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# Suppression of Rac1 activity at the apical membrane of MDCK cells is essential for cyst structure maintenance

*Shunsuke Yagi*[1], *Michiyuki Matsuda*[1,2] & *Etsuko Kiyokawa*[2†+]

[1]Laboratory of Bioimaging and Cell Signaling, Graduate School of Biostudies, and [2]Department of Pathology and Biology of Diseases, Graduate School of Medicine, Kyoto University, Yoshida Konoe-cho, Kyoto, Japan

**Using MDCK cells that constitutively express a Förster resonance energy transfer biosensor, we found that Rac1 activity is homogenous at the entire plasma membrane in early stages of cystogenesis, whereas in later stages Rac1 activity is higher at the lateral membrane than at the apical plasma membrane. If Rac1 is activated at the apical membrane in later stages, however, the monolayer cells move into the luminal space. In these cells, tight junctions are disrupted, accompanied by mislocalization of polarization markers and disorientation of cell division. These observations indicate that Rac1 suppression at the apical membrane is essential for the maintenance of cyst structure.**

Keywords: cell division axis; FRET; polarity; Rho family GTPases; tight junction

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## INTRODUCTION

Rac1, a member of the Rho-family GTPases, has a pivotal role in cell–cell adherence. For example, in Madin–Darby canine kidney (MDCK) cells cultured on a two-dimensional sheet, Rac1-mediated signalling was shown to be involved in the initiation of tight or adherence junctions [1–3], and Rac1 has been shown to be activated upon the establishment of cadherin-based cell junctions [4]. In the higher-order structure of MDCK cells cultured in gel, Rac1 mediated laminin accumulation on the basal surface of MDCK cysts to orientate apico-basal polarity [5]. These conclusions were mainly reached based on the exogenous expression of mutant proteins, which are either constitutively active or dominant negative with respect to the endogenous protein. A recent study raised the caveat that these overexpressed proteins might destabilize other Rho GTPases by competing with RhoGDI [6].

[1]Laboratory of Bioimaging and Cell Signaling, Graduate School of Biostudies, and [2]Department of Pathology and Biology of Diseases, Graduate School of Medicine, Kyoto University, Yoshida Konoe-cho, Kyoto 606-8501, Japan
[†]Present address: Department of Oncologic Pathology, Kanazawa Medical University, 1-1 Daigaku, Ishikawa 920-0293, Japan
[+]Corresponding author. Tel: +81 76 286 8116; Fax: +81 76 286 6926;
E-mail: kiyokawa@kanazawa-med.ac.jp

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Studies of clinical materials have implicated Rac1 in carcinogenesis. For example, Rac1b, a splice variant of Rac1, showed the biochemical properties of a constitutively active GTPase [7], and was preferentially expressed in patients with colon or breast cancer [8,9]. Although these findings suggested that active Rac1 might correlate with at least some epithelial malignancies, the activation pattern of intrinsic Rac1 in epithelial organs, or the aberrant mechanisms of Rac1 activation driving the morphological malignancy, has not been fully investigated.

Here, we used a Förster resonance energy transfer (FRET)-based live imaging system to generate a Rac1 activity map during cystogenesis of MDCK cells. In addition, a rapamycin-inducible oligomer system allowed the induction of endogenous Rac1 activation at a precise period of interest, and we report the consequences of such activation during cystogenesis.

## RESULTS AND DISCUSSION

To investigate the spatio-temporal activation pattern of Rac1 during epithelial structure formation, we established stable cell lines expressing FRET biosensors for Rho-family GTPases, Raichu-Rac1, Raichu-RhoA and Raichu-Cdc42 (Fig 1A; supplementary Fig S1A online). Establishing cell lines with constitutively expressed FRET biosensors has historically been complicated by intra-genomic recombination of the CFP-derived sequence with the yellow fluorescent protein-derived sequence of the Raichu probe. To overcome this problem, CFP from the conventional Raichu probe was replaced with TFP1, which shows a spectral sensitivity similar to that of CFP. As TFP1 is derived from *Clavularia* coral, and its nucleotide sequence shows little homology to green fluorescent protein (GFP; [10]), we expected no intra-genomic recombination of the Raichu biosensor containing TFP1 [11]. The lack of recombination was validated, and these newly developed Raichu biosensors were stably expressed in MDCK cells after retrovirus-mediated gene transfer. The resulting cell lines were designated as MDCK-Raichu-Rac1, MDCK-Raichu-RhoA and MDCK-Raichu-Cdc42. The subcellular activity maps were identical to those previously reported for cells with transient expression of the prototype Raichu biosensors (supplementary Fig S1A online; [12,13]).

MDCK cells expressing the biosensors were cultured in Matrigel to form cysts, spherical monolayers of the cells that

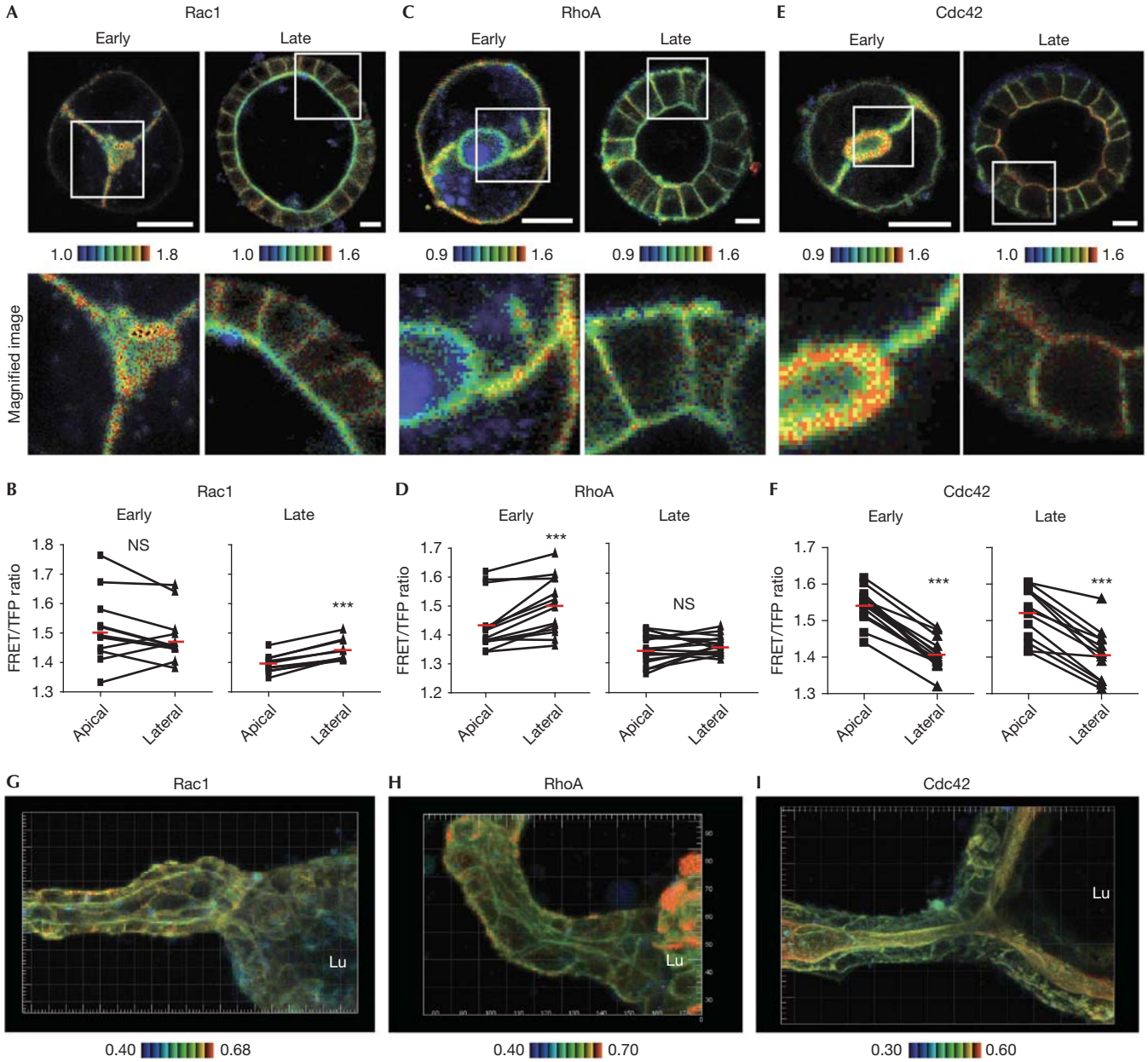


**Fig 1** | Activity map of Rho-family GTPases in cystogenesis. (**A,C,E**) Various Madin–Darby canine kidney (MDCK)-Raichu cells were embedded in Matrigel for 2 days (early) and over 8 days (late), observed by confocal microscopy, and images were processed as described in supplementary information online. The calculated Förster resonance energy transfer (FRET) efficiency is differentiated by colour in intensity-modulated display (IMD) modes shown at the bottom. The upper and lower limits of the ratio range are also shown at the bottom of the panel. Scale bar, 10 μm. Enlarged images from white squares in the top panels are shown at the bottom. (**B,D,F**) Quantitative measurements of FRET/TFP ratios in the apical and lateral membranes of cysts. FRET/TFP ratios of the apical and lateral membrane of MDCK-Raichu cysts were analysed as described in supplementary methods online. Red bars indicate the average. ***$P<0.001$ by two-tailed paired *t*-test against the apical membrane. (**G–I**) MDCK-Raichu cysts were treated with hepatocyte growth factor and observed under a confocal microscope. Images were obtained by serial sectioning along the *z* axis and stacked into a single plane, and processed to FRET/TFP ratio images. Lu indicates lumen position in cysts. NS, not significant; TFP, teal fluorescent protein.

enclose a central lumen, and were observed under a scanning confocal microscope (supplementary Fig S1B–E online). As shown in Fig 1A, the polarity of Rac1 activity was not obvious in the early stages; however, in the late stages of cystogenesis, Rac1 activity at the lateral membrane became higher than that at the apical membrane (Fig 1A,B). In contrast, RhoA activity was higher at the lateral membrane than at the apical membrane in the early stages (Fig 1C,D), but this polarized distribution of RhoA activity disappeared in the late stages. Cdc42 showed significantly higher activity at the apical membrane than at the lateral plasma

membrane throughout cystogenesis (Fig 1E,F). In migrating cells, it has been reported that Rac1 and Cdc42 are both activated at the leading edge [12], and show a positive feedback cycle in epidermal growth factor-stimulated cells [14]. These discrepancies might be the result of cell-type-specific features or microenvironment-dependent mechanisms. Epithelial organs, such as mammary gland, submandibular gland, kidney and pancreas, contain cysts and tubules, which are both lumen-enclosing structures, but tubules are cylindrical instead of spherical [15]. It has been established that MDCK cysts are stimulated to form branching tubules on treatment with hepatocyte growth factor. We visualized the spatial distributions of Rac1, RhoA and Cdc42 activities in such tubules and found that they were similar to those in cysts (Fig 1G–I), supporting previous findings that cysts and tubules are topologically equivalent [15].

In this study, we focused on the role of polarized Rac1 activity in the maintenance of cyst structure. For this purpose, we established a rapamycin-inducible membrane translocation system of guanine nucleotide exchange factor (GEF) and GTPase-activating protein in MDCK cells, which is based on a previously characterized method [16,17]. In this system, rapamycin induces the hetero-dimerization of FK506-binding protein (FKBP)-fused proteins and FKBP12-rapamycin-binding domain (FRB)-fused proteins. In cells expressing Lyn-FRB, an FRB protein anchored at the plasma membrane, the FKBP-fused protein translocates from the cytosol to the plasma membrane upon rapamycin (dimerizer) treatment (Fig 2A). We first used a Venus (a yellow fluorescent protein derivative)-tagged FKBP protein for the validation of this system (Fig 2B). On dimerizer treatment, cytosolic Venus-FKBP migrated to the plasma membrane within 1 min, as expected. Importantly, dimerizer treatment led to an even distribution of Venus-FKBP protein at both the apical and basolateral plasma membranes. Additional control experiments tested for any effects of dimerizer and the expression of FRB and FKBP on establishing cellular polarity. For this, parent MDCK cells were transduced with apical and basolateral markers, GPI-mCherry and GFP-syntaxin4, respectively, and grown in Matrigel in the presence of dimerizer for 8 days. Both markers were localized at the expected subcellular regions, without any sign of abnormal morphology (supplementary Fig S2 online), excluding the effect of dimerizer (that is, perturbation of mTOR signalling) on cell polarity.

We next prepared MDCK cell lines expressing FKBP-Tiam1 and FKBP-β2-chimaerin, which contain GEF and GTPase-activating protein domains specific for Rac1, respectively, for the regulation of the Rac1 activity in a dimerizer-dependent manner. We confirmed that Rac1 activity was rapidly increased or decreased upon the application of dimerizer in cells plated on dishes (Fig 2C,D). Further, FKBP-Tiam1- or FKBP-β2-chimaerin-expressing cells were transduced with the polarity markers and grown for 11 days in Matrigel in the absence of dimerizer (Fig 2E,F, upper panels). Again, these cells formed cysts comparable in size, morphology and polarity to the parent MDCK cells (supplementary Fig S2 online).

We next examined the effect of Rac1 activation on the maintenance of the cyst structure (Fig 2E). One day after the addition of dimerizer, the apical marker at the plasma membrane diffused into the lateral membrane and within the cytoplasm. At the same time, the basolateral marker also diffused into the apical membrane, which appears as a white area in the merged image. These observations indicated the loss of epithelial polarity on Rac1 activation in the mature cysts. Despite aberrant polarity, cells in the cyst wall were organized in a monolayer forming the central lumen at this stage. On day 2, the lumen of the cysts was narrowed because of multilayering. The percentages of cysts with luminal filling were 71 % ($n=275$) in the presence of dimerizer, compared with 14 % ($n=305$) in the absence of dimerizer. The Rac1 activation by the dimerizer-induced translocation of FKBP-Tiam1 was mostly limited to the Rac1 at the apical membrane, when Rac1 activity was examined in MDCK-Raichu-Rac1 (supplementary Fig S3 online). FKBP-Tiam1 is the only tool to induce the Rac1 activation system at this moment; therefore, we could not rule out the possibility that the observed phenotype is specific for Tiam1-mediated Rac1 activation. Using the Cdc42 FRET biosensor, we found that expression of FKBP-Tiam1 *per se* induced Cdc42 activation at the lateral membrane. However, on dimerizer treatment, we could not observe further activation of Cdc42 (data not shown), negating the possibility that Cdc42 is involved in the Rac1-induced phenotype. On day 3 (the bottom), the lumen of the cyst was almost completely filled by cells. This observation raised another interesting possibility that Rac1 activation might have accelerated cell proliferation, which caused the growth of cells within the cyst. Thus, we collected cysts from the Matrigel to count cell numbers. We could not find a significant difference in the number of cells between cysts grown in the presence or in the absence of dimerizer, negating the possibility that Rac1 activation accelerated cell proliferation (supplementary Fig S4 online). Collectively, these results indicate that the Rac1 activation at the apical plasma membrane of the mature cysts causes aberrant cell movement into the lumen.

The effect of Rac1 inactivation on the cyst structure was similarly examined in FKBP-β2-chimaerin-expressing cysts (Fig 2F). During the 3-day observation period, the morphology was well conserved in comparison with the control cysts (data not shown). However, after Rac1 inactivation, a substantial portion of the apical marker was dispersed in the cytosol, especially beneath the apical membrane, whereas localization of the basolateral marker was not affected. We confirmed that Rac1 was significantly inactivated both in the apical and lateral membranes on rapamycin treatment (supplementary Fig S3 online). These results indicate that the Rac1 inactivation at the plasma membrane in the mature cysts specifically perturbed the transport of the apical protein, but that GPI-protein redistribution is not the principal determinant of aberrant cell morphology.

To examine the effect of Rac1 misregulation in the early stages of cystogenesis, cells were grown in the continuous presence of dimerizer from day 0–8. Under this condition, neither cells expressing FKBP-Tiam1 nor FKBP-β2-chimaerin could form mature cysts with luminal space (supplementary Fig S5 online). The latter phenotype is similar to the MDCK cysts expressing the dominant-negative Rac1 for 4 days in the early stages [5].

To investigate the mode of Rac1-induced luminal filling of MDCK cell cysts, the nuclei of cells expressing dKeima-tagged Histone-H1 in FKBP-Tiam1-were tracked. In the absence of dimerizer, when the chromosomes were aligned on the telophase plate, spindles were properly oriented parallel to the cyst wall (Fig 3A; supplementary Movie 1 online). In contrast, in the presence of dimerizer the direction of cell division was mostly

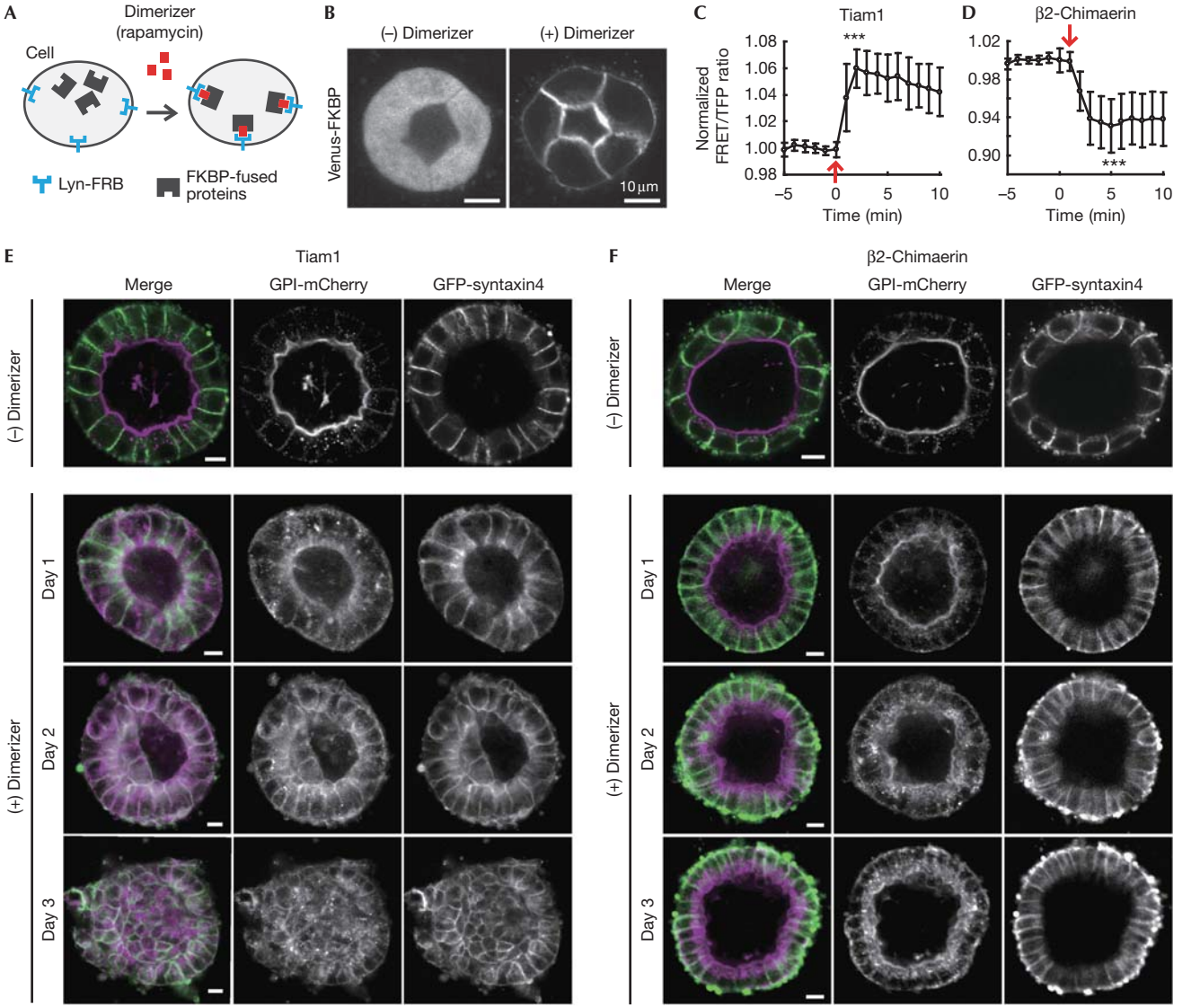


**Fig 2** | Redistribution of polarity markers on Rac1 activity manipulation in mature cysts. (**A**) Schematic representation of a rapamycin-inducible Rac1 activity-manipulating system. (**B**) The representative confocal images of a cyst expressing Lyn-FKBP12-rapamycin-binding domain (FRB) and Venus-FK506-binding protein (FKBP) proteins treated with 100 nM of dimerizer. (**C,D**) Dimerizer-induced activity change of Rac1 in FKBP-Tiam1-expressing or -β2-chimaerin-expressing cells. Madin–Darby canine kidney (MDCK) cells expressing the Raichu-Rac1, Lyn-FRB and FKBP-fused proteins indicated above were plated onto glass-bottom dishes, treated with 100 nM dimerizer at 0 min (indicated by red arrows) and observed using an epifluorescence microscope. The Förster resonance energy transfer (FRET)/TFP ratios were normalized by the average value before stimulation. Results were obtained with FKBP-Tiam1 ($n = 14$) and -β2-chimaerin ($n = 14$). Bars = s.d. ***$P < 0.001$ (against the 0 min time point, by two-tailed paired $t$-test). (**E,F**) Representative images of cysts expressing polarity markers. MDCK cells expressing the apical protein GPI-mCherry (magenta) and the basolateral green fluorescent protein (GFP)-syntaxin4 (green) together with FKBP-Tiam1 (**E**) or -β2-chimaerin (**F**) were cultured without dimerizer for 11 days (top panels). Similarly, MDCK cells expressing these polarity markers together with FKBP-Tiam1 or -β2-chimaerin were cultured without dimerizer for 11 days and further cultured with 100 nM dimerizer for indicated periods. Scale bar, 10 μm.

perpendicular to the cyst wall (Fig 3B; supplementary Movie 2 online). Live imaging also showed that the misalignment of the spindle pole in the monolayer cells occurred before the luminal cell filling became evident, suggesting a causal role of spindle pole misalignment for luminal cell filling. Quantitative analysis of the spindle angle (α °) in cells undergoing telophase confirmed this finding (Fig 3C). Spindle orientation was also visualized by expressing GFP-tubulin. Again, the axis of mitotic spindles was mostly perpendicular to the cyst wall in the presence of dimerizer (Fig 3D, E), indicating that Rac1 activation in mature cysts induced aberrant mitosis orientation. In three-dimensional culture, knock-down of Cdc42 [18,19], or its GEF Tuba [20] or intersectin 2 [21], also disrupts the mitotic spindle orientation. However, knock-down of these molecules leads to multi-lumen formation rather

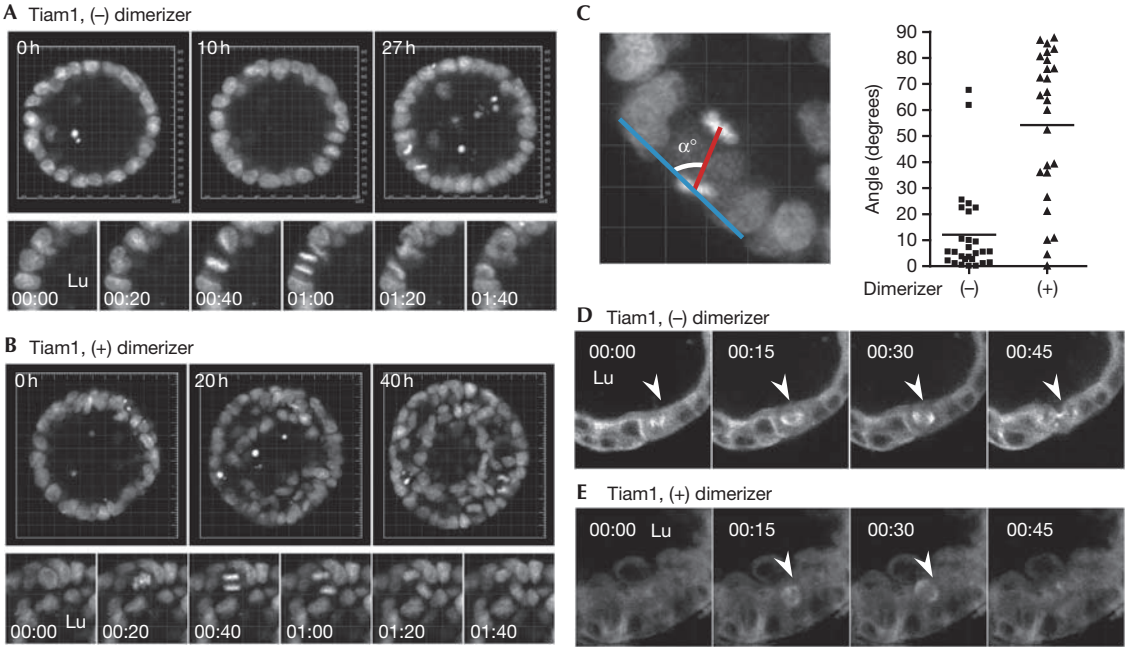


**Fig 3** | Aberrant mitosis orientation on Rac1 activation in mature cysts. (**A**,**B**) Representative Z-stack images of mitosis in a mature cyst. A cyst expressing Lyn-FKBP12-rapamycin-binding domain and FK506-binding protein (FKBP)-Tiam1 together with H1-Keima in the absence (**A**) and presence (**B**) of 100 nM dimerizer was imaged every 20 min with a time-lapse confocal microscope. In the upper panel, the numbers indicate elapsed time after the imaging was started. Lower panels show magnified images of mitosis, and elapsed time (hours: minutes) is indicated. (**C**) The spindle angle (left; α °) was measured in telophase cells. The blue line indicates the axis parallel to the cyst walls. The red line was drawn by connecting the centre of two chromosomes. The spindle angle was measured by the MetaMorph Software, and plotted (right). (**D**,**E**) Representative time-lapse Z-stack images of Madin–Darby canine kidney cysts expressing green fluorescent protein-tubulin and FKBP-Tiam1 in the absence (**D**) and presence of dimerizer (**E**). Arrowheads denote mitotic cells. Lu indicates cyst lumen. Elapsed time (hours: minutes) is indicated. Scale bar, 10 μm.

than luminal filling as observed in this study. Thus, Rac1 activation and Cdc42 inactivation seem to cause disorientation of the cell division axis by different mechanisms.

Tight junctions and other protein complexes involved in cell–cell adherence are known to be maintained during mitosis in epithelial sheets of MDCK cells [22,23]. It has been reported that the dynein–dynactin complex, which is involved in spindle positioning, is found in a circumferential ring below the tight junction [24]. Furthermore, tight junctions are retained during mitosis to separate the apical and basolateral membrane domains for epithelial integrity [23]. We therefore hypothesized that the aberrant distribution of the basolateral marker is brought about by the disappearance of the tight junction, and that tight-junction disruption triggers the aberrant redistribution of polarity markers, inappropriately oriented cell division axes and luminal filling.

To test this, GFP-tagged occludin, a tight-junction marker, was introduced into MDCK cells. As shown in Fig 4A, GFP-occludin was localized at cell–cell interfaces and accumulated at regions close to the apical membrane during mitosis. In dimerizer-treated cells expressing Lyn-FRB and FKBP-Tiam1 (Fig 4B), GFP-occludin accumulated in the cytoplasm, beneath the apical membrane of MDCK cells treated with dimerizer for 20 hours. After 40 hours of treatment, cysts showed the loss of tight junctions and showed luminal filling. As predicted from the observations of Fig 2F, Rac1 inactivation did not induce GFP-occludin dislocation (Fig 4C). To confirm disruption of the tight junctions, FKBP-Tiam1-expressing cysts with or without dimerizer were fixed and stained with the antibody to ZO-1, a tight-junction component [25]. As shown in Fig 4D, ZO-1 localized to the tight junction in the absence of dimerizer. On dimerizer treatment, however, ZO-1 staining was absent in the double-layered cells, although ZO-1 localization was retained in the monolayered cells. As expected, ZO-1 staining was not affected on Rac1 inactivation (supplementary Fig S6 online). These observations indicate that Rac1 activation at the apical plasma membrane disrupts tight junctions. However, the molecular mechanism causing tight-junction disruption has not been identified. In the previous report, expression of dominant-negative E-Cadherin in MDCK sheets disrupted adhesion complex distribution and spindle orientation in a leucine–glycine–asparagine repeat protein (LGN), nuclear and mitotic apparatus protein (NuMA) and dynein-independent manner [26]. Another report indicated that LGN was necessary for proper spindle orientation in cysts [27]. Further experiments will elucidate the mechanism of Rac1 activation-induced tight-junction disruption.

Our observations are summarized in Fig 4E. The forced activation of Rac1 at the apical membrane disrupts cyst morphology by reorienting cell division axes into the luminal space. In contrast, suppression of Rac1 at the basolateral plasma membrane did not significantly affect the morphology of mature cysts. Thus, the suppression of Rac1 at the apical plasma

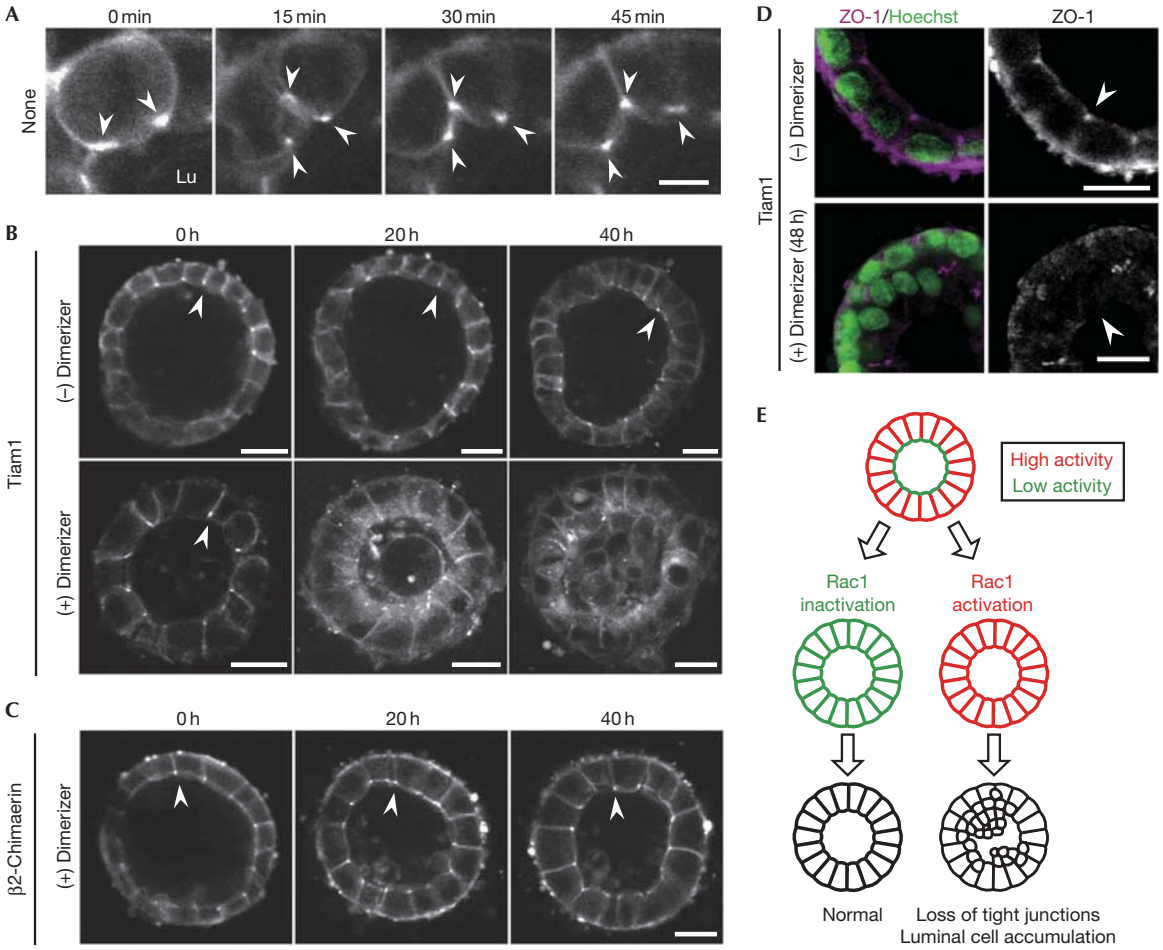


**Fig 4** | Loss of tight junctions on Rac1 activation in mature cysts. Localization of green fluorescent protein (GFP)-occludin during mitosis in a mature cyst. (**A**) Time-lapse confocal images of an Madin–Darby canine kidney (MDCK) cyst expressing GFP-occludin. (**B**,**C**) MDCK cysts expressing FK506-binding protein (FKBP)-Tiam1 (**B**) and FKBP-β2-chimaerin (**C**) together with GFP-occludin were imaged using a confocal microscope at 0, 20 and 40 h without or with 100 nM dimerizer. Arrowheads indicate retained and newly formed tight junctions. Lu indicates a cyst lumen. Scale bar, 10 μm. (**D**) Cysts expressing FKBP-Tiam1 were cultured for 11 days and further cultured in the absence and presence of 100 nM dimerizer, fixed and stained with anti-ZO-1 antibody (magenta) and Hoechst33258 (green). Scale bar, 10 μm. (**E**) A model of Rac1 activity regulation for morphological change during cystogenesis.

membrane is critical for the maintenance of cyst structure. Our observation suggests that aberrant activation of Rac1 might underlie the abnormal morphology of cancer cells. Considering the fact that the most of the sporadic cancers arise from such polarized tissues, disruption of the regulation of polarized Rac1 activity might be a common step in the initiation of carcinoma.

## METHODS

Subcloning, retroviral gene transfer, genesis of cysts and tubules, microscopy and image analysis were performed using standard protocols. For details on plasmid construction, as well as microscopy, see supplementary information online.

**Supplementary information** is available at EMBO *reports* online (http://www.emboreports.org).

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*Author contributions:* E.K. performed DNA design and constructs, and established the Madin–Darby canine kidney cyst protocol at the initial stage of the study. S.Y. carried out all other experiments and processed the Figures. S.Y., M.M. and E.K. wrote the manuscript. E.K. was responsible for the overall study design and for writing the manuscript.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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