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Functional analysis of FilGAP in epithelial tubulogenesis

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Abstract

Epithelial cells form a globular organ-like multi-cellular structure called cyst when cultured in extracellular matrix. The cyst generates extension followed by cell chains and tubules in response to hepatocyte growth factor (HGF). The Rho family small GTPases play essential roles for tubulogenesis. FilGAP, a Rac specific Rho GTPase-activating protein, is highly expressed in kidney. In this study, we examined the role of FilGAP in the tubulogenesis of Madin-Darby Canine Kidney (MDCK) epithelial cells. HGF induces basolateral extensions from cysts. Depletion of FilGAP by siRNA increased the number of extensions in response to HGF, whereas forced expression of FilGAP decreased the number of the extensions. FilGAP is phosphorylated and activated downstream of Rho-ROCK-signaling. Overexpression of phospho-mimic FilGAP (ST/D) mutant blocked formation of the membrane extensions induced by HGF in the presence of ROCK inhibitor, Y-27632. On the other hand, treatment of the tubules with Y-27632 induced scattering of the cells, but FilGAP (ST/D) blocked cell scattering and promoted lumen formation. Taken together, our study suggests that FilGAP may suppress formation of extensions whereas stabilize tubule formation downstream of Rho-ROCK-signaling.

Introduction

Epithelial organs, such as kidney, mammary gland, pancreas, and lung, contain two types of building blocks, cysts and tubules. Cysts are spherical monolayer of polarized cells that enclose a central lumen. Tubules are also lumen-enclosing structures, but are cylindrical. Connecting cysts and tubules can generate complex structures and therefore the processes of cyst and tubule development, also referred as cystogenesis and tubulogenesis, are essential processes for development of epithelial organs [1-4].

Madin-Darby Canine Kidney (MDCK) cells are well-established model for the study of cystogenesis and tubulogenesis [1, 2, 5]. They develop cysts when grown in a three-dimensional culture condition such as collagen type I matrix. A single MDCK cell proliferates to form a spherical monolayer of polarized cells enclosing a central lumen. The organization of the cysts resembles that of epithelia in vivo. The cysts develop tubules when stimulated by hepatocyte growth factor (HGF). Tubulogenesis can be divided into distinct processes [2, 6]. After addition of HGF, the cysts produce extensions of their basolateral surfaces. Then, cells migrate out to form short chains. This process is referred as partial EMT because cells lose basolateral polarity and cell-cell contact is differentially regulated [7]. Further migration led a formation of cords, which contain multiple lumina between the cells. The cells in cords re-polarize and form mature tubules. Tubulogenesis requires coordinated cellular behaviors including cell migration, proliferation, adhesion, and polarization [7, 8].

Rho family small GTPases (Rho GTPases) are involved in the control of actin cytoskeleton and regulate morphogenetic processes including cell adhesion, migration, polarization, and differentiation [9-13]. The GTP-bound active form stimulates downstream effectors, whereas hydrolysis of GTP inactivates Rho GTPases. Two classes of proteins mainly regulate the cycle between an inactive GDP-bound state and an active GTP-bound state. Guanine-nucleotide-exchange factors (GEFs) activate Rho GTPase by catalyzing the exchange of GDP to GTP. GTPase-activating proteins (GAPs) stimulate the intrinsic GTPase activity and inactivate them [14-18].

Because Rho GTPases are involved in the control of actin cytoskeleton, they play important roles in the regulation of epithelial cystogenesis and tubulogenesis [19-22]. Expression of dominant-negative Rac1 induced the inversion of cell polarity in cysts [23]. Inhibition of RhoA-ROCK-myosin II pathway reorients the inverted polarity by expression of DNRac1 [22, 24]. Inactivation of ROCK stimulates the formation of extension and partial EMT [25]. A recent study has demonstrated that activation of RhoA-ROCK- myosin II pathway is required for later steps of tubulogenesis[26].

FilGAP (also known as ARHGAP24) is a Rac-specific GAP that suppresses Rac-dependent lamellae formation and cell spreading [27-30]. FilGAP is phosphorylated by ROCK, and the phosphorylation stimulates its RacGAP activity. FilGAP mediates the antagonism of Rac by Rho that suppresses a mesenchymal morphology and promotes amoeboid migration of carcinoma cells[11, 29, 30]. FilGAP also stabilizes cell-cell adhesion through inactivation of Rac at adherence junctions of MDCK cells [31].

In this study, we demonstrate that FilGAP is involved in initial and later stages of tubulogenesis downstream of Rho-ROCK signaling. We found that FilGAP suppressed HGF-induced formation of extension whereas FilGAP stabilized cord and tubule formation.

Materials and Methods

Cell culture and transfection

MDCK II cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma, St Louis, MO) containing 10% (v/v) fetal bovine serum (FBS) and 50 U/ml penicillin/streptomycin at 37°C under 5% CO_2. Cells were transfected with plasmid DNA or siRNA for 24 hr using Lipofectamin 2000 or Lipofectamin RNAiMAX (Invitrogen, Carlsbad, CA). MDCK cell lines stably expressing FilGAP were used after transfection of plasmid DNA followed by selection with 1.2 mg/ml G418 (Gibco by life technologies).

Cyst culture and tubulogenesis

MDCK cells were trypsinized into a single cell suspension containing 2% Matrigel (Corning, Corning, NY). This suspension was plated into well coated with 100% Matrigel and grown for 2–8 days. To induce cysts extension and tubulogenesis, the Matrigel overlay was removed on day 5 by PBS containing 0.02% EDTA for 1hr at 4°C and embedded in collagen matrix. After gelling, cells were cultured for 24-72 hr in the presence of indicated concentrations of HGF (Millipore, Billerica, MA). MDCK cells were trypsinized into a single cell suspension containing 2 mg/ml atelocollagen I (Koken, Tokyo, Japan), and 20 mM HEPES. This suspension was plated into well and culture for 5 days. For tubulogenesis, cells were treated with indicated concentrations of HGF for 24-48 hr.

Microscopy

For immunofluorescence staining, cells cultured on coverslips were washed with PBS, and fixed with 4% paraformaldehyde in PBS for 30 min at room temperature or ice-cold methanol for 5 min on ice. The fixed cells were washed with PBS, and permeabilized with blocking buffer containing 0.5% Triton X-100 and house serum (HS) in PBS for 30 min at room temperature. Cells were washed with PBS, and immunostained with primary antibodies in blocking buffer for 16 hr at 4°C. Cells were then washed with PBS, and incubated with 2nd antibody solutions in blocking buffer for 3 hr at room temperature. Cells were washed with PBS, and observed under an Olympus IX81 fluorescence microscope (Olympus, Tokyo, Japan). Images were acquired by an EMCCD camera (iXon3; Andor, South Windsor, CT) and analyzed by IMAGEJ. Confocal images were obtained using Olympus DSU (Disk scanning unit)-IX81with a 60× objectives. For time-lapse microscopic observation, images were acquired at 30 min intervals and analyzed by IMAGEJ. Trajectory of cell migration was generated by tracking cell nuclei.

Antibodies and reagents

Mouse monoclonal anti-α-tubulin (Sigma), anti-β1-Integrin (Santa cruz biotechnology, Dallas, Texas), anti-E-cadherin (BD biosciences, Bedford, MA), and anti-ZO-1 (Invitrogen) antibodies were purchased from commercial sources. Rabbit polyclonal anti-FilGAP antibody was prepared as described previously (Ohta et al., 2006). Secondary antibodies conjugated to Alexa Flour 488 or 568 or Alexa-Flour-568 phalloidin were prepared from Invitrogen. Y-27632 was from Wako (Osaka, Japan).

Plasmids and siRNA

pIRES2-AcGFP1 FilGAP (wild type or ST/D) vectors were generated by PCR. siRNA oligonucleotide duplexes targeting human FilGAP was purchased from Invitrogen. The targeting sequence was as follows: FilGAP KD 5'-AAGAUAGAGUAUGAGUCCAGGAUAA-3' (nt 1975-1999)

Statistical analysis

The statistical significance was accessed by two-tailed unpaired Welch's t-test or one-way analysis of variance (ANOVA). Differences were considered to be statistically significant at $P<0.05$. Error bars (s.e.m.) and P values were determined from results of at least three experiments.

Results

Part 1. FilGAP localizes at the basal membrane in MDCK cysts.

First, we examined expression and localization of FilGAP during cystogenesis of MDCK cells. MDCK cells were cultured in Matrigel and expression level of FilGAP protein was monitored by immunoblotting (Fig. 1A). FilGAP protein expression increased gradually during cystogenesis and reached plateau after 6 days (Fig. 1B). The maximal content is more than 10 fold compared to that at day 1 of 3D culture (Fig. 1B). This is in agreement with the observation that, among various RacGAPs, the mRNA levels of FilGAP and beta-chimaerin most increased during cystogenesis of MDCK cells [32]. In contrast to the localization of beta-chimaerin at the apical membrane of mature cysts [32], FilGAP was localized mainly at the basal surface of cysts (Fig. 2B). siRNA targeting FilGAP reduced the expression of endogenous FilGAP in MDCK cells (Fig. 2A). Depletion of FilGAP by siRNA abolished the signal of FilGAP at basal surface of cysts (Fig. 2B) demonstrating the specific localization. We found that FilGAP-depleted cells developed into normal cysts with a single lumen (Fig. 3A and B). Thus, FilGAP does not seem to be essential for development and maturation of cysts.

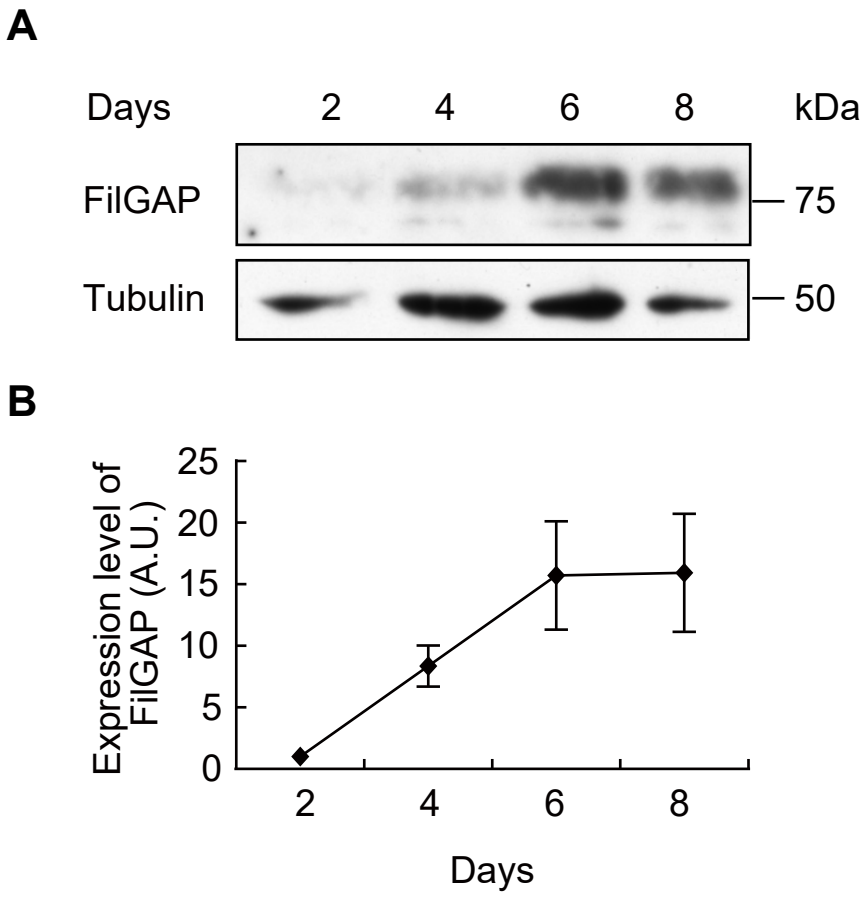


Figure 1. Expression of FilGAP protein increased accirding during cystogenesis.
(A) MDCK cells were cultured in Matrigel for 2, 4, 6, and 8 days. The cell lysates were prepared and expression of FilGAP was analyzed by immunoblotting using anti-FilGAP antibody. Tubulin was used as a loading control. **(B)** Quantification analysis of immunoblots (N=3).

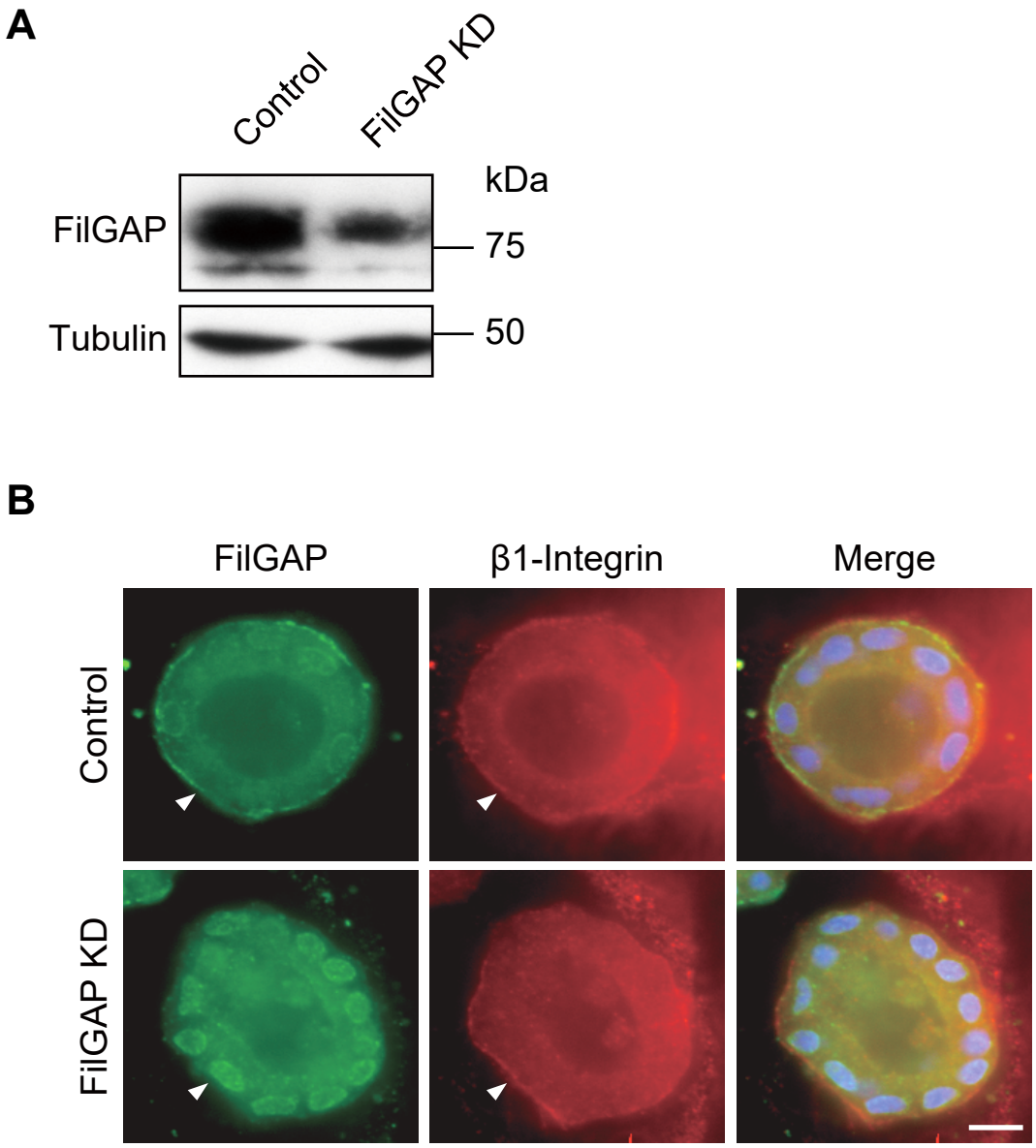


Figure 2. FilGAP is localized at the basal membrane.
(A) MDCK cells transfected with control or FilGAP siRNA were cultured for 24 hr. Control or transfected cells were embedded in Matrigel and cultured for 6 days. The cell lysates were prepared and analyzed by immunoblotting with anti-FilGAP antibody. Tubulin was used as a loading control. **(B)** Control or FilGAP-depleted MDCK cells were cultured in Matrigel for 5 days. Cysts were fixed and stained with anti-FilGAP antibody (green), anti-β1-Integrin antibody (red) and Hoechst 33258 for nuclei (blue). β1-Integrin was used as a marker for basal membrane. Scale bar, 10 μm. Merged images are also shown. Arrowheads show the presence or absence of FilGAP at the basal membrane.

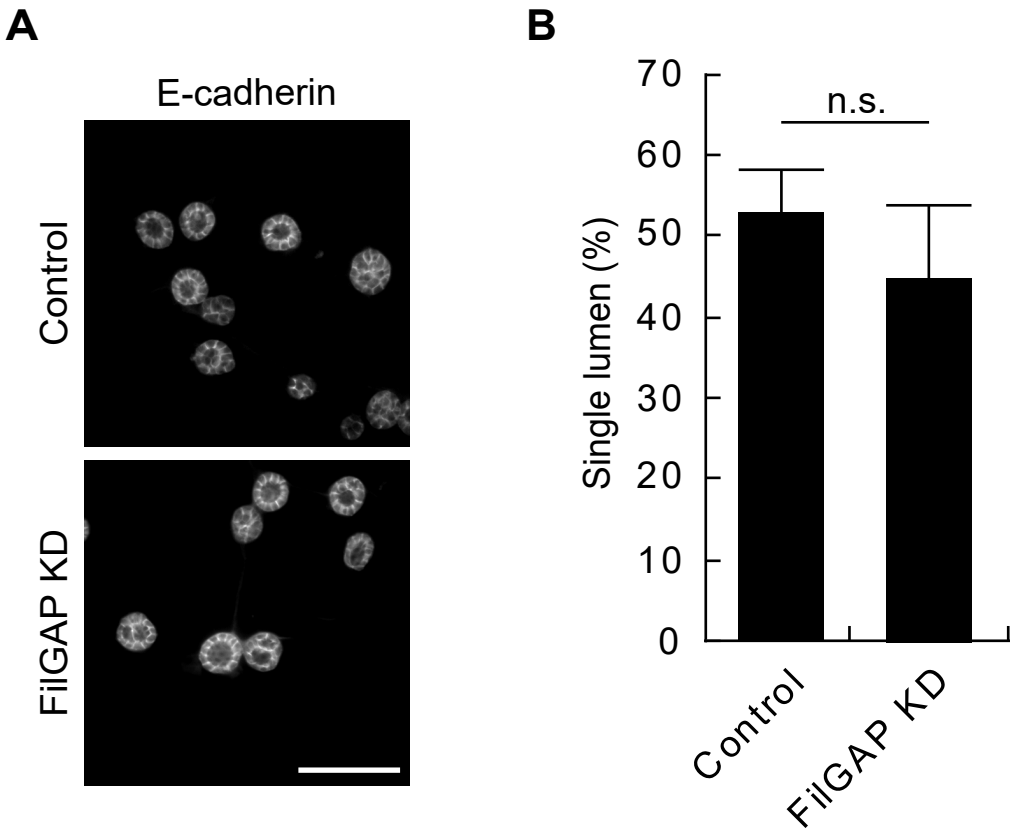


Figure 3. Depletoin of FilGAP did not block lumen formation.
(A) Control or FilGAP-depleted MDCK cells were cultured in Matrigel for 5 days. Cysts were fixed and stained with anti-E-cadherin antibody. Scale bar, 200 μm. **(B)** Quantification analysis of the percentage of normal cysts. Data are the means +/- s. e. m. (N=3).

Part 2. FilGAP blocks HGF-induced formation of extensions downstream of Rho-ROCK-signaling.

We next examined the effect of FilGAP knock down on HGF-induced tubulogenesis. When MDCK cysts were cultured in collagen I and treated with HGF for 24 hr, they produced thin F-actin rich extensions around basal membranes (Fig. 4A). Knock down of FilGAP increased both the number and length of extensions (Fig. 4B and C). The result suggests that FilGAP may suppress the formation of extensions in MDCK cysts in response to HGF treatment.

FilGAP is phosphorylated by ROCK, an effector of RhoA, and the phosphorylation stimulates its Rac GAP activity. Previous studies have demonstrated that Rho-ROCK-signaling is inhibitory for the formation of extensions in MDCK cysts induced by HGF. Therefore, we investigated if FilGAP is located downstream of ROCK to block the formation of extensions induced by HGF. We stably transfected wild-type FilGAP or phospho-mimic FilGAP (ST/D) mutant into MDCK cells (Fig. 5-1A). Control or FilGAP-transfected cells were cultured in Matrigel and mature cysts were prepared. Then, the cysts were treated with HGF for 24 hr and the morphology of the cysts were examined. Forced expression of FilGAP decreased the number of extensions induced by HGF (Fig. 5-1B). We found that phospho-mimic FilGAP (ST/D)-expressing cells blocked the formation of extensions more strongly compared to control or wild-type FilGAP-expressing cells (Fig. 5-2C and D). Moreover, phospho-mimic FilGAP (ST/D)-expressing cells did not produce long extensions after treatment with HGF and ROCK inhibitor Y-27632 (Fig. 5-1B). In contrast, control or wild-type FilGAP-expressing cells produced abundant extensions in response to HGF and Y-27632 treatment (Fig. 5-2C and D). These results suggest that FilGAP may suppress the formation of extensions downstream of ROCK.

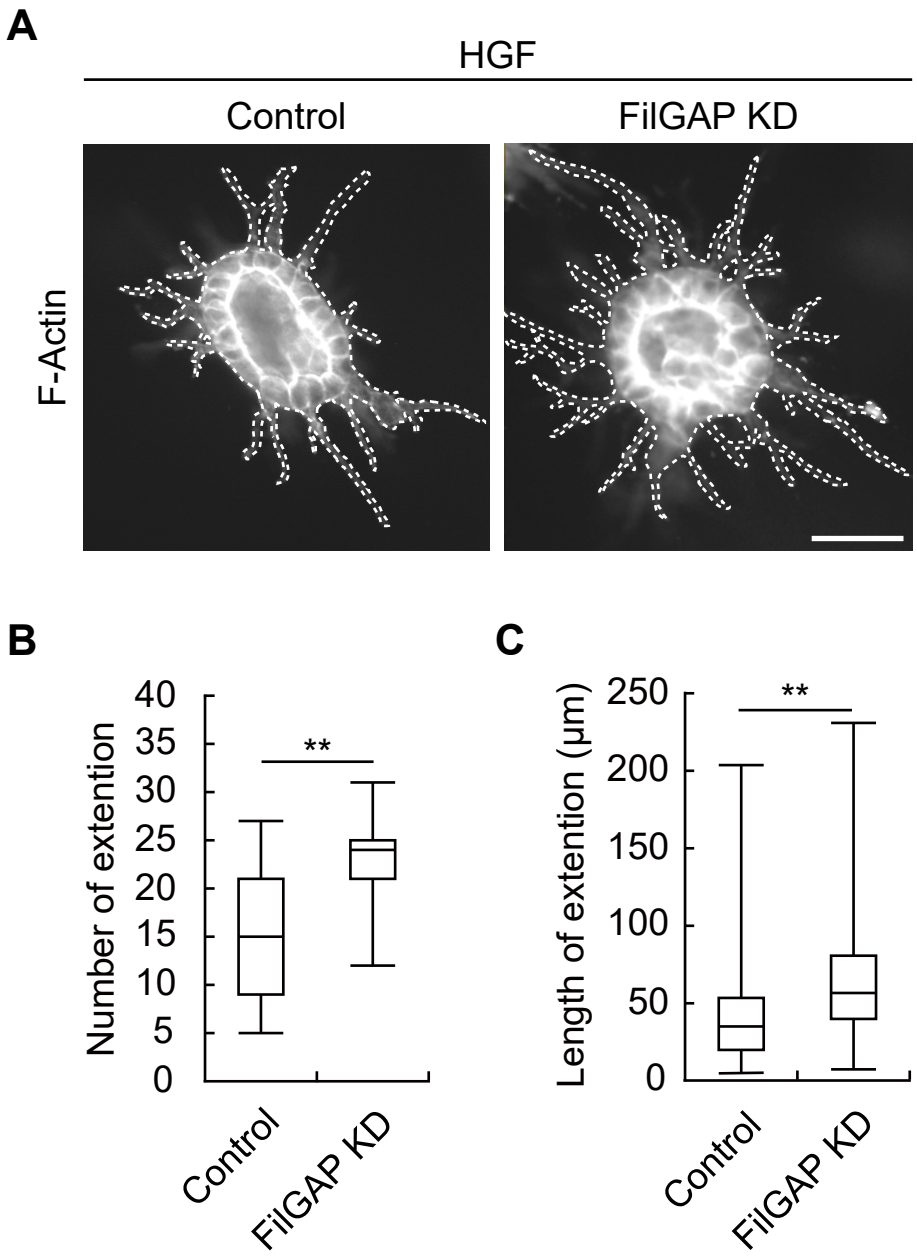


Figure 4. FilGAP blocks HGF-induced fomation of extensions.
(A) Control or FilGAP-depleted MDCK cells were cultured in type I collagen for 5 days and then treated with HGF (20 ng/ml) for 24 hr. Cysts were fixed and stained with Alexa 568 conjugated phalloidin for F-actin. Cell outlines are shown by white dotted lines. Scale bars, 100 μm. **(B)** Quantification of HGF-induced extension. Numbers of extensions per cyst (n=25) are shown as box and whisker plot (N=3, **p<0.01). **(C)** Quantification of HGF-induced extension. Lengths of extensions of cysts (n > 370) are shown as box and whisker plot (N=3, **p<0.01).

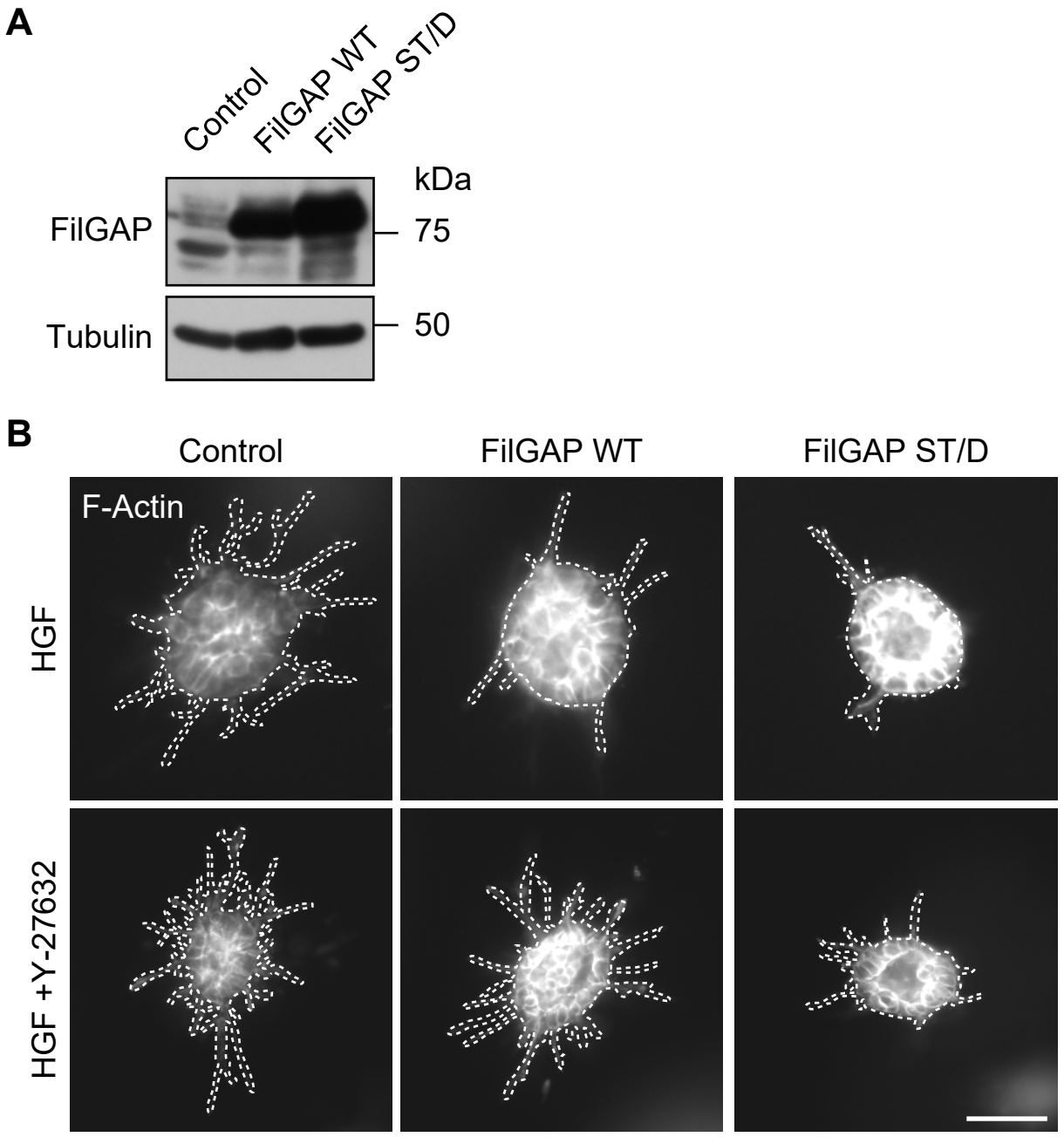
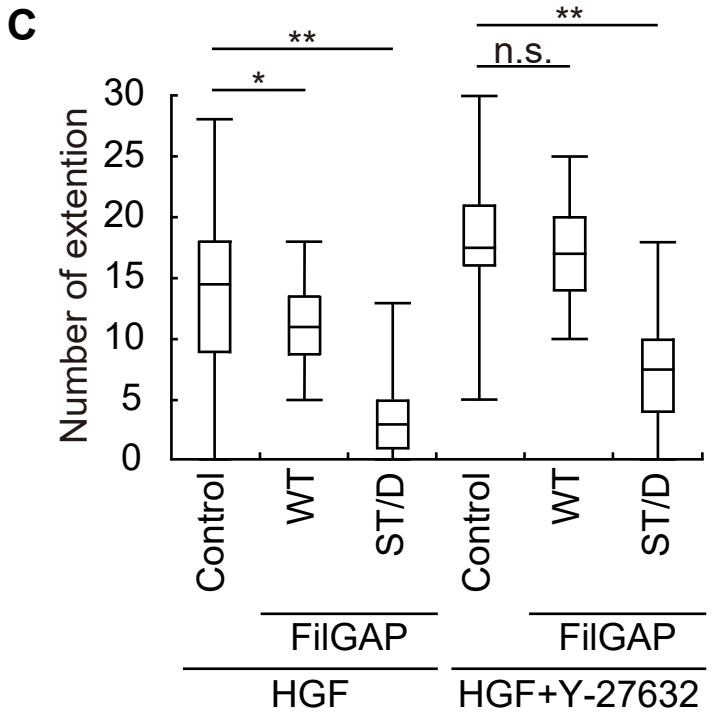


Figure 5-1. FilGAP blocks HGF-induced fomation of extensions downstream of Rho-ROCK-signaling.
(A) The cell lysates of control or FilGAP (WT or ST/D)-stably expressing MDCK cells were prepared and the expression of FilGAP was analyzed by immunoblotting with anti-FilGAP antibody. Tubulin was used as a loading control. **(B)** Control or FilGAP (WT or ST/D)-stably expressing MDCK cells were cultured in Matrigel for 5 days. Cysts were transferred to type I collagen and then treated with HGF (20 ng/ml) in the absence or presence of Y-27632 (10 μM) for 24 hr. Cysts were fixed and stained with Alexa 568 conjugated phalloidin for F-actin. Cell outlines are shown by white dotted lines. Scale bar, 100 μm.



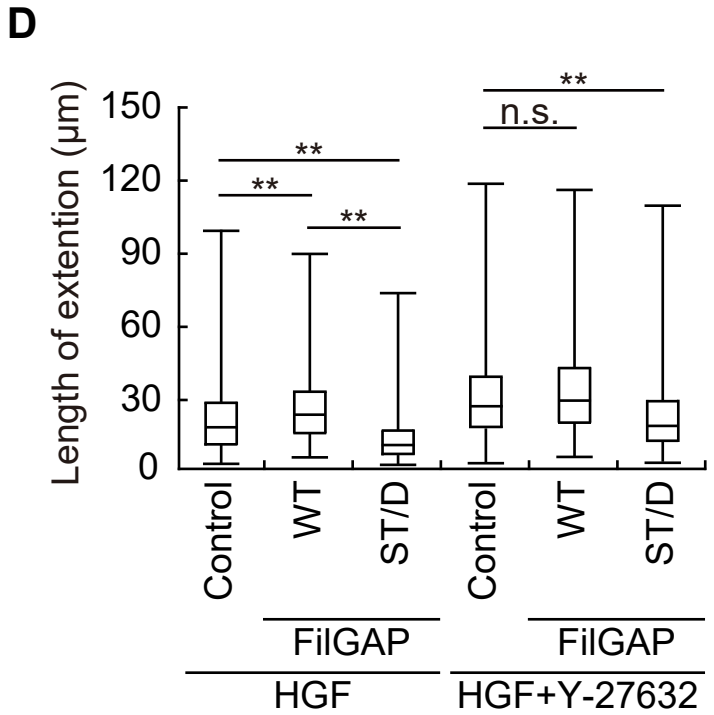


Figure 5-2. FilGAP blocks HGF-induced fomation of extensions downstream of Rho-ROCK-signaling.
(C) Quantification of HGF-induced extension.Numbers of extensions per cyst (n=25) are shown as box and whisker plot (N=3, **P<0.01). Statistical significance was determined by one-way ANOVA. **(D)** Quantification of HGF-induced extension. Lengths of extensions of cyst (n > 350) are shown as box and whisker plot (N=3, **P<0.01). Statistical significance was determined by one-way ANOVA.

Part 3. FilGAP is required for HGF-induced chain formation.

After treatment of the cysts with HGF for 2 days, control cysts produced chains of cells that are connected to the cysts whereas depletion of FilGAP resulted in the scattering of cells and the cysts failed to produce chains (Fig. 6A and B). The scattering of the cells was also observed when the cysts were treated with HGF in the presence of ROCK-inhibitor, Y-27632 (Fig. 7A) [25]. The cysts expressing phospho-mimic mutant FilGAP (ST/D) produced chains of cells and the morphology and the length distribution were similar to those of the control cysts (Fig. 7A). However, FilGAP ST/D mutant expressing cysts were able to produce chains of cells in the presence of Y-27632 (Fig. 7A and B). Thus, FilGAP is indispensable for the formation of chains of cells after stimulation with HGF.

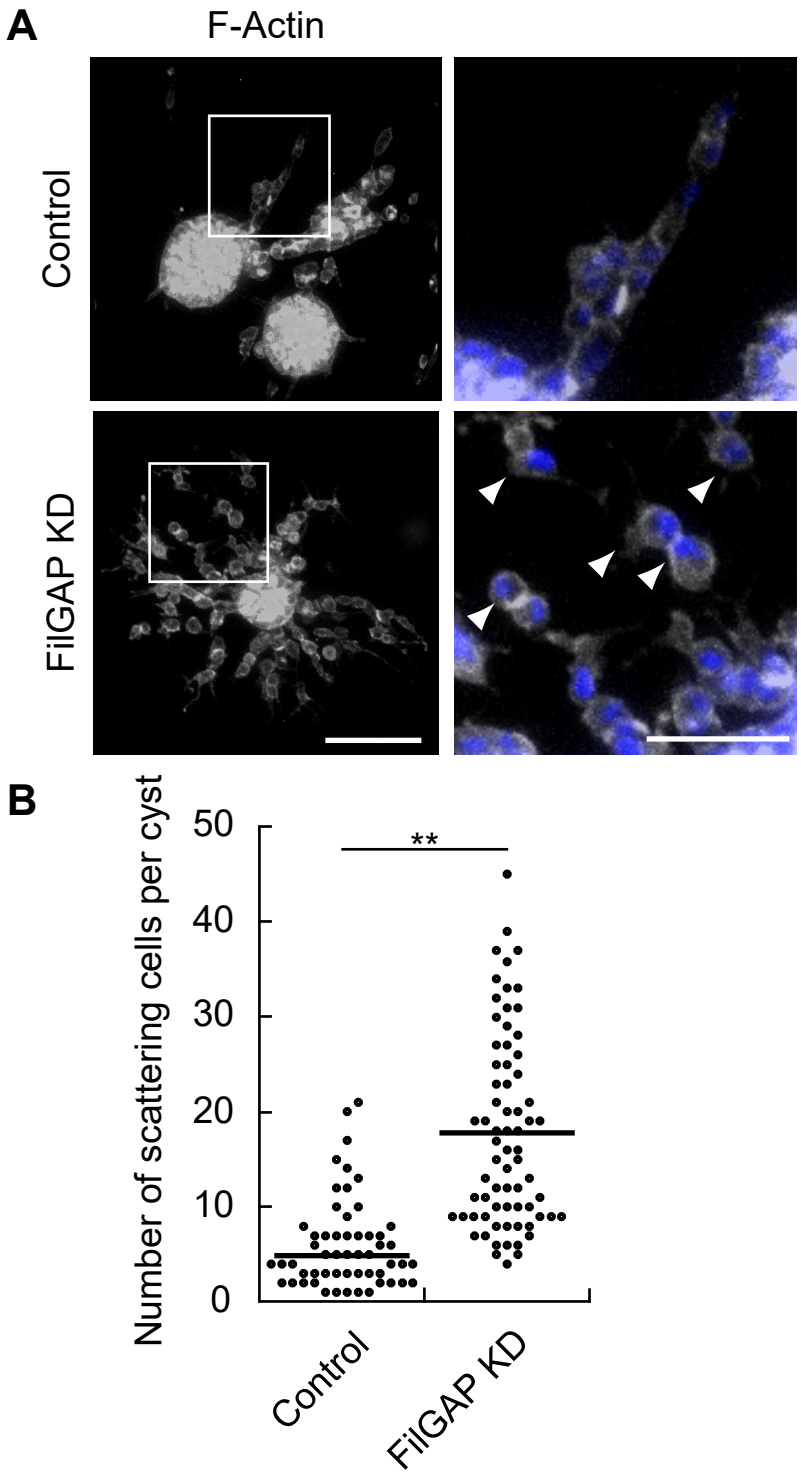
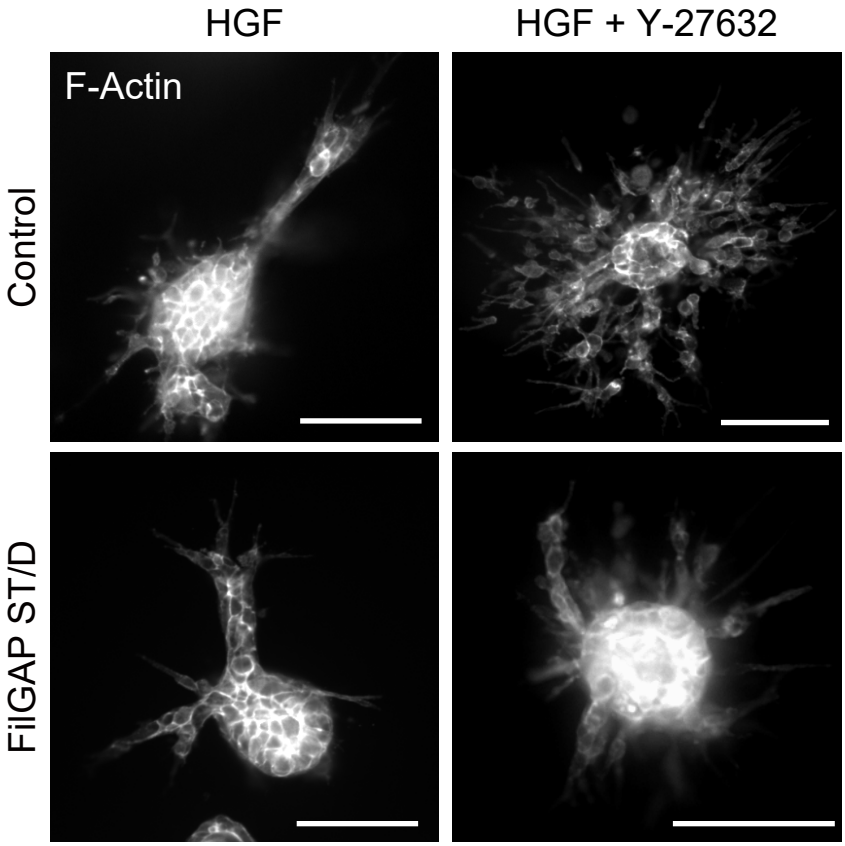


Figure 6. FilGAP is required for HGF-induced chain formation.
(A) Control or FilGAP-depleted MDCK cells were cultured in type I collagen for 5 days and then treated with HGF (10 ng/ml) for 48 hr. Cysts were fixed and stained with Alexa 568 conjugated phalloidin for F-actin and Hoechst 33258 for nuclei (blue). 2D images along z-series are piled up and flattened. Scale bar, 100 μm. Enlarged images of cells indicated by white squares are shown with nuclei (blue). Scale bar, 50 μm. Arrowheads indicate the scattered cells. **(B)** Quantification of HGF-induced cell scattering. Numbers of scattering cells per cyst (n=20) are shown as dot plot (N=3, **$p<0.01$).

A


HGF
HGF + Y-27632
F-Actin
Control
FilGAP ST/D

B

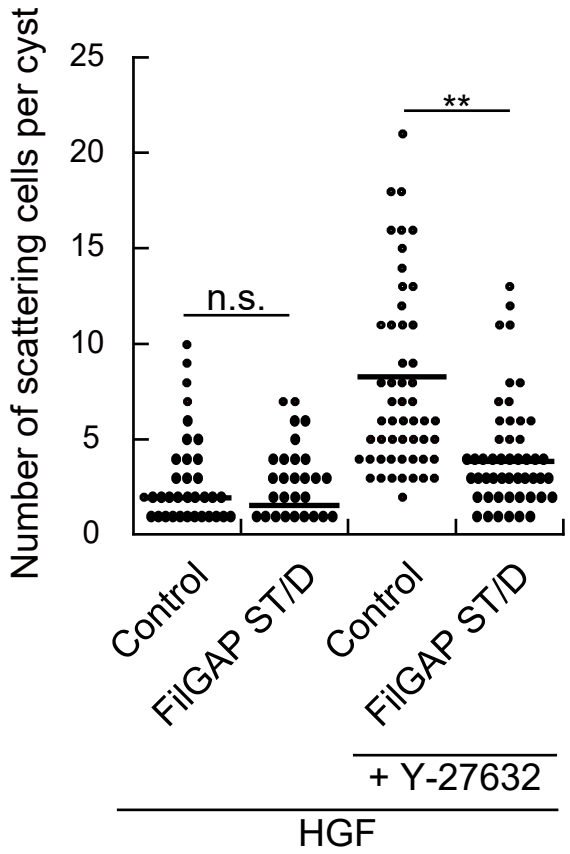

Number of scattering cells per cyst
25
20
15
10
5
0
n.s.
**
Control
FilGAP ST/D
Control
FilGAP ST/D
+ Y-27632
HGF

Figure 7. FilGAP stabilizes the cell chain downstream of Rho-ROCK-signaling.
(A) Control or FilGAP (ST/D)-stably expressing MDCK cells were cultured in Matrigel for 4 days. The cysts were transferred into type I collagen gels and then treated with HGF (20 ng/ml) in the absence or presence of Y-27632 (10 μM) for 48 hr. Cysts were fixed and stained with Alexa 568 conjugated phalloidin for F-actin. Scale bar, 100 μm. **(B)** Quantification of HGF-induced cell scattering. Numbers of scattering cells per cyst (n=20) are shown as dot plot (N=3, **$p<0.01$). Statistical significance was determined by one-way ANOVA.

Part 4. Role of FilGAP in HGF-induced tubule formation.

Following production of chains of cells, the cysts developed chords and mature tubules with single lumen (Fig. 8A) [5, 7]. After incubation of the cysts with HGF for 72 hr, the length distribution of mature tubules varies from 50 to 300 μm but the expression of phospho-mimic mutant FilGAP (ST/D) resulted in the formation of longer tubules compared to control cysts (Fig. 8B). Moreover, expression of FilGAP (ST/D) resulted in the increased in the number of tubules with single lumen compared to control (Fig. 8C). Thus, FilGAP seems to stabilize the tubule structure.

We performed the time-lapse video microscopic analysis to study the cell behavior during later stage (60-72 hr) of HGF-mediated tubulogenesis. Both control and FilGAP (ST/D)-expressing cells migrate collectively and protrude tubules (Fig. 9-1A). Tracking of individual cells in the tube demonstrated that FilGAP (ST/D)-expressing cells migrated faster and longer than control cells (Fig. 9-2B-D) although their persistency of migration did not change compared to that of control cells (Fig. 9-2E).

Rho-ROCK pathway plays a critical role for the tubulogenesis of MDCK cells induced by HGF [26]. Therefore, we examined whether FilGAP may play a role in tubulogenesis downstream of ROCK-signaling. MDCK cysts were incubated with HGF for 48 hr to produce tubules. Then, the cells were treated with ROCK-inhibitor Y-27632 for additional 24 hr and the effect of ROCK inhibition was observed. We found addition of Y-27632 to the tubules resulted in the dissociation of cells from the tubules (Fig. 10A). On the other hand, expression of phospho-mimic mutant FilGAP (ST/D) blocked the scattering of cells in the presence of Y-27632 (Fig. 10A). Quantitative analysis demonstrated that the number of scattering cells significantly decreased in tubules expressing FilGAP (ST/D) in the presence of Y-27632 (Fig. 11-1A). The result suggests that FilGAP may suppress EMT downstream of Rho-ROCK-signaling to promote collective cell migration during later stage of tubulogenesis of MDCK cells.

We performed the time-lapse video microscopic analysis to study the cell behavior during later stage (60-72hr) of HGF-mediated tubulogenesis. Addition of Y-27632 increased the number of cells detaching form the tubules in control cells. Expression of phospho-mimic mutant FilGAP (ST/D) significantly decreased the number of cells detaching from tubules (Fig. 11-1B and 11-2C). Taken together, activated FilGAP may stabilize the cell-cell junctions during lumen formation.

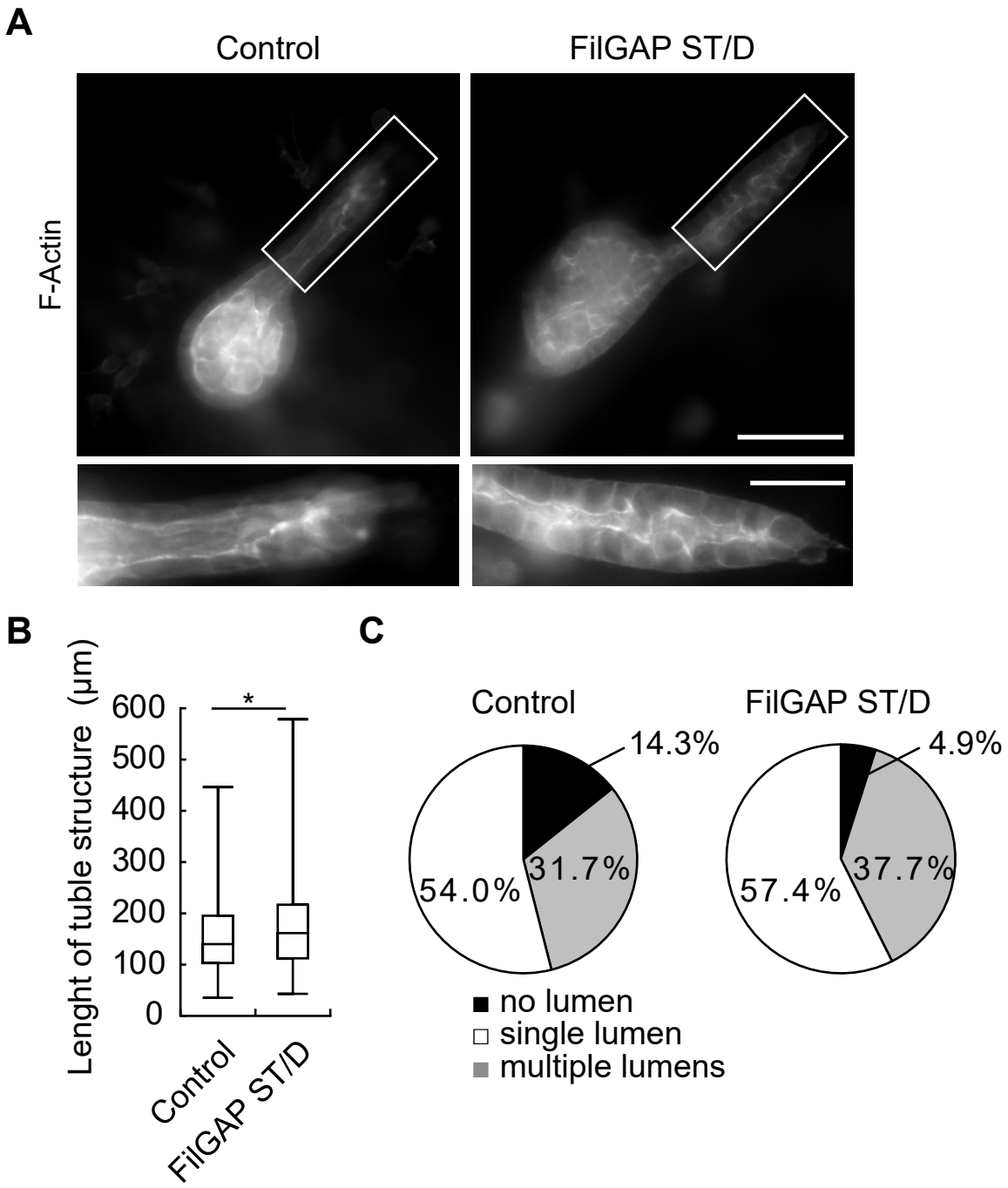


Figure 8. FilGAP is required for HGF-induced chain formation.
(A) Control or FilGAP (ST/D)-stably expressing cysts were treated with HGF (10 ng/ml) for 72 hr in type I collagen gels. Then, the cytsts were fixed and stained with Alexa 568 conjugated phalloidin for F-actin. Scale bar, 100 μm. Enlarged images of cells indicated by white squares are shown. Scale bar, 50 μm. **(B)** Distribution of length of tubules (n > 63) after the treatment of the cysts with HGF for 72 hr (N=3). **(C)** The total number of lumens in tubules (n > 23) were calculated and the tubules were categorized as having no lumen, single lumen, or multiple lumens (N=3).

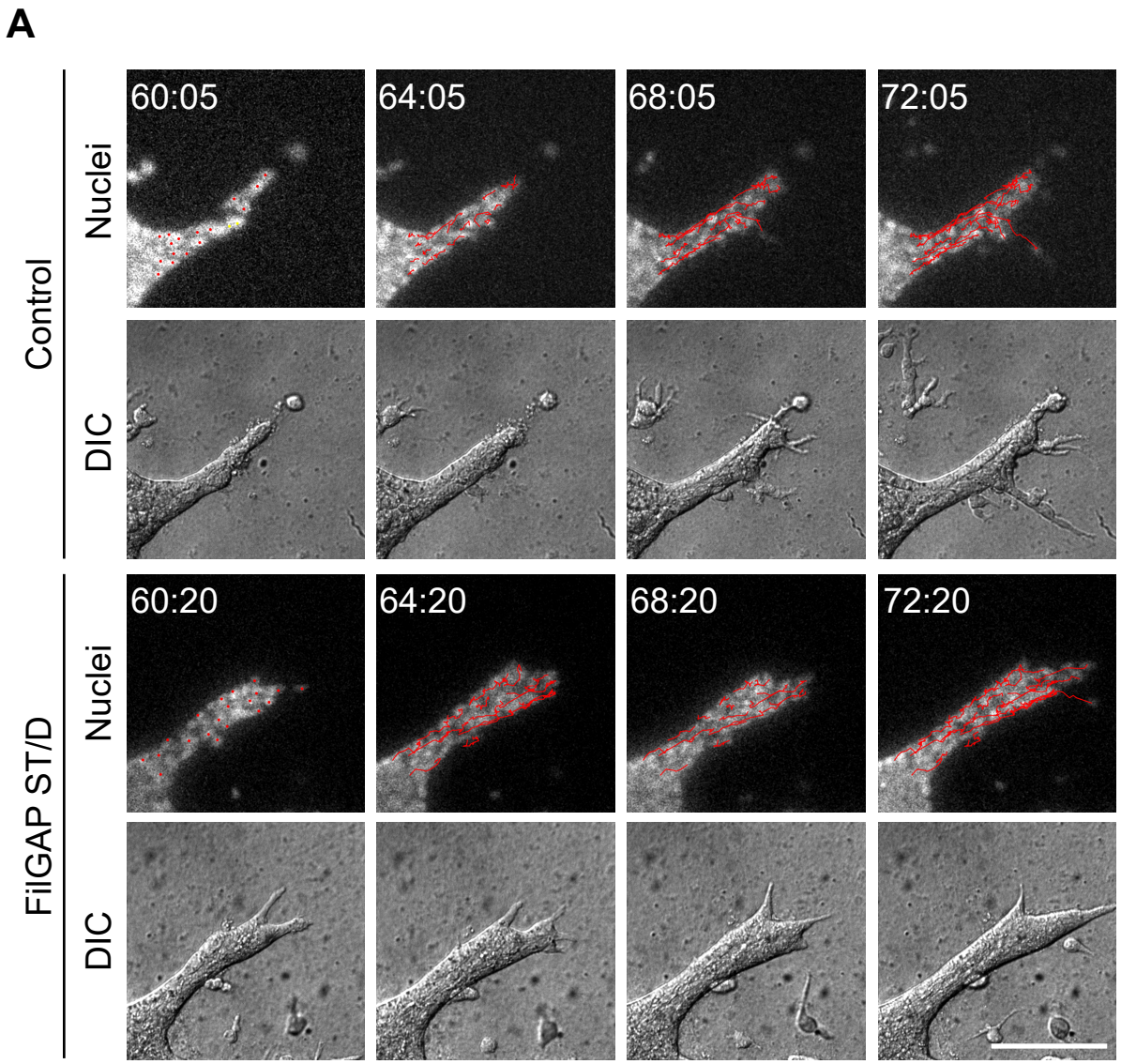


Figure 9-1. FilGAP promotes cell migration during the tubule formation.
(A) Time-lapse images of migrating control and FilGAP (ST/D)-stably expressing cells. Control or FilGAP (ST/D)-stably expressing cysts were treated with HGF (10 ng/ml). After 60 hr, Tubulogenesis was monitored by time-lapse imaging. Images were shown from selected 4 hr intervals. Red lines show trajectory of the cells. Scale bar, 100 μm.

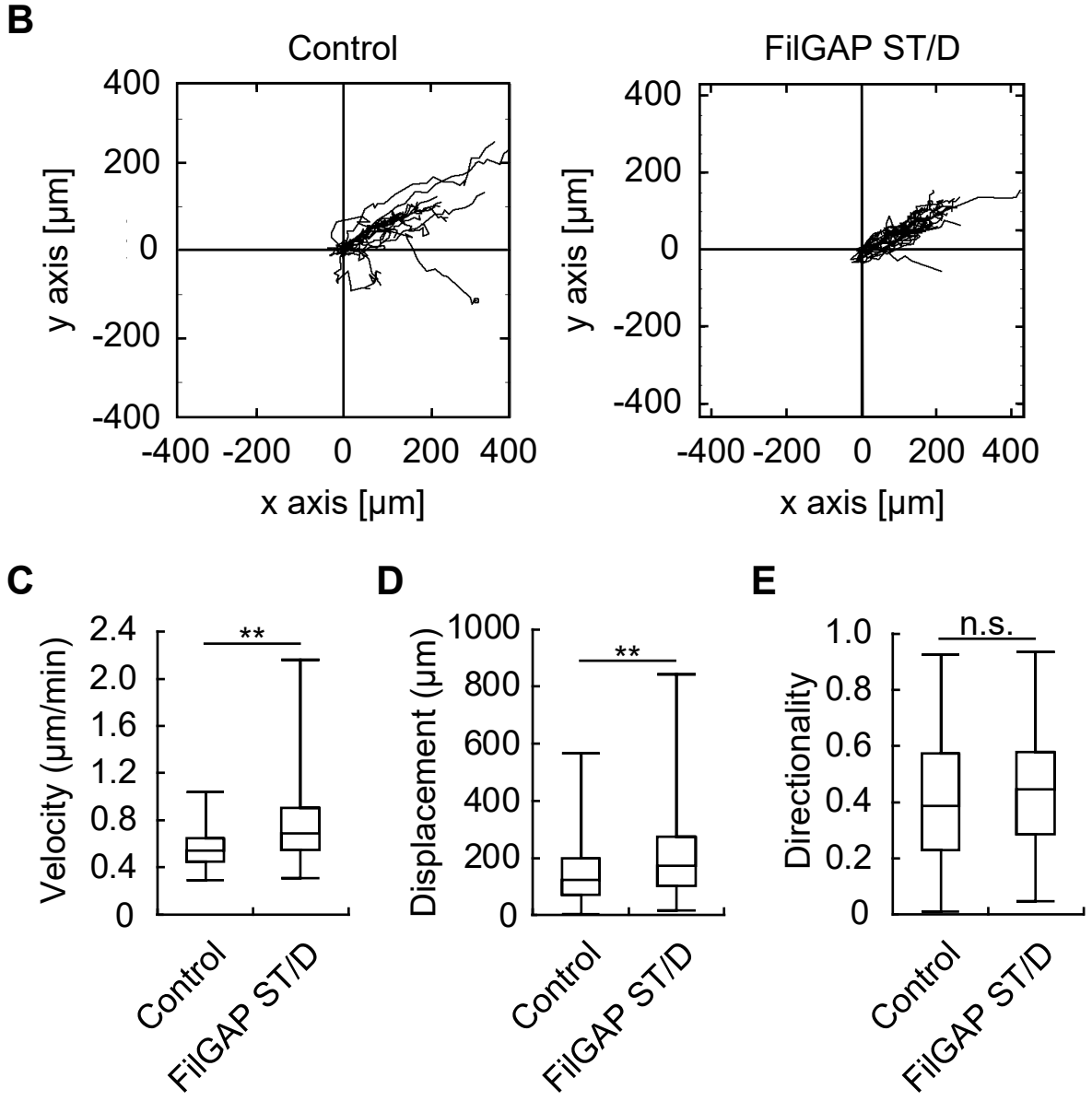


Figure 9-2. FilGAP promotes cell migration during the tubule formation.
(B) Migration tracks of cells are displayed as displacement plots. For control and FilGAP (ST/D)-stably expressing cells, the trajectories of cells (n=18 for control and n=20 for FilGAP (ST/D)-expressing) at 30 min intervals over 12 hr are presented. **(C)** Cell speed of migrating MDCK cells. **(D)** Displacement of migrating MDCK cells. **(E)** Directionality of cell movements of control and FilGAP (ST/D)-stably expressing cells.

A

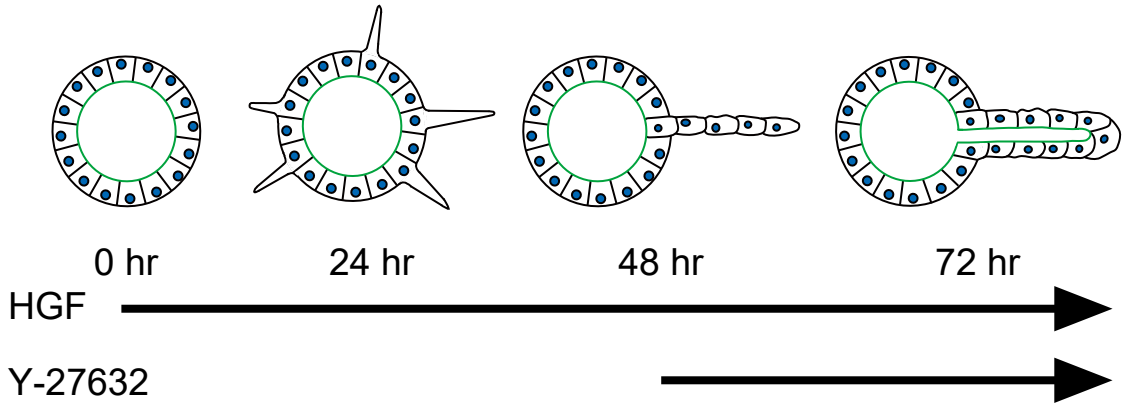
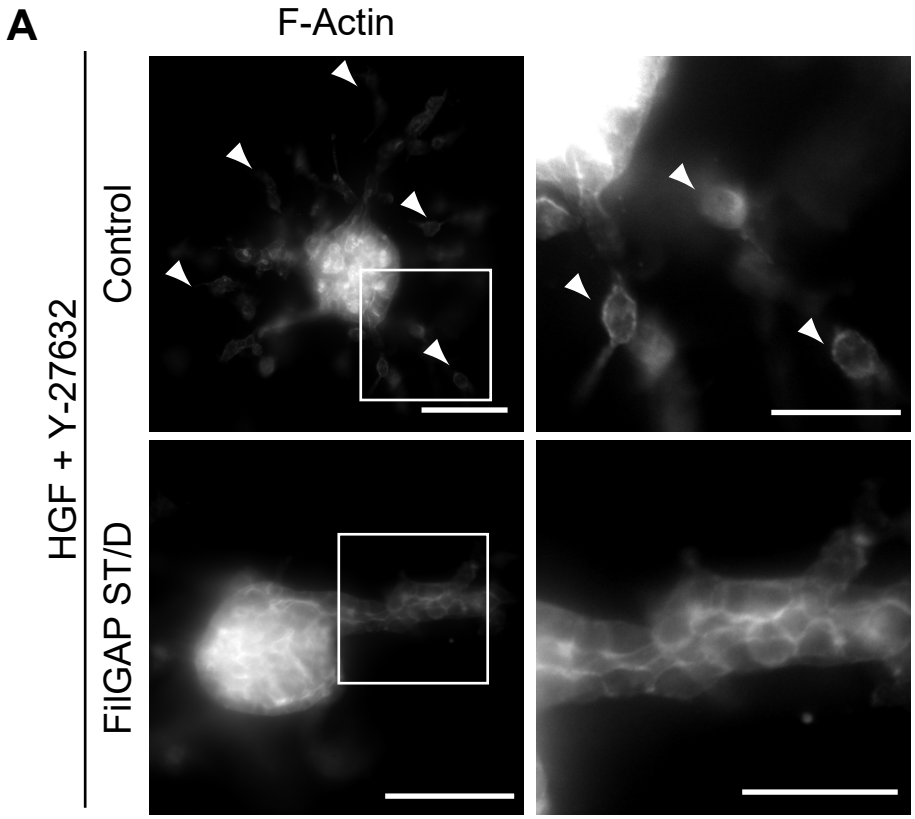


Figure 10. Stages of HGF-induced MDCK tubulogenesis.
(A) MDCK cysts were incubated with HGF for 48 hr and then treated with Y-27632 to inhibit ROCK activity.


A
F-Actin
HGF + Y-27632
Control
FilGAP ST/D

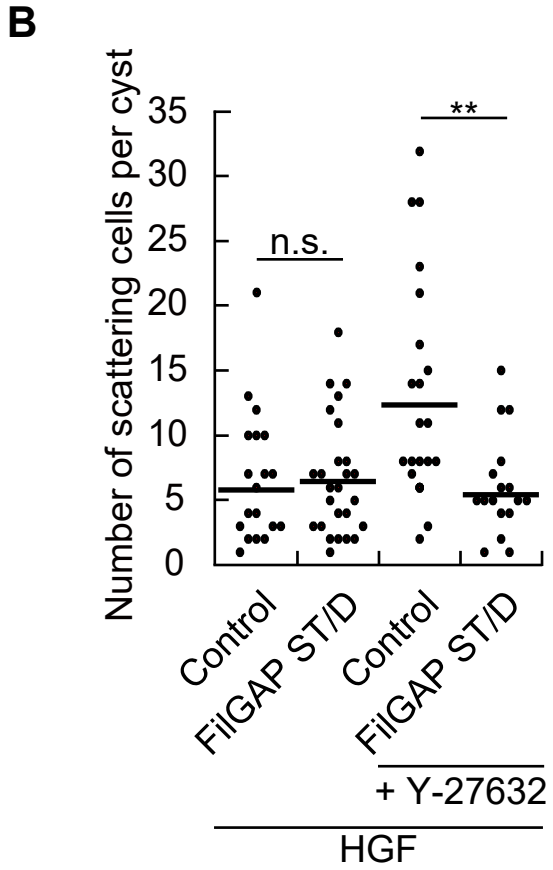

B
Number of scattering cells per cyst
35
30
25
20
15
10
5
0
n.s.
**
Control
FilGAP ST/D
Control
FilGAP ST/D
+ Y-27632
HGF

Figure 11-1. FilGAP stabilizes tubular lumen formation downstream of Rho-RCOK-signaling.
(A) Control and FilGAP (ST/D)-expressing MDCK cells were cultured in Matrigel for 4 days to form cysts. Then, the cysts were transferred into type I collagen gels and treated with HGF (10 ng/ml). After 48 hr, the cysts were further treated with HGF in the presence of Y-27632 (10 μM) for 24 hr. Then, the cysts were fixed and stained with Alexa 568 conjugated phalloidin for F-actin. Scale bar, 100 μm. Enlarged images of cells indicated by white squares are shown. Scale bar, 50 μm. Arrowheads indicate the scattered cells. **(B)** The number of scattering cells from the cysts (n=20) were calculated and plotted.

C

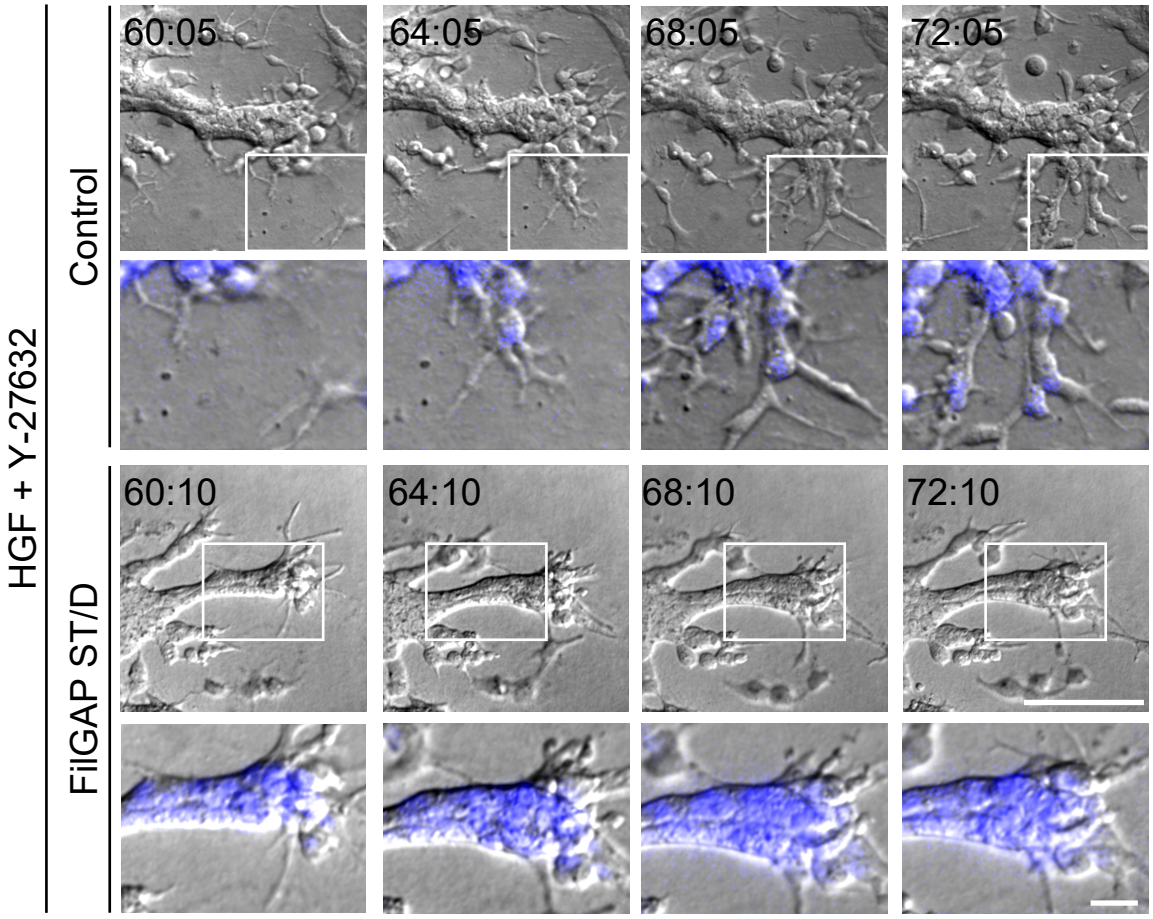


Figure 11-2. FilGAP stabilizes tubular lumen formation downstream of Rho-RCOK-signaling.
(C) Time-lapse images of migrating control and FilGAP (ST/D)-stably expressing cells. The cysts were treated with HGF and Y-27632 as in **(A)**. After 12 hr of treatment with Y-27632 (10 μM), tubulogenesis was monitored by time-lapse imaging. Selected images of movies of tubulogenesis were shown. Scale bar, 100 μm. Enlarged images of cells indicated by white squares are shown with nulei (blue). Scale bar, 20 μm.

Part 5. FilGAP may regulate E-Cadherin localization.

We hypothesized that cell dissociation form the tubules in the presence of ROCK inhibitor Y-27632 may be due to the loss of cell-cell contact. E-cadherin plays a major role for the stabilization of epithelial cell interaction. Therefore, we examined if FilGAP has any role in the localization of E-cadherin during tubulogenesis. We found that E-cadherin is localized at cell-cell junctions in tubules induced by HGF for 72 hr. The localization of E-cadherin at cell-cell junctions did not change in FilGAP (ST/D)-expressing cells compared to control cells (Fig. 12-1A). However, addition of Y-27632 to control cells decreased the localization of E-cadherin at cell-cell junctions at the tips of tubules (Fig. 12-2B). On the other hand, E-cadherin is detectable at cell-cell junctions around tips of tubules expressing FilGAP (ST/D). Localizations of E-cadherin at cell-cell junctions of control and FilGAP (ST/D)-expressing cells after treatment with Y-27632 are clearly shown in 3D renderings of tubules from different viewpoints. Therefore, FilGAP seems to stabilize cell-cell interaction through targeting E-cadherin at cell-cell junctions downstream of Rho-ROCK-signaling. In agreement with this hypothesis, phosphorylated FilGAP is co-localized with E-cadherin at cell-cell junctions (Fig. 13A and B).

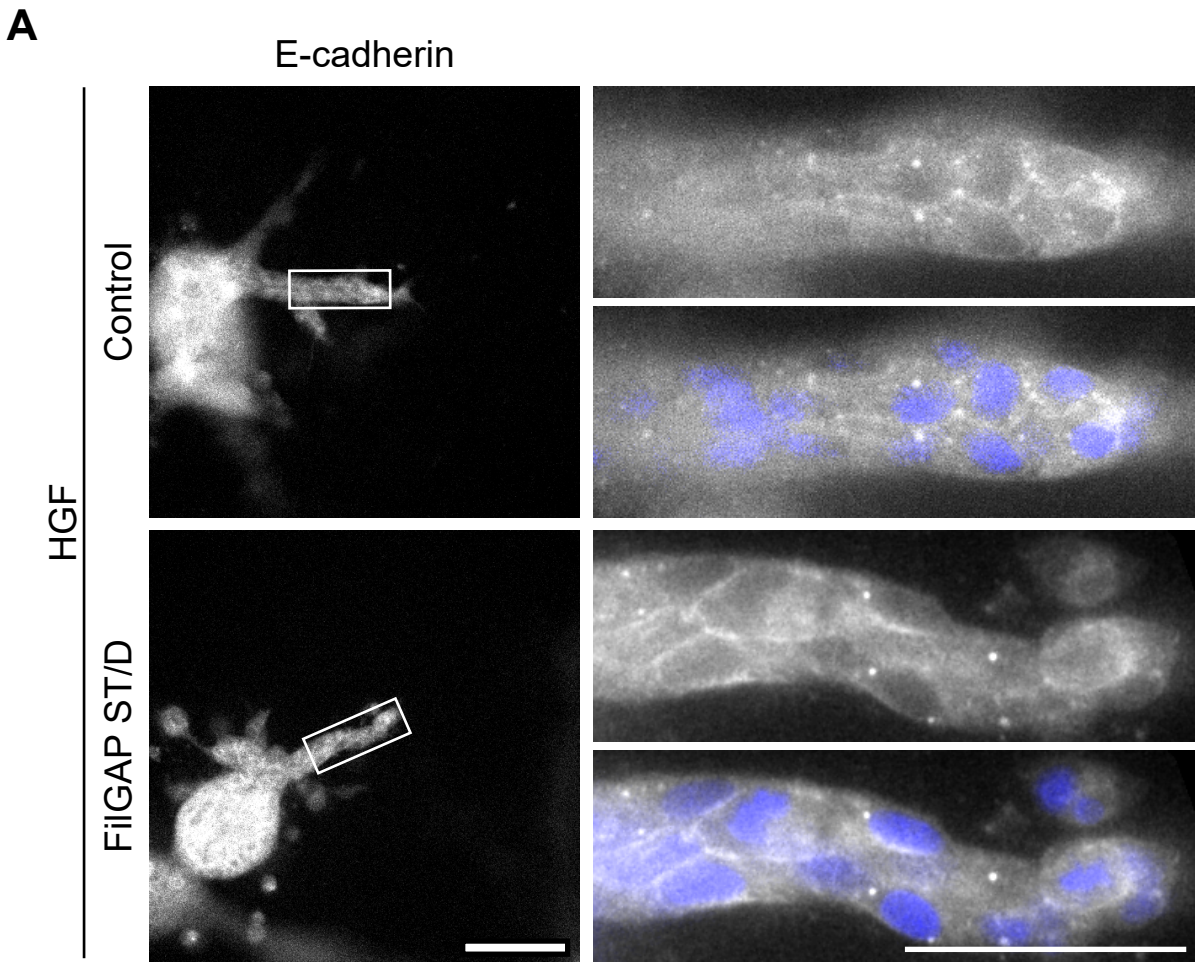


Figure 12-1. FilGAP regulates localization of E-cadherin at cell-cell junctions.
(A) Control and FilGAP (ST/D)-stably expressing MDCK cells were cultured in Matrigel for 4 days to form cysts. Then, the cysts were transferred into type I collagen gels and treated with HGF (10 ng/ml). After 48 hr, the cysts were further treated with HGF in the absence. Then, the cysts were fixed and stained with anti-E-cadherin antibodies. Scale bar, 100 μm. Enlarged images of cells indicated by white squares are shown. Scale bar, 50 μm.

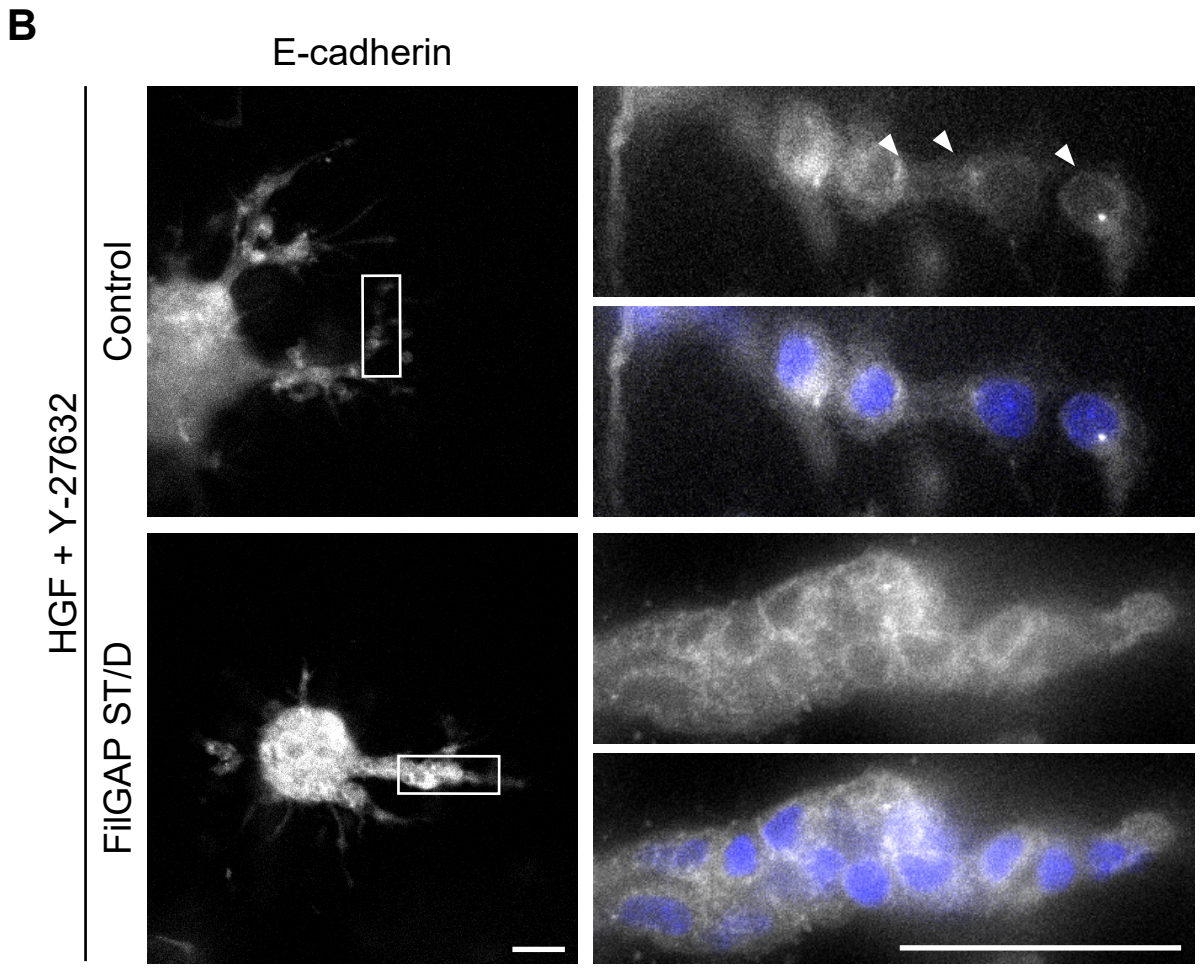


Figure 12-2. FilGAP regulates localization of E-cadherin at cell-cell junctions.
(B) Control and FilGAP (ST/D)-stably expressing MDCK cells were cultured in Matrigel for 4 days to form cysts. Then, the cysts were transferred into type I collagen gels and treated with HGF (10 ng/ml). After 48 hr, the cysts were further treated with HGF in the presence of Y-27632 for 24 hr. Then, the cysts were fixed and stained with anti-E-cadherin antibodies. Scale bar, 100 μm. Enlarged images of cells indicated by white squares are shown. The loss of E-cadherin localization at cell-cell junctions is indicated by arrowheads. Scale bar, 50 μm.

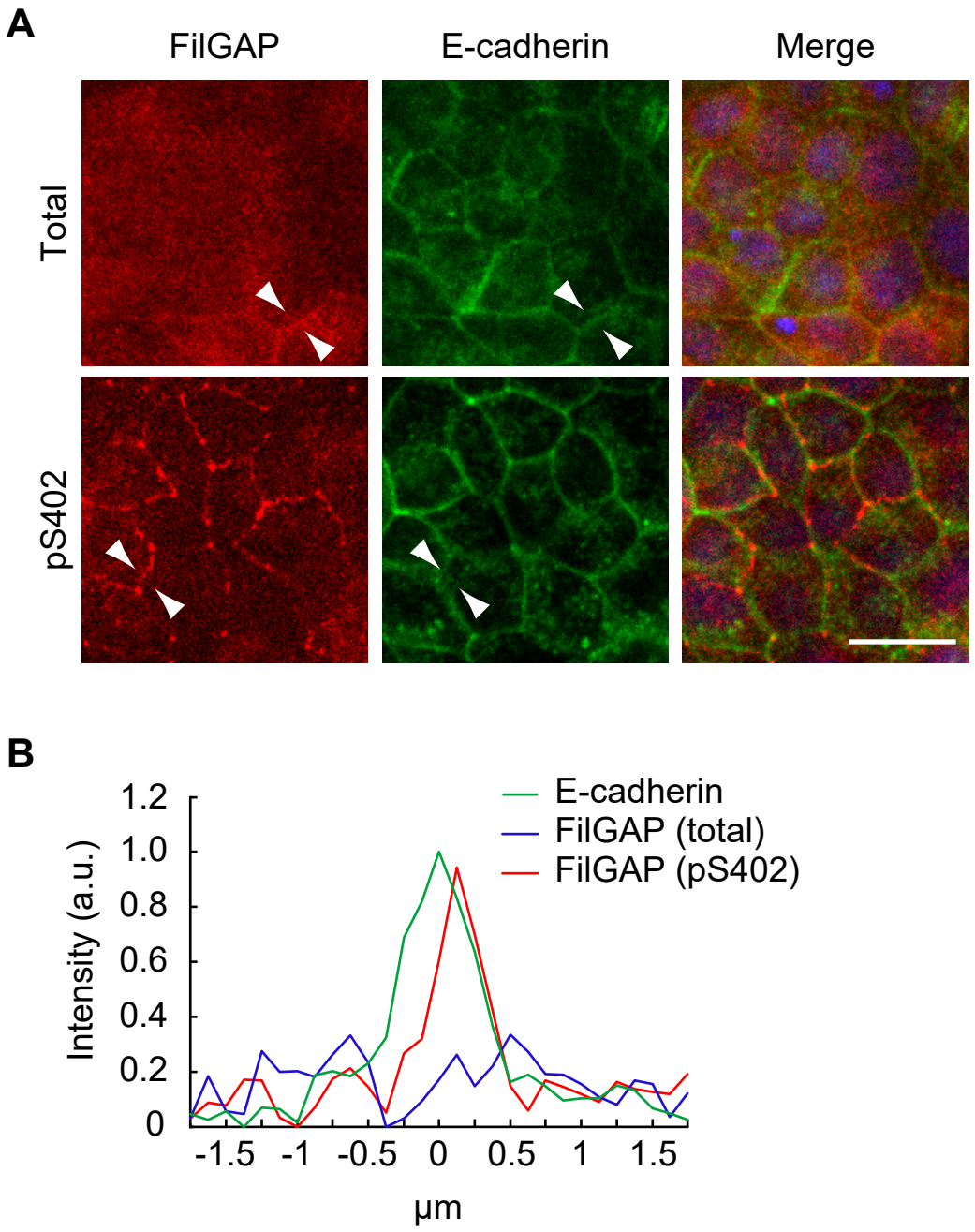


Figure 13. Phosphorylated FilGAP is co-localized with E-cadherin at cell-cell junctions.
(A) FilGAP (WT)-stably expressing MDCK cells are fixed and stained with anti-FilGAP antibody (Total) and anti-S402 phospho-FilGAP antibody (pS402) and anti-E-cadherin antibody. Merged images are also shown. Scale bar, 20 μm. **(B)** Line plot of total FilGAP (blue) or S402 phospho-FilGAP (red) and E-cadherin (green) fluorescence intensities are shown.

Discussion

In this study, we examined the role of FilGAP in cystogenesis and tubulogenesis of MDCK cells. Our study suggests that FilGAP may play a role in the initial and later stages of tubulogenesis downstream of Rho-ROCK-signaling.

We showed that overexpression nor depletion of FilGAP had no effects on the organization of cyst. Although expression of DNRac1 in MDCK cells resulted in the inversion of cell polarity in cysts [27], it has been shown that the inhibition of RhoA-ROCK-myosin pathway reorients the inverted polarity by expression of DNRac1 and, therefore, activation of RhoA as a result of inactivation of Rac by DNRac1 seems to be responsible for the inversion of cell polarity in cysts [22, 24]. Presumably, FilGAP does not seem to modulate RhoA activity to affect cell polarity in cysts.

Our study suggests that FilGAP may suppress formation of extension in response to HGF addition. First, depletion of FilGAP by siRNA increased the number and length of extensions induced by HGF. Second, forced expression of FilGAP suppressed the formation of extensions induced by HGF. FilGAP is phosphorylated by ROCK, and the phosphorylation increases its RacGAP activity [27]. In this study, we found that phospho-mimic FilGAP (ST/D) mutant blocked more efficiently than wild-type FilGAP. Moreover, FilGAP (ST/D) was able to suppress formation of extensions in the presence of ROCK inhibitor Y-27632. It has been reported that inhibition of ROCK stimulated the formation of extensions [25]. Therefore, FilGAP may play a role responsible for blocking the formation of extensions downstream of Rho-ROCK-signaling.

Although FilGAP blocks induction of extensions induced by HGF, FilGAP appears to be required for chain and chord formation. Knockdown of FilGAP resulted in the induction of cell scattering and the cysts failed to form chains and chords. On the other hand, expression of phospho-mimic FilGAP (ST/D) stabilized the structure and the formation of chains was detectable in the presence of ROCK inhibitor. This is consistent with the earlier observation that ROCK is required for chain formation [25].

We further examined if FilGAP is involved in the regulation of later stages of tubulogenesis. After formation of chains and chords, the cysts produce branching tubules through migration and repolarization of the cells[2, 6, 8]. We found that the cells stably transfected with wild-type FilGAP produced tubules without any defects compared to control cells. We were unable to determine the effect of knockdown of FilGAP on the later stages of tubulogenesis because depletion of FilGAP induced scattering of the cells when the cysts were treated with HGF and the cysts failed to produce chains and cords.

Rho-ROCK-myosin II-pathway is critically involved in the regulation of later stages of tubulogenesis [8]. Consistent with the previous study, time-lapse video microscopy revealed that inhibition of ROCK resulted in the loss of directed migration of the cells [8]. However, in our study, inhibition of ROCK did not block cell movement but induced cell scattering when the cysts were treated with HGF and Y-27632 at the same time. The reason of the discrepancy is unclear. We have adjusted concentrations of reagents (HGF and Y-27632) and used similar 3D culture conditions as described [8]. It is possible that our MDCK cells may have distinct phenotypes compared to the cells used by other groups. Further study is necessary to reveal the reason of the discrepancy.

Nevertheless, our study clearly showed that FilGAP might play a role in later stage of tubulogenesis downstream of Rho-ROCK-signaling. Treatment of the tubules with Y-27632 induced cell scattering in control and FilGAP WT-expressing cells. However, tubules expressing phospho-mimic FilGAP (ST/D) protein are more resistant to the treatment of Y-27632. We have shown previously that, in 2D culture, FilGAP induces accumulation of E-cadherin at adherence junctions of MDCK cells and stabilizes adherence junctions downstream of Rho-ROCK-signaling [31]. We showed in this study that phosphorylated FilGAP is co-localized with E-cadherin at cell-cell junctions. Consistent with the findings, inhibition of ROCK induced the dissociation of E-cadherin at cell-cell junctions of tubules but phospho-mimic FilGAP (ST/D) blocked the dissociation. Localization of E-cadherin is dynamically regulated during tubulogenesis. E-cadherin is lost during extension and regained its distinct epithelial localization at later stage of tubulogenesis [6]. Moreover, E-cadherin plays a role in the regulation of collective cell migration [33] and depletion of E-cadherin blocked lumen formation[34]. FilGAP may stabilize cell-cell junctions through accumulation of E-cadherin to promote cell migration and lumen formation. It is also possible that inactivation of Rac-dependent signaling by FilGAP may play a role in the regulation of tubulogenesis. For example, expression of DN-PAK induced tubules through regulation of integrin-dependent adhesion[35]. Further study is necessary how FilGAP regulate later stages of tubulogenesis.

In summary, we show that FilGAP might function to maintain integrity of cell-cell junctions to regulate cell migration during later stage of tubulogenesis. In addition to ROCK-myosin pathway, FilGAP might be crucially involved downstream of Rho-ROCK-signaling to regulate directed cell motility during tubulogenesis.

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