

Rab5-regulated endocytosis plays a crucial role in apical extrusion of transformed cells

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Newly emerging transformed cells are often eliminated from epithelial tissues. Recent studies have revealed that this cancer-preventive process involves the interaction with the surrounding normal epithelial cells; however, the molecular mechanisms underlying this phenomenon remain largely unknown. In this study, using mammalian cell culture and zebrafish embryo systems, we have elucidated the functional involvement of endocytosis in the elimination of RasV12-transformed cells. First, we show that Rab5, a crucial regulator of endocytosis, is accumulated in RasV12-transformed cells that are surrounded by normal epithelial cells, which is accompanied by up-regulation of clathrin-dependent endocytosis. Addition of chlorpromazine or coexpression of a dominant-negative mutant of Rab5 suppresses apical extrusion of RasV12 cells from the epithelium. We also show in zebrafish embryos that Rab5 plays an important role in the elimination of transformed cells from the enveloping layer epithelium. In addition, Rab5-mediated endocytosis of E-cadherin is enhanced at the boundary between normal and RasV12 cells. Rab5 functions upstream of epithelial protein lost in neoplasm (EPLIN), which plays a positive role in apical extrusion of RasV12 cells by regulating protein kinase A. Furthermore, we have revealed that epithelial defense against cancer (EDAC) from normal epithelial cells substantially impacts on Rab5 accumulation in the neighboring transformed cells. This report demonstrates that Rab5-mediated endocytosis is a crucial regulator for the competitive interaction between normal and transformed epithelial cells in mammals.

cell competition | endocytosis | apical extrusion | Rab5 | RasV12

At the initial stage of carcinogenesis, transformation occurs in a single cell within the epithelium. However, it is not clearly understood what happens at the interface between the newly emerging transformed cells and surrounding normal epithelial cells. In previous studies, we and other groups have demonstrated that when cells with an oncogenic mutation, such as RasV12 or v-Src, are surrounded by normal epithelial cells, the transformed cells are apically extruded from the epithelial monolayer (1–5). During this process, normal epithelial cells can recognize and actively eliminate the neighboring transformed cells through dynamic regulation of the cytoskeletal protein filamin, a phenomenon called EDAC (epithelial defense against cancer) (6), implying a notion that the normal epithelium has antitumor activity that does not involve immune systems. In addition, EPLIN (epithelial protein lost in neoplasm) is accumulated in transformed cells when they are surrounded by normal epithelial cells, and the EPLIN accumulation plays a crucial role in apical extrusion of the transformed cells (7). However, the molecular mechanisms of elimination of transformed cells still remain largely unknown, including the link between EDAC and EPLIN accumulation.

Endocytosis is a process by which cells take up macromolecules from the plasma membrane and extracellular space. Through endocytosis, internalized molecules are first transported into early endosomes and often further targeted into late endosomes and

lysosomes, leading to degradation of the cargo molecules. This endocytic event is involved in various cellular processes, such as cell motility, cell proliferation, and oncogenesis (8–11). Previous studies in *Drosophila* have demonstrated that endocytosis also plays a crucial role in cell competition where cells with different properties compete with each other for survival (12–14). However, it is not known whether and how endocytosis is involved in the interaction between normal and transformed epithelial cells in vertebrates.

In this study, using mammalian cultured cells and zebrafish embryos, we have demonstrated that Rab5-mediated endocytosis is enhanced in Ras-transformed cells that are surrounded by normal epithelial cells, which positively regulates the elimination of the transformed cells from epithelia by linking EDAC and EPLIN.

Results

Rab5 Accumulates in Ras- or Src-Transformed Cells That Are Surrounded by Normal Cells. To explore the involvement of endocytosis in the interaction between normal and transformed epithelial cells, we first examined the localization of Rab5 that plays a crucial role in the internalization and transport of endocytic vesicles to early endosomes and in the endosomal fusion (15–17). To this end, we used

Significance

At the initial stage of carcinogenesis, transformation occurs in a single cell within the epithelium. However, it is not clearly understood what happens at the interface between the newly emerging transformed cells and the surrounding normal epithelial cells. Here, using mammalian cell culture and zebrafish embryo systems, we demonstrate that Rab5, an important regulator of endocytosis, is accumulated and that endocytosis is enhanced in RasV12-transformed cells surrounded by normal cells. The elevation of endocytosis disrupts E-cadherin-based cell-cell adhesions with the surrounding normal cells and modulates signaling pathways, eventually leading to apical elimination of the transformed cells. This report demonstrates that endocytosis plays a crucial role in cell competition between normal and transformed epithelial cells in mammals.

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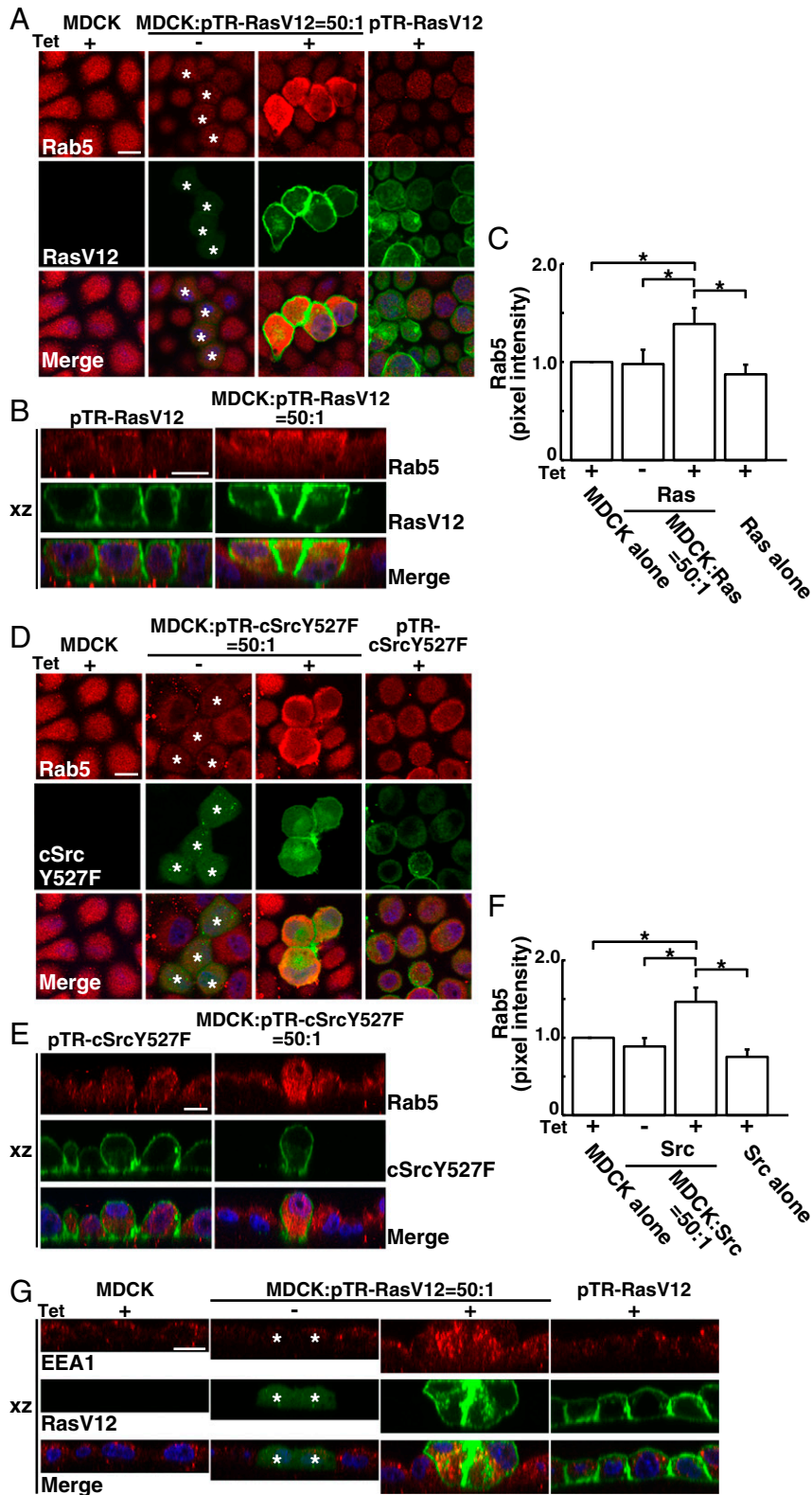


Fig. 1. Rab5 is accumulated in Ras- or Src-transformed cells when they are surrounded by normal epithelial cells. (*A*, *B*, *D*, and *E*) Immunofluorescence of Rab5 shown by confocal images of xy (*A* and *D*) or xz (*B* and *E*) sections. The confocal images are xy images if not indicated. MDCK-pTR GFP-RasV12 cells (*A* and *B*) or MDCK-pTR cSrcY527F-GFP cells (*D* and *E*) were mixed with normal MDCK cells or cultured alone on collagen gels. Cells were fixed after 16-h incubation with tetracycline and stained with anti-Rab5 antibody (red) and Hoechst (blue). For Tet (-), MDCK-pTR GFP-RasV12 cells or MDCK-pTR cSrcY527F-GFP cells were preincubated with CMFDA (green; white asterisks), and the mixture of cells was incubated without tetracycline. (Scale bars, 10 μ m.) (*C* and *F*) Quantification of the fluorescence intensity of Rab5 for *A* and *D*, respectively. Data are mean \pm SD from four (*C*) or three (*F*) independent experiments. **P* < 0.05; *n* = 156, 147, 156, and 155 cells (*C*) or *n* = 155, 103, 154, and 153 cells (*F*). Values are expressed as a ratio relative to MDCK cells. (*G*) Immunofluorescence of EEA1 shown by confocal images of xz sections. Cells were incubated in the same way as described above and stained with anti-EEA1 antibody (red) and Hoechst (blue). (Scale bar, 10 μ m.)

Madin-Darby canine kidney (MDCK) cells stably expressing GFP-tagged oncogenic Ras (RasV12) in a tetracycline-inducible manner (1). We found that Rab5 was substantially accumulated in RasV12-transformed cells when they were surrounded by normal epithelial cells (Fig. 1 *A–C* and Fig. S14). The accumulation of Rab5 was not observed when normal cells or RasV12-transformed cells were cultured alone, or expression of RasV12 was not induced in the absence of tetracycline (Fig. 1 *A–C*). The analyses of confocal images at higher magnification showed that the number and total immunofluorescence intensity of intracellular Rab5 puncta were significantly increased in RasV12 cells surrounded by normal cells, compared with those in RasV12 cells cultured alone (Fig. S1 *B* and *C*). Comparable noncell-autonomous accumulation was also observed for exogenously expressed Rab5 (Fig. S1 *D* and *E*). These data indicate that the

presence of surrounding normal epithelial cells induces accumulation of Rab5⁺ vesicles in RasV12-transformed cells. In addition, the non-cell-autonomous Rab5 accumulation also occurred in Src-transformed cells that were surrounded by normal epithelial cells (Fig. 1 *D–F*). Furthermore, we found that immunofluorescence intensity of the early endosomal marker EEA1 was enhanced in RasV12 cells surrounded by normal cells (Fig. 1*G*), suggesting that endosomal formation is elevated in a noncell-autonomous fashion. In contrast, the Golgi marker GM130 or the recycling endosome marker Rab11 was not accumulated in RasV12-transformed cells that were surrounded by normal cells (Fig. S2), indicating the specific accumulation of Rab5.

Rab5 Plays a Positive Role in the Apical Extrusion of RasV12-Transformed Cells. We next examined the endocytic uptake by analyzing the incorporation of transferrin. Cells were incubated with

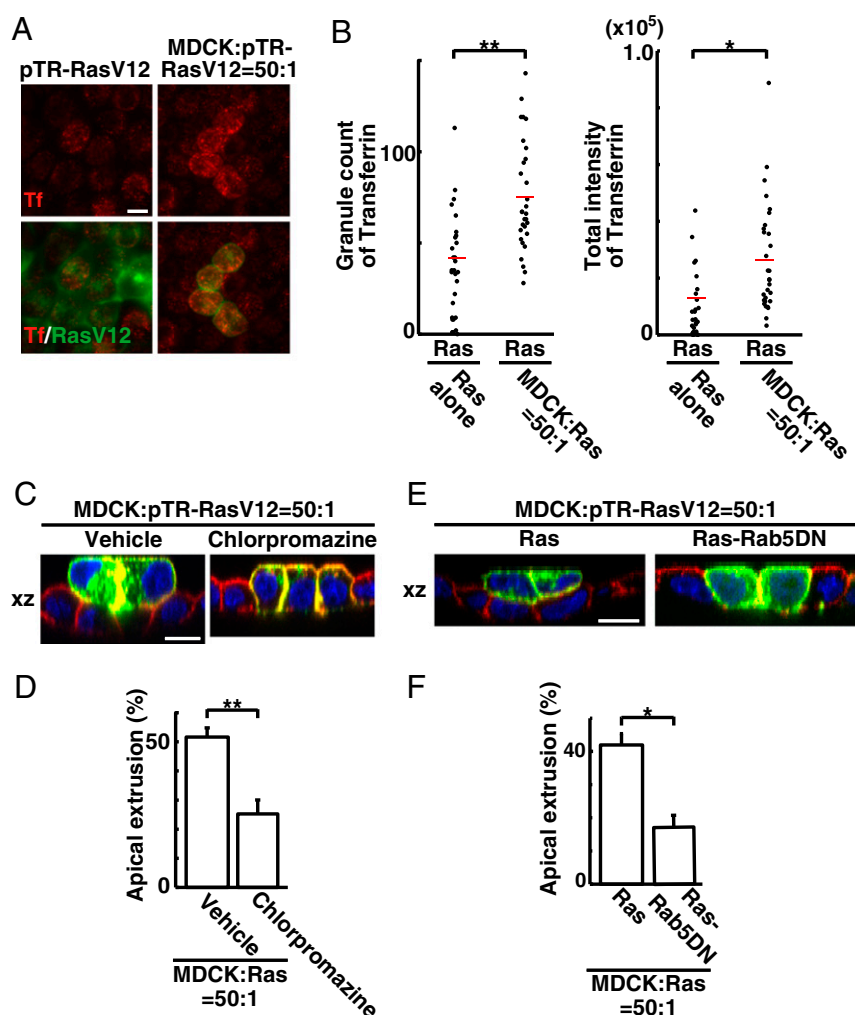


Fig. 2. Rab5-regulated endocytosis plays a positive role in apical extrusion of RasV12-transformed cells. (*A* and *B*) Internalization of Alexa Fluor-647-conjugated transferrin into MDCK-pTR GFP-RasV12 cells that are cultured alone or mixed with normal MDCK cells. After 16 h of tetracycline addition, cells were incubated with Alexa Fluor-647-conjugated transferrin, followed by acid-wash to remove surface-attached transferrin. (*A*) Fluorescence images of internalized Alexa Fluor-647-conjugated transferrin (Tf; red). (Scale bar, 10 μ m.) (*B*) Quantification of internalized transferrin. The number of transferrin granules (*Left*) or the total fluorescence intensity (*Right*) in each RasV12 cell was depicted as a dot. The red bars indicate the mean of the results. ** $P < 0.001$, * $P < 0.05$; $n = 30$ cells for each condition. (*C* and *D*) Effect of chlorpromazine on apical extrusion of MDCK-pTR GFP-RasV12 cells surrounded by normal MDCK cells. (*C*) After incubation with tetracycline in the presence or absence of chlorpromazine for 24 h, cells were stained with Alexa Fluor-568 phalloidin (red) and Hoechst (blue). (Scale bar, 10 μ m.) (*D*) Quantification of apical extrusion of RasV12 cells. Data are mean \pm SD from three independent experiments. ** $P < 0.001$; $n = 280$ and 292 cells. (*E* and *F*) Effect of expression of Rab5DN on apical extrusion of MDCK-pTR GFP-RasV12 cells. MDCK-pTR GFP-RasV12 cells or MDCK-pTR GFP-RasV12-HA-Rab5S34N (Rab5DN) cells (clone 1) were mixed with normal MDCK cells, and incubated with tetracycline for 24 h. (*E*) Cells were stained with Alexa Fluor-568 phalloidin (red) and Hoechst (blue). (Scale bar, 10 μ m.) (*F*) Quantification of apical extrusion of RasV12 cells. Data are mean \pm SD from four independent experiments. * $P < 0.05$; $n = 230$ cells for each condition. As to MDCK-pTR GFP-RasV12-HA-Rab5S34N (Rab5DN) cell lines, clone 1 and clone 2 showed comparable phenotypes (e.g., Fig. S2*C*); thus, hereafter the data with clone 1 are presented.

fluorescence-conjugated transferrin, and intracellularly incorporated transferrin was quantified (18). We found that both the number and total intensity of transferrin granules were significantly elevated in RasV12-transformed cells surrounded by normal cells, compared with those in RasV12 cells cultured alone (Fig. 2*A* and *B*), suggesting that clathrin-dependent endocytosis is enhanced under the former condition. We further studied whether the noncell-autonomous up-regulation of endocytosis influences the behavior of transformed cells. Incubation with chlorpromazine, an inhibitor of clathrin-mediated endocytosis, suppressed apical extrusion of RasV12 cells that were surrounded by normal cells (Fig. 2*C* and *D* and Fig. S3*A*). In addition, expression of a dominant-negative mutant of Rab5 (Rab5S34N, hereafter referred to as Rab5DN) in RasV12 cells substantially diminished their apical extrusion (Fig. 2*E* and *F* and Fig. S3*A–C*), whereas expression of a dominant-negative mutant of Rab11 did not (Figs. S2*C* and S3*D* and *E*).

In the previous studies, we have demonstrated that Src-transformed cells are apically extruded from a monolayer of the enveloping layer (EVL), the outermost epithelium of zebrafish embryos (2, 6). By using this experimental system, we demonstrated that Rab5 was accumulated in Src-transformed cells that emerged in a mosaic-manner within the normal epithelium (Fig. 3*A*), and that coexpression of Rab5DN significantly suppressed apical extrusion of the transformed cells (Fig. 3*B* and *C*). Collectively, these data suggest that Rab5-mediated endocytosis plays a positive role in the elimination of transformed cells from epithelia.

Vesicle Transport Through Late Endosomes Is Also Involved in Apical Extrusion of Transformed Cells. Some of the cargo molecules transported into early endosomes are destined for lysosomal degradation via vesicle transport through multivesicular bodies/late endosomes (11, 19). Tsg101 localizes at the multivesicular body/late endosome

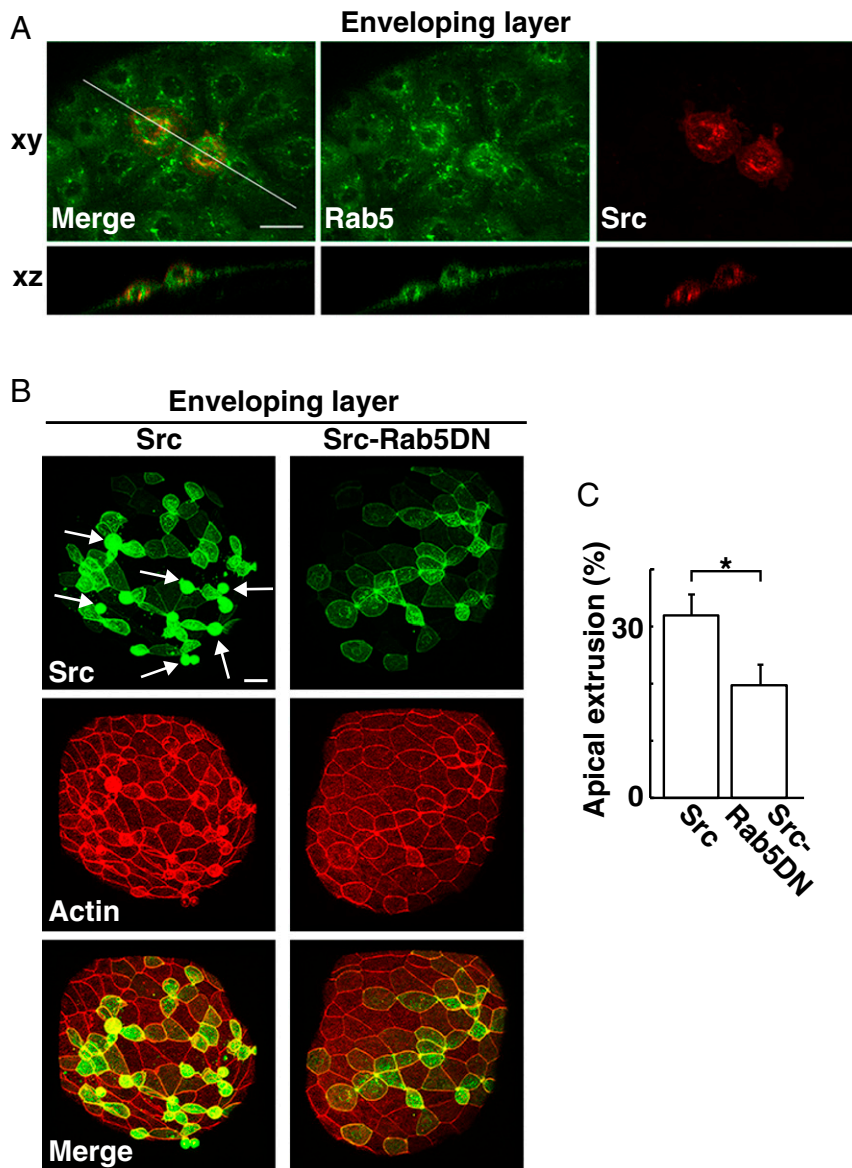


Fig. 3. Rab5 plays a crucial role in apical extrusion of v-Src-expressing cells in the EVL of zebrafish embryos. (A) Confocal images of a Rab5-YFP-expressing zebrafish embryo (at 10–11 h postfertilization, 3 h after tamoxifen treatment) with RFP-v-Src in a mosaic manner. (Scale bar, 20 μ m.) (B and C) Effect of expression of Rab5DN on apical extrusion of v-Src-expressing cells. Embryos were injected with the GFP-v-Src-expressing vector with or without the Rab5DN cassette. Representative images are shown in B. Arrows indicate apically extruded v-Src-expressing cells. (Scale bar, 10 μ m.) (C) Quantification of apical extrusion of v-Src-expressing cells. Data are mean \pm SD from four independent experiments. * $P < 0.05$; $n = 490$ and 364 cells.

and is involved in maturation of late endosomes as a component of the endosomal sorting complex required for transport (ESCRT)-I complex (20). We found that Tsg101 was accumulated in RasV12-transformed cells surrounded by normal epithelial cells, but not in RasV12 cells cultured alone (Fig. 4A). Comparable noncell-autonomous accumulation was also observed for another ESCRT-I component, Vps28 (Fig. S4A). Expression of Rab5DN significantly suppressed Tsg101 accumulation (Fig. 4B and C), suggesting that accumulation of Tsg101 is dependent on Rab5 activity. Rab7 is another late endosome marker, which is required for cargo transport from the late endosome to the lysosome (11). Rab7 was also specifically accumulated in RasV12 cells surrounded by normal cells (Fig. S4B), and the accumulation was diminished by expression of Rab5DN (Fig. S4C). Bafilomycin inhibits the activity of a V-type ATPase in the endosome membrane, thereby increasing pH within endosomal compartments. Treatment with bafilomycin blocks the transport of endocytosed cargos from early to late endosomes (21). We showed that addition of bafilomycin significantly suppressed apical extrusion of RasV12-transformed cells that were surrounded by normal cells (Fig. 4D and E). Next, we established Tsg101-knockdown RasV12 cells (Fig. 4F). Knockdown of Tsg101 strongly attenuated Vps28

accumulation (Fig. S4D) (22), and substantially suppressed apical extrusion of RasV12-transformed cells (Fig. 4G). Taken together, these data suggest that vesicle transport through late endosomes also plays an important role in apical extrusion of transformed cells.

Rab5-Regulated Endocytosis Acts Upstream of EPLIN in the Interaction Between Normal and Transformed Epithelial Cells. In the previous studies, we have reported that E-cadherin-based cell-cell adhesions are dynamically modulated at the boundary between normal and transformed cells (1, 2). Moreover, E-cadherin has been reported to be endocytosed via Rab5 (23–26). Thus, we next examined the link between Rab5-mediated endocytosis and E-cadherin. When normal or RasV12-transformed cells were cultured alone, E-cadherin mainly localized at cell-cell contact sites (Fig. 5A) (1). However, when RasV12 cells were surrounded by normal cells, cytoplasmic puncta of E-cadherin substantially increased (Fig. 5A and B), which partially overlapped EEA1 immunofluorescence (Fig. S5A). Cytoplasmic puncta were not observed for the tight junction marker occludin or the cell-adhesion marker integrin α V β 3 (Fig. S5B and C), suggesting the specific effect on E-cadherin, at least to a certain extent. By superresolution microscopic analyses, we further demonstrated

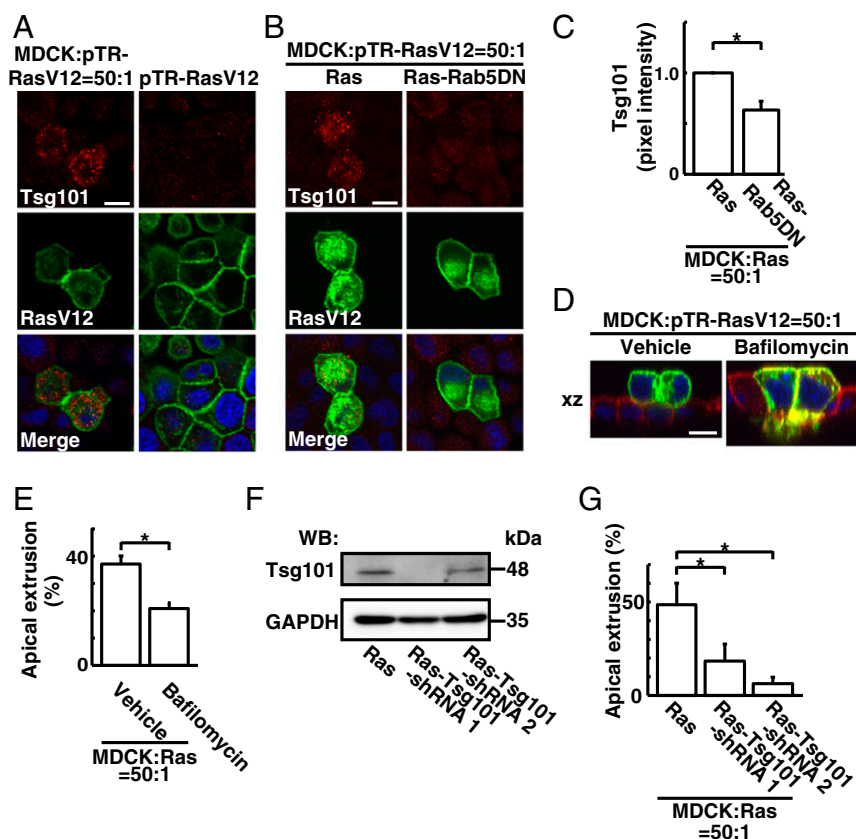


Fig. 4. Vesicle transport through late endosomes is involved in apical extrusion of transformed cells. (A) Accumulation of Tsg101 in RasV12-transformed cells that are surrounded by normal epithelial cells. MDCK-pTR GFP-RasV12 cells were mixed with normal MDCK cells or cultured alone on collagen gels. Cells were fixed after 16-h incubation with tetracycline and stained with anti-Tsg101 antibody (red) and Hoechst (blue). (Scale bar, 10 μ m.) (B and C) Effect of coexpression of Rab5DN on the accumulation of Tsg101. MDCK-pTR GFP-RasV12 cells or MDCK-pTR GFP-RasV12-Rab5DN cells were mixed with normal MDCK cells, and incubated with tetracycline for 16 h. (B) Cells were stained with anti-Tsg101 antibody (red) and Hoechst (blue). (Scale bar, 10 μ m.) (C) Quantification of the fluorescence intensity of Tsg101 for B. Data are mean \pm SD from three independent experiments. * P < 0.02; n = 104 and 110 cells. Values are expressed as a ratio relative to RasV12 cells. (D and E) Effect of bafilomycin on apical extrusion of RasV12-transformed cells surrounded by normal epithelial cells. MDCK-pTR GFP-RasV12 cells were mixed with normal MDCK cells, and incubated with tetracycline in the presence or absence of bafilomycin for 24 h. (D) Cells were stained with Alexa Fluor-568 phalloidin (red) and Hoechst (blue). (Scale bar, 10 μ m.) (E) Quantification of apical extrusion of RasV12 cells. Data are mean \pm SD from three independent experiments. * P < 0.002; n = 376 and 306 cells. (F) Establishment of MDCK-pTR GFP-RasV12 cells stably expressing Tsg101-shRNA. Cell lysates were examined by Western blotting with the indicated antibodies. (G) Effect of Tsg101-knockdown on apical extrusion of RasV12 cells. MDCK-pTR GFP-RasV12 cells or MDCK-pTR GFP-RasV12 Tsg101-shRNA 1 or -shRNA 2 cells were mixed with normal MDCK cells, and incubated with tetracycline for 24 h. Data are mean \pm SD from three independent experiments. * P < 0.05; n = 316, 319, and 316 cells.

that the number and total immunofluorescence intensity of intracellular E-cadherin puncta were significantly increased in RasV12 cells surrounded by normal cells, compared with those in RasV12 cells cultured alone (Fig. 5 C and D). On the other hand, intracellular localization of E-cadherin was substantially suppressed in Rab5DN-expressing RasV12 cells (Fig. 5 A and B). In addition, E-cadherin immunofluorescence became often blurred at cell–cell contact sites between normal and RasV12 cells, but remained intact between normal and Rab5DN-expressing RasV12 cells (Fig. S64). These data suggest that E-cadherin undergoes internalization via Rab5-mediated endocytosis, which is—at least partly—involved in the dynamic regulation of cell–cell adhesions between normal and

transformed cells. Intracellular E-cadherin puncta were also observed in about 20% of the surrounding normal cells (Fig. S6B), which may be passively induced by the loss of E-cadherin from the plasma membrane on the adjacent transformed cell.

Next, we examined EPLIN, which forms a complex with E-cadherin at cell–cell adhesions (27). EPLIN has also been reported to play an active role in apical extrusion of the transformed cells through the activation of protein kinase A (PKA) (7, 28). When normal or RasV12-transformed cells were cultured alone, EPLIN was mainly localized at cell–cell adhesions (Fig. 6A) (7, 27). In contrast, in RasV12-transformed cells that were surrounded by normal cells, EPLIN was translocated from cell–cell contacts to the

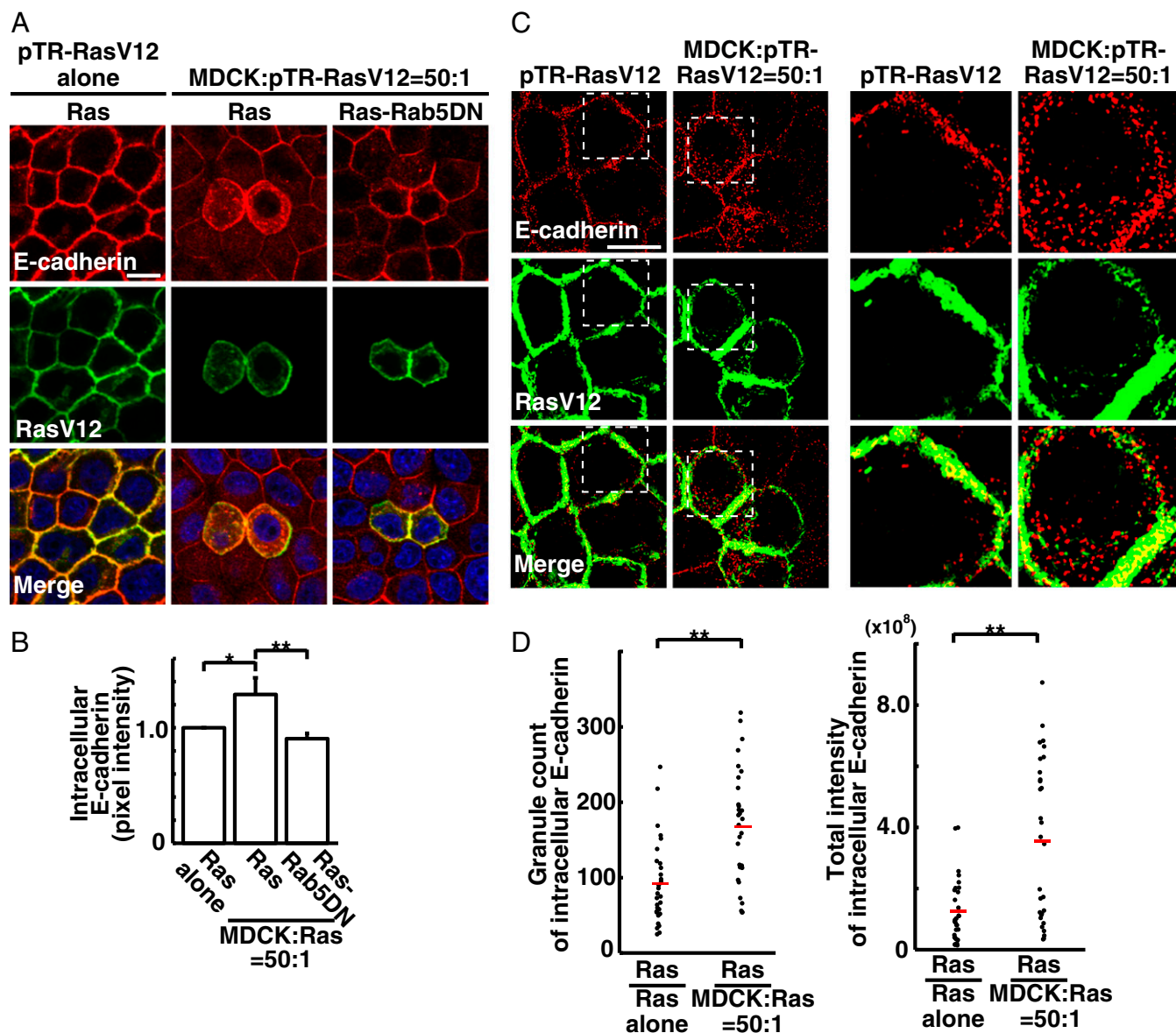


Fig. 5. Rab5-mediated endocytosis of E-cadherin is enhanced in RasV12-transformed cells surrounded by normal cells. (A and B) Effect of coexpression of Rab5DN on cytoplasmic E-cadherin puncta in RasV12-transformed cells that are surrounded by normal epithelial cells. MDCK-pTR GFP-RasV12 cells or MDCK-pTR GFP-RasV12-Rab5DN cells were mixed with normal MDCK cells or cultured alone on collagen gels. (A) Cells were fixed after 16-h incubation with tetracycline and stained with anti-E-cadherin antibody (red) and Hoechst (blue). (Scale bar, 10 μ m.) (B) Quantification of the intracellular fluorescence intensity of E-cadherin. Data are mean \pm SD from four independent experiments. * P < 0.05, ** P < 0.02; n = 126, 128, and 127 cells. (C and D) Superresolution microscopic analyses of intracellular E-cadherin puncta. (C) Fluorescence images of intracellular E-cadherin puncta (red). Cells were fixed after 16-h incubation with tetracycline and stained with anti-E-cadherin antibody (red). The area in the white dashed box is shown at 2.5-fold magnification (Right). (Scale bar, 10 μ m.) (D) Quantification of intracellular E-cadherin. The number of E-cadherin granules (Left) or the total fluorescence intensity (Right) in each RasV12 cell was depicted as a dot. The red bars indicate the mean of the results. ** P < 0.01; n = 30 cells for each condition.

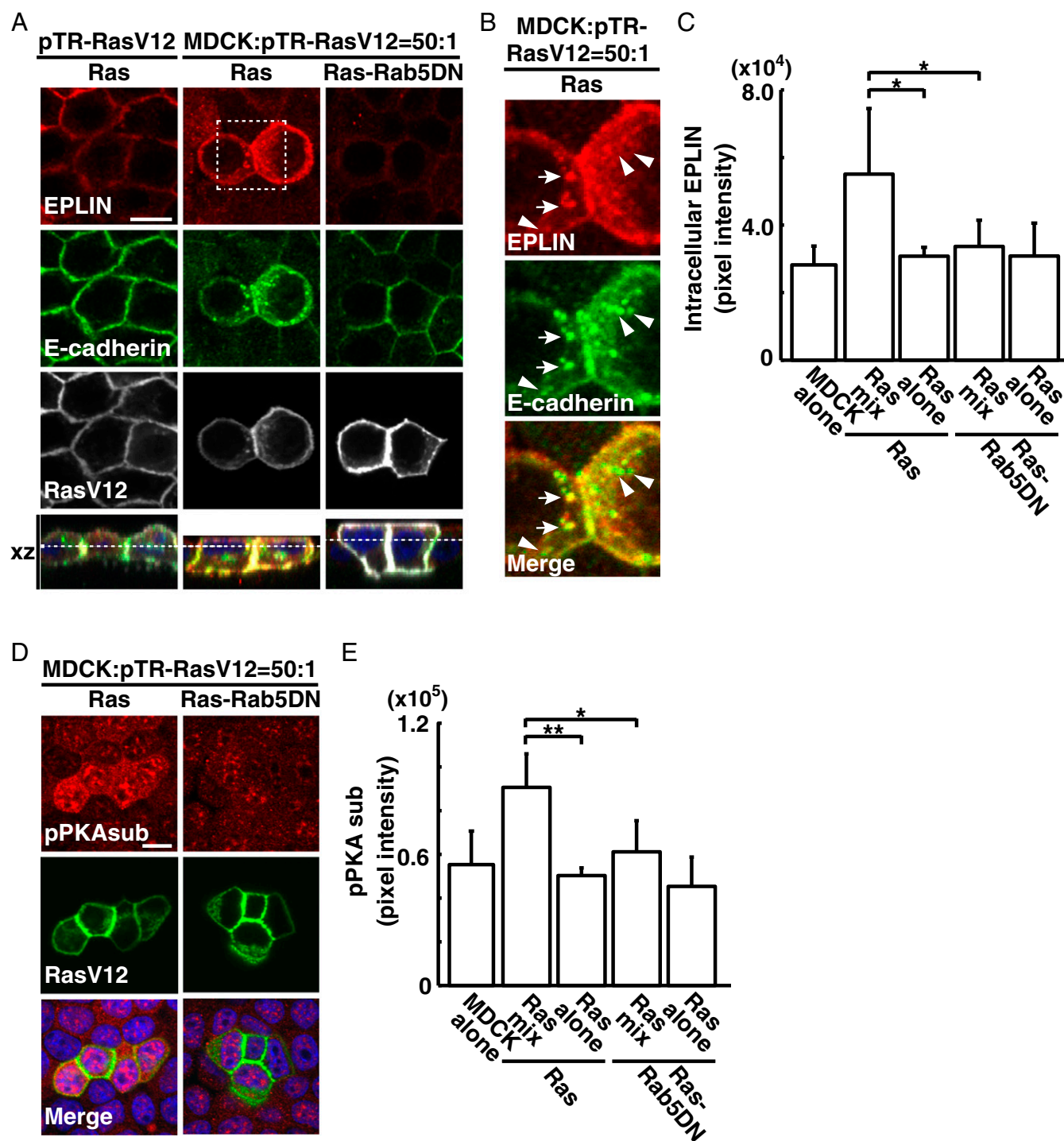


Fig. 6. Rab5-regulated endocytosis acts upstream of the EPLIN/pKA pathway in the interaction between normal and transformed epithelial cells. (A–C) Effect of coexpression of Rab5DN on EPLIN accumulation in RasV12-transformed cells that are surrounded by normal epithelial cells. MDCK-pTR GFP-RasV12 cells or MDCK-pTR GFP-RasV12-Rab5DN cells were mixed with normal MDCK cells or cultured alone on collagen gels. (A) Cells were fixed after 16-h incubation with tetracycline and stained with anti-EPLIN (red) and anti-E-cadherin (green) antibodies and Hoechst (blue). GFP-RasV12 is shown in white. The white dashed line in the xz panel denotes the cross-sections represented in the above xy panels. The confocal images are xy images if not indicated. (Scale bar, 10 μ m.) The area in the white dashed box is shown at 2.3-fold magnification in B. (B) Arrows and arrowheads indicate cytoplasmic E-cadherin puncta with or without colocalization with EPLIN, respectively. (C) Quantification of the intracellular fluorescence intensity of EPLIN. Data are mean \pm SD from four independent experiments. * $P < 0.05$; $n = 137, 133, 138, 134,$ and 135 cells. (D and E) Effect of coexpression of Rab5DN on PKA-catalyzed phosphorylation in RasV12 cells surrounded by normal cells. (D) Cells were incubated as described above except tetracycline treatment for 20 h, and stained with anti-phospho-PKA substrate (pPKAsub) antibody (red) and Hoechst (blue). (Scale bar, 10 μ m.) (E) Quantification of the fluorescence intensity of pPKAsub. Data are mean \pm SD from three independent experiments. * $P < 0.05$, ** $P < 0.02$; $n = 103, 101, 101, 101,$ and 101 cells.

cytoplasmic region (Fig. 6 *A–C*) (7). Intracellularly accumulated EPLIN was only partially colocalized with the cytoplasmic E-cadherin puncta (Fig. 6*B*). The quantitative immunofluorescence analysis showed that 45% of EPLIN⁺ vesicles were not costained with E-cadherin ($n = 1,800$ EPLIN⁺ intracellular puncta). In addition, 81% of Rab7/E-cadherin double-positive vesicles were EPLIN[−] [$n = 100$ Rab7 (+) E-cadherin (+) puncta], suggesting that during endocytosis, EPLIN often dissociates from the E-cadherin complex. We then found that coexpression of Rab5DN significantly suppressed intracellular accumulation of EPLIN in RasV12 cells surrounded by normal cells (Fig. 6 *A* and *C*). Addition of chlorpromazine also diminished the accumulation of EPLIN (Fig. *S7A*). Furthermore, PKA activity was elevated in RasV12 cells surrounded by normal cells (Fig. 6 *D* and *E*) (7), but was significantly suppressed by coexpression of Rab5DN (Fig. 6 *D* and *E*). PKA inhibitor KT5720 significantly suppressed apical extrusion of RasV12 cells (Fig. *S7B*), indicating a crucial role of PKA in this process. In contrast, knockdown of EPLIN did not affect Rab5 accumulation (Fig. *S7C* and *D*). Collectively, these data suggest that Rab5-regulated endocytosis functions upstream of the EPLIN/PKA pathway.

EDAC from the Surrounding Normal Cells Induces Rab5 Accumulation in RasV12-Transformed Cells. We have previously reported that normal epithelial cells can recognize and actively eliminate the neighboring transformed cells through dynamic regulation of the cytoskeletal protein filamin, a process called EDAC (6). Accumulation of filamin in normal cells at the boundary with the neighboring transformed cells induces up-regulation of EPLIN in the transformed cells, eventually leading to their apical extrusion (7). In addition to filamin, E-cadherin in the surrounding normal cells also plays a vital role in apical extrusion of transformed cells (1). We found that when RasV12-transformed cells were surrounded by filamin-knockdown cells, accumulation of Rab5 was significantly suppressed (Fig. 7 *A–C*). Similarly, knockdown of E-cadherin in the surrounding normal cells also substantially diminished Rab5 accumulation in RasV12 cells (Fig. 7 *D* and *E*). Taken together, these data suggest that EDAC from normal epithelial cells plays a positive role in the regulation of endocytosis in the neighboring transformed cells.

Discussion

In this study, we have presented several lines of evidence indicating that Rab5-mediated endocytosis and the following transport to late endosomes are enhanced and play an active role in the apical elimination of the transformed cells. First, Rab5 is accumulated in RasV12 cells surrounded by normal cells where clathrin-dependent endocytosis is elevated. Second, addition of chlorpromazine or coexpression of Rab5DN profoundly suppresses apical extrusion of transformed cells. Third, in the EVL of zebrafish embryos, Rab5 is accumulated in v-Src-expressing epithelial cells, and coexpression of Rab5DN significantly attenuates their apical delamination. Fourth, Tsg101 and Rab7 are accumulated in RasV12 cells surrounded by normal cells. Fifth, addition of bafilomycin or knockdown of Tsg101 suppresses the elimination of transformed cells. Collectively, these data demonstrate that endocytosis is a crucial regulator for the interaction between normal and transformed epithelial cells, which substantially influences the behavior and fate of transformed cells in the cell community.

It has been previously reported in *Drosophila* that endocytosis is involved in the interaction between normal and transformed epithelial cells and affects the outcome of cell competition between them (12–14). For example, Igaki et al. reported that in the eye disk epithelium, Rab5-mediated endocytosis is enhanced in *scribble* mutant cells that are surrounded by wild-type cells and that coexpression of Rab5DN strongly suppresses apoptosis of *scribble* mutant cells (13). Moreno and Basler showed that overexpression of Rab5 in wild-type epithelial cells diminishes their apoptosis at the interface with the neighboring myc-overexpressing cells in the wing

discs (12). In addition, Ballesteros-Arias et al. reported that knockdown of Rab5 can induce cell competition in the wing discs in a cell-context-dependent manner (14). Herein, we demonstrate that endocytosis plays an important role in cell competition between normal and transformed epithelial cells in vertebrates as well. The data from *Drosophila* show that the Jun N-terminal kinase (JNK) pathway acts downstream of endocytosis (13, 14); however, we have not observed the noncell-autonomous activation of this pathway, suggesting the involvement of distinct cellular processes or signaling pathways in the elimination of RasV12-transformed cells. Here, we have revealed three molecular mechanisms whereby endocytosis regulates cell competition: (i) involvement of EDAC: filamin accumulation in the surrounding normal cells induces elevated endocytosis of transformed cells in a non-cell-autonomous fashion; (ii) dynamic modulation of cell–cell adhesions: endocytosis of E-cadherin is enhanced in transformed cells surrounded by normal cells; and (iii) specific up-regulation of E-cadherin endocytosis at the interface: endocytosis of other cell-adhesion proteins, such as occludin and integrin, is not enhanced. Hence, these findings provide insights into still enigmatic phenomena of cell competition and shed light on the unexplored events at the initial stage of carcinogenesis.

We show that E-cadherin is one of the cargo proteins via Rab5-mediated endocytosis, as reported by other groups using the different experimental conditions (23–26). In addition to E-cadherin, localization of EPLIN is also regulated by endocytosis. In a steady status, EPLIN forms a protein complex with E-cadherin via β -catenin and α -catenin at cell–cell adhesions (27). However, in RasV12 cells surrounded by normal cells, EPLIN translocates into the cytoplasm where it just partially colocalizes with internalized E-cadherin (Fig. 6*B*). Together with other data, these results show that it is plausible that after internalization, EPLIN dissociates from the E-cadherin complex, which is consistent with the previous reports that a certain fraction of catenins dissociates from E-cadherin during endocytosis (29–31). Free EPLIN would then form a complex with other binding partners, thereby activating signaling pathways, such as PKA, and promoting the apical extrusion event (Fig. 7*F*). However, it is likely that there are a number of proteins, in addition to E-cadherin, of which endocytosis is modulated by Rab5 in transformed cells; there could be additional adhesion molecules and signaling pathways that are regulated by endocytosis, and the sum of those overall orchestrated effects would modulate the behavior of RasV12-transformed cells.

In addition to PKA, EPLIN functions upstream of myosin-II; the activity of myosin-II is elevated in RasV12-transformed cells in a noncell-autonomous fashion, which is regulated by EPLIN (7). The activation of myosin-II generates pulling-forces exerted at the interface between normal and transformed cells, thereby promoting apical extrusion of the transformed cells (6). Along the same line, recent studies have revealed that physical forces or mechanical tensions can play a crucial role in cell competition between normal and transformed epithelial cells (32, 33). Thus, the EPLIN–myosin-II pathway could be another endocytosis-mediated regulatory mechanism for apical extrusion.

Another question is: What are the molecular mechanisms that induce the noncell-autonomous up-regulation of endocytosis? Our results suggest that EDAC from the surrounding normal cells regulates the elevation of Rab5-mediated endocytosis, implying that transformed cells sense the modulated conditions in the neighboring normal cells and accordingly respond to them by activating the endocytic pathways. To explore the molecular mechanisms whereby EDAC induces Rab5-mediated endocytosis, we have examined the effect of various inhibitors on Rab5 accumulation in RasV12-transformed cells that are surrounded by normal cells (Table S1). Among the tested inhibitors, the MEK inhibitor U0126 diminishes accumulation of Rab5. MEK is a component of the MAPK signaling pathway that is the cell-autonomous downstream target of Ras. Thus, this result suggests that among the downstream pathways of Ras, the MAPK pathway is involved in

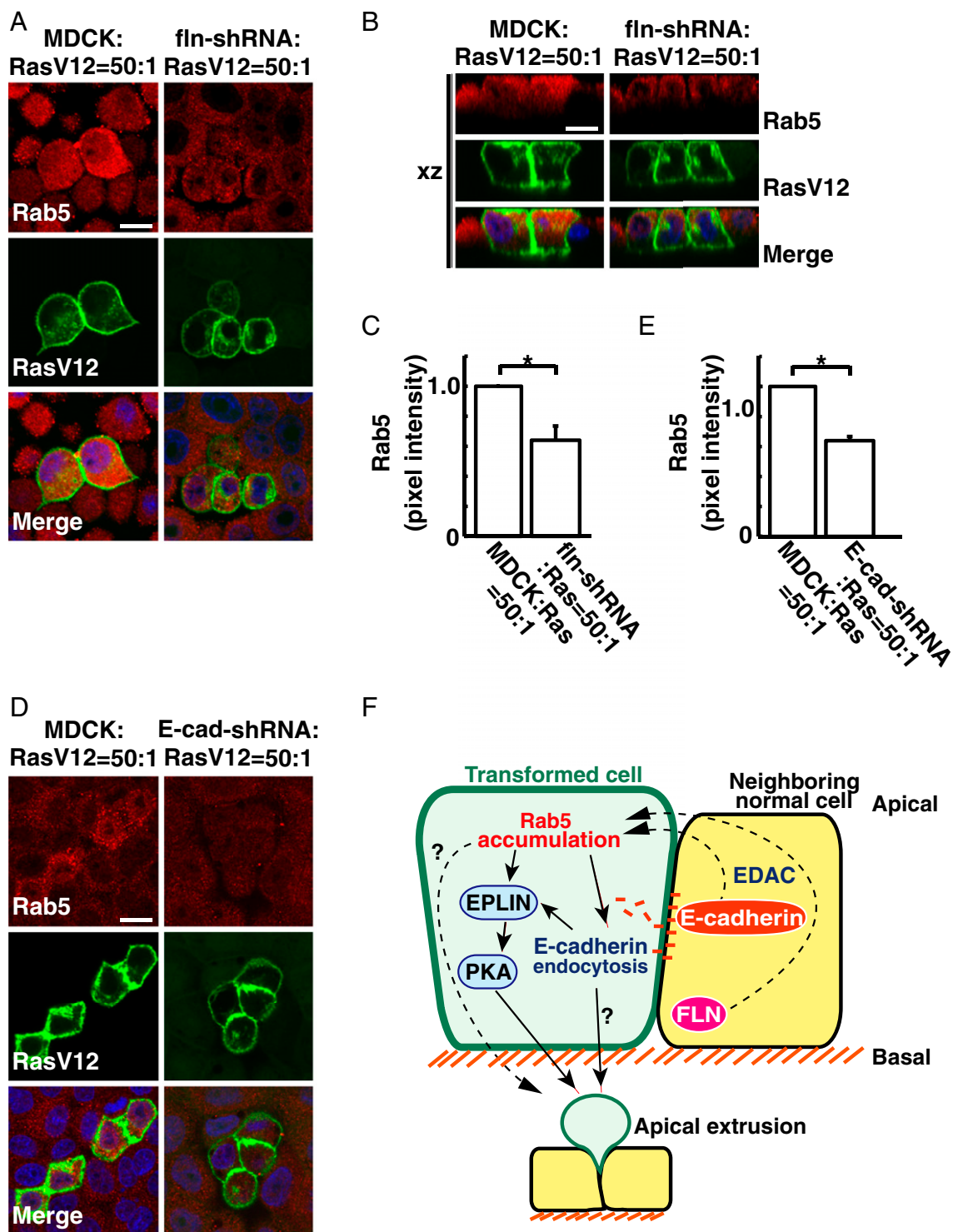


Fig. 7. EDAC from the surrounding normal cells induces Rab5 accumulation in RasV12-transformed cells. (A–C) Effect of filamin-knockdown in normal cells on Rab5 accumulation in the neighboring RasV12-transformed cells. MDCK-pTR GFP-RasV12 cells were mixed with normal or filamin-knockdown MDCK cells and incubated with tetracycline for 16 h. (A and B) Cells were stained with anti-Rab5 antibody (red) and Hoechst (blue). (Scale bars, 10 μ m.) (C) Quantification of the fluorescence intensity of Rab5 in RasV12 cells. Data are mean \pm SD from three independent experiments. $*P < 0.05$; $n = 101$ and 93 cells. Values are expressed as a ratio relative to MDCK:Ras = 50:1. (D and E) Effect of E-cadherin knockdown in normal cells on Rab5 accumulation in the neighboring RasV12 cells. MDCK-pTR GFP-RasV12 cells were mixed with normal or E-cadherin knockdown MDCK cells and incubated with tetracycline for 16 h. (D) Cells were stained with anti-Rab5 antibody (red) and Hoechst (blue). (Scale bar, 10 μ m.) (E) Quantification of the fluorescence intensity of Rab5 in RasV12 cells. Data are mean \pm SD from three independent experiments. $*P < 0.05$; $n = 104$ and 98 cells. Values are expressed as a ratio relative to MDCK:Ras = 50:1. (F) A schematic model of the molecular regulation at the interface between transformed and the neighboring normal cells. When transformed cells are surrounded by normal cells, Rab5-mediated endocytosis is up-regulated in the transformed cells, leading to E-cadherin endocytosis, EPLIN accumulation, and PKA activation, thereby inducing apical elimination of transformed cells. In addition, EDAC from the neighboring normal cells, via E-cadherin-based cell–cell adhesions and filamin accumulation, positively regulates Rab5-mediated endocytosis.

the accumulation of Rab5. Still, it is not clear at present whether the MAPK pathway is also directly involved in the further downstream, noncell-autonomous processes. The other inhibitors have no effect on Rab5 accumulation (Table S1); thus, so far we have been unable to identify a key signaling pathway that plays a direct role in the up-regulation of Rab5-mediated endocytosis, which needs to be identified in future studies.

Several studies have reported that Rab5 is involved in cancer development and progression. Elevated expression of Rab5 is observed in various types of cancer (34–36), and Rab5 plays a positive role in tumor invasion/metastasis (37–39). Our results demonstrate that Rab5-mediated endocytosis is also involved in the apical extrusion of transformed cells from the normal epithelium at the initial stage of carcinogenesis. Thus, what is the fate of the apically extruded transformed cells? For transformed cells to metastasize into distant organs, they have to leave the epithelium basally and invade the underlying matrix. In turn, in the apical lumen, cells are generally subjected to harsh physical conditions (e.g., stool, urine, digestive fluids). Hence, apical extrusion is the opposite direction from metastasis, and thus can be regarded as a cancer-preventive process. Indeed, our recent result using a cell competition mouse model has demonstrated that apically extruded cells do not form a tumorous mass and eventually disappear from the intestine tissues (40), although the pathological consequence of apical extrusion of transformed cells still remains controversial at present (4). To fully address

this issue, the fate of apically extruded cells needs to be extensively studied in various epithelial tissues in vivo. By further clarifying the functional significance and molecular mechanisms of the Rab5-mediated endocytosis, it is expected that we could develop a novel type of cancer preventive medicine.

Materials and Methods

A complete description of the methods is provided in *SI Materials and Methods*. This description includes antibodies and materials, cell culture, immunofluorescence and Western blotting, transferrin-uptake assay, superresolution microscopy, microinjection, and confocal imaging of zebrafish embryos, and data analyses.

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