundance. Against this possibility, our results show that PKD1 overexpression lead to a reduced quantity of Paneth cells in the crypt-like domains of enteroids (Figure 2*D* and Supplementary Figure 1). We verified a similar increase in olfactomedin 4–positive cells and a decrease in Paneth cells in primary crypts isolated from PKD1-Tg mice (Figure 2*E* and *F*), indicating that the increase in ISCs and the decrease in Paneth cell numbers found in enteroids is not an artifact of the culture conditions. Collectively, our results show that PKD1-mediated signaling increases enteroid area and complexity and modulates the balance of ISCs and Paneth cells. The findings indicate that PKD1 contributes to the regulation of ISC proliferation and differentiation.

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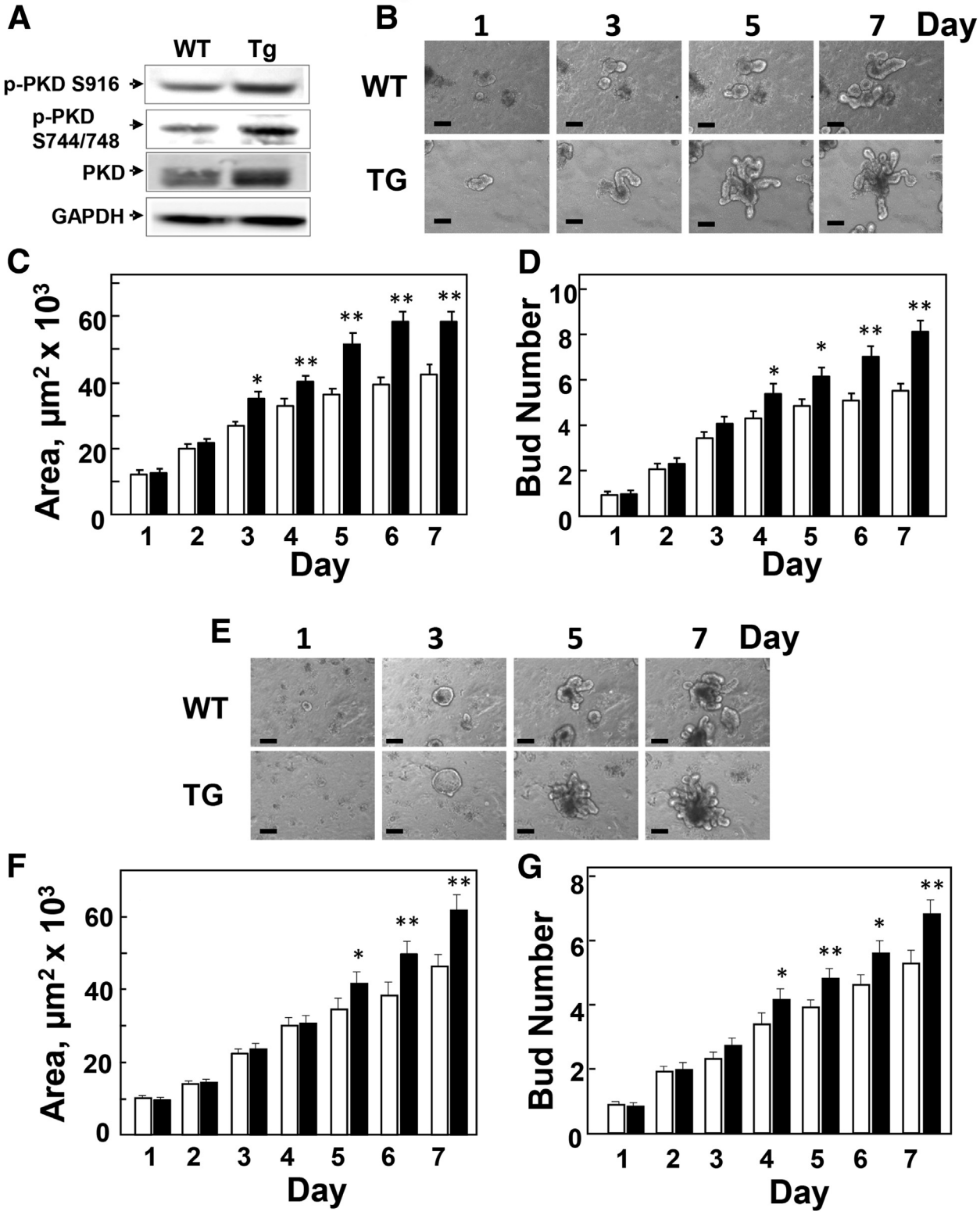

Figure 1. PKD1 overexpression increases the area and complexity of enteroids. (*A*) Western blot analysis of PKD1 expression and activity. (*B–G*) Representative images and quantification of the size and bud number of enteroids derived from wild-type (WT, *open bars*) and PKD1-Tg (TG, *solid bars*) crypts in either (*B–D*) passage 4 enteroids or (*E–G*) primary enteroids. Values are means ± SEM of at least 30 enteroids. Similar results were obtained in (*C* and *D*) 18 and (*F* and *G*) 7 independent experiments. *Scale bars*: 100 μm. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. *$P < .05$; **$P < .01$; TG vs WT.

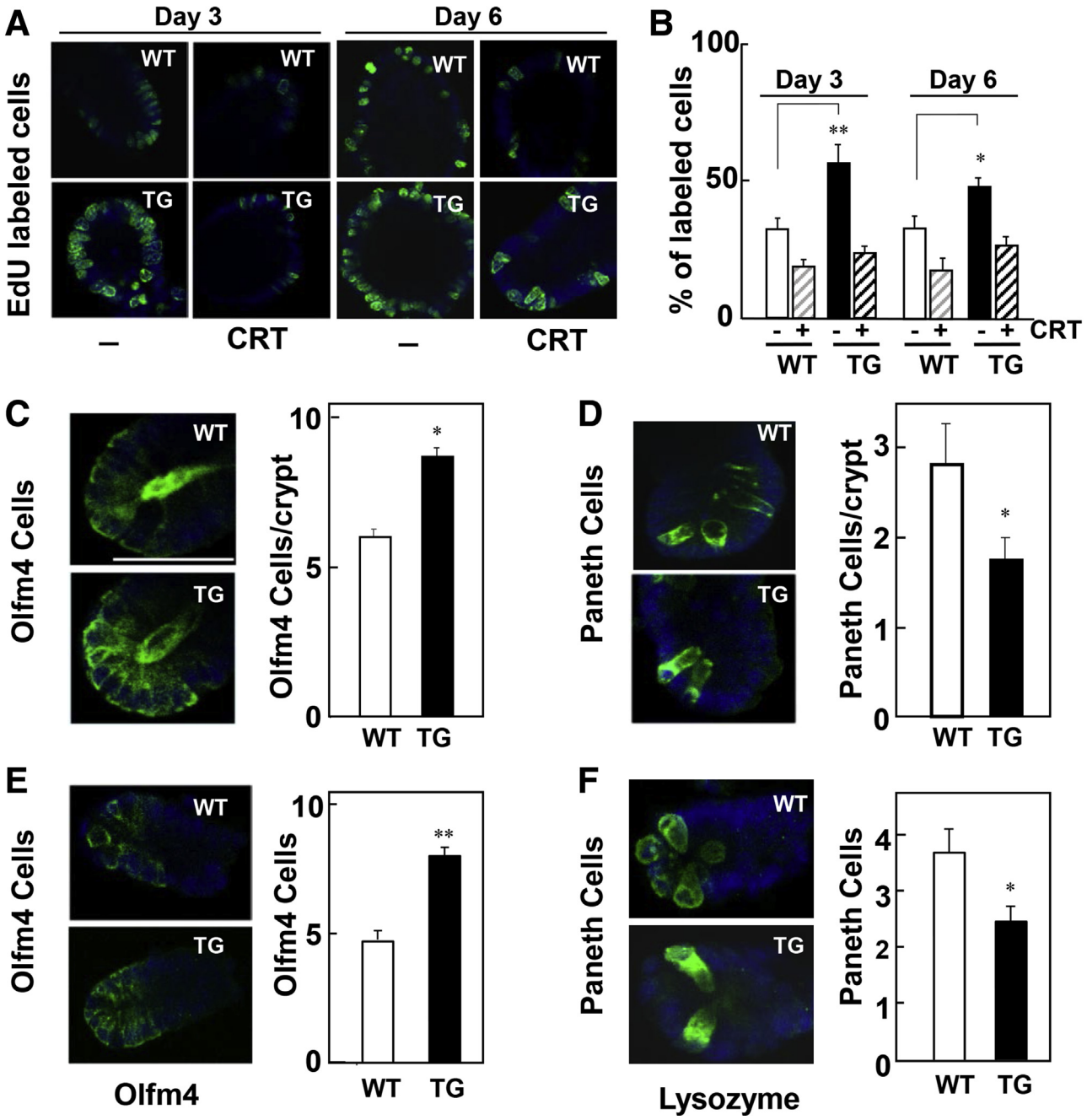

Figure 2. Impact of PKD1 overexpression on cell proliferation and abundance of ISCs and Paneth cells. (*A*) 5-Ethynyl-2′-deoxyuridine (EdU) incorporation in enteroids derived from wild-type (WT, *open bars*) and PKD1-Tg (TG, *solid bars*) crypts in the absence or presence of CRT0066101 (1.0 μmol/L, added 24 h before EdU). (*B*) Values are means ± SEM; n = 10 buds. Similar results were obtained in 3 (day 3) and 4 (day 6) independent experiments. Supplementary Figure 4 shows a different assay of EdU incorporation. (*C–F*) Representative images of (*C* and *E*) olfactomedin 4 (Olfm4) and (*D* and *F*) lysozyme staining from (*C* and *D*) WT (*open bars*) and PKD1-Tg (*solid bars*) mice–derived enteroids at passage 4, or (*E* and *F*) freshly isolated crypts. *Bars* represent means ± SEM; n = 7–8 buds. Similar results were obtained in 4 independent experiments. *$P < .05$; **$P < .01$; TG vs WT.

References

1. Koo B-K, et al. Gastroenterology 2014;147:289–302.
2. Ootani A, et al. Nat Med 2009;15:701–706.
3. Sato T, et al. Nature 2009;459:262–265.
4. Sato T, et al. Nature 2011;469:415–418.
5. Rozengurt E, et al. J Biol Chem 2005;280:13205–13208.
6. Rozengurt E. Physiology 2011;26:23–33.
7. Sinnett-Smith J, et al. J Biol Chem 2011;286:511–520.
8. Wang J, et al. Am J Physiol Cell Physiol 2016;310:C542–C557.
9. van der Flier LG, et al. Gastroenterology 2009;137:15–17.
10. Schuijers J, et al. Stem Cell Rep 2014;3:234–241.

Abbreviations used in this letter: **ISC, intestinal stem cell; PKD1, protein kinase D1; Tg, transgenic**

Most current article

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Conflicts of interest

The authors disclose no conflicts.

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Supplementary Materials and Methods

Crypt Isolation and Enteroid Culture

To assess the effect of PKD1 on enteroid formation, we used Tg mice that express increased PKD1 protein in the intestinal epithelium. The generation of PKD1-Tg mice has been described elsewhere.[1] Tg mice were identified by polymerase chain reaction using genomic tail DNA and specific primers.[1,2] Overexpression of PKD1 in the ileum was verified using epithelial cells isolated sequentially along the crypt–villus axis by timed incubations in EDTA–phosphate-buffered saline solutions.[3] To isolate crypts from PKD1-Tg and non-Tg mice, the distal 6 cm of small intestines (ileum) were harvested and flushed with ice-cold phosphate-buffered saline. The ileums were cut into 2- to 4-mm fragments and washed 5 times with ice-cold phosphate-buffered saline. The fragments then were incubated in 2.5 mmol/L EDTA with gentle shaking at 4°C for 30 minutes.[4] The fragments were transferred to cold phosphate-buffered saline and the crypts were released by manual shaking for 1 minute. The supernatant was collected and the procedure was repeated 6 more times. The fractions containing crypts (usually fractions 3 to 5 of the supernatant) were centrifuged at 100*g* for 2 minutes, resuspended in 1 mL of 10% fetal bovine serum/phosphate-buffered saline. The fractions containing crypts were combined, filtered through 100-μm and 70-μm cell strainers, transferred into a 15-mL tube, and centrifuged at 100*g* for 2 minutes. The pellet was resuspended in 5 mL of Advanced Dulbecco's modified Eagle medium/F12 (Invitrogen, Carlsbad, CA), and centrifuged at 84*g* to remove single cells and tissue debris, this step was repeated 3 times. The resulting crypt-enriched pellet was dissociated and embedded directly in Matrigel (growth factor reduced; Corning, Tewksbury, MA), at a concentration of 500 crypts/50 μL in a 24-well plate. After polymerization (15 min, 37°C), the gels were overlaid with 500 μL of Advanced Dulbecco's modified Eagle medium/F12 containing Glutamax (Invitrogen), HEPES, penicillin-streptomycin, B27, N2 (Invitrogen), and 1 μmol/L N-acetylcysteine [MilliporeSigma, St. Louis, MO]), supplemented with epidermal growth factor (50 ng/mL; R&D), Noggin (100 ng/mL; R&D), and R-spondin (500 ng/mL; R&D, Systems, Inc, Minneapolis, MN). Epidermal growth factor, Noggin, and R-spondin were added every other day and the complete media was changed every 4 to 5 days. The primary enteroids were disassociated mechanically using a p1000 pipette (Gilson, Middleton, WI) and pipetting (with a bended tip) 50–100 times. The cells were centrifuged, resuspended in Matrigel, and plated as described earlier.

Enteroid Measurements

For enteroid size evaluation, 5–8 (4×) and/or 10–12 (10×) nonoverlapping images were acquired using an inverted microscope (Axio Observer.A1; Zeiss, White Plains, NY). Enteroid perimeters for surface area were defined manually using ImageJ software (version 1.52j; National Institutes of Health, Bethesda, MD). The same images were used to count the number of crypt-like buds per enteroid.

5-Ethynyl-2′-Deoxyuridine Incorporation

The rate of cell proliferation was measured using the Click-iT 5-ethynyl-2′-deoxyuridine assay (C10337; Thermo Fisher Scientific), and the enteroid cultures were incubated either on day 3 or day 6 with 10 μmol/L 5-ethynyl-2′-deoxyuridine for 30 minutes. The cultures then were fixed with 3.7% formaldehyde in phosphate-buffered saline for 15 minutes at room temperature. The cultures were washed (2×) with 3% bovine serum albumin in phosphate-buffered saline before permeabilization with 0.5% Triton X-100 (MilliporeSigma) in phosphate-buffered saline for 20 minutes at room temperature, washed with 3% bovine serum albumin in phosphate-buffered saline, and then incubated with a Click-iT reaction cocktail, containing Click-iT reaction buffer, CuSO4, Alexa Fluor 488 Azide, and reaction buffer additive for 30 minutes while protected from light. The cultures were washed once more with phosphate-buffered saline and stained for DNA with 5 ug/mL Hoechst 33342 for 3 minutes. All other details were as described in the manufacturer's protocol.

Immunofluorescence

Enteroid cultures were collected and transferred to a 1.5-mL tube by breaking up the Matrigel with 500 μL of cold phosphate-buffered saline and kept on ice for 5 minutes. The enteroids were centrifuged at 100*g* for 2 minutes and fixed with 4% paraformaldehyde for 30 minutes at room temperature, centrifuged (100*g*, 2 min), washed with NH_4Cl (50 mmol/L), and then permeabilized with 0.1% Triton X-100 in phosphate-buffered saline for 30 minutes at room temperature. The enteroids then were washed (2×) with 1% bovine serum albumin in phosphate-buffered saline and incubated with the either olfactomedin 4 (1:400 dilution, D6Y5A 39141; Cell Signaling Technologies) or lysozyme polyclonal antibody (1:100, PA5-16668; Thermo Fisher Scientific) overnight at 4°C. The enteroids then were washed 2 times with 1 mL phosphate-buffered saline and then incubated with goat anti-rabbit IgG secondary antibody and Alexa Fluor 488 (1:500 dilution, A-11034; Thermo Fisher Scientific) for 2 hours at room temperature, and then washed 2 times with phosphate-buffered saline and stained for DNA with 5 ug/mL Hoechst 33342 for 3 minutes. The samples then were washed 3 times with phosphate-buffered saline and the final pellets were resuspended in Matrigel (10–20 μL) and plated onto a microscope chamber slide (Nunc Lab-Tek II Chamber Slide 12-565-6; Thermo Fisher Scientific), after polymerization the Matrigel was covered with phosphate-buffered saline. Confocal microscopy was performed using a Zeiss LSM 710 confocal microscope with an Achroplan 40/.75W objective (Zeiss).

Western Blot

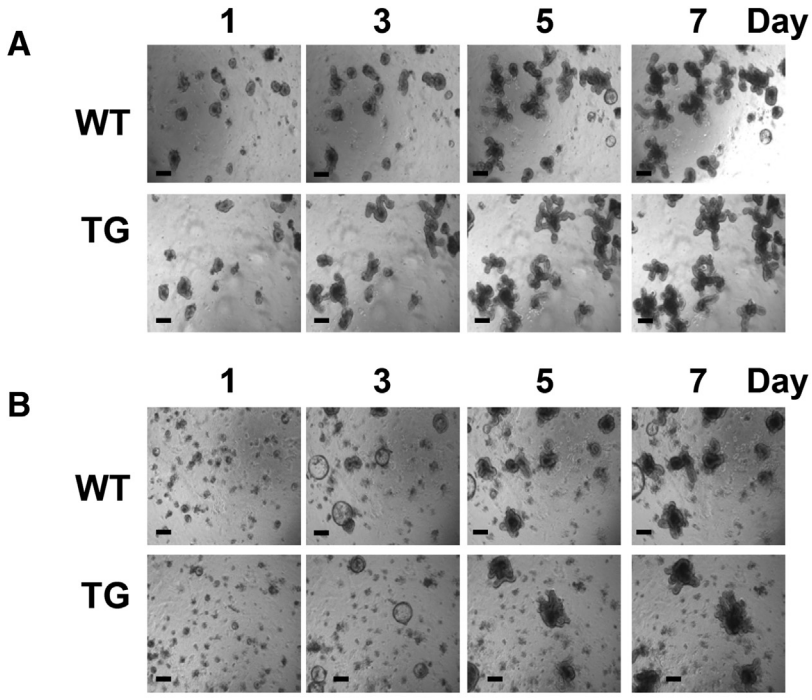
Enteroid cultures were collected and transferred to a 1.5-mL tube by breaking up the Matrigel with 500 μL of cold phosphate-buffered saline and kept on ice for 5 minutes. The enteroids were centrifuged at 100*g* for 2 minutes, the supernatant was removed, and the pellet was lysed with 2× sodium dodecyl sulfate–polyacrylamide gel electrophoresis sample buffer (200 mmol/L Tris-HCl [pH 6.8], 2 mmol/L EDTA, 0.1 mol/L Na_3VO_4, 6% sodium dodecyl sulfate, 10% glycerol, and 4% 2-mercaptoethanol), followed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on 4%–15% gels and transfer to Immobilon-P membranes (Millipore, Billerica, MA). For detection of proteins, membranes were blocked using 5% nonfat dried milk in phosphate-buffered saline, pH 7.2, and then incubated overnight with the following rabbit antibodies from Cell Signaling Technologies: (phospho-PKD-Ser916) antibody (1:1000, 2051), phospho-PKD (Ser744/748) antibody (1:1000, 2054), PKD (D4J1N) (1:1000, 90039), and a mouse monoclonal glyceraldehyde-3-phosphate dehydrogenase antibody (G-9) (1:1000, sc-365062) diluted in phosphate-buffered saline containing 0.1% Tween. Primary antibodies bound to immunoreactive bands were visualized by enhanced chemiluminescence detection with horseradish-peroxidase–conjugated anti-mouse, anti-rabbit antibody and a FUJI LAS-4000 mini luminescent image analyzer (FUJIFILM Corp. Akasaka, Tokyo).

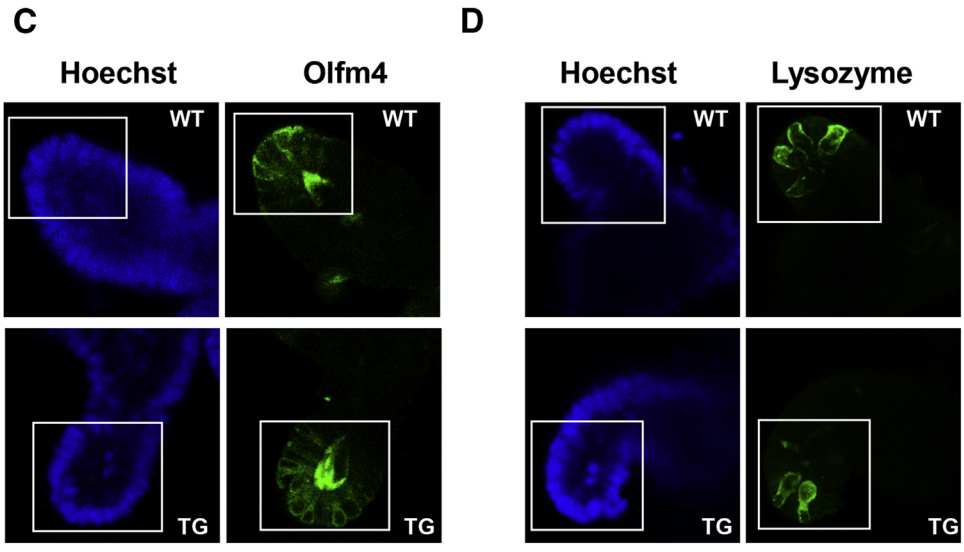
Statistical Analysis

For all studies, results from quantitative experiments were expressed as means ± SEM and were analyzed using SigmaPlot computer software (Systat Software, Inc, San Jose, CA). Where appropriate, significance was calculated by Student *t* test or the Mann–Whitney rank sum test.

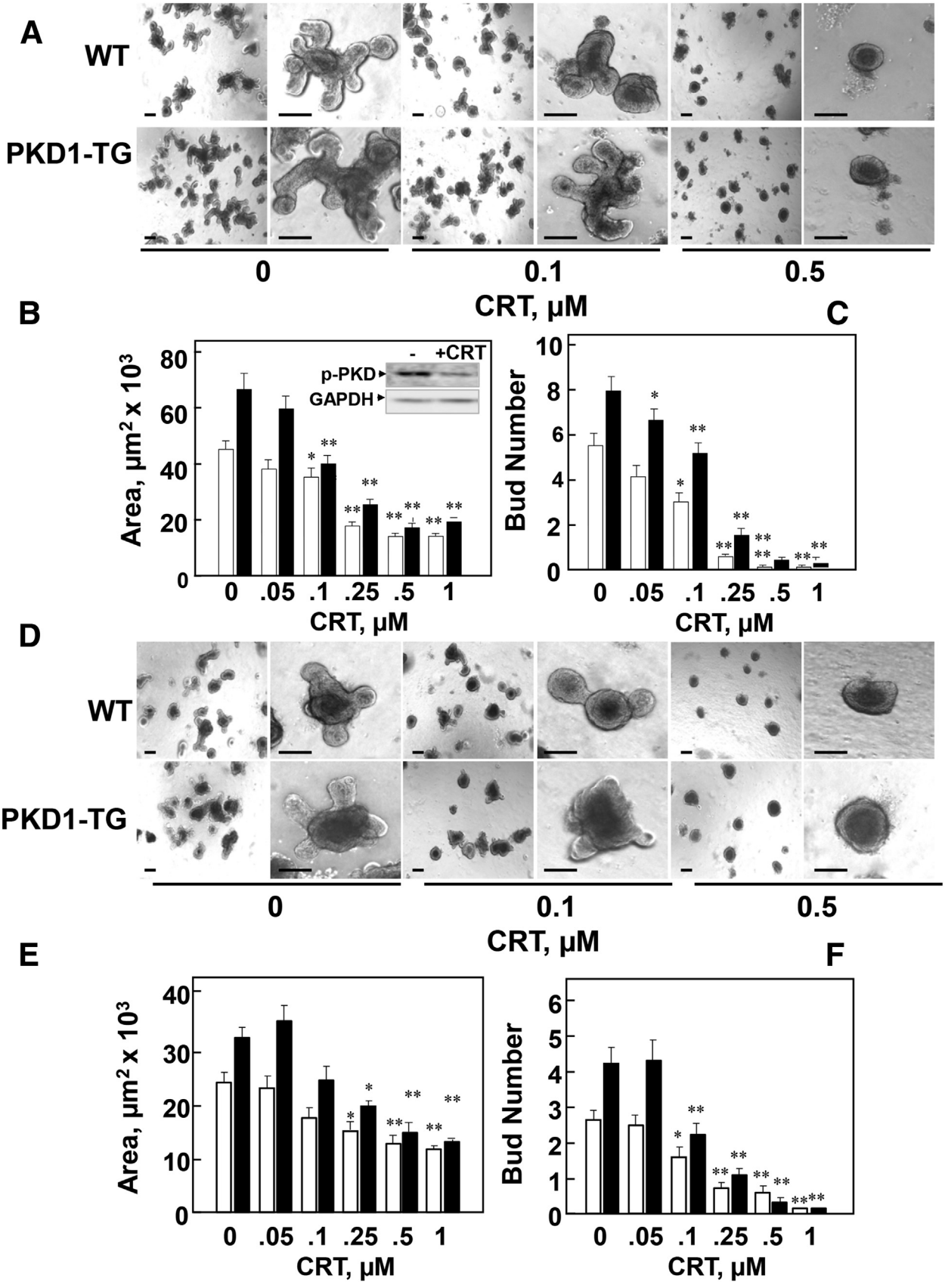
Supplementary References

1. Sinnett-Smith J, et al. J Biol Chem 2011; 286:511–520.
2. Wang J, et al. Am J Physiol Cell Physiol 2016; 310:C542–C557.
3. Young SH, et al. Am J Physiol Gastrointest Liver Physiol 2012;303:G356–G366.
4. Wang B, et al. Cell Stem Cell 2018; 22:206–220.e4.
5. Harikumar KB, et al. Mol Cancer Ther 2010; 9:1136–1146.





Supplementary Figure 1. (*A* and *B*) PKD1 overexpression increases the area and complexity of enteroids. Representative low-magnification images of enteroids derived from the ileum of wild-type (WT) or PKD1 Tg (*A*) at passage 4 or (*B*) primary enteroids. These images complement the high-magnification images of enteroids shown in Figure 1. (*C* and *D*) Impact of PKD1 overexpression on abundance of ISCs and Paneth cells. Additional representative (*C*) olfactomedin 4 (Olfm4) and (*D*) lysozyme staining from WT and PKD1-Tg mice–derived enteroids at passage 4. Hoechst staining is overexposed to show nuclei in the enteroids. *Boxes* represent the portion of enteroids used for quantification and shown in Figure 2. Similar results were obtained in 4 independent experiments.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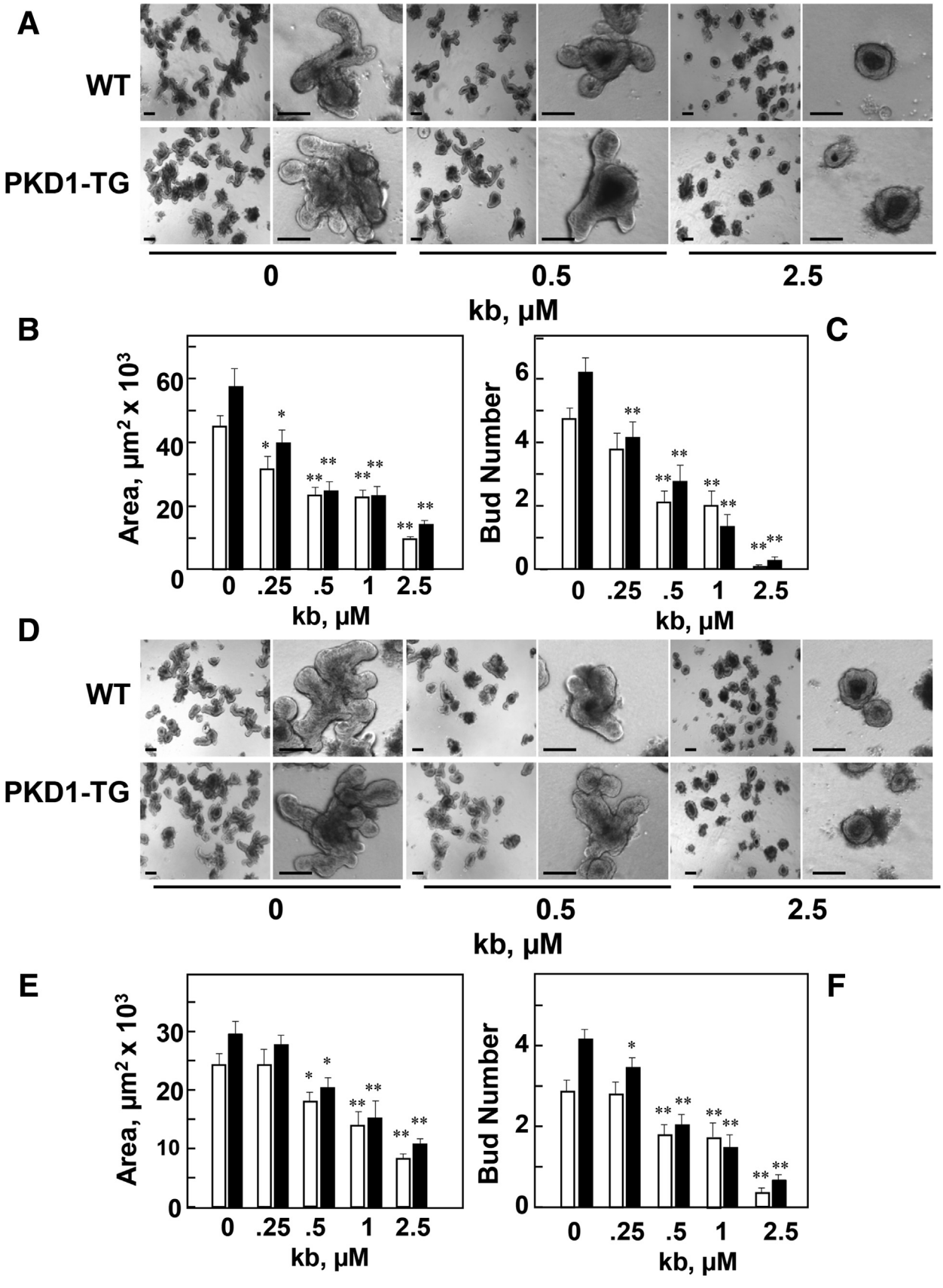
Supplementary Figure 2. (***See previous page***). **The selective PKD family inhibitor CRT0066101 (CRT) reduced the size and the number of crypt-like buds in enteroids from PKD1-Tg and non-Tg mice.** (*A*) Crypts isolated from the ileum of PKD1-Tg and non-Tg mice were cultured in the 3-dimensional system, as described in the Supplementary Materials and Methods section, either in the absence or presence of increasing concentrations of the selective PKD family inhibitor CRT0066101.[5] For enteroid area measurements, 5–8 (4×) or 10–12 (10×) nonoverlapping images were acquired using an inverted microscope (Axio Observer.A1; Zeiss). Enteroid surface areas were defined manually by outlining the perimeter with the polygon function in ImageJ software (National Institutes of Health). The same images were used to count the number of buds per enteroid. Note that addition of CRT0066101 strikingly decreased the area of enteroids grown for 6 days in a dose-dependent manner (typical images at low and high magnification are shown in panel *A*, quantification is shown in panel *B*). At a concentration as low as 0.1 μmol/L, CRT0066101 significantly reduced the area of enteroids isolated from PKD1-Tg and non-Tg littermates and abolished the increase in area shown by enteroids of PKD-Tg. The addition of CRT0066101 also reduced the number of crypt-like buds from the enteroids (images are shown in panel *A*, quantification is shown in panel *C*). Similar effects of CRT0066101 on the area and number of crypt-like buds were obtained with enteroids treated for 3 days with CRT0066101 (typical images at low and high magnification are shown in panel *D*, quantification is shown in panels *E* and *F*) instead of 6 days in culture. Statistical analysis was as described in the Materials and Methods section. $^{*}P < .05$, $^{**}P < .01$, as compared with untreated control. The bars represent the means ± SEM; (*B* and *C*) n = 50 and (*E* and *F*) n = 30. Similar results were obtained in 3 independent experiments. *Scale bars*: 100 μm. (B) *Insert*: Western blot of PKD1 activity. Enteroids were treated on day 2 of culture without or with 1 μmol/L CRT0066101 for 24 hours, the cultures then were lysed and analyzed by Western blot with phospho-PKD Ser^{916} and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Note that CRT0066101 markedly inhibited PKD activity (scored by autophosphorylation on Ser^{916}) when added to the growing enteroids. Similar results were obtained in 5 independent experiments. p-PKD, phospho-Protein Kinase D; WT, wild-type.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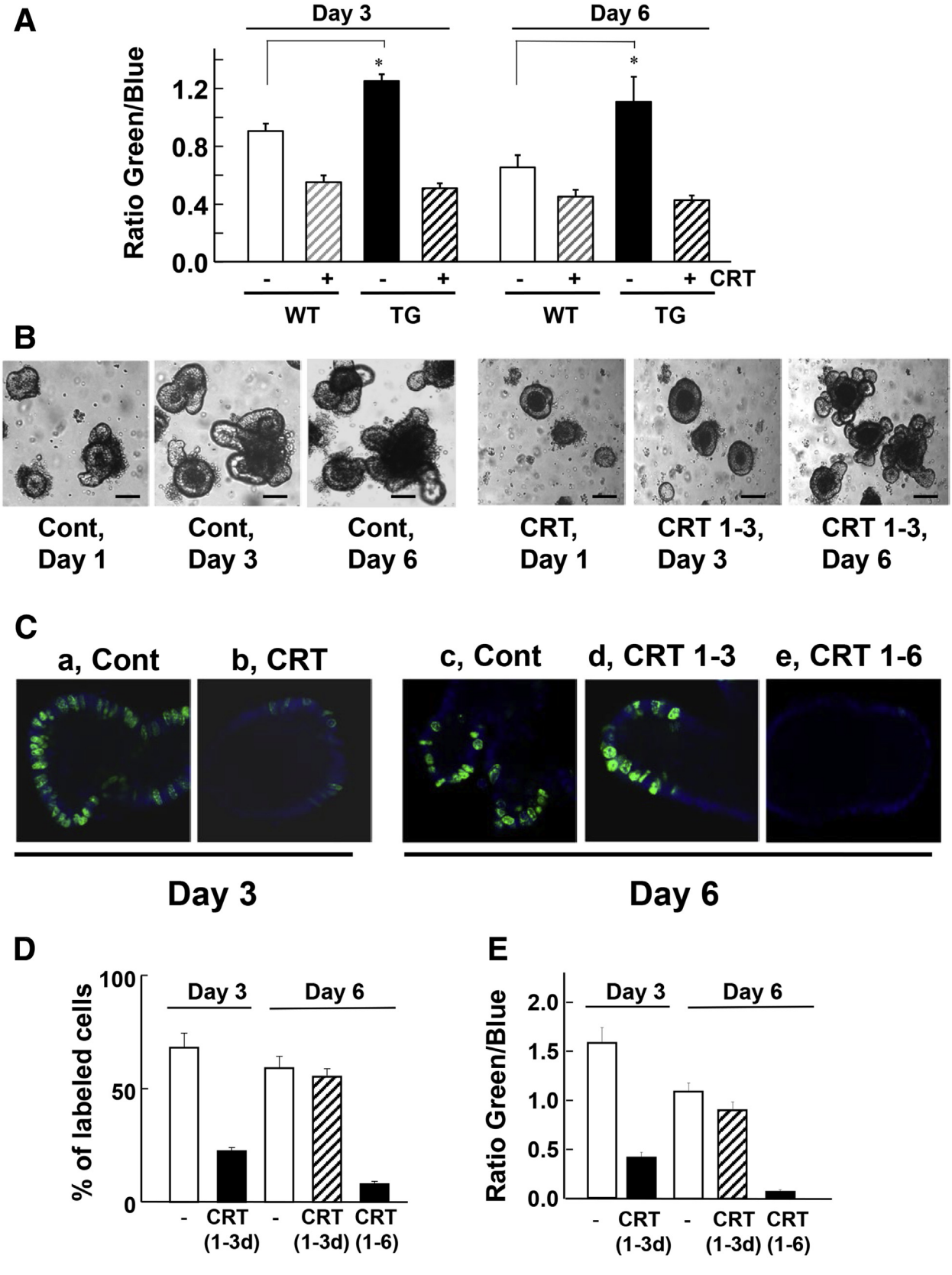
Supplementary Figure 3. (***See previous page***). **Impact of kb NB 142-70, a PKD family inhibitor structurally unrelated to CRT0066101, on enteroid size and complexity.** Crypts isolated from PKD1-Tg and non-Tg mice were cultured in the 3-dimensional system in the absence or presence of increasing concentrations of kb NB 142-70, as described in the Materials and Methods section. Measurements of the enteroid area and number of buds were performed as described in the Materials and Methods section and in Supplementary Figure 2. The addition of kb NB 142-70 markedly decreased the area of enteroids grown for 6 days in a dose-dependent manner (typical images at low and high magnification are shown in panel *A*, quantification is shown in panel *B*). (*C*) The addition of kb NB 142-70 also reduced the number of crypt-like buds from the enteroids. Similar effects of kb NB 142-70 on the area and number of crypt-like buds were obtained with enteroids treated for 3 days (typical images at low and high magnification are shown in panel *D*, quantification is shown in panels *E* and *F*) instead of 6 days in culture. $^{*}P < .05$, $^{**}P < .01$, as compared with untreated control. *Bars* represent the means $\pm$ SEM; n $=$ 40. *Scale bars*: 100 μm. Similar results were obtained in 3 independent experiments. WT, wild-type.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Supplementary Figure 4. (***See previous page***). **The inhibitory effect of CRT0066101 on DNA synthesis in enteroids is reversible.** (*A*) Fluorescent ratios of green (5-ethynyl-2′-deoxyuridine)/blue (Hoechst 33342) were determined with Photoshop (Adobe, San Jose, CA) using the histogram function to determine the mean fluorescent values of green and blue from confocal images. All images were captured with identical exposures in each experiment. The values shown represent the mean ratio ± SEM; n = 10 buds. Similar results were obtained in 4 independent experiments. **P* < .05; ***P* < .01, PKD1-TG (TG, *solid bars*) vs Wild Type (WT, *open bars*). (*B*) Typical images of enteroids from PKD1-Tg mice grown for 1, 3, or 6 days in the absence (control [cont] or presence of 1 μmol/L CRT0066101 for 1 or 3 days (CRT day 1 and CRT day 3, respectively). Parallel enteroids exposed to 1 μmol/L CRT0066101 for 3 days were washed and transferred to medium without the inhibitor for 3 additional days (CRT days 1–3, day 6). Similar results were obtained in 4 independent experiments. *Scale bars*: 100 μm. (*C*) In line with the results shown, addition of CRT0066101 (1 μmol/L) markedly inhibited the incorporation of EdU into epithelial cells of the crypt-like domain of enteroids derived from PKD1-Tg mice (compare panels *B* and *A* at day 3). After 3 days of exposure to CRT0066101, the media of parallel cultures of enteroids were replaced with fresh media supplemented with or without 1 μmol/L CRT0066101. Enteroids continuously exposed to CRT0066101 for 6 days show a striking inhibition of EdU incorporation (compare panels *E* and *C* at day 6). In contrast, enteroids exposed to CRT0066101 for 3 days followed by 3 days without the inhibitor showed a striking recovery of DNA synthesis (compare panels *D* and *C* at day 6). (*D*) Percentage of EdU-labeled cells shown in panel *C*. Values are means ± SEM; n = 10 buds. Similar results were obtained in 3 independent experiments. (*E*) Ratios of green/blue fluorescence. Values are means ± SEM; n = 10 buds. Similar results were obtained in 3 independent experiments. Collectively, the results indicate that CRT0066101 reversibly arrests DNA synthesis in enteroids of PKD1-Tg mice.