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EPLIN is a crucial regulator for extrusion of RasV12-transformed cells

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ABSTRACT

At the initial stage of carcinogenesis, a mutation occurs in a single cell within a normal epithelial layer. We have previously shown that RasV12-transformed cells are apically extruded from the epithelium when surrounded by normal cells. However, the molecular mechanisms underlying this phenomenon remain elusive. Here, we demonstrate that Cav-1-containing microdomains and EPLIN (also known as LIMA1) are accumulated in RasV12-transformed cells that are surrounded by normal cells. We also show that knockdown of Cav-1 or EPLIN suppresses apical extrusion of RasV12-transformed cells, suggesting their positive role in the elimination of transformed cells from epithelia. EPLIN functions upstream of Cav-1 and affects its enrichment in RasV12-transformed cells that are surrounded by normal cells. Furthermore, EPLIN regulates non-cell-autonomous activation of myosin-II and protein kinase A (PKA) in RasV12-transformed cells. In addition, EPLIN substantially affects the accumulation of filamin A, a vital player in epithelial defense against cancer (EDAC), in the neighboring normal cells, and vice versa. These results indicate that EPLIN is a crucial regulator of the interaction between normal and transformed epithelial cells.

KEY WORDS: Apical extrusion, Ras, Epithelial cell, Cav-1, EPLIN

INTRODUCTION

More than 80% of malignant tumors arise from the epithelium in humans. During the process of carcinogenesis, epithelial cells progressively acquire multiple oncogenic mutations to become invasive malignant cancers. However, the initial step of this process is not clearly understood and remains a ‘black box’ in cancer biology; when the first mutation occurs in a single cell within epithelia, what happens next? Recent studies have revealed that Ras-, Src- or ErbB2-transformed cells are apically extruded from a monolayer of normal epithelial cells (Grieve and Rabouille, 2014; Hogan et al., 2009; Kajita et al., 2010; Leung and Brugge, 2012; Wu et al., 2014) and that perturbation of proper epithelial organization suppresses this anti-oncogenic process (Hogan et al., 2009; Kajita et al., 2014). During the process of apical extrusion, various signaling pathways are activated in both normal and transformed cells in a non-cell-autonomous fashion. For example, myosin-II and protein kinase A (PKA) are activated in transformed cells when they are surrounded by normal cells (Anton et al., 2014; Hogan et al., 2009; Kajita et al., 2010). In addition, filamin and vimentin are accumulated in normal cells at the interface with the neighboring transformed cells, and they play an active role in the elimination of the transformed cells from the epithelium (Kajita et al., 2014). These results imply that at the early stage of carcinogenesis, normal epithelial cells act as ‘immunity’ against transformed cells, and we have named this process ‘epithelial defense against cancer’ (EDAC) (Kajita et al., 2014). In Drosophila, it has been also shown that normal and transformed epithelial cells often compete with each other for survival, and this phenomenon is called cell competition (Amoyel and Bach, 2014; Baker, 2011; Moreno and Rhiner, 2014). However, detailed molecular mechanisms regulating these non-cell-autonomous processes still remain largely unknown.

Caveolae are one of the lipid rafts with characteristic flask-shaped invaginations of the plasma membrane where cholesterol, glycolipids and sphingomyelin are enriched (Coskun and Simons, 2010; Staubach and Hanisch, 2011). Caveolin-1 (Cav-1), a major component of caveolae, plays a vital role in the function of caveolae, including the regulation of endocytosis and signaling pathways (Lajoie et al., 2009; Parton and Simons, 2007). Several reports suggest that reduced expression of Cav-1 is observed in breast, colon and ovarian cancers (Bender et al., 2000; Sloan et al., 2004; Wiechen et al., 2001), although its functional role in oncogenesis is not clearly understood.

Epithelial protein lost in neoplasm (EPLIN, also known as LIMA1) was originally identified as a protein of which expression is often downregulated or lost in various types of cancers (Jiang et al., 2008; Maul and Chang, 1999; Zhang et al., 2010). EPLIN interacts with actin and regulates its dynamics by cross-linking and stabilizing actin filaments (Maul et al., 2003). In addition, EPLIN interacts with α-catenin, thereby linking the cadherin–catenin complex and actin filaments, and thus plays a crucial role in the establishment of adherens junctions (Abe and Takeichi, 2008).

In this study, we show that Cav-1 and EPLIN accumulate in RasV12-transformed cells that are surrounded by normal cells. EPLIN regulates Cav-1 and multiple signaling pathways, and positively regulates the apical extrusion of the transformed cells.
RESULTS

Cav-1-containing microdomains are enriched in RasV12-transformed cells surrounded by normal cells and play a crucial role in the apical extrusion of transformed cells

We started this study by examining the possible involvement of lipid rafts in the interaction between normal and transformed epithelial cells. To this end, we first performed immunofluorescence analyses using anti-Cav-1 antibody. We found that Cav-1 accumulated at both the apical and lateral membrane domains in MDCK-pTR GFP–RasV12 cells (hereafter referred to as RasV12 cells) surrounded by normal Madin-Darby canine kidney (MDCK) cells (Fig. 1A,B). Co-immunostaining with anti-ZO-1 antibody showed that Cav-1 was often localized at the cell–cell contact sites basal to tight junctions (supplementary material Fig. S1A). By contrast, gp135 (also known as podocalyxin), an apical marker protein, was localized exclusively at the apical membrane domain (supplementary material Fig. S1B), indicating that the accumulation of Cav-1 is not merely due to the expansion of apical membrane domains in RasV12 cells surrounded by normal cells. By contrast, when RasV12 cells alone were cultured, Cav-1 localized mainly at the apical membrane, and the immunofluorescence intensity was weaker than that in RasV12 cells surrounded by normal cells (Fig. 1A,B). In addition, Cav-1 was also accumulated at both the apical and lateral membrane domains in Src-transformed cells surrounded by normal cells (supplementary material Fig. S1C). Cav-1 is localized at caveolae where various molecules are also enriched, including Forssman antigen (a glycolipid heterophil protein) and sphingomyelin (Zurzolo et al., 1994). Immunofluorescence with anti-Forssman antibody showed that glycolipids co-accumulated with Cav-1 at the lateral membrane domain in RasV12 cells surrounded by normal cells (Fig. 1C,D). Similarly, staining with fluorescence-conjugated lysenin (a sphingomyelin-specific binding protein) demonstrated that sphingomyelin also co-localizes with Cav-1 in RasV12 cells surrounded by normal cells (supplementary material Fig. S1D,E). These data indicate that Cav-1-containing microdomains are enriched in transformed cells surrounded by normal cells. Next, we examined the effect of disruption of lipid rafts on the fate of RasV12-transformed cells surrounded by normal cells. DL-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) and methyl-β-cyclodextrin (MbCD) are the chemical compounds that disrupt lipid rafts by blocking the biosynthesis of glucosylceramide and by removing cholesterol from the plasma membrane, respectively. Addition of PDMP or MbCD diminished the accumulation of Cav-1 (supplementary material Fig. S2A; data not shown), and significantly suppressed the apical extrusion of RasV12-transformed cells surrounded by normal cells (Fig. 2A–D). Interestingly, the addition of PDMP...
or MbCD also promoted the basal protrusion of transformed cells (Fig. 2A,C; arrowheads). Furthermore, when Cav-1 was knocked down in RasV12 cells (Fig. 2E; supplementary material Fig. S2B,C), accumulation of Forssman antigen and sphingomyelin was severely diminished (supplementary material Fig. S2D,E), and apical extrusion was significantly suppressed (Fig. 2F,G; supplementary material Fig. S2F).

Collectively, these results suggest that Cav-1-containing microdomains are the crucial regulator for the behavior of transformed cells that are surrounded by normal cells.

**EPLIN also plays an active role in the apical extrusion of RasV12-transformed cells**

To understand the molecular mechanisms of Cav-1-mediated apical extrusion, we performed immunoprecipitation using an anti-Cav-1 antibody with cell lysates from three different culture conditions – normal cells alone, RasV12 cells alone and a mixture of normal and RasV12 cells. We found that multiple proteins were co-immunoprecipitated with Cav-1 specifically under the mixed culture condition (Fig. 3A). Mass spectrometric analysis revealed that one of the Cav-1-binding proteins was EPLIN (Fig. 3A; arrowheads). We confirmed by western blotting that EPLIN binds to Cav-1 predominantly when normal and RasV12 cells interact with each other (Fig. 3B; supplementary material Fig. S3A). We then examined the subcellular localization of EPLIN by immunofluorescence. In RasV12 cells surrounded by normal cells, EPLIN was accumulated at both the apical and lateral membrane domains where Cav-1 was partially colocalized (Fig. 3C). In addition, EPLIN was also localized at the cytosol and intercellular regions between RasV12 cells where Cav-1 was absent (Fig. 3C; arrowheads). By contrast, when RasV12 cells were cultured alone, EPLIN was localized at the lateral membrane domain to a lesser extent (Fig. 3C). Comparable non-cell-autonomous accumulation of EPLIN was also observed in Src-transformed cells (supplementary material Fig. S3B). EPLIN was shown to interact with the α-catenin–β-catenin complex (Abe and Takeichi, 2008). We found that comparable accumulation of α-catenin or β-catenin was not observed in RasV12 cells, except at the cell–cell contact sites between RasV12 cells (supplementary material Fig. S3C), suggesting that accumulation of EPLIN might be regulated independently of the cell–cell adhesion complex.

Next, we established RasV12 cells stably expressing EPLIN short hairpin (sh)RNA (Fig. 4A; supplementary material Fig. S3D,E). EPLIN knockdown in RasV12 cells significantly suppressed apical extrusion (Fig. 4B,C; supplementary material Fig. S3F,G), indicating that EPLIN also plays a crucial role in the elimination of the transformed cells.
EPLIN functions upstream of Cav-1

Furthermore, we analyzed the functional relationship between Cav-1 and EPLIN. We found that accumulation of EPLIN was not significantly affected by Cav-1 knockdown in RasV12 cells (Fig. 5A,B). Consistently, addition of PDMP did not substantially influence the accumulation of EPLIN (supplementary material Fig. S4A). By contrast, when EPLIN was knocked down in RasV12 cells, accumulation of Cav-1 at the lateral membrane domain was substantially suppressed, and patchy localization was observed at the apical membrane domain (Fig. 5C,D). EPLIN knockdown also suppressed accumulation of lysenin (supplementary material Fig. S4B), suggesting that EPLIN functions, at least partially, upstream of Cav-1-containing microdomains and regulates their localization in transformed cells surrounded by normal cells. EPLIN knockdown did not substantially affect the localization of gp135 (supplementary material Fig. S4C), suggesting that the effect on Cav-1 is not due to perturbation in cell morphology or cell polarity.

EPLIN is a crucial regulator of the activation and/or localization of various molecules in both RasV12-transformed and the neighboring normal cells

During the apical extrusion of transformed cells, various signaling pathways are activated, which plays a positive role in this process (Fujita, 2011; Hogan et al., 2011). To examine the functional involvement of Cav-1 or EPLIN in these signaling pathways, we examined the effect of various inhibitors on the apical extrusion and on the localization of Cav-1 or EPLIN (supplementary material Table S1). Among the signaling pathways downstream of active Ras, the MAPK pathway plays a crucial role in the apical extrusion of RasV12-transformed cells (Hogan et al., 2009). Upon addition of the MEK inhibitor U0126, accumulation of Cav-1 at the lateral membrane domain was diminished, and Cav-1 was predominantly localized at the apical membrane domain in a punctate manner (Fig. 6A). U0126 also induced punctate accumulation of EPLIN at the apical side of RasV12 cells (Fig. 6B). By contrast, the PI3 kinase inhibitor LY294002 did not substantially affect their localization (Fig. 6A,B). These data show that activity of the MAPK pathway regulates non-cell-autonomous enrichment of Cav-1 and EPLIN. Cytochalasin D, which blocks actin polymerization, strongly perturbed the enrichment of Cav-1 and EPLIN (Fig. 6A,B), indicating that the dynamics of actin filaments are required for the establishment and/or maintenance of their accumulation. In previous studies, we have reported that activity of myosin-II and PKA is enhanced in RasV12-transformed cells surrounded by normal cells (Anton et al., 2014; Hogan et al., 2009) (also shown in Fig. 7A,D; supplementary material Fig. S4D). The myosin-II inhibitor blebbistatin diminished Cav-1 localization at the lateral membrane domain, and induced punctate accumulation of EPLIN at the apical side of RasV12 cells (Fig. 6B). By contrast, the PI3 kinase inhibitor LY294002 did not substantially affect their localization (Fig. 6A,B). These data show that activity of the MAPK pathway regulates non-cell-autonomous enrichment of Cav-1 and EPLIN. Cytochalasin D, which blocks actin polymerization, strongly perturbed the enrichment of Cav-1 and EPLIN (Fig. 6A,B), indicating that the dynamics of actin filaments are required for the establishment and/or maintenance of their accumulation. In previous studies, we have reported that activity of myosin-II and PKA is enhanced in RasV12-transformed cells surrounded by normal cells (Anton et al., 2014; Hogan et al., 2009) (also shown in Fig. 7A,D; supplementary material Fig. S4D). The myosin-II inhibitor blebbistatin diminished Cav-1 localization at the lateral membrane domain, and induced punctate accumulation of EPLIN at the apical side (Fig. 6A,B). PKA inhibitor KT5720 did not affect the accumulation of Cav-1 or EPLIN (Fig. 6A,B).

Next, we examined whether EPLIN or Cav-1 regulates the activity of these signaling pathways or vice versa. EPLIN knockdown in RasV12-transformed cells significantly suppressed the activation of myosin-II (Fig. 7A,B). The height of RasV12 cells along the apicobasal axis increases when they are surrounded by normal cells (reflecting the increased contractility), which occurs in a myosin-II-dependent manner (Hogan et al., 2009). Consistently, we found that EPLIN knockdown significantly reduced the height of RasV12 cells.
that were surrounded by normal cells (supplementary material Fig. S4E). By contrast, Cav-1 knockdown did not affect myosin-II activity (Fig. 7C). In addition, expression of a dominant-negative form of myosin light chain (MLCAA) did not influence EPLIN or Cav-1 enrichment (supplementary material Fig. S4F,G). Similarly, the activity of PKA was substantially repressed by EPLIN knockdown, but not by Cav-1 knockdown (Fig. 7D–F). Collectively, these data indicate that EPLIN plays a crucial role in the non-cell-autonomous upregulation of myosin-II and PKA, independently of Cav-1 (Fig. 8E).

We have recently reported that filamin accumulates in normal cells at the interface with RasV12-transformed cells and plays a vital role in EDAC (Kajita et al., 2014). We found that when filamin A was knocked down in the surrounding normal cells, the enrichment of EPLIN or Cav-1 in RasV12-transformed cells was substantially suppressed (Fig. 8A,B). In addition, when EPLIN or Cav-1 was knocked down in RasV12-transformed cells, the accumulation of filamin in the surrounding normal cells was significantly diminished (Fig. 8C,D). These data indicate that there exist mutual regulatory mechanisms between EPLIN

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**Fig. 4.** EPLIN also plays a crucial role in the apical extrusion of RasV12-transformed cells. (A) Establishment of RasV12-transformed cells stably expressing EPLIN shRNA1. Knockdown of EPLIN protein was analyzed by western blotting (WB) using the anti-EPLIN antibody. Equal protein loading was confirmed using the anti-GAPDH antibody. (B) Knockdown of EPLIN suppresses the apical extrusion of RasV12-transformed cells. Normal MDCK cells were mixed with MDCK-pTR GFP–RasV12 cells (upper panels, green) or MDCK-pTR GFP–RasV12-EPLIN shRNA1 cells (lower panels, green), and incubated with tetracycline for 24 hours. Cells were stained with Hoechst 33342 (blue), anti-EPLIN antibody (red) and Alexa-Fluor-647-conjugated phalloidin (white). Scale bars: 10 μm. (C) Quantification of the apical extrusion for B. Data are the mean±s.d. from three independent experiments. *P<0.05; n=89 and 102 cells.

**Fig. 5.** EPLIN functions upstream of Cav-1. (A,C) Immunofluorescence images of EPLIN in Cav-1-knockdown RasV12-transformed cells (A) or of Cav-1 in EPLIN-knockdown RasV12-transformed cells (C). Cells were stained with Hoechst 33342 (blue) and anti-Cav-1 (red) and anti-EPLIN (white) antibodies. Arrowheads indicate patchy localization of Cav-1. Scale bars: 10 μm. (B,D) Quantification of EPLIN enrichment in Cav-1-knockdown RasV12-transformed cells (B) or Cav-1 enrichment in EPLIN-knockdown RasV12-transformed cells (D). Data are the mean±s.d. from two (B) or three (D) independent experiments. *P<0.01; n=64 and 62 cells (B); 79 and 119 cells (D).
and Cav-1 in RasV12-transformed cells and filamin in the surrounding normal cells (Fig. 8E).

**DISCUSSION**

In this study, we present several lines of evidence demonstrating that EPLIN is a crucial regulator of the extrusion of RasV12-transformed cells (Fig. 8E). First, EPLIN is accumulated at the apical and lateral membrane domains as well as in the cytosol in RasV12-transformed cells when they are surrounded by normal cells. Second, EPLIN knockdown in RasV12-transformed cells suppresses their apical extrusion from the monolayer of normal epithelial cells. Third, EPLIN functions upstream of Cav-1 and affects its localization in RasV12-transformed cells surrounded by normal cells. Forth, EPLIN regulates non-cell-autonomous activation of myosin-II and PKA in RasV12-transformed cells. Fifth, EPLIN in RasV12-transformed cells substantially affects the accumulation of filamin in the neighboring normal cells and vice versa. In previous studies, we have shown that myosin-II is activated in transformed cells that are surrounded by normal cells and that enhanced myosin-II activity leads to increased cellular elasticity in the transformed cells, which induces the accumulation of the mechanosensor filamin in the neighboring normal cells (Hogan et al., 2009; Kajita et al., 2014). Collectively, our data suggest that EPLIN functions upstream of myosin-II in this process, thereby promoting apical extrusion through filamin. EPLIN interacts with &egr;-catenin, and thus links the cadherin–catenin complex to F-actin (Abe and Takeichi, 2008). However, &egr;-catenin is not colocalized with EPLIN at the apical membrane domain or in the cytosol, suggesting that the non-cell-autonomous accumulation of EPLIN occurs by an &egr;-catenin-independent mechanism. In future studies, upstream or downstream regulators of EPLIN need to be explored to further reveal the molecular mechanisms for apical extrusion of transformed cells.

We also demonstrate that Cav-1-containing microdomains are non-cell-autonomously accumulated in transformed cells and that Cav-1 knockdown significantly suppresses apical extrusion of the transformed cells. PDMP treatment abolishes Cav-1 accumulation (supplementary material Fig. S2A) and Cav-1 knockdown diminishes the enrichment of Forssman antigen or lysenin (supplementary material Fig. S2D,E). In addition, EPLIN knockdown perturbs the accumulation of Cav-1 and lysenin (Fig. 5C,D; supplementary material Fig. S4B). These data indicate that Cav-1 and lipid rafts are co-regulated in transformed cells that are surrounded by normal cells. We have tried to identify molecules that function downstream of Cav-1, but knockdown of Cav-1 does not affect several tested signaling pathways including myosin-II or PKA (Fig. 7C,F). The data shown in Fig. 3A suggest that Cav-1 binds to multiple proteins when normal and transformed cells are co-cultured. Identification and analyses of these Cav-1-binding proteins would clarify how Cav-1-containing microdomains positively regulate the process of apical extrusion.

EPLIN was originally identified as a tumor suppressor protein, and downregulation of EPLIN expression was observed in various types of cancers (Jiang et al., 2008; Maul and Chang, 1999; Zhang et al., 2011). In addition, expression of Cav-1 is often reduced in multiple cancers (Bender et al., 2000; Sloan et al., 2004; Wiechen et al., 2001). However, the roles of EPLIN and Cav-1 in carcinogenesis still remain elusive. Our results demonstrate that knockdown of EPLIN or Cav-1 substantially suppresses apical extrusion of transformed cells, implying that the expression of these molecules is required for the elimination of newly emerging transformed cells from the epithelium. The functional involvement of EPLIN or Cav-1 at the initial stage of carcinogenesis needs to be determined in *in vivo* experimental systems in future studies. The elucidation of molecular mechanisms upstream or downstream of EPLIN and/
or Cav-1 would lead to the establishment of novel types of cancer prevention and treatment that enhance the ability of surrounding normal cells to fight against cancer cells or attenuate the defense of cancer cells against neighboring normal cells.

MATERIALS AND METHODS
Antibodies and materials
Mouse anti-EPLIN antibody was from Santa Cruz Biotechnology (Heidelberg, Germany). Rabbit anti-caveolin-1 antibody was from Abcam (Cambridge, UK). Mouse anti-GAPDH antibody was from Millipore (Darmstadt, Germany). Rabbit anti-phospho-myosin light chain 2 (ppMLC; Thr18/Ser19) and rabbit anti-phospho-(Ser/Thr) PKA substrate antibodies were from Cell Signaling Technology (Danvers, MA). Mouse anti-filamin antibody was from Sigma-Aldrich (Gillingham, Dorset, UK). Mouse anti-α-catenin, mouse anti-β-catenin and mouse anti-ZO-1 antibodies were from Life Technologies (Paisley, UK). Alexa-Fluor-568-conjugated anti-β-catenin or anti-ZO-1 antibody was prepared using Zenon H Mouse IgG Labeling Kit (Life Technologies) according to the manufacturer’s instructions. Alexa-Fluor-568- or -647-conjugated phalloidin (Life Technologies) was used at 1.0 U ml⁻¹. Alexa-Fluor-568- and -647-conjugated secondary antibodies were from Life Technologies. Hoechst 33342 (Life Technologies) was used at a dilution of 1:5000. Monomeric red fluorescent protein–lysenin (amino acids 161–298) was expressed in Escherichia coli and purified by affinity chromatography using TALON metal affinity resin (Clontech, Palo Alto, CA) as described previously (Ikenouchi et al., 2012). The culture supernatants of hybridoma cells producing anti-Forssman glycosphingolipid (clone 12B12) were used for immunofluorescence as described previously (Zinkl et al., 1996). Mouse anti-gp135 antibody was kindly provided by Dr. George K. Ojakian (SUNY Downstate Medical Center, NY). For immunofluorescence, all primary antibodies were used at 1:100, except for anti-Forssman antibody, which was used at 1:10, and all secondary antibodies were used at 1:200. The inhibitors bisindolylmaleimide (BIM)-I (10 μM), (S)-(−)-blebbistatin (30 μM), CK666 (100 μM), IPA-3 (40 μM), KT5720 (4 μM), LY294002 (10 μM), PP2 (20 μM) and Y27632 (10 μM) were from Millipore. Cytochalasin D (4 μM) and methyl-β-cyclodextrin (MbCD, 10 mM) were from Sigma-Aldrich. U0126 (10 μM) was from Promega (Madison, WI), and DL-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP, 30 μM) was from Cayman Chemical (Ann Arbor, MI).

Cell culture
MDCK and MDCK-pTR GFP–RasV12 cells were cultured as described previously (Hogan et al., 2009). To establish MDCK-pTR GFP–cSrcY527F cells, the cDNA of cSrcY527F was excised from pEGFP-N1–cSrcY527F (a kind gift from Dr Margaret Frame, Edinburgh Cancer Research Centre, UK) and was cloned into the Not1 site of pcDNA4/TO (Life Technologies). MDCK-pTR cells were then transfected with pcDNA/TO/cSrcY527F-GFP using Lipofectamine 2000 (Life Technologies), followed by selection in medium containing 5 μg ml⁻¹.
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Immunoprecipitation and western blotting

For immunoprecipitation, MDCK and MDCK-pTR GFP–RasV12 cells were trypsinized and plated at a density of 1.6×105 cells in 14.5-cm dishes (Greiner-Bio-One, Longwood, FL) and cultured at 37°C for 9–12 hours until a monolayer was formed. Tetracycline was added to induce RasV12 expression for 16 hours. Cells were washed with ice-cold PBS containing 1 mM Na2VO4 and lysed for 30 min in Triton X-100 lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM Na2VO4 and 10 mM NaF) or RIPA lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 1 mM Na2VO4 and 10 mM NaF) containing 5 μg ml−1 leupeptin, 1 mM phenylmethylsulfonyl fluoride and 7.2 trypsin inhibitor units of aprotinin. The former and the latter buffers were used for immunoprecipitation with anti-Cav-1 and anti-EPLIN antibodies, respectively. After centrifugation at 21,500 g at 4°C for 10 minutes, the supernatants were subjected to immunoprecipitation for 1 hour with Dynabeads® Protein G (Life Technologies) conjugated to anti-caveolin-1 antibody (5 μg) or anti-EPLIN antibody (5 μg).

Immunoprecipitated proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by SYPRO® Ruby protein gel staining (Life Technologies) or western blotting with the indicated antibodies. SYPRO Ruby protein staining was performed according to the manufacturer’s instructions. Western blotting was performed as described previously (Hogan et al., 2009). Primary antibodies were used at 1:1000. Stained gels and western blotting data were analyzed using ImageQuant™ LAS4010 (GE healthcare, Chalfont St Giles, UK). Protein bands were identified by mass spectrometry as described previously (Kajita et al., 2014).

Immunofluorescence

For immunofluorescence, MDCK-pTR GFP–RasV12 or MDCK-pTR GFP–SrfYaY527F cells were mixed with MDCK cells at a ratio of 1:50 and cultured on the collagen matrix as described previously (Hogan et al., 2009). The mixture of cells was incubated for 8–12 hours, followed by tetracycline treatment for 16 hours, except for analyses of apical extrusions that were examined after 24 hours of tetracycline treatment. Cells were fixed with 4% paraformaldehyde in PBS, except for filamin A protein, MDCK-pTR filamin A shRNA cells were incubated with 2 μg ml−1 tetracycline (Sigma-Aldrich) to induce the expression of GFP-RasV12 or GFP–SrfYaY527F. For Fig. 8A,B, to induce sufficient knockdown of filamin A protein, MDCK-pTR filamin A shRNA cells were incubated with 2 μg ml−1 tetracycline for 40 hours prior to co-incubation with MDCK-pTR GFP–RasV12 cells. For immunofluorescence, cells were plated onto collagen-gel-coated coverslips. Type-I collagen (Cellmatrix) was used at 1:1000. Stained gels and western blotting data were analyzed using ImageQuant™ LAS4010 (GE healthcare, Chalfont St Giles, UK). Protein bands were identified by mass spectrometry as described previously (Kajita et al., 2014).

Fig. 8. Mutual regulatory mechanisms between EPLIN–Cav-1 in RasV12-transformed cells and filamin in the surrounding normal cells. (A,B) Effect of knockdown of filamin A (Fln) in the surrounding normal cells on the enrichment of EPLIN (A) or Cav-1 (B) in RasV12-transformed cells. (C,D) Quantification of the effect of knockdown of EPLIN (C) or Cav-1 (D) in RasV12-transformed cells on the filamin accumulation in the neighboring normal cells. Data are the mean±s.d. from three independent experiments. *P<0.05, **P<0.005, ***P<0.001; n = 143 and 147 cells (A); 141 and 139 cells (B); 131 and 133 cells (C); 125 and 126 cells (D). (E) A schematic model of the molecular regulations at the interface between transformed and the surrounding normal cells. When transformed cells are surrounded by normal cells, EPLIN is upregulated in the transformed cells, leading to Cav-1 accumulation and activation of myosin-II and PKA. EPLIN and Cav-1 in transformed cells positively regulate the accumulation of filamin in the neighboring normal cells, which in turn affects the accumulation of EPLIN and Cav-1.

GTTTCCCTTTTTCC-3′ and 5′-TCGAGAAAAAGGAAACGTGTTGATAGAGAAATCTTGGATTCTCACTACAG-3′; EPLIN shRNA2, 5′-GATCCCCGCACACCAAGGAAACGTGTTGATAGAGAAATCTTGGATTCTCACTACAG-3′ and 5′-TCGAGAAAAAGGAAACGTGTTGATAGAGAAATCTTGGATTCTCACTACAG-3′) or EPLIN shRNA sequences (EPLIN shRNA1, 5′-GATCCC-CCCGAAGGTAAGAGAATCCATTCTTCACAGCCTGCCG-3′ and 5′-TCGAGAAAAAGGAAACGTGTTGATAGAGAAATCTTGGATTCTCACTACAG-3′) were cloned into the BglII and XhoI site of puSuer.neo+gfp (Oligoengine, Seattle, WA). MDCK-pTR GFP–RasV12 cells were transfected with puSuer.neo+gfp caveolin-1 shRNA or EPLIN shRNA using Lipofectamine 2000, followed by selection in medium containing 5 μg ml−1 blasticidin, 800 μg ml−1 G418 (Life Technologies) and 400 μg ml−1 zeocin. MDCK cells stably expressing filamin A shRNA in a tetracycline-inducible manner or MDCK-pTR GFP–RasV12 cells stably expressing mCherry–MLC-AA were established as described previously (Kajita et al., 2014). For tetracycline-inducible MDCK-pTR GFP–RasV12 or MDCK-pTR GFP–SrfYaY527F cell lines, 2 μg ml−1 tetracycline (Sigma-Aldrich) was used to induce the expression of GFP-RasV12 or GFP–SrfYaY527F. For Fig. 8A,B, to induce sufficient knockdown of filamin A protein, MDCK-pTR filamin A shRNA cells were incubated with 2 μg ml−1 tetracycline for 40 hours prior to co-incubation with MDCK-pTR GFP–RasV12 cells. For immunofluorescence, cells were plated onto collagen-gel-coated coverslips. Type-I collagen (Cellmatrix Type I-A) was obtained from Nitta Gelatin (Osaka, Japan) and was neutralized on ice to a final concentration of 2 mg ml−1 according to the manufacturer’s instructions.
Data analyses
For data analyses, two-tailed Student’s t-tests were used to determine P-values. For quantification of apical extrusion of RasV12 cells using confocal microscopy, >40 RasV12 cells were analyzed for each experimental condition.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
A.O. conceived and performed the experiments, and analyzed the results. J.I. designed and helped with the experiments on the lipid rafts. M.I. and T.S. performed mass-spectrometric analysis. M.K., Y.Y., S.K., S.I. and S.I. contributed to different aspects of experimental work and data interpretation. Y.F. conceived and designed the study. The manuscript was written by A.O. and Y.F. with assistance from the other authors.

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Supplementary material
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References


Supplementary material Fig. 1. Cav-1-containing microdomains are enriched not only in RasV12– but also in Src-transformed cells surrounded by normal cells. (A,B) Cav-1 is localized at the cell-cell contact sites basal to tight junctions. MDCK-pTR GFP-RasV12 cells (green) were mixed with normal MDCK cells (upper panels) or cultured alone (lower panels), and incubated with tetracycline for 16 hours. Cells were stained with anti-Cav-1 (A, red) or anti-gp135 (B, red), and anti-ZO-1 (white) antibodies. Arrowheads indicate the position of tight junctions. Scale bars: 10 μm. (C) Immunofluorescence images of Cav-1 in Src-transformed cells that are surrounded by normal cells. MDCK-pTR GFP-cSrcY527F cells (green) were mixed with normal MDCK cells (upper panels) or cultured alone (lower panels), and incubated with tetracycline for 16 hours. Cells were stained with Hoechst 33342 (blue), anti-Cav-1 antibody (red), and Alexa-Fluar-647-conjugated phalloidin (white). Scale bars: 10 μm. (D) Sphingolipids are co-accumulated with Cav-1 in RasV12-transformed cells that are surrounded by normal cells. Cells were stained with Hoechst 33342 (blue), Lysenin (red), and anti-Cav-1 antibody (white). Scale bars: 10 μm. (E) Quantification of Lysenin–enriched MDCK-pTR GFP-RasV12 cells. The percentage of Lysenin–enriched cells was measured. Data are mean ± s.d. from three independent experiments. *P<0.05; n=157 and 1,104 cells.
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Supplementary material Fig. 2. Cav-1-containing microdomains regulate the apical extrusion of RasV12-transformed cells surrounded by normal cells. (A) Immunofluorescence images of Forssman and Cav-1 in RasV12-transformed cells in the absence (upper panels) or presence (lower panels) of PDMP. MDCK-pTR GFP-RasV12 cells (green) were mixed with normal MDCK cells, and incubated with tetracycline in the absence or presence of PDMP for 16 hours. Cells were stained with Hoechst 33342 (blue), anti-Forssman (red), and anti-Cav-1 (white) antibodies. Scale bars: 10 μm. (B) Immunofluorescence images showing the effect of knockdown of Cav-1. MDCK-pTR GFP-RasV12 cells (upper panels) or MDCK-pTR GFP-RasV12-Cav-1 shRNA1 cells (lower panels) were cultured alone and stained with Hoechst 33342 (blue), anti-Cav-1 antibody (red), and Alexa-Fluor-647-conjugated phalloidin (white). Scale bars: 10 μm. (C) Establishment of MDCK-pTR GFP-RasV12 cells stably expressing Cav-1 shRNA2. Knockdown of Cav-1 protein was detected by western blotting using anti-Cav-1 antibody. Equal protein loading was confirmed using anti-GAPDH antibody. (D,E) Immunofluorescence images of Forssman (D) or Lysenin (E) in RasV12-transformed cells (upper panels) or Cav-1-knockdown RasV12-transformed cells (lower panels). MDCK-pTR GFP-RasV12 cells or MDCK-pTR GFP-RasV12-Cav-1 shRNA1 cells (green) were mixed with normal MDCK cells, and incubated with tetracycline for 16 hours. Cells were stained with Hoechst 33342 (blue), anti-Forssman antibody (D, red) or Lysenin (E, red), and anti-Cav-1 antibody (white). Scale bars: 10 μm. (F) Quantification of the apical extrusion of MDCK-pTR GFP-RasV12 cells or MDCK-pTR GFP-RasV12-Cav-1 shRNA2 cells from a monolayer of normal MDCK cells. Data are mean ± s.d. from four independent experiments. *P<0.001; n=135 and 142 cells.
MDCK-pTR GFP-cSrcY527F cells (green) were mixed with normal MDCK cells (upper panels) or cultured alone (lower panels), and incubated with tetracycline for 16 hours. Cells were stained with Hoechst 33342 (blue), anti-EPLIN antibody (red), and Alexa-Fluor-647-conjugated phalloidin (white). Scale bars: 10 μm. (C) Immunofluorescence images of EPLIN, α-catenin, and β-catenin in RasV12-transformed cells that are surrounded by normal cells. Cells were stained with Hoechst 33342 (blue), anti-β-catenin (red), and anti-EPLIN (upper panels, white) or anti-α-catenin (lower panels, white) antibodies. Scale bars: 10 μm. (D) Establishment of MDCK-pTR GFP-RasV12 cells stably expressing EPLIN shRNA1. MDCK-pTR GFP-RasV12 cells (upper panels) or MDCK-pTR GFP-RasV12-EPLIN shRNA1 cells (lower panels) were cultured alone and stained with Hoechst 33342 (blue), anti-EPLIN antibody (red), and Alexa-Fluor-647-conjugated phalloidin (white). Scale bars: 10 μm. (E) Establishment of RasV12-transformed cells stably expressing EPLIN shRNA2. Knockdown of EPLIN protein was analyzed by western blotting using anti-EPLIN antibody. Equal protein loading was confirmed using anti-GAPDH antibody. (F) Quantification of the apical extrusion of MDCK-pTR GFP-RasV12 cells or MDCK-pTR GFP-RasV12-EPLIN shRNA2 cells from a monolayer of normal MDCK cells. Data are mean ± s.d. from four independent experiments. *P<0.005, n=157 and 160 cells. (G) Immunofluorescence images of actin in RasV12-transformed cells (upper panels) or EPLIN-knockdown RasV12-transformed cells (lower panels) surrounded by normal cells. MDCK-pTR GFP-RasV12 cells or MDCK-pTR GFP-RasV12-EPLIN shRNA1 cells (green) were mixed with normal MDCK cells, and incubated with tetracycline for 16 hours. Cells were stained with Hoechst 33342 (blue), anti-EPLIN antibody (red), and Alexa-Fluor-647-conjugated phalloidin (white). Scale bars: 10 μm. It should be noted that EPLIN knockdown does not induce apparent actin cytoskeletal changes.
Supplementary material Fig. 4. Expression of dominant negative myosin-II in RasV12-transformed cells does not affect accumulation of EPLIN or Cav-1. (A) Immunofluorescence images of Forssman and EPLIN in RasV12-transformed cells in the absence (upper panels) or presence (lower panels) of PDMP. MDCK-pTR GFP-RasV12 cells (green) were mixed with normal MDCK cells, and incubated with tetracycline in the absence or presence of PDMP for 16 hours. Cells were stained with Hoechst 33342 (blue), anti-Forssman (red), and anti-EPLIN (white) antibodies. Scale bars: 10 μm. (B) Immunofluorescence images of Lysenin in RasV12-transformed cells (upper panels) or EPLIN-knockdown RasV12-transformed cells (lower panels) surrounded by normal cells. MDCK-pTR GFP-RasV12 cells or MDCK-pTR GFP-RasV12-EPLIN shRNA1 cells (green) were mixed with normal MDCK cells, and incubated with tetracycline for 16 hours. Cells were stained with Hoechst 33342 (blue), Lysenin (red), and anti-EPLIN antibody (white). Scale bars: 10 μm. (C) Immunofluorescence images of gp135 in RasV12-transformed cells (upper panels) or EPLIN-knockdown RasV12-transformed cells (lower panels) surrounded by normal cells. MDCK-pTR GFP-RasV12 cells or MDCK-pTR GFP-RasV12-EPLIN shRNA1 cells (green) were mixed with normal MDCK cells, and incubated with tetracycline for 16 hours. Cells were stained with anti-gp135 antibody (red). Scale bars: 10 μm. (D) Immunofluorescence images of ppMLC in RasV12-transformed cells surrounded by normal cells in the presence of DMSO (left panels) or Rho kinase inhibitor Y27632 (right panels). Cells were stained with anti-ppMLC anti(red). Scale bars: 10 μm. This result indicates that the ppMLC immunofluorescence indeed correlates with MLC activity. (E) Quantification of cell height of MDCK cells, MDCK-pTR GFP-RasV12 cells, and MDCK-pTR GFP-RasV12-EPLIN shRNA1 cells under the single or mix culture condition. Data are mean ± s.d. from three independent experiments. *P<0.05; **P<0.005; n= 80, 62, 64, 96, and 82 cells. (F,G) Quantification of EPLIN (F) or Cav-1 (G) enrichment in MDCK-pTR GFP-RasV12 cells with or without expression of dominant negative myosin-II (MLCAA) that are surrounded by normal cells. Data are mean ± s.d. from three independent experiments. n=191 and 173 cells (F); 174 and 148 cells (G).
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Supplementary material Table 1. Effect of various inhibitors on the apical extrusion of RasV12-transformed cells and on Cav-1 or EPLIN enrichment in RasV12-transformed cells that are surrounded by normal cells. *: statistically significant; †: with dotty apical accumulation; ND: not done; Grey box: our published observations.