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Identification of EPLIN as a Crucial Regulator for Extrusion of RasV12-transformed Cells

(Ras 変異細胞の正常上皮細胞層からの逸脱における
重要な制御因子としてのEPLIN の同定)

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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 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 PKA in RasV12-transformed cells. In addition, EPLIN substantially affects accumulation of filamin, a vital player in EDAC (Epithelial Defense Against Cancer), in the neighboring normal cells, and *vice versa*. These results indicate that EPLIN is a crucial regulator for the interaction between normal and transformed epithelial cells.

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

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LIST OF ABBREVIATIONS

MDCK: Mardin-Darby canine kidney

MAPK: Mitogen-activated protein kinase

shRNA: Short hairpin RNA

v-Src: Sarcoma virus Src

c-Src: cellular-Src

Cav-1: Caveolin-1

EPLIN: Epithelial Protein Lost in Neoplasm

LIM: Lin-1, Isl-1, and Mec-3

PKA: Protein kinase A

Dpp: Decapentaplegic

JNK: c-jun N-terminal kinase

Hid: Head involution defective

Ras: Rat sarcoma

GTPase: guanosine triphosphatase

Cdc42: Cell division control protein 42

FAK: Focal adhesion kinase

MMP: Matrix metalloproteinase

PDMP: DL-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol

MbCD: methyl- β -cyclodextrin

EDAC: Epithelial Defense Against Cancer

1. INTRODUCTION

1.1. Background of this thesis

More than 80% of malignant tumors arise from the epithelium in human. In addition, it is known as that multiple transformation is occurred in oncogenes or suppressor genes in detectable carcinogenesis at clinical detection. Since first oncogene *Src* was identified in the end of 1970's, a variety of oncogenes and tumor suppressor genes have been found, and downstream signaling pathways of the encoded proteins have been revealed ^{1,2}. Furthermore, it has been defined that these proteins involve various processes, such as cell proliferation, cell differentiation and cell death. It largely progressed in the effect of transformation of oncogene proteins and tumor suppressor proteins on cells cell-autonomously. However, in these studies, It has not been looked back very much about social nature of carcinogenesis which cancer cells occur from normal epithelial cells, and then proliferate under surrounded by them. Thus, 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, then what happens (Fig. 1)? Do surrounding normal cells, for example, recognize the transformation that has occurred in their neighbor? What is the fate of the transformed cell when surrounded by normal cells?

Some of these questions have been addressed by our laboratory using a mammalian tissue culture system. For example, we have shown that when cells transformed by overexpression of an oncogene (RasV12 or v-Src) are surrounded by normal cells, the transformed cells are apically extruded from the

monolayer of normal epithelial cells ^{3,4}. In addition, when cells transformed by depletion of a tumor suppressor (Scribble) are surrounded by normal cells, the transformed cells undergo apoptosis and are apically extruded from the epithelium ⁵. This elimination phenomenon resembles cell competition in *Drosophila* (described in section 1.2) where less fit mutant cells are removed from imaginal discs of a developing embryo in order to maintain the integrity of the whole organ. A similar mechanism may function in mammalian epithelial monolayers leading to the removal of randomly appearing transformed cells. These studies shed light on unexplored processes occurring at the initial step of carcinogenesis. Although we have shown that various signaling pathways are activated in transformed cells surrounded by normal cells, leading to their extrusions (described in section 1.3), molecular mechanisms for the apical extrusion of transformed cells are largely unknown. How does the recognition between normal and transformed cells occur? What is the crucial molecule for extrusion of transformed cells? The aim of my thesis is to identify novel proteins that are important for extrusion of transformed cells and to understand their roles.

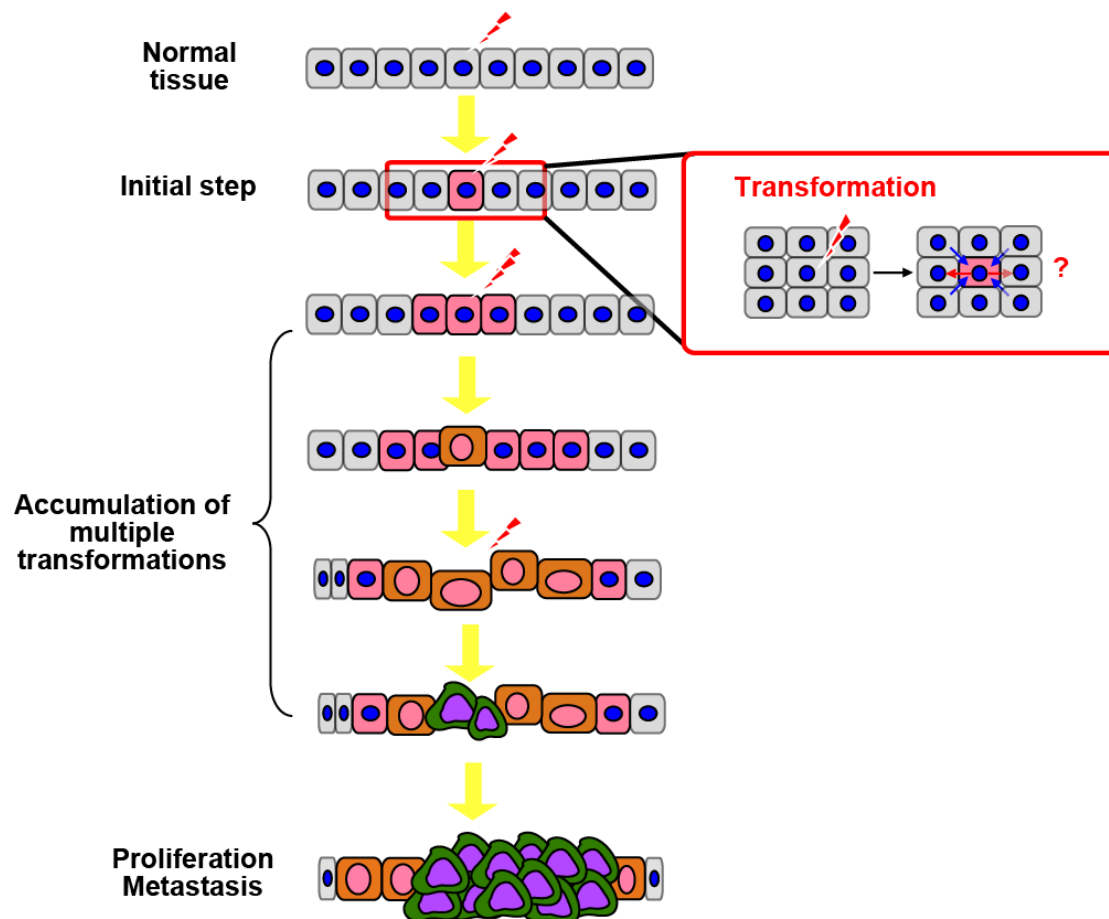


Fig. 1. Multistep model of carcinogenesis. At the initial stage of carcinogenesis, transformation occurs in a single cell within a normal epithelial layer, and the transformed cells grow while being surrounded by neighbouring normal epithelial cells.

1.2. Cell competition in *Drosophila*

In studies of *Drosophila*, it has been revealed that several phenomena occur at the interface between normal and transformed cells (Table 1). In recent years, the modest fruit fly *Drosophila melanogaster* has become a popular model system for the analysis of cellular growth control and organ size determination during animal development. For their studies, many biologists have focused on the so-called wing imaginal discs, the precursor organs of adult wings and thoraxes. Wing imaginal discs originate from a group of 40 to 50 cells that are set aside at the end of embryogenesis. During the 4 days of larval development, these cells multiply 1,000-fold to form the mature imaginal disc, which consists mainly of a columnar epithelial monolayer⁶. The cellular growth and cell cycle characteristics during this proliferative phase closely resemble those of vertebrate cells, inasmuch as similar regulatory proteins have been found to function in both situations. In contrast to cells cultured on plastic, however, imaginal disc cells are embedded in an intact tissue and subject to physiologic short- and long-range signals that cannot be observed *in vitro*.

1.2.1. *Minute*

Firstly, in 1975, Morata et al reported the interesting phenomena which occur when normal cells and transformed cells co-survival⁷. *Minutes* are mutations in ribosomal protein genes⁸ that are characterized by recessive lethality and by a dominant growth defect. *Drosophila* having homozygous *Minute* can not synthesis protein, so that it is dead. On the other hands, the phenotype of

heterozygous *Minute* only shows that delay of growth rates and the size of hair are smaller than normal body. When normal cells were mixed with *Minute* cells, *Minute* cells were eliminated by apoptosis and normal cells proliferated until keeping the size of body. They considered to the results of competition during the process of development, and then they named “cell competition”. In this phenomenon, normal cells and *Minute* cells are called winner and loser, respectively. The most important point is that cell competition does not occur when *Minute* cells cultured alone, while it occurs when *Minute* cells are surrounded by normal cells. Therefore, cell competition is cell-context-dependent manner.

1.2.2. *Myc*

Myc has also been shown as a key regulator of cell competition. In 2004, two papers clearly demonstrated that this phenomenon was triggered by the difference in *Myc* expression levels between the two adjacent cell populations^{9,10}. *Myc* is a conserved transcription factor regulating multiple downstream targets involved in cell growth and ribosome biogenesis^{11,12}. Clones expressing high levels of *dmyc* overgrew at the expense of the surrounding tissue until they filled the compartment^{9,10}. Clone expansion required the elimination of the surrounding cells by apoptosis, which was induced through Dpp deprivation, Jun N-terminal kinase (JNK) activation¹⁰, and induction of the apoptotic activator Hid in the loser cells⁹. On the contrary, down-regulation of *Myc* in clones led to their elimination, similar to *Minute* mutations⁹⁻¹¹. *dmyc*

competition could be genetically related to *Minute*, as the *Minute* mutation suppressed the supercompetitor phenotype of *dmyc* overexpression¹⁰. The clones expressing Myc grew to a significantly larger size than control wild-type clones induced in parallel. However, the sister clones were at a significant disadvantage and were smaller than control wild-type clones induced in parallel. This was shown to depend on an increase in apoptosis in the clones of wild-type cells (called “losers”) that were growing adjacent to the Myc-expressing clones (called “winners”).

Mutations	Phenomena	Reference
<i>Drosophila</i>		
Minute	Apoptosis of <i>Minute</i> -mutant cells	Morata and Ripoll, 1975
Myc	Apoptosis of cells expressing lower amount of <i>Myc</i>	Moreno and Basler, 2004 de la Cova et al., 2004
Src (Csk)	Basal extrusion and apoptosis of <i>Src</i> -transformed cells	Vidal et al., 2006
Scribble	Apoptosis of <i>Scribble</i> -knockdown cells	Brumby and Richardson, 2003
Ras	Apical or basal extrusion of <i>Ras</i> -transformed cells	Hogan et al., 2009
Lgl/Mahjong	Apoptosis of <i>Mahjong</i> - or <i>Lgl</i> -knockout cells	Grzeschik et al., 2007 Tamori et al., 2010
Mammals		
Ras	Apical extrusion or basal protrusion of <i>Ras</i> -transformed cells	Hogan et al., 2009
Src	Apical extrusion of <i>Src</i> -transformed cells	Kajita et al., 2010
Scribble	Apoptosis of <i>Scribble</i> -knockdown cells	Norman et al., 2012
Mahjong	Apoptosis of <i>Mahjong</i> -knockdown cells	Tamori et al., 2010
ErbB2	Apical extrusion of <i>ErbB2</i> -overexpressing cells	Leung and Brugge, 2012

Table 1. Interactions between normal and transformed epithelial cells in *Drosophila* and mammals.

1.3. Cell competition in mammals

Recent studies using mammalian cell culture systems have revealed that various phenomena can occur at the interface between normal and transformed epithelial cells (Table 1) ^{3-5,13,14}. In these studies, we have established Madin-Darby canine kidney (MDCK) epithelial cell lines expressing oncoproteins or shRNA of tumor suppressor proteins in a tetracycline- or temperature-inducible manner ^{3-5,13}.

1.3.1. *Ras*

Ras is one of the small GTPase superfamily that regulates many cellular processes including cell proliferation, differentiation, viability and motility, and is frequently mutated to its active forms in various types of human cancers ¹⁵. We established MDCK cells expressing constitutively active, oncogenic Ras (RasV12) in a tetracycline-inducible manner (MDCK-pTR GFP-RasV12 cells, hereafter referred as RasV12-transformed cells) ³. Firstly, RasV12-transformed cells were mixed with normal MDCK cells at a ratio of 1:100, and cultured on collagen matrix in the absence of tetracycline until a monolayer was formed. Subsequently, expression of GFP-RasV12 was induced with tetracycline. Under these conditions, a number of molecules such as Cdc42 and myosin-II in RasV12-transformed cells surrounded by normal cells, and within 24 hours after addition of tetracycline, 80% of RasV12-transformed cells were apically extruded from the monolayer of normal cells, which occurred in an apoptosis-independent manner. On the other hands, apical extrusion did not

occur in 20% of RasV12-transformed cells surrounded by normal cells. They found that non-extruded RasV12-transformed cells formed large, dynamic basal protrusions beneath the surrounding normal cells, and eventually delaminated basally and invaded a collagen matrix. Importantly, apical extrusion was not observed when RasV12-transformed cells were cultured alone, suggesting that activation of autonomous Ras signaling pathways alone is not sufficient to induce apical extrusion of RasV12-transformed cells, but that the interaction with the surrounding normal cells is also required.

1.3.2. *Src*

Rous sarcoma virus *Src* gene (*v-Src*) was the first identified oncogene ¹⁶, and its cellular counterpart c-*Src* is overexpressed and highly activated in various human cancers ¹⁷. *v-Src* and c-*Src* are non-receptor tyrosine kinases that phosphorylate multiple proteins on tyrosine residues and thereby regulate the actin cytoskeleton, cell adhesions, cell proliferation and other cellular processes ^{18,19}. They observed the phenomena occurring at the interface between normal and *Src*-transformed epithelial cells using MDCK cells expressing a temperature-sensitive mutant of *v-Src* (hereafter referred as *Src* cells) ⁴. They found that activity of myosin-II and focal adhesion kinase (FAK) is increased in *Src* cells surrounded by normal cells, leading to activation of the downstream mitogen-activated protein kinase (MAPK), and 80% of *Src* cells were apically extruded from the monolayer of normal cells after *Src* activation was induced. However, *Src* cells do not form the basal protrusions that are observed in

RasV12 cells. Collectively, these results demonstrate that these two systems share some common signaling pathways, but other distinct signaling pathways are also regulated.

1.3.3. *Scribble*

Scribble is a neoplastic tumor suppressor gene that was identified in *Drosophila*. Scribble has also been shown to function as a tumor suppressor protein in mice²⁰, and decreased Scribble expression is observed in human colon and breast cancers^{21,22}. In *Drosophila*, when clones of homozygous *scrib* mutant cells are surrounded by wild-type cells in eye imaginal discs, *scrib* mutant cells are eliminated from the epithelium by JNK pathway-mediated apoptosis²³. The authors established MDCK cell lines that stably express Scribble shRNA in a tetracycline-inducible manner (MDCK-pTR Scribble shRNA)⁵. Using this cell line, the authors tested for involvement of Scribble in cell competition. When Scribble-knockdown cells were surrounded by normal cells, they died and were extruded from the apical surface of the monolayer. Furthermore, they demonstrated that apoptosis of Scribble-knockdown cells depends on activation of p38 MAPK, not JNK. Importantly, Scribble-knockdown cells did not die when Scribble-knockdown cells were cultured alone, suggesting that the presence of surrounding normal cells induces death of Scribble-knockdown cells.

1.3.4. *ERBB2*

ERBB2 is an oncogenic receptor tyrosine kinase and is overexpressed in 30% of breast cancers ²⁴. MCF10A cells, a non-transformed human mammary epithelial cell line, develop into polarized, growth-arrested acinar structures containing a hollow lumen when cultured on reconstituted basement membrane (Matrigel). Leung and Brugge established a 3D culture system which induces mosaic expression of ERBB2 in mature MCF-10A acini infected with low-dose lentiviral vectors driving the tetracycline-inducible bicistronic expression of oncogenes ¹⁴. When ERBB2 was overexpressed in sporadic cells within 3D acini, ERBB2-overexpressing cells were translocated into the lumen, followed by clonal expansion. The MAPK pathway was involved in luminal translocation of ERBB2-overexpressing cells. Interestingly, MAPK has also been shown to play a crucial role in apical extrusion of RasV12- or Src-transformed cells that are surrounded by normal epithelial cells ^{3,4}. Thus, MAPK seems to be a general regulator for apical extrusion of transformed cells from the epithelium. Furthermore, they showed that inhibition of matrix metalloproteinases (MMPs) substantially blocks ERBB2-mediated luminal translocation and suppresses proliferation of the ERBB2-overexpressing cells that stayed in the epithelial layer. These data indicate that the neighboring normal cells control proliferation of ERBB2-overexpressing cells.

1.4. Proteins involved in this thesis

In this thesis, We focused on Caveolae and Caveolin-1 (Cav-1). Furthermore, Epithelial Protein Lost In Neoplasm (EPLIN) was identified as one of

Cav-1-binding proteins under the mix culture condition with normal and RasV12-transformed cells. Caveolae, Cav-1 and EPLIN are introduced below.

1.4.1. Caveolae and Caveolin-1

Caveolae are one of the lipid rafts with characteristic flask-shaped invagination of the plasma membrane where cholesterol, glycolipids, and sphingomyelin are enriched^{25,26}. Owing to their specific lipid composition, caveolae concentrate several signaling molecules involved in cellular processes or trafficking events from the cell surface; therefore, they are recognized as a platform for preassembled complexes of receptors, signal components, and their targets, facilitating efficient and specific cellular responses²⁷⁻²⁹. The shape and structural organization of caveolae result from the presence of their signature protein, Caveolin-1 (Cav-1), that binds cholesterol and self-assembles into high-mass oligomers to form caveolae. Cav-1 plays a vital role in the function of caveolae including the regulation of endocytosis and signaling pathways^{30,31}. There are now three known members of the Caveolin gene family: Caveolin-1 (two isoforms α and β)^{32,33}, Caveolin-2 (three isoforms α , β and δ)³⁴ and Caveolin-3³⁵ (Fig.2A). The N- and C-termini of Caveolins are cytoplasmic, suggesting that they form a 'hairpin' structure in the membrane, and they are palmitoylated on several residues³⁶. Caveolins-1 and -2 have a wide, overlapping distribution: they (and therefore caveolae) are present in most cell types, being most abundant in endothelial cells, fibroblasts, adipocytes, pneumocytes and epithelial cells^{37,38}. In contrast, Caveolin-3 is almost solely

expressed in smooth and skeletal muscle ³⁹. Cav-1 and -2 share similar chromosomal location (7q31.1) whereas Cav-3 is located on a different chromosome (3p25). The Caveolins are highly conserved among species ⁴⁰ with Cav-1 and Cav-3 sharing 65% identity and 85% similarity in protein sequence. Caveolin deficiency leads to caveolar loss, which is accompanied by alterations in signaling responses ^{41,42}. Cav-1 (21-24kDa) is an integral membrane protein, expressing two isoforms (alpha and beta) of different length and distinct potential in caveolae formation. The full length Cav-1 (isoform alpha, 178 amino acids) has a hairpin-like structure spanning the plasmatic membrane, both C- and N-termini facing the cytosol. The beta isoform is 31 amino acids shorter and is translated from the same mRNA as the longer form, but at divergent translation initiation sites. Both isoforms have two hydrophilic domains at the C- and N-termini, that flank a hydrophobic central domain. Several functional domains were defined (Fig.2B). The membrane attachment domains are located at the N- (residues 82-101) and C-termini (residues 135-150). Cav-1 contains palmitoylation sites on Cys 133, 143 and 156, involved in membrane anchorage. The central region (residues 102-134) (TMD) was first suggested to be the transmembrane domain, but, after predicting its beta-sheet rather than alpha-helix conformation, it was suggested that it is involved in hetero-oligomerization of Cav-1 with Cav-2 and in specific interactions with other proteins. The caveolin scaffolding domain (CSD), located at the N-terminus (amino acids 82-101), is involved in the binding and inhibition of proteins containing a defined caveolin binding motif, such as

ω XXXX ω XX ω or ω X ω XXXX ω - where ω is an aromatic amino acid (Trp, Phe or Tyr). The oligomerization domain (amino acids 61-101) contains CSD and directs the formation of homo-oligomers (14-16 Cav-1 molecules), which interact with cholesterol and signaling molecules ⁴³.

In addition, several reports suggest that reduced expression of Cav-1 is observed in breast, colon, and ovarian cancers ⁴⁴⁻⁴⁶, though its functional role in oncogenesis is not clearly understood.

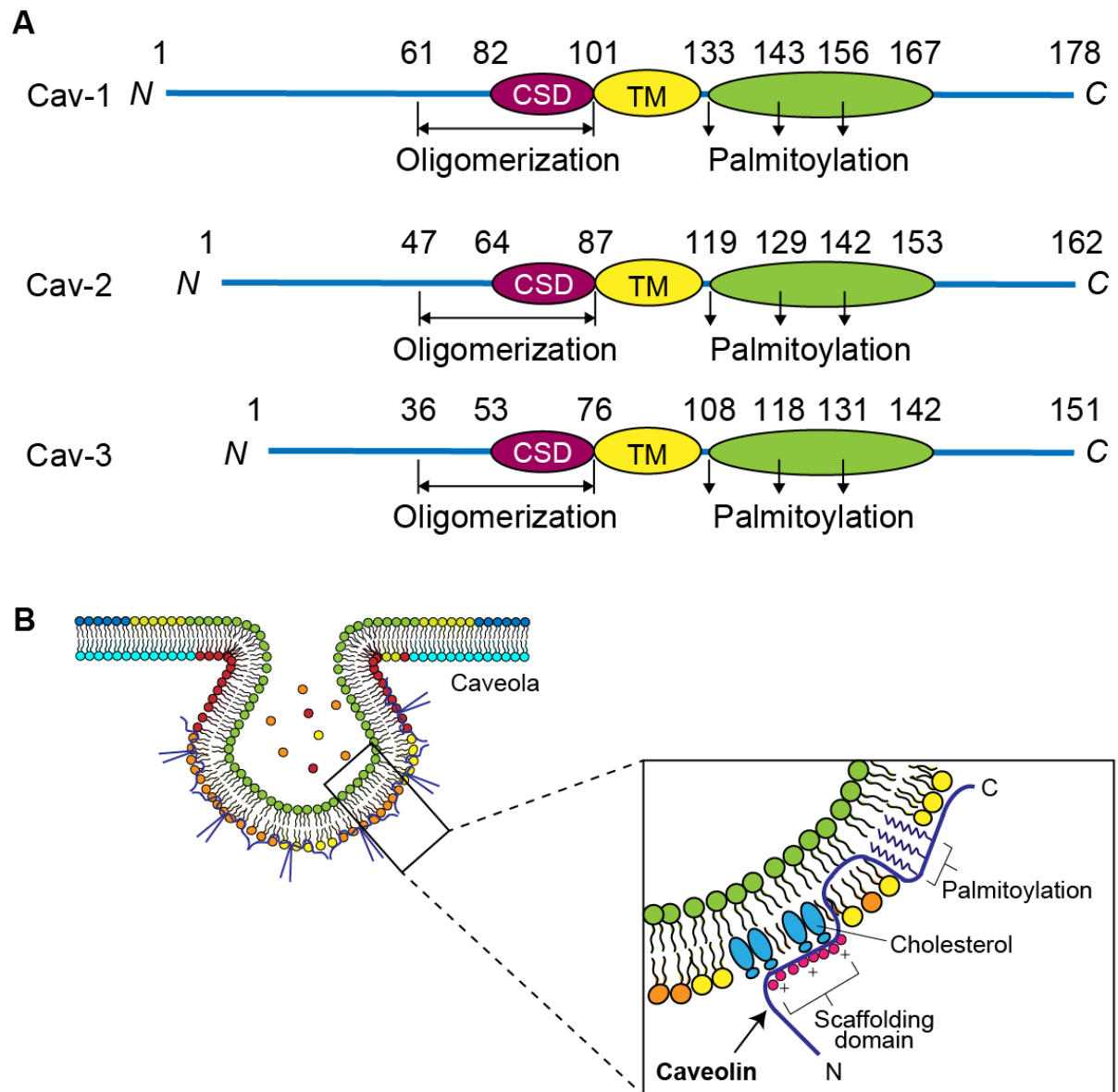


Fig. 2. Caveolae and Caveolin. (A) Schematic presentation of the structural similarities of the caveolin family of proteins. The caveolin gene family has 3 members: Cav-1, Cav-2, and Cav-3. Both Cav-1 and Cav-2 exist in different isoforms. The N-terminal region contains the CSD (Caveolin scaffolding domain), which is essential for both the formation of caveolin oligomers and the interaction of caveolins with other proteins. TM indicates the transmembrane domains. (B) The diagrams show the main features of caveolae and caveolins (blue line). Panel indicates how caveolin is inserted into the caveolar membrane, with the N and C termini facing the cytoplasm and a putative 'hairpin' intramembrane domain embedded within the membrane bilayer. The scaffolding domain, a highly conserved region of caveolin, might have a role in cholesterol interactions through conserved basic (+) and bulky hydrophobic residues (red circles). The C-terminal domain, which is close to the intramembrane domain, is modified by palmitoyl groups that insert into the lipid bilayer.

1.4.2. EPLIN

Epithelial Protein Lost In Neoplasm (EPLIN), also known as Lima-1, was originally identified as a protein of which expression is often down-regulated or lost in various types of cancers⁴⁷⁻⁴⁹. Two isoforms of EPLIN, designated as EPLIN α and EPLIN β , are generated from this gene by two distinct promoters⁴⁷. The sequences of these two isoforms differ by the addition of a 160 amino acid extension at the N terminus of the α isoform (Fig.3A). EPLIN belongs to the family of LIM domain proteins as it contains a centrally located LIM domain known to form two closely packed zinc-binding subdomains⁵⁰. Additionally, EPLIN exhibits two functional actin binding sites, one on each side of the centrally located LIM domain, that give it the ability to cross-link and bundle actin filaments⁵¹. *In vitro*, EPLIN stabilizes actin filaments by preventing their depolymerization and blocks the formation of branched filaments by inhibiting actin nucleation by Arp2/3⁵¹. Based on these properties, EPLIN is implicated in different actin-related processes, such as cell motility and migration, cytokinesis, and intercellular junctions^{48,52,53}. Subsequent *in vivo* studies confirmed the down-regulation of EPLIN α in a number of human epithelial cancer cells and tissues, suggesting that the loss of EPLIN α could contribute to the transformed phenotype. This indicates that EPLIN may act as a tumor suppressor⁴⁸. In addition, EPLIN can bind to α -catenin associated with the cadherin- β -catenin complex; and through this interaction with α -catenin, EPLIN serves to link this complex to the actin fibers (Fig. 3B). E-cadherin is stabilized at the cell surface by its link to the actin cytoskeleton via β -catenin, α -catenin, and EPLIN. Thus, EPLIN plays a crucial role in the establishment of adherens junctions⁵³.

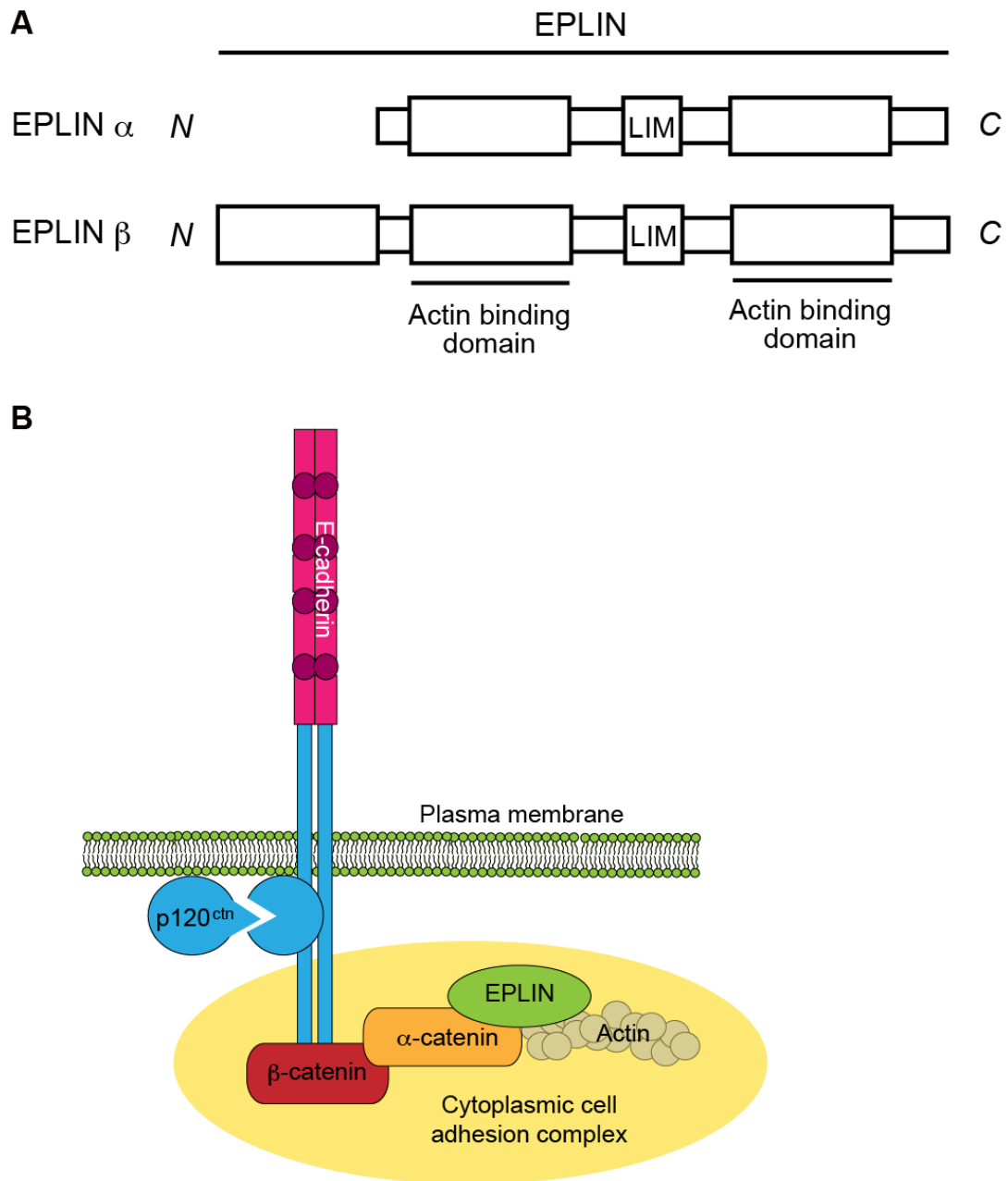


Fig. 3. Schematic structure of EPLIN. (A) The diagrams show two isoforms of EPLIN. EPLIN has a central LIM domain and N- and C-terminal domains involved in actin binding. (B) E-cadherin and the Cytoplasmic Cell Adhesion Complex. E-cadherin is stabilised at the cell surface by its link to the actin cytoskeleton via β -catenin, α -catenin, and, possibly, Epithelial Protein Lost in Neoplasm (EPLIN). p120 catenin (p120^{ctn}) stabilises the cytoplasmic cell adhesion complex by preventing clathrin-mediated endocytosis.

1.5. The aim of this thesis

We hypothesized that RasV12 cells are apically extruded as a result of recognizing the difference between normal and transformed epithelial cells each other via Cav-1-containing microdomains-mediated signaling pathways. The aim of this study is to identify and clarify the role of Cav-1 or Cav-1-associated proteins in the interaction between normal and transformed epithelial cells.

1.6. Summary

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

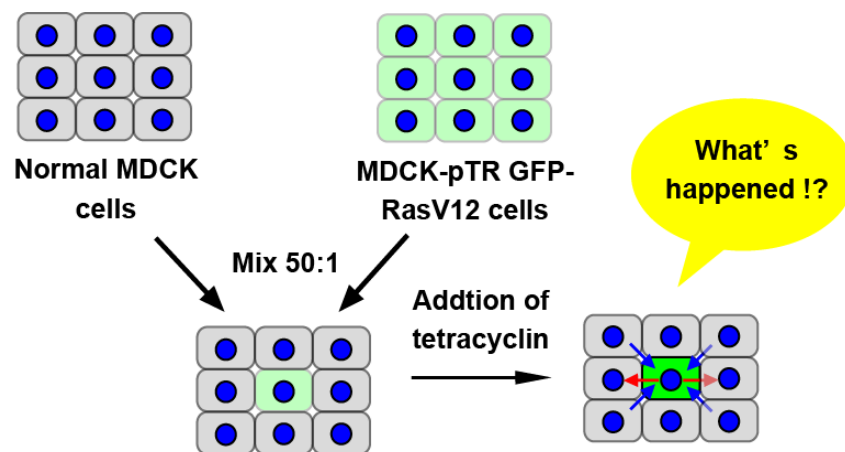


Fig. 4. Experimental procedure. Used epithelial cells in this study: (normal cells) MDCK cells that form a polarized epithelial monolayer; (transformed cells) MDCK cells expressing GFP-tagged constitutively active, oncogenic RasV12 in a tetracycline-inducible manner.

2. MATERIALS AND METHODS

2.1. 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; Thr 18/Ser 19), rabbit anti-cleaved caspase-3, 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, mouse anti-ZO-1, and rabbit anti-Src (pY418) antibodies were from Life Technologies (Paisley, UK). Alexa-Fluor-568-conjugated anti- β -catenin or anti-ZO-1 antibody was prepared using Zenon[®] 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 1:5,000. Monomeric red fluorescent protein-lysenin (161 aa–298 aa) was expressed in *Escherichia coli*, and purified by affinity chromatography using TALON metal affinity resin (Clontech, Palo Alto, CA) as previously. The culture supernatants of hybridoma cells producing anti-Forssman glycosphingolipid (clone 12B12) were used for immunofluorescence. Mouse anti-gp135 antibody was kindly provided by G. K. Ojakian. For immunofluorescence, all primary antibodies were used at 1:100,

except for anti-Forssman antibody that was used at 1:10, and all secondary antibodies were used at 1:200. The following inhibitors, Bisindolylmaleimide (BIM)-I (10 μ M), (S)-(-)-blebbistatin (30 μ M), CK666 (100 μ M), IPA-3 (40 μ M), KT5720 (4 μ M), LY294002 (10 μ M), PP2 (20 μ M), and Y27632 (10 μ M) were from Millipore. Cytochalasin D (4 μ M) and Methyl- β -cyclodextrin (MbCD, 10 mM) were from Sigma-Aldrich. U0126 (10 μ M) was from Promega (Madison, WI), and DL-*threo*-1-Phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP, 30 μ M) was from Cayman Chemical (Ann Arbor, MI).

2.2. Cell culture

MDCK cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovin serum (FBS) and penicillin/streptomycin at 37°C in ambient air supplemented with 5% CO₂. To establish MDCK cells stably expressing GFP-RasV12 in an inducible manner, a Tet-ON system was used. Briefly, MDCK cells were transfected with pcDNA6/TR using Lipofectamine 2000 (Life Technologies), followed by selection in medium containing 5 μ g ml⁻¹ of blasticidin (Invivogen, California, USA).

pcDNA4/TO/GFP-RasV12 was then used for the second transfection and the doubly transfected cells were selected in medium containing 10% FBS, 5 μ g ml⁻¹ of blasticidin and 400 μ g ml⁻¹ of zeocin (Invivogen). To establish MDCK-pTR GFP-cSrcY527F cells, the cDNA of cSrcY527F was excised from pEGFP-N1-cSrcY527F (a kind gift from M. Frame) and was cloned into the

NotI site of pcDNA4/TO (Life technologies). MDCK-pTR cells were then transfected with pcDNA/TO/cSrcY527F-GFP using Lipofectamine 2000, followed by selection in medium containing 5 $\mu\text{g ml}^{-1}$ of blasticidin and 400 $\mu\text{g ml}^{-1}$ of zeocin. MDCK-pTR GFP-RasV12 cells stably expressing Caveolin-1 shRNA or EPLIN shRNA were established as follows; Caveolin-1 shRNA oligonucleotides (Caveolin-1 shRNA1: 5'-

GATCCCCGCAGTTGTGCCGTGCATTATTCAAGAGATAATGCACGGCA
CAACTGCTTTTTC-3' and 5'-

TCGAGAAAAAGCAGTTGTGCCGTGCATTATCTCTTGAATAATGCACG
GCACAACTGCGGG-3' or Caveolin-1 shRNA2: 5'-

GATCCCCGCACACCAAGGAAATCGACTTCAAGAGAGTCGATTTCCTT
GGTGTGCTTTTTC-3' and 5'-

TCGAGAAAAAGCACACCAAGGAAATCGACTCTCTTGAAGTCGATTT
CCTTGGTGTGC GGG-3') or EPLIN shRNA oligonucleotides (EPLIN
shRNA1: 5'-

GATCCCCGGAAACGTGTGTAGGAGAATTCAAGAGATTCTCCTACAC
ACGTTTCCTTTTTC-3' and

5'-TCGAGAAAAAGGAAACGTGTGTAGGAGAATCTCTTGAATTCTCCT
ACACACGTTTCCGGG-3' or EPLIN shRNA2:

5'-GATCCCCGCACCTGCAAGAGAGACTTTTCAAGAGAAAGTCTCTCT
TGCAGGTGC TTTTTC-3' and 5'-

TCGAGAAAAAGCACCTGCAAGAGAGACTTTCTCTTGAAAAGTCTCTC
TTGCAGGTGCGGG-3') were cloned into the BglII and XhoI site of

pSUPER.neo+gfp (Oligoengine, Seattle, WA). MDCK-pTR GFP-RasV12 cells were transfected with pSUPER.neo+gfp Caveolin-1 shRNA or EPLIN shRNA using Lipofectamine 2000, followed by selection in medium containing 5 $\mu\text{g ml}^{-1}$ of blasticidin, 800 $\mu\text{g ml}^{-1}$ of G418 (Life Technologies), and 400 $\mu\text{g ml}^{-1}$ of zeocin. To construct pmCherry-N1-MLC-AA, the cDNA of MLC-AA was inserted into the EcoRI/KpnI site of pmCherry-N1 (gift from R. Y. Tsien). To establish MDCK-pTR GFP-RasV12 cells stably expressing mCherry-MLC-AA, MDCK-pTR GFP-RasV12 cells were transfected with pmCherry-N1-MLC-AA as described above, followed by selection in medium containing 5 $\mu\text{g ml}^{-1}$ of blasticidin, 400 $\mu\text{g ml}^{-1}$ of zeocin and 800 $\mu\text{g ml}^{-1}$ of G418. MDCK cells stably expressing filamin A short hairpin RNA (shRNA) in a tetracycline-inducible manner were established as follows; filamin A shRNA oligonucleotides (filamin A shRNA: 5'-

GATCCCCGCTGGAGTGCCAGCTGAATTTCAAGAGAATTCAGCTGGCACTCCAGCTTTTTC-3' and 5'-

TCGAGAAAAAGCTGGAGTGCCAGCTGAATTCTCTTGAAATTCAGCTGCACTCCAGCGGG-3') were cloned into the BglII and XhoI site of

pSUPERIOR.neo+gfp (Oligoengine). MDCK-pTR cells⁸ were transfected with pSUPERIOR.neo+gfp filamin A shRNA or vimentin shRNA using

Lipofectamine 2000, followed by selection in medium containing 5 $\mu\text{g ml}^{-1}$ of blasticidin and 800 $\mu\text{g ml}^{-1}$ of G418. For tetracycline-inducible MDCK-pTR

GFP-RasV12 or MDCK-pTR GFP-cSrcY527F cell lines, 2 $\mu\text{g ml}^{-1}$ of tetracycline (Sigma-Aldrich) was used to induce expression of GFP-RasV12 or

GFP-cSrcY527F. For Fig. 21A and C, to induce sufficient knockdown of filamin A protein, MDCK-pTR filamin A shRNA cells were incubated with 2 $\mu\text{g ml}^{-1}$ of tetracycline for 40 hours prior to co-incubation with MDCK-pTR GFP-RasV12 cells. For immunofluorescence, cells were plated onto collagen gel-coated cover slips. 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.

2.3. Immunoprecipitation and western blotting

For immunoprecipitation, MDCK and MDCK-pTR GFP-RasV12 cells were trypsinized and plated at a density of 1.6×10^7 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 Na_3VO_4 , 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 Na_3VO_4 , 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 Na_3VO_4 , and 10 mM NaF) containing 5 $\mu\text{g ml}^{-1}$ leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 7.2 trypsin inhibitor units of aprotinin. The former and latter was used for immunoprecipitation with anti-Cav-1 and anti-EPLIN antibody, respectively. After centrifugation at 21,500 x g for 10 min, the supernatants were subjected to immunoprecipitation for 1 hour with Dynabeads[®] Protein G (Life Technologies) conjugated with

anti-Caveolin-1 antibody (5 μ g) or anti-EPLIN antibody (5 μ g).

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. For Western blotting, SDS-PAGE gels were transferred onto polyvinylidene difluoride membranes. After incubation with primary and secondary antibodies, proteins were visualized by enhanced chemiluminescence (ECL⁺; Perkin-Elmer Life Sciences, Boston, Mass.). Primary antibodies were used at 1:1,000. Stained gels and western blotting data were analyzed using ImageQuant[™] LAS4010 (GE healthcare, Chalfont St Giles, UK). Protein bands were identified by mass-spectrometry.

2.4. Immunofluorescence

For immunofluorescence, MDCK-pTR GFP-RasV12 or MDCK-pTR GFP-cSrcY527F cells were mixed with MDCK cells at a ratio of 1:50, and cultured on the collagen matrix. 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% PFA in PBS, except for filamin accumulation where cells were fixed in methanol for 2.5 min at -20°C, and permeabilized in 0.5% Triton X-100-PBS for 15 min, followed by blocking in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum for 1 h. They were further

incubated in primary antibody for 1 h, washed three times in PBS, incubated in secondary antibody solution for 1 h, and washed three times in PBS. The coverslip was then mounted onto Mowiol on a glass slide. Immunofluorescence images were analyzed by Olympus FV1000 system and Olympus FV10-ASW software. Images were quantified by MetaMorph software (Molecular Devices).

2.5. 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, more than 40 RasV12 cells were analyzed for each experimental condition.

3. The effect of localization or knockdown of Cav-1 on apical extrusion of RasV12-transformed cells

3.1. Introduction

Apical extrusion of RasV12-transformed cells has been discovered and briefly characterized in our laboratory. Although some of the pathways downstream of Ras activation as well as a few proteins regulating cytoskeletal rearrangements have been both implicated or excluded from the process, answers to many questions remain unknown. Up to date, we cannot explain how normal and transformed cells recognize each other, what receptors mediate recognition, what signaling pathways are activated before and during extrusion, what is the mechanism and nature of this process and finally, what is the significance and fate of extruded transformed cells in an organism.

Caveolae are one of the lipid rafts with characteristic flask-shaped invagination of the plasma membrane where cholesterol, glycolipids, and sphingomyelin are enriched^{25,26}. Owing to their specific lipid composition, caveolae concentrate several signaling molecules involved in cellular processes or trafficking events from the cell surface; therefore, they are recognized as a platform for preassembled complexes of receptors, signal components, and their targets, facilitating efficient and specific cellular responses²⁷⁻²⁹. Therefore, I hypothesize that caveolae-mediated pathways involve in the recognition of the difference between normal and transformed cells. Cav-1, a major component of caveolae, plays a vital role in the function of caveolae including the regulation of endocytosis and signaling pathways^{30,31}. Several reports suggest that reduced

expression of Cav-1 is observed in breast, colon, and ovarian cancers⁴⁴⁻⁴⁶, though its functional role in oncogenesis is not clearly understood.

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. Then, we also examined the behavior of Cav-1-containing microdomains using anti-Forsmann antibody (a glycolipid heterophil protein) or fluorescence-conjugated lysenin (sphingomyelin-specific binding protein). In addition, we examined the effect of disruption of lipid rafts on the fate of RasV12-transformed cells surrounded by normal cells in the presence or absence of DL-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) and methyl- β -cyclodextrin (MbCD) which are the chemical compounds that disrupt lipid rafts by blocking biosynthesis of glucosylceramide and by removing cholesterol from the plasma membrane, respectively. We established Cav-1-knockdown RasV12-transformed cells, and also examined the effect of knockdown of Cav-1 on the fate of RasV12-transformed cells surrounded by normal cells. Then, to examine the functional involvement of Cav-1 in these signaling pathways, we examined the effect of various inhibitors on the apical extrusion and on the localization of Cav-1. Furthermore, 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.

3.2 Results

3.2.1. Cav-1 is enriched at the apical and lateral membrane domains in RasV12-transformed cells surrounded by normal cells

To examine the localization of caveolin-1 in normal and RasV12 transformed cells, RasV12 cells were mixed with normal or RasV12 cells at a ratio of 1 to 50. The mixture of cells was then cultured on a collagen matrix. GFP-RasV12 expression was induced with tetracycline, and cells were fixed after 16 hours and stained with an anti-Cav-1 antibody (Fig. 4). When RasV12 transformed cells were surrounded by normal cells, Cav-1 was enriched at the apical and lateral membrane domains in RasV12 cells surrounded by normal cells (Fig. 5A). Co-immunostaining with anti-ZO-1 antibody showed that Cav-1 was often localized at the cell-cell contact sites basal to tight junctions (Fig. 6A). In contrast, gp135, an apical marker protein, was localized exclusively at the apical membrane domain (Fig. 6B), 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. In contrast, when RasV12 transformed 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. 5A,B). In addition, Cav-1 was also accumulated at both the apical and lateral membrane domains in Src-transformed cells surrounded by normal cells (Fig. 7). These data indicate that the enrichment of Cav-1 in RasV12 cells occurs in the presence of neighboring normal cells.

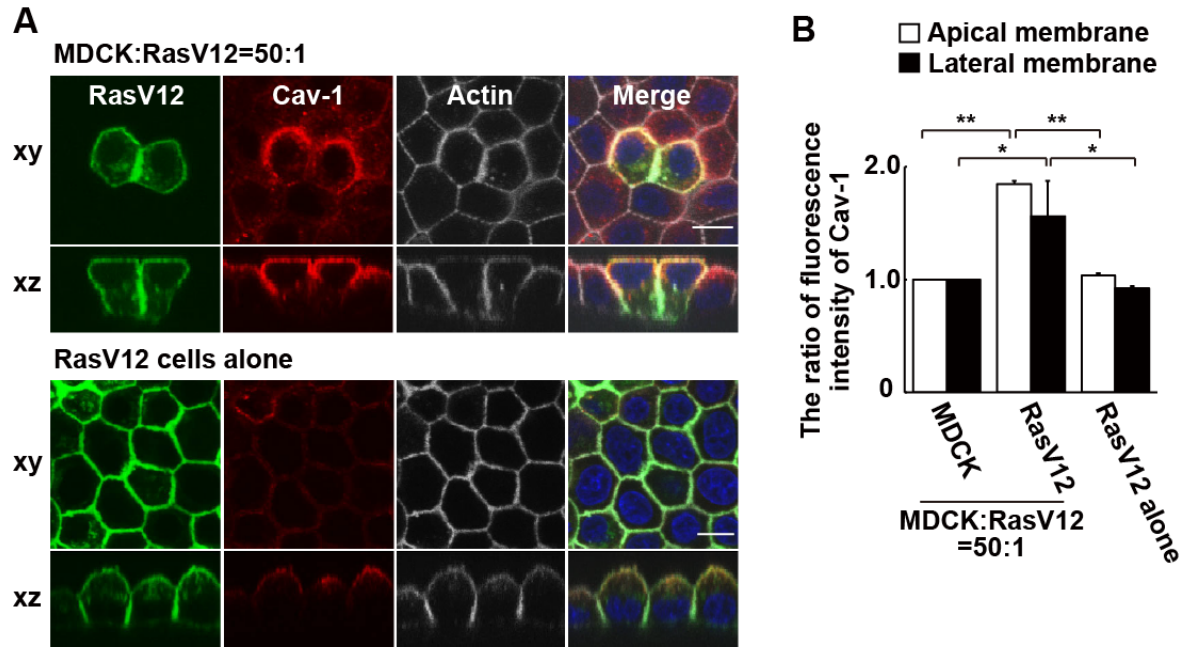


Fig. 5. Cav-1 is enriched at the apical and lateral membrane domains in RasV12-transformed cells surrounded by normal cells. (A) Cav-1 is accumulated at both the apical and lateral membrane domains in RasV12-transformed cells that are surrounded by normal cells. MDCK-pTR GFP-RasV12 cells (green) were mixed with normal MDCK cells (upper panels) or cultured alone (Lower panels), and incubated with tetracycline for 16 hours. Cells were stained with Hoechst33342 (blue), anti-Cav-1 antibody (red), and Alexa-Fluor-647-conjugated phalloidin (white). Scale bars: 10 μ m. (B) Quantification of the fluorescence intensity of Cav-1 at the apical or lateral membrane domain. Data are mean $\pm$ s.d. from three independent experiments. Values are expressed as a ratio relative to MDCK cells. * P <0.05, ** P <0.005; n=85, 80, 81, 74, 81, and 85 cells.

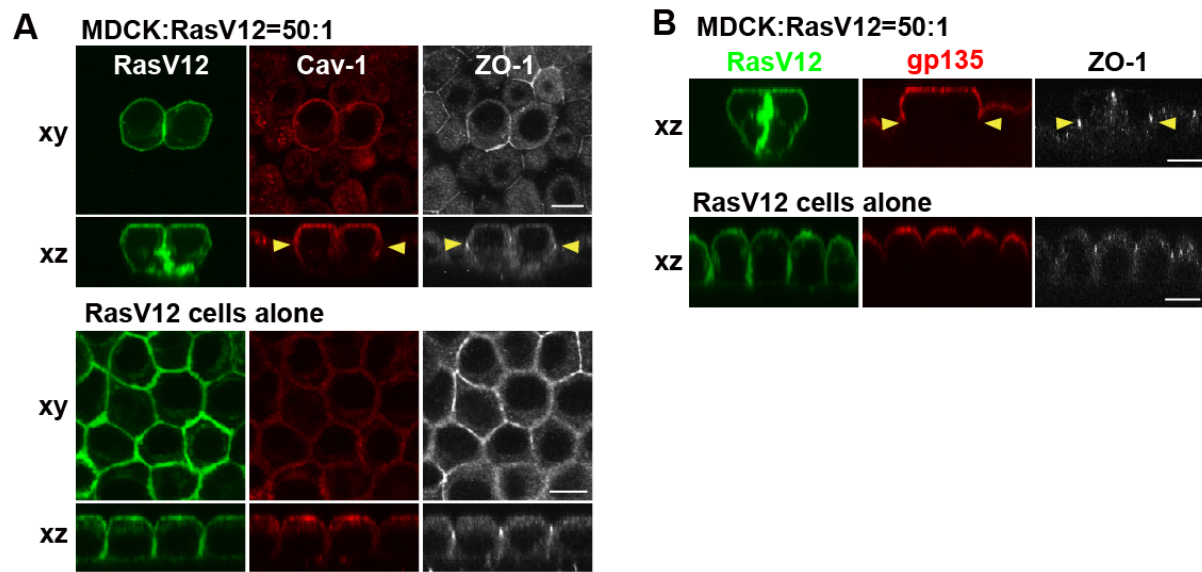


Fig. 6. The accumulation of Cav-1 is not merely due to the expansion of apical membrane domains in RasV12 cells surrounded by normal cells.. (A,B) Cav-1 is localized at the cell-cell contact sites basal to tight junctions. MDCK-pTR GFP-RasV12 cells (green) were mixed with normal MDCK cells (upper panels) 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 μ m.

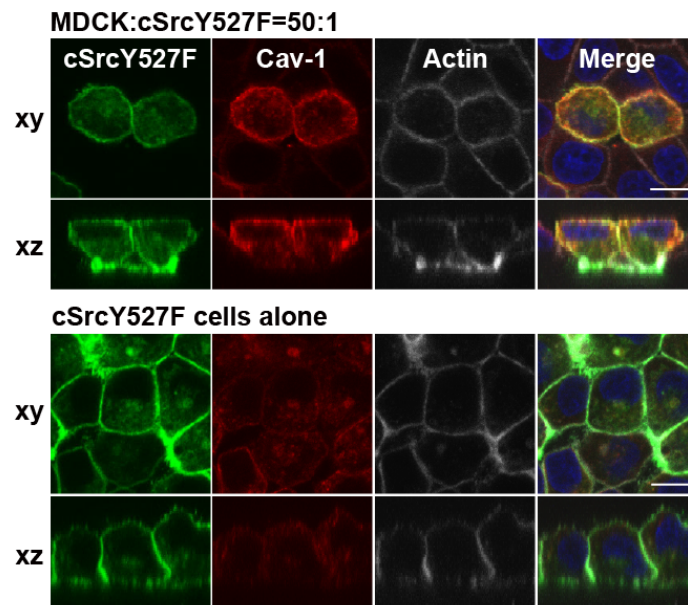


Fig. 7. Cav-1 is also enriched at the apical and lateral membrane domains in Src-transformed cells surrounded by normal cells. (A) Cav-1 is accumulated at both the apical and lateral membrane domains in cSrcY527F-transformed cells that are surrounded by normal cells. MDCK-pTR GFP-RasV12 cells (green) were mixed with normal MDCK cells (upper panels) or cultured alone (Lower panels), and incubated with tetracycline for 16 hours. Cells were stained with Hoechst33342 (blue), anti-Cav-1 antibody (red), and Alexa-Fluor-647-conjugated phalloidin (white). Scale bars: 10 μ m.

3.2.2. Cav-1-containing microdomains are enriched in

RasV12-transformed cells surrounded by normal cells

Cav-1 is localized at caveolae where various molecules are also enriched, including Forssman antigen (a glycolipid heterophil protein) and sphingomyelin. Immunofluorescence with anti-Forssman antibody showed that glycolipids were co-accumulated with Cav-1 at the lateral membrane domain in RasV12 cells surrounded by normal cells (Fig. 8A,B). 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 (Fig. 8C,D). These data indicate that Cav-1-containing microdomains are enriched in transformed cells surrounded by normal cells.

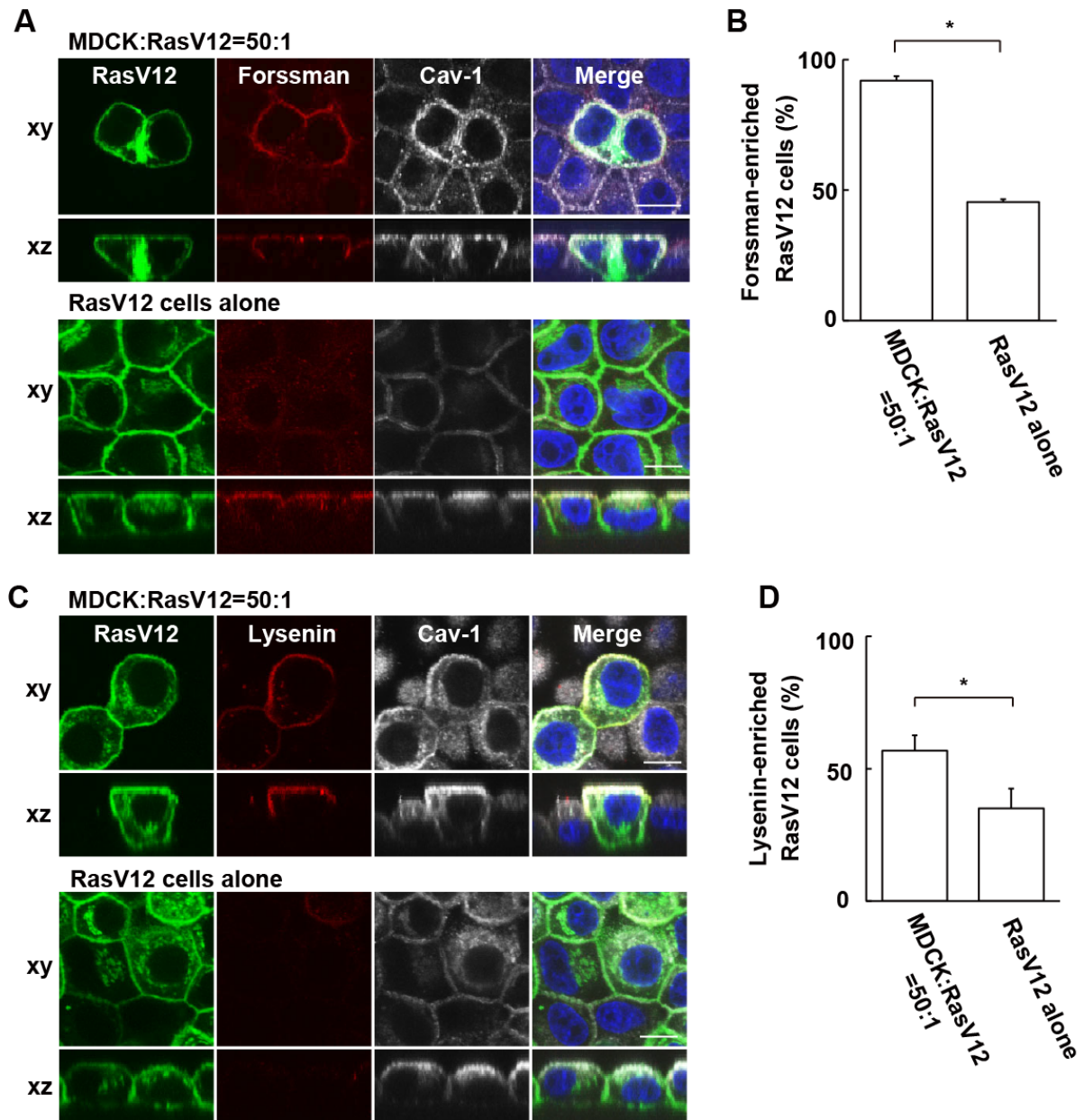


Fig. 8. Cav-1-containing microdomains are enriched in RasV12-transformed cells surrounded by normal cells. (A) Glycolipids are co-accumulated with Cav-1 in MDCK-pTR GFP-RasV12 cells that are surrounded by normal cells. Cells were stained with Hoechst 33342 (blue), anti-Forssman (red), and anti-Cav-1 (white) antibodies. Scale bars: 10 μ m. (B) Quantification of Forssman-enriched RasV12-transformed cells. The percentage of RasV12 cells in which Forssman is accumulated not only at the apical membrane domain but also at the lateral membrane domain was measured. Data are mean $\pm$ s.d. from three independent experiments. * $P < 0.0001$; $n = 95$ and 1,283 cells. (C) 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 μ m. (D) Quantification of Lysenin-enriched MDCK-pTR GFP-RasV12 cells. The percentage of Lysenin-enriched cells was measured. Data are mean $\pm$ s.d. from three independent experiments. * $P < 0.05$; $n = 157$ and 1,104 cells.

3.2.3. Cav-1-containing microdomains play a crucial role in the apical extrusion of transformed 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 biosynthesis of glucosylceramide and by removing cholesterol from the plasma membrane, respectively. Addition of PDMP or MbCD diminished the accumulation of Cav-1 (Fig. 9A, and data not shown), and significantly suppressed apical extrusion of RasV12-transformed cells surrounded by normal cells (Fig. 9B-E). Interestingly, addition of PDMP or MbCD also promoted the basal protrusion of transformed cells (Fig. 9B,D; arrowheads). Furthermore, when Cav-1 was knocked down in RasV12 cells (Fig. 10A-D), accumulation of Forssman antigen and sphingomyelin was severely diminished (Fig. 11A,B), and apical extrusion was significantly suppressed (Fig. 12A-D). 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.

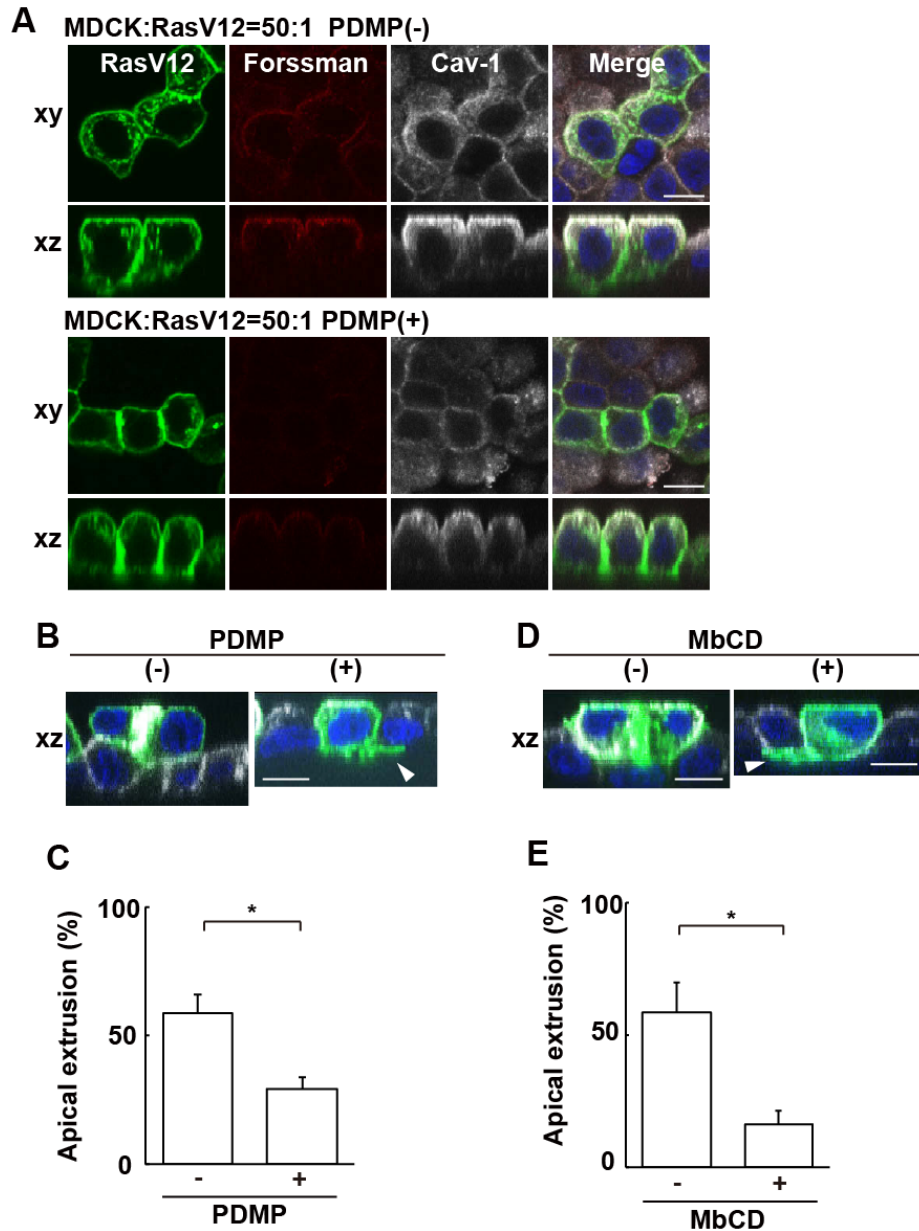


Fig. 9. Cav-1-containing microdomains play a crucial role in the apical extrusion of transformed cells. (A) Immunofluorescence images of Forssman and Cav-1 in RasV12-transformed cells in the absence (upper panels) or presence (lower panels) of PDMP. MDCK-pTR GFP-RasV12 cells (green) were mixed with normal 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 μ m. (B,D) Immunofluorescence images of XZ sections of MDCK-pTR GFP-RasV12 cells (green) that are surrounded by normal MDCK cells in the absence or presence of PDMP (B) or MbCD (D). Cells were stained with Hoechst 33342 (blue) and Alexa-Fluor-647-conjugated phalloidin (white). Arrowheads indicate basal protrusion of RasV12 cells that are surrounded by normal cells. Scale bars: 10 μ m. (C,E) Quantification of the apical extrusion of RasV12 cells from a monolayer of MDCK cells in the absence or presence of PDMP (C) or MbCD (E). Data are mean $\pm$ s.d. from three independent experiments. * $P < 0.05$; $n = 163$ and 168 cells (C); 295 and 309 cells (E).

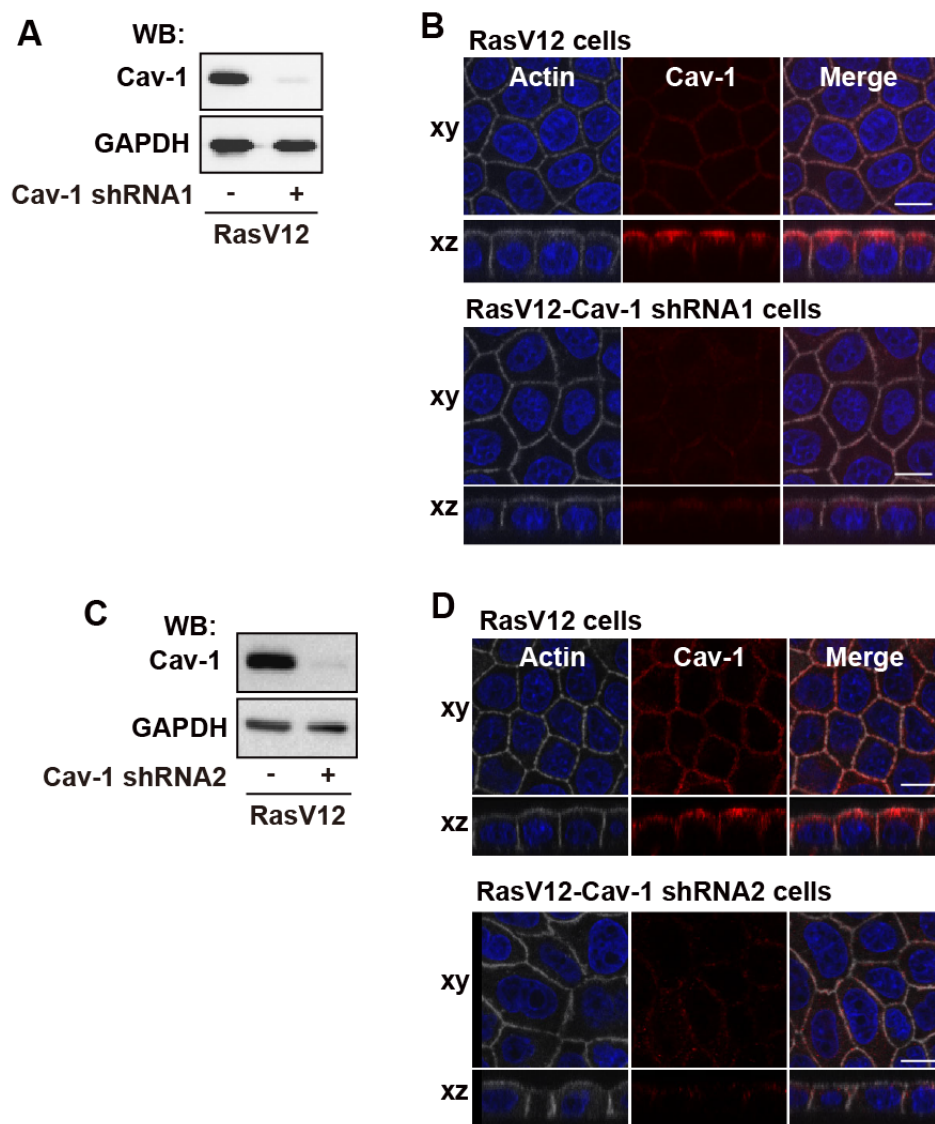


Fig. 10. Establishment of Cav-1-knockdown RasV12-transformed cells. (A,C) Establishment of MDCK-pTR GFP-RasV12 cells stably expressing Cav-1 shRNA1 (A) or shRNA2 (C). Knockdown of Cav-1 protein was analyzed by western blotting using anti-Cav-1 antibody. Equal protein loading was confirmed using anti-GAPDH antibody. (B,D) 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 (B) or shRNA2 (D) 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 μ m.

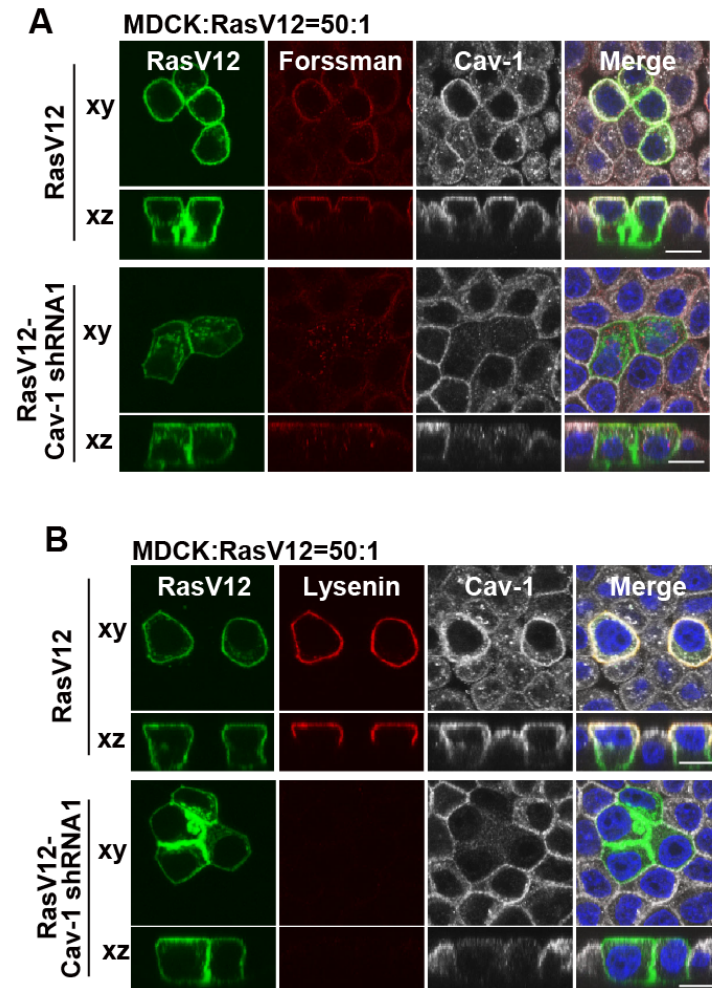


Fig. 11. Cav-1-containing microdomains are diminished when Cav-1 is knocked down in RasV12 cells. (A,B) Immunofluorescence images of Forssman (A) or Lysenin (B) in RasV12-transformed cells (upper panels) or Cav-1-knockdown RasV12-transformed cells (lower panels). MDCK-pTR GFP-RasV12 cells or MDCK-pTR 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 (A, red) or Lysenin (B, red), and anti-Cav-1 antibody (white). Scale bars: 10 μ m.

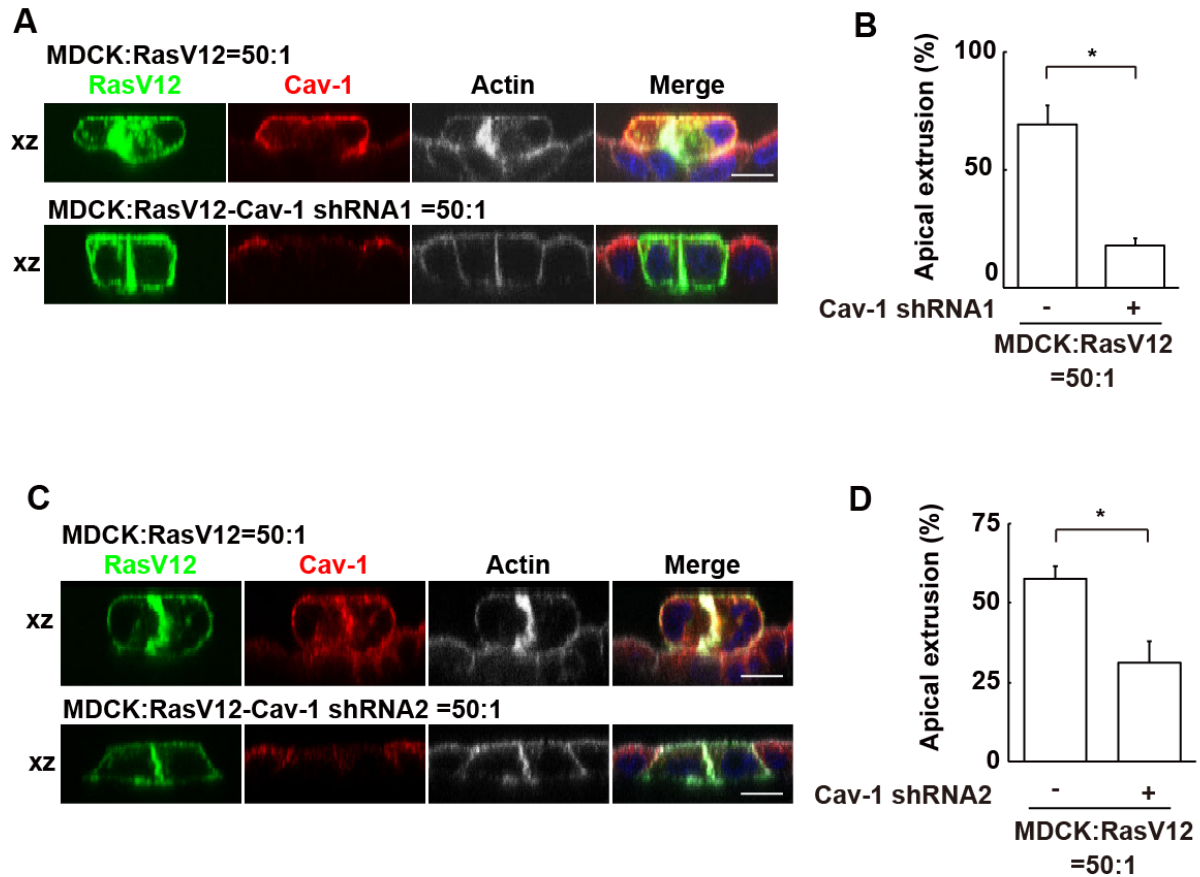


Fig. 12. Cav-1-containing microdomains play a crucial role in the apical extrusion of transformed cells. (A,C) Knockdown of Cav-1 suppresses the apical extrusion of RasV12-transformed cells. Normal MDCK cells were mixed with MDCK-pTR GFP-RasV12 cells (upper panels, green) or with MDCK-pTR GFP-RasV12-Cav-1 shRNA1 (A) or shRNA2 (C) cells (lower panels, green), and incubated with tetracycline for 24 hours. Cells were stained with Hoechst 33342 (blue), anti-Cav-1 antibody (red), and Alexa-Fluor-647-conjugated phalloidin (white). Scale bars: 10 μm. (B,D) Quantification of the apical extrusion for (A), (C), respectively.

3.2.4. MAPK, myosin-II and dynamics of actin filaments regulate accumulation of Cav-1

During the apical extrusion of transformed cells, various signaling pathways are activated, which plays a positive role in this process^{54,55}. To examine the functional involvement of Cav-1 in these signaling pathways, we examined the effect of various inhibitors on the apical extrusion and on the localization of Cav-1 (Table 2). Among the signaling pathways downstream of active Ras, the MAPK pathway plays a crucial role in the apical extrusion of RasV12-transformed cells³. Upon addition of MEK inhibitor U0126, accumulation of Cav-1 at the lateral membrane domain was diminished, and they were predominantly localized at the apical membrane domain in a dotted manner (Fig. 13). In contrast, PI3 kinase inhibitor LY294002 did not substantially affect its localization (Fig. 13). These data show that activity of the MAPK pathway regulates non-cell autonomous enrichment of Cav-1. Cytochalasin D, which blocks actin polymerization, strongly perturbed the enrichment of Cav-1 (Fig. 13), indicating that dynamics of actin filaments are required for the establishment and/or maintenance of their accumulation. In the previous studies, we have reported that activity of myosin-II and PKA is enhanced in RasV12-transformed cells surrounded by normal cells^{3,56} (also shown in Fig. 23A; Fig. 24A). Myosin-II inhibitor blebbistatin diminished Cav-1 localization at the lateral membrane domain (Fig. 13A). PKA inhibitor KT5720 did not affect the accumulation of Cav-1 (Fig. 13A).

Inhibitor	Target	RasV12	
		Extrusion	Cav-1 enrichment
BIM-I	PKC	ND	↓*
Blebbistatin	Myosin-II	↓*	↓*
CK666	Arp2/3	No effect	↓*
Cytochalasin D	Actin polymerization	↓*	↓*
IPA-3	PAK	ND	No effect
KT5720	PKA	ND	No effect
LY294002	PI3K	No effect	No effect
MbCD	Cholesterol	↓*	↓*
PDMP	Glucosylceramide synthase	↓*	↓*
PP2	Src-family tyrosin kinases	↓*	No effect
U0126	MAPK	↓*	↓*
Y27632	Rho kinase	↓*	No effect

Table 2. Effect of various inhibitors on the apical extrusion of RasV12-transformed cells and on Cav-1 enrichment in RasV12-transformed cells that are surrounded by normal cells. *: statistically significant; †: with dotted apical accumulation; ND: not done; Grey box: our published observations

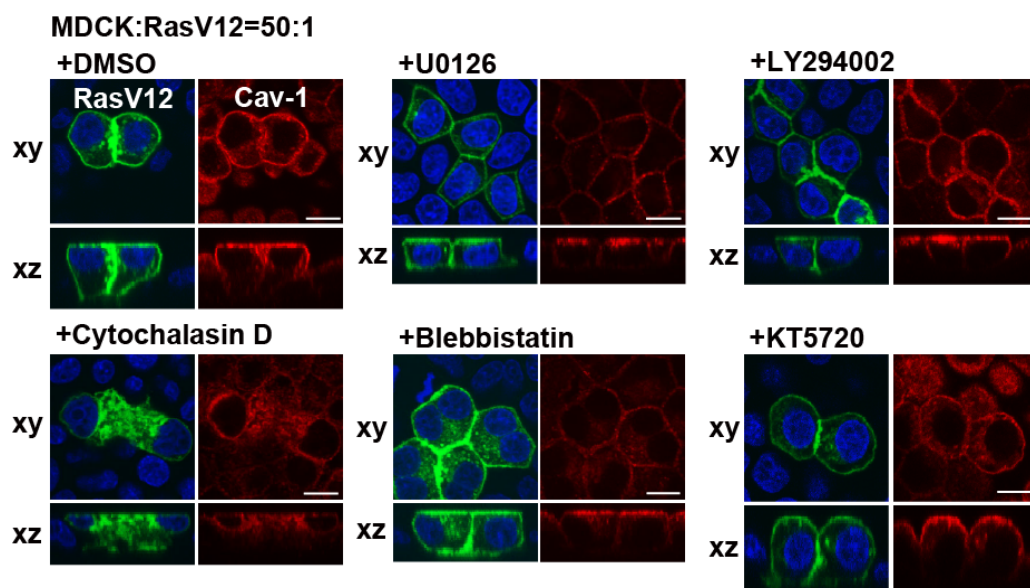


Fig. 13. Effect of various inhibitors on the non-cell autonomous accumulation of Cav-1.

Immunofluorescence images of Cav-1 in RasV12-transformed cells that are surrounded by normal cells in the presence of various inhibitors. Cells were stained with Hoechst 33342 (blue) and anti-Cav-1 (red) antibody. Scale bars: 10 μ m.

3.2.5. EPLIN is identified as one of the Cav-1-binding proteins when normal and RasV12-transformed cells interact with each other

To understand molecular mechanisms for Cav-1-mediated apical extrusion, we performed immunoprecipitation using anti-Cav-1 antibody with cell lysates from three different culture conditions: normal cells alone, RasV12 cells alone, and the mixture of normal and RasV12 cells. We found that multiple proteins were co-immunoprecipitated with Cav-1 specifically under the mix culture condition (Fig. 14A). Mass spectrometric analysis revealed that one of the Cav-1-binding proteins is EPLIN (Fig. 14A; arrowheads). We confirmed by western blotting that EPLIN binds to Cav-1 predominantly when normal and RasV12 cells interact with each other (Fig. 14B,C). These data suggest that the formation of novel protein complexes specifically occurs when normal and transformed epithelial cells interact with each other.

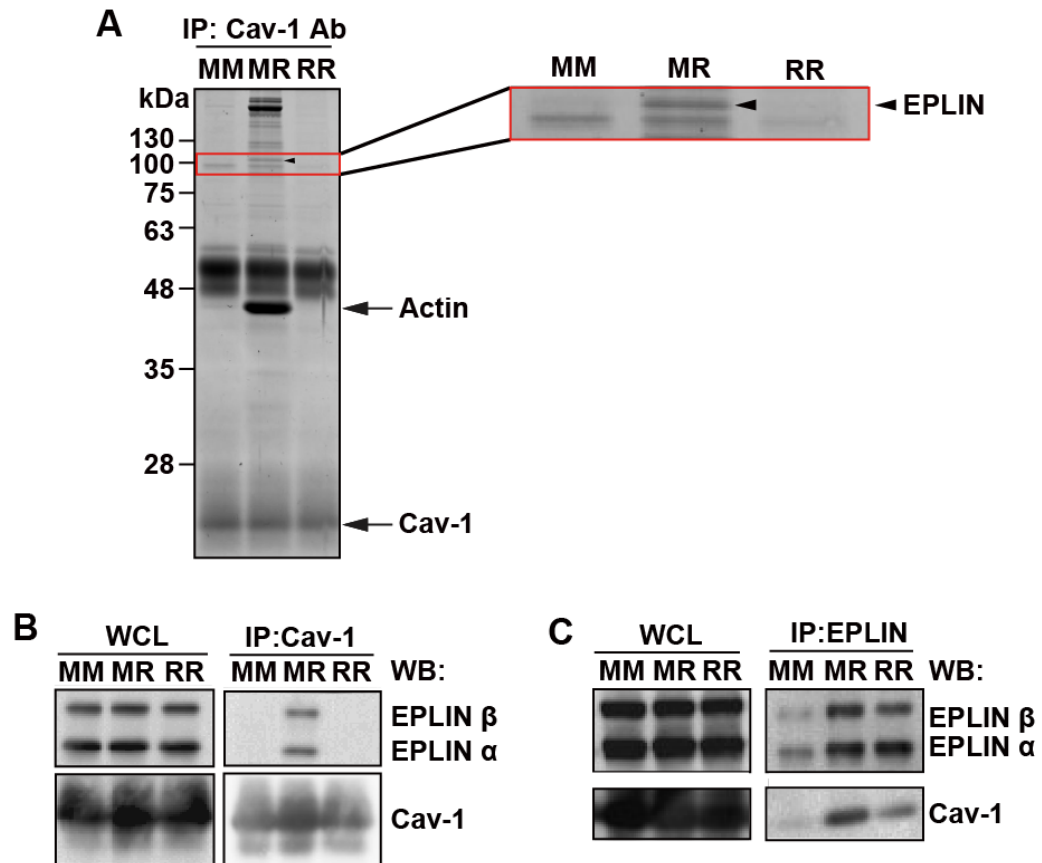


Fig. 14. Identification of proteins that specifically bind to Cav-1 when normal and RasV12-transformed cells interact with each other. (A) SYPRO Ruby protein staining of the immunoprecipitated proteins with anti-Cav-1 antibody. Black arrowheads indicate the band for EPLIN. Note that identification or validation of the other bands has not been completed yet. MM, normal MDCK cells cultured alone; MR, 1:1 mix culture of normal MDCK and MDCK-pTR GFP-RasV12 cells; RR, MDCK-pTR GFP-RasV12 cells cultured alone. (B,C) Co-immunoprecipitation of EPLIN and Cav-1 with anti-Cav-1 antibody (B) or anti-EPLIN antibody (C).

4. The influence of EPLIN identified as one of Cav-1-binding proteins on Cav-1

4.1. Introduction

We identified EPLIN that specifically binds to Cav-1 when normal and RasV12-transformed cells interact with each other. Epithelial Protein Lost In Neoplasm (EPLIN) was originally identified as a protein of which expression is often down-regulated or lost in various types of cancers⁴⁷⁻⁴⁹. EPLIN interacts with actin and regulates its dynamics by cross-linking and stabilizing actin filaments⁵¹. 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 junctions⁵³.

To investigate the function of EPLIN at the interface between normal and RasV12-transformed cells, we first examined subcellular localization of EPLIN by immunofluorescence analysis. Then, we established EPLIN-knockdown RasV12-transformed cells, and also examined the effect of knockdown of EPLIN on the fate of RasV12-transformed cells surrounded by normal cells. Furthermore, we analyzed functional relationship between Cav-1 and EPLIN. In addition, to examine the functional involvement of EPLIN in these signaling pathways, we examined the effect of various inhibitors on the apical extrusion and on the localization of EPLIN.

4.2. Results

4.2.1. EPLIN partially co-localizes with Cav-1 at the plasma membrane, and is also accumulated in RasV12-transformed cells surrounded by normal cells non-cell autonomously.

To investigate the function of EPLIN at the interface between normal and RasV12-transformed cells, we first examined subcellular localization of EPLIN by immunofluorescence analysis. We found that, in RasV12 cells surrounded by normal cells, EPLIN was accumulated at both the apical and lateral membrane domains where Cav-1 was partially co-localized (Fig. 15A). In addition, EPLIN was also localized at the cytosol (Fig. 15A,B) and intercellular regions between RasV12 cells where Cav-1 was absent (Fig. 15A; arrowheads). In contrast, when RasV12 cells were cultured alone, EPLIN was localized at the lateral membrane domain to a lesser extent (Fig. 15A). Comparable non-cell autonomous accumulation of EPLIN was also observed in Src-transformed cells (Fig. 15C). EPLIN was shown to interact with the α -catenin- β -catenin complex⁵³. 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 (Fig. 16), suggesting that accumulation of EPLIN may be regulated independently of the cell-cell adhesion complex.

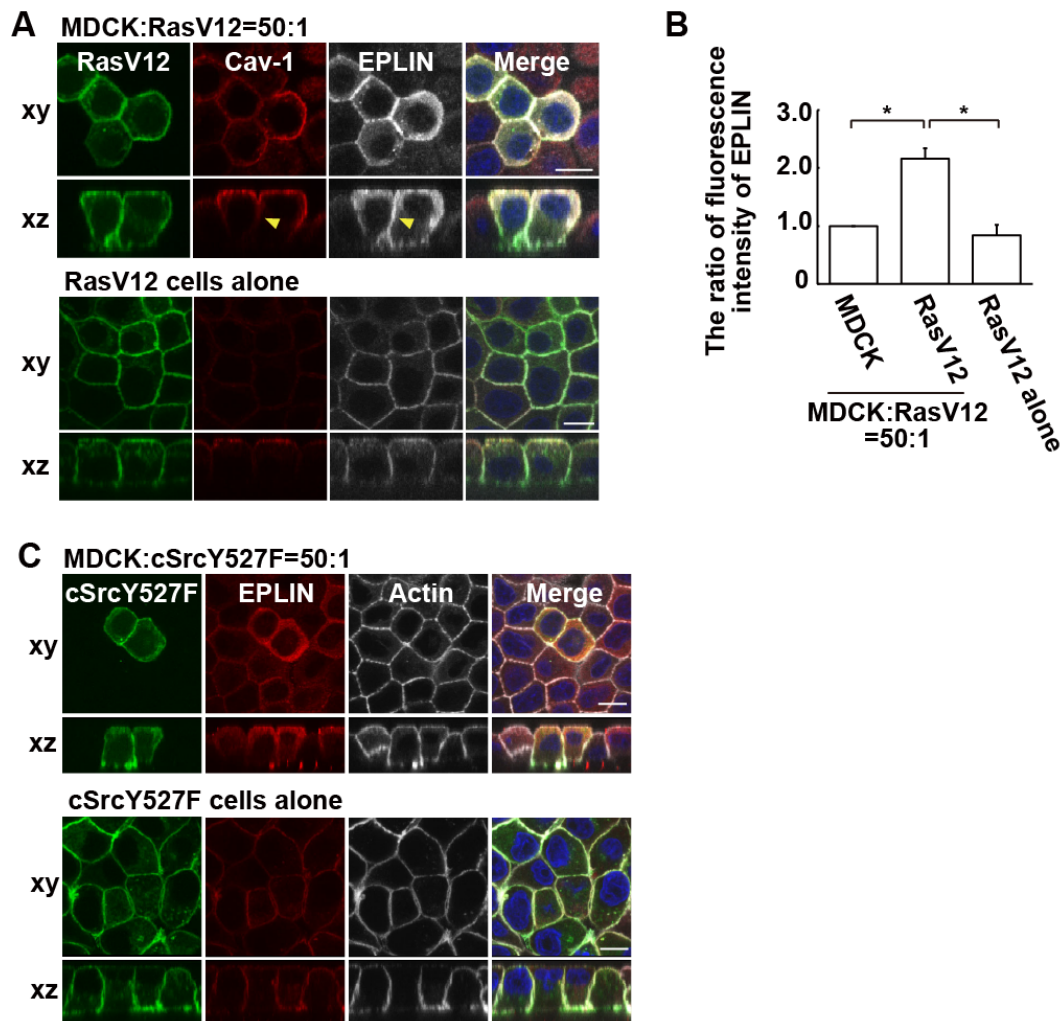


Fig. 15. EPLIN is accumulated at the cytosol and membrane domains in RasV12-transformed cells surrounded by normal cells. (A) Cav-1 partially co-localizes with EPLIN at the apical and lateral membrane domains in RasV12-transformed cells that are surrounded by normal cells. MDCK-pTR GFP-RasV12 cells (green) were mixed with normal MDCK cells (upper panels) or cultured alone (lower panels), and incubated with tetracycline for 16 hours. Cells were stained with Hoechst 33342 (blue), anti-Cav-1 (red), and anti-EPLIN (white) antibodies. Arrowheads indicate EPLIN accumulation at the intercellular regions between RasV12-transformed cells where Cav-1 was absent. Scale bars: 10 μ m. (B) Quantification of the fluorescence intensity of EPLIN at the cytosol. Data are mean $\pm$ s.d. from three independent experiments. Values are expressed as a ratio relative to MDCK cells. * $P < 0.005$; $n = 83, 86$, and 75 cells. Data are mean $\pm$ s.d. from three independent experiments. * $P < 0.01$; $n = 158$ and 176 cells. (B) Immunofluorescence images of EPLIN 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-EPLIN antibody (red), and Alexa-Fluor-647-conjugated phalloidin (white). Scale bars: 10 μ m.

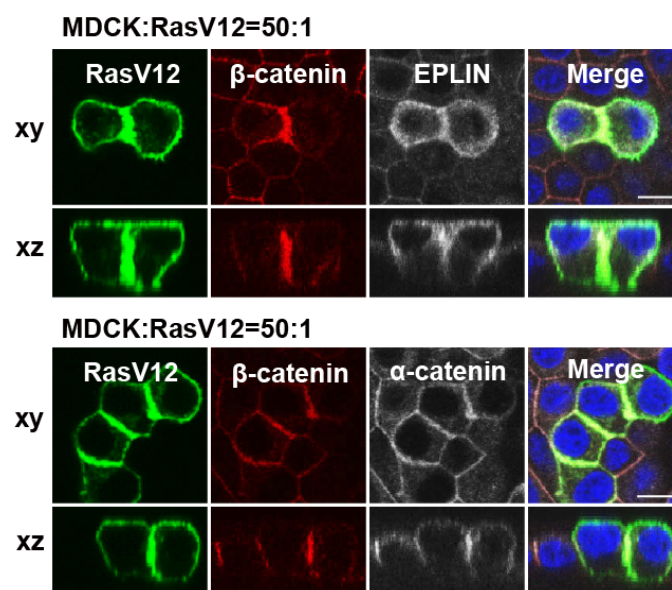


Fig. 16. Accumulation of EPLIN is regulated independently of the cell-cell adhesion complex. Immunofluorescence images of EPLIN, α -catenin, and β -catenin in RasV12-transformed cells that are surrounded by normal cells. Cells were stained with Hoechst 33342 (blue), anti- β -catenin (red), and anti-EPLIN (upper panels, white) or anti- α -catenin (lower panels, white) antibodies. Scale bars: 10 μ m.

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

Next, we established RasV12 cells stably expressing EPLIN shRNA (Fig. 17A-D). EPLIN knockdown in RasV12 cells significantly suppressed apical extrusion (Fig. 18A-D), indicating that EPLIN also plays a crucial role in the elimination of the transformed cells.

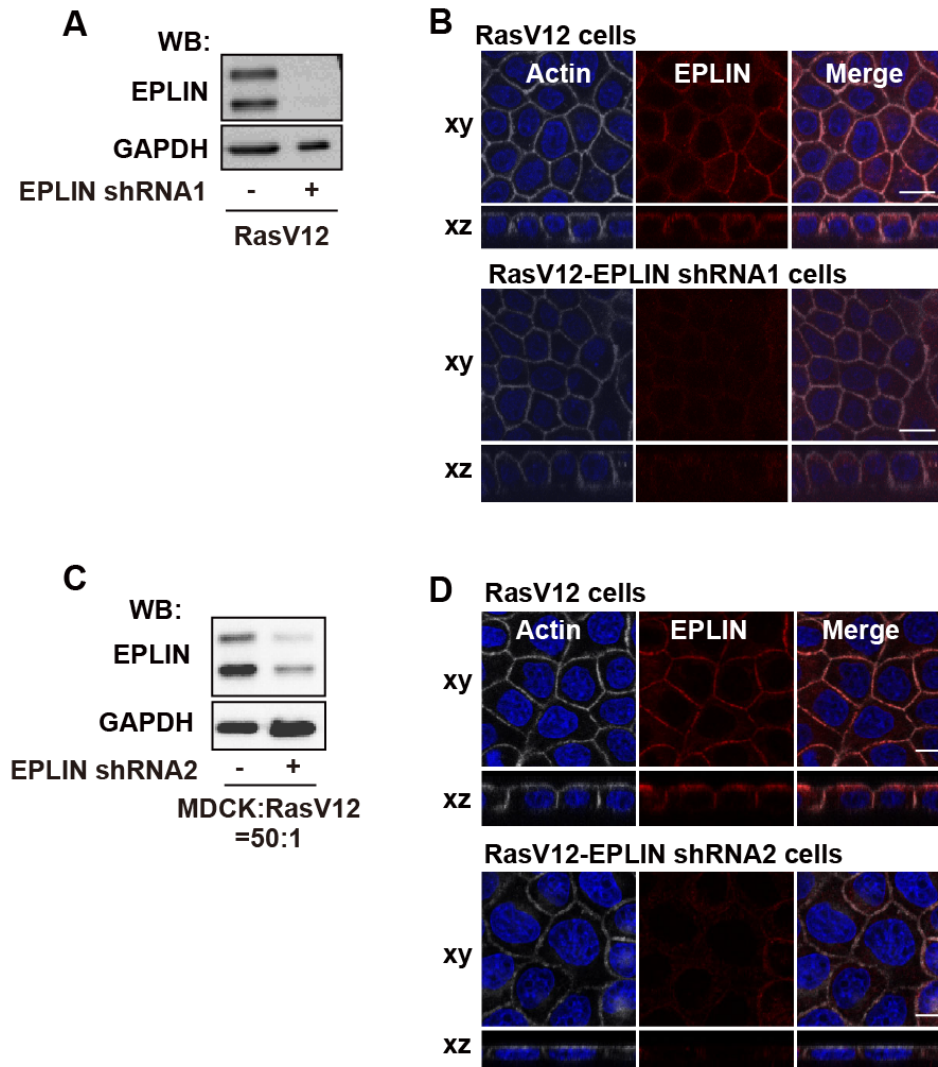


Fig. 17. Establishment of EPLIN-knockdown RasV12-transformed cells.. (A,C) Establishment of MDCK-pTR GFP-RasV12 cells stably expressing EPLIN shRNA1 (A) or shRNA2 (C). Knockdown of EPLIN protein was analyzed by western blotting using anti-EPLIN antibody. Equal protein loading was confirmed using anti-GAPDH antibody. (B,D) Immunofluorescence images showing the effect of knockdown of EPLIN. MDCK-pTR GFP-RasV12 cells (upper panels) or MDCK-pTR GFP-RasV12-EPLIN shRNA1 (B) or shRNA2 (D) 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 μ m.

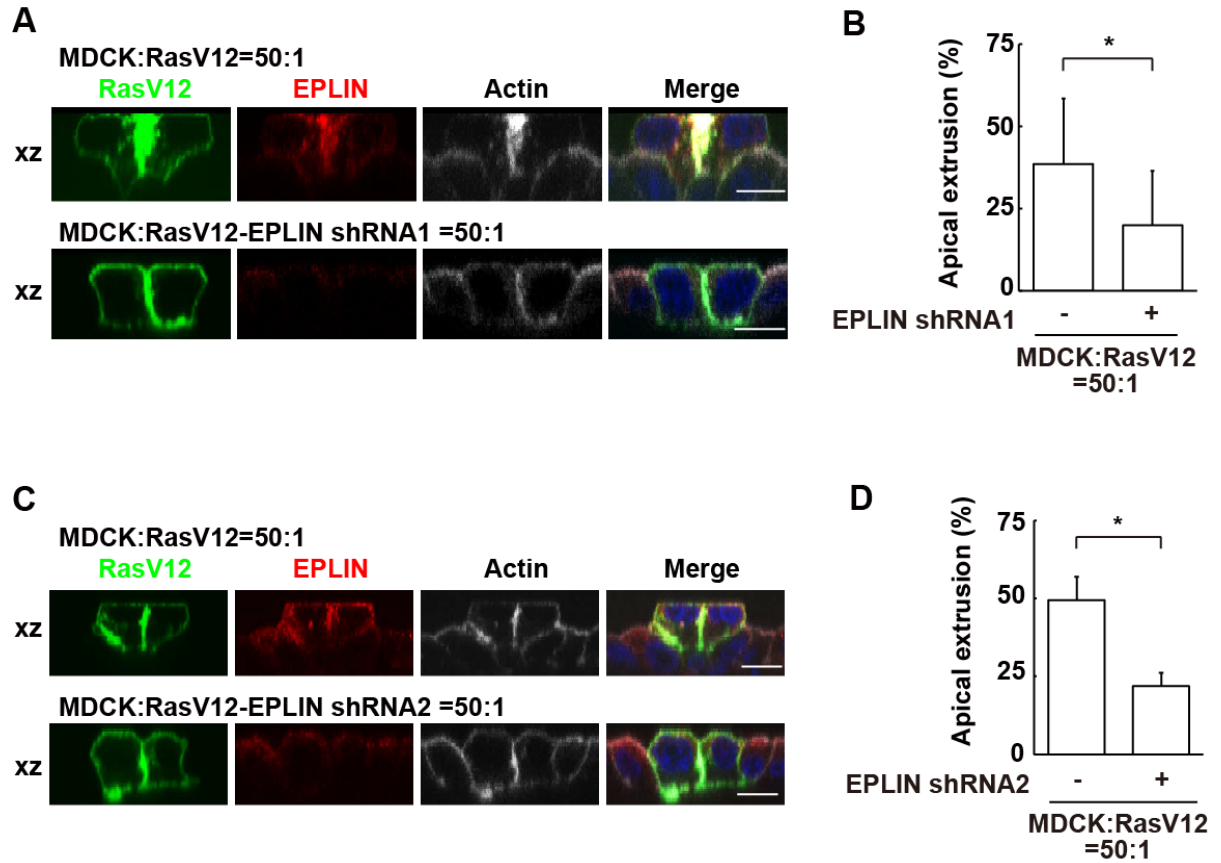


Fig. 18. EPLIN also plays an active role in the apical extrusion of transformed cells. (A,C) Knockdown of EPLIN suppresses the apical extrusion of RasV12-transformed cells. Normal MDCK cells were mixed with MDCK-pTR GFP-RasV12 cells (upper panels, green) or with MDCK-pTR GFP-RasV12-EPLIN shRNA1 (A) or shRNA2 (C) 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 μ m. (B,D) Quantification of the apical extrusion for (A), (C), respectively.

4.2.3. EPLIN functions upstream of Cav-1

Furthermore, we analyzed 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. 19A,B). Consistently, addition of PDMP did not substantially influence the accumulation of EPLIN (Fig. 20A). In 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. 19C,D). EPLIN knockdown also suppressed accumulation of Lysenin (Fig. 20B), 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 localization of gp135 (Fig. 20C), suggesting that the effect on Cav-1 is not due to perturbation in cell morphology or cell polarity.

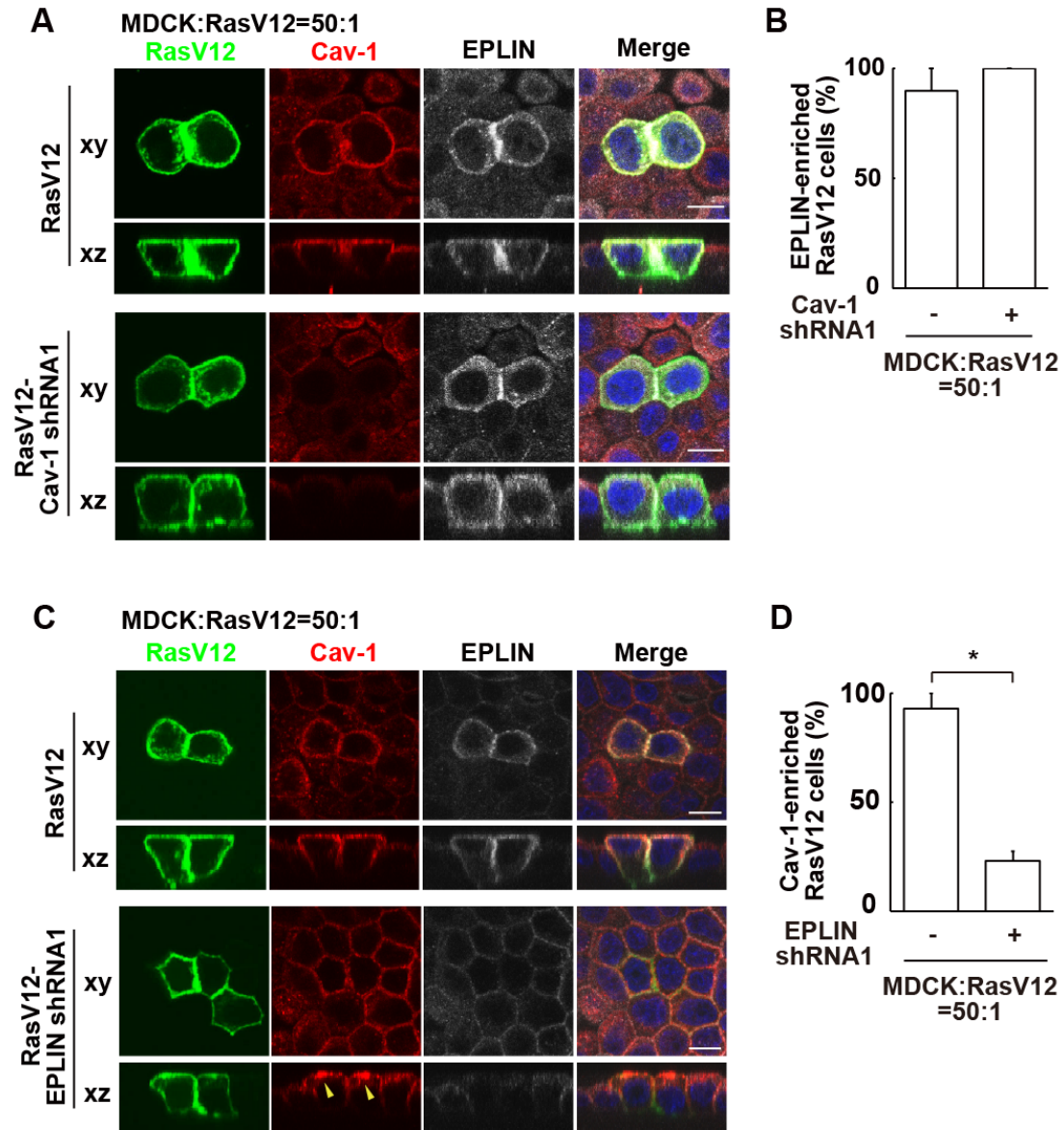


Fig. 16. EPLIN functions upstream of Cav-1. (A,C) Immunofluorescence images of EPLIN in Cav-1-knockdown RasV12-transformed cells (A) or of Cav-1 in EPLIN-knockdown RasV12-transformed cells (C). Cells were stained with Hoechst 33342 (blue), anti-Cav-1 (red), and anti-EPLIN (white) antibodies. Arrowheads indicate patchy localization of Cav-1. Scale bars: 10 μ m. (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 mean $\pm$ s.d. from two (B) or three (D) independent experiments. * P <0.01; n=64 and 62 cells (B); 79 and 119 cells (D).

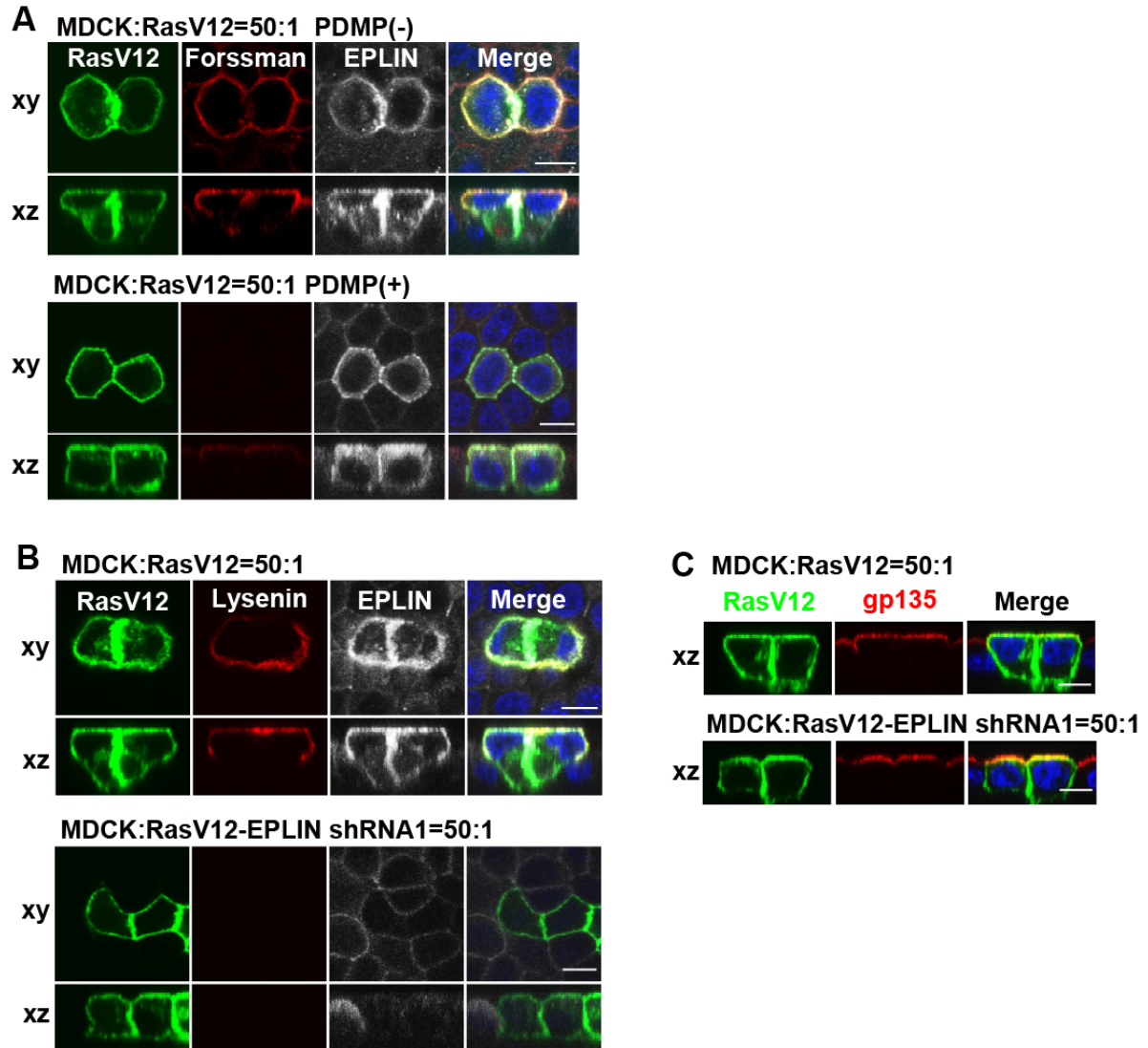


Fig. 20. EPLIN functions upstream of Cav-1-containing domains. (A) Immunofluorescence images of Forssman and EPLIN in RasV12-transformed cells in the absence (upper panels) or presence (lower panels) of PDMP. MDCK-pTR GFP-RasV12 cells (green) were mixed with normal MDCK cells, and incubated with tetracycline 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 μ m. (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 μ m. (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 μ m.

4.2.4. MAPK and dynamics of actin filaments regulate accumulation of EPLIN

During the apical extrusion of transformed cells, various signaling pathways are activated, which plays a positive role in this process^{54,55}. To examine the functional involvement of EPLIN in these signaling pathways, we examined the effect of various inhibitors on the apical extrusion and on the localization of EPLIN (Table 3). Among the signaling pathways downstream of active Ras, the MAPK pathway plays a crucial role in the apical extrusion of RasV12-transformed cells³. Upon addition of MEK inhibitor U0126, it also induced dotted accumulation of EPLIN at the apical side of RasV12 cells (Fig. 21). In contrast, PI3 kinase inhibitor LY294002 did not substantially affect EPLIN localization (Fig. 21). These data show that activity of the MAPK pathway regulates non-cell autonomous enrichment of EPLIN. Cytochalasin D, which blocks actin polymerization, strongly perturbed the enrichment of EPLIN (Fig. 21), indicating that dynamics of actin filaments are required for the establishment and/or maintenance of EPLIN accumulation. Myosin-II inhibitor blebbistatin induced dotted accumulation of EPLIN at the apical side (Fig. 21). PKA inhibitor KT5720 did not affect the accumulation of EPLIN (Fig. 21).

Inhibitor	Target	RasV12	
		Extrusion	EPLIN enrichment
BIM-I	PKC	ND	No effect
Blebbistatin	Myosin-II	↓*	No effect [†]
CK666	Arp2/3	No effect	No effect
Cytochalasin D	Actin polymerization	↓*	↓*
IPA-3	PAK	ND	No effect
KT5720	PKA	ND	No effect
LY294002	PI3K	No effect	No effect
MbCD	Cholesterol	↓*	No effect
PDMP	Glucosylceramide synthase	↓*	No effect
PP2	Src-family tyrosin kinases	↓*	No effect
U0126	MAPK	↓*	No effect [†]
Y27632	Rho kinase	↓*	No effect

Table 3. Effect of various inhibitors on the apical extrusion of RasV12-transformed cells and on EPLIN enrichment in RasV12-transformed cells that are surrounded by normal cells. *: statistically significant; †: with dotted apical accumulation; ND: not done; Grey box: our published observations.

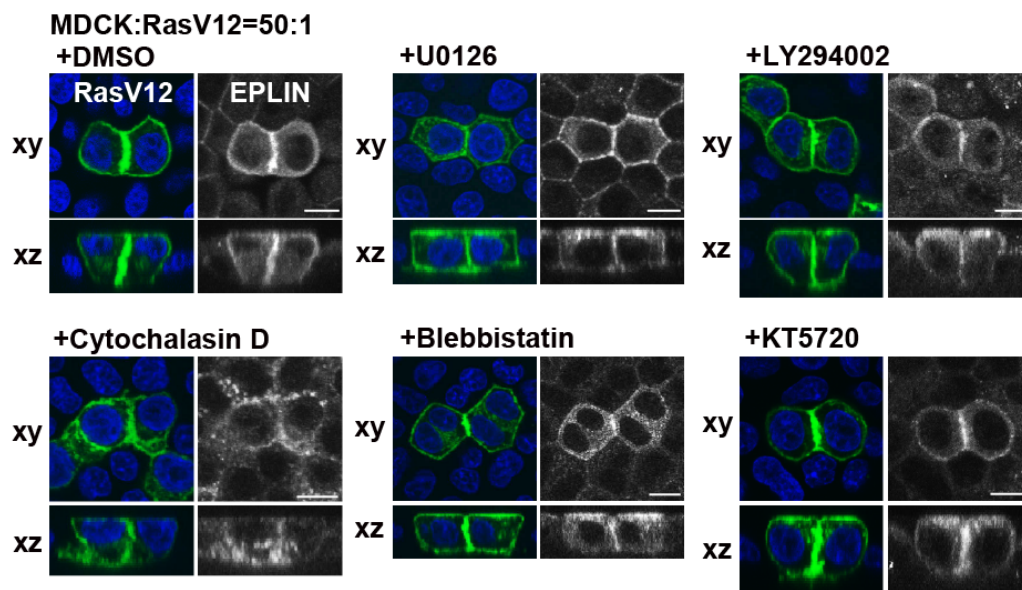


Fig. 21. Effect of various inhibitors on the non-cell autonomous accumulation of EPLIN.

Immunofluorescence images of EPLIN in RasV12-transformed cells that are surrounded by normal cells in the presence of various inhibitors. Cells were stained with Hoechst 33342 (blue) and anti-EPLIN (white) antibody. Scale bars: 10 μ m.

5. Clarification of multiple molecular mechanisms controlled by EPLIN

5.1. Introduction

In the previous studies, we have reported that activity of myosin-II and PKA is enhanced in RasV12- transformed cells surrounded by normal cells ^{3,56}(also shown in Fig. 23A; Fig. 24A). When transformed cells are surrounded by normal cells, myosin-II activity is enhanced and the transformed cells become round and tall, suggesting the elevated tensile forces within the transformed cells ^{3,4} (Fig. 22). Furthermore, reduction of myosin-II activity in the transformed cells diminishes these morphological changes ³. Collectively, the pulling forces at the interface are likely to be mediated by enhanced myosin-II activity in the transformed cells, which would physically drive the apical extrusion process. Phosphorylation of VASP at serine 239 was specifically upregulated in RasV12-transformed cells interacting with normal cells. VASP phosphorylation was required for the apical extrusion of RasV12-transformed cells and occurred downstream of PKA ⁵⁶. The activation of PKA occurred in a non-cell-autonomous fashion, indicating that RasV12-transformed cells recognize and respond to the presence of normal cells, resulting in the stimulation of PKA in the transformed cells (Fig. 22).

Furthermore, we have recently reported that filamin accumulates in normal cells at the interface with RasV12-transformed cells and plays a vital role in EDAC (Epithelial Defense Against Cancer) which is the process that at the early stage of carcinogenesis, normal epithelial cells act as ‘immunity’

against transformed cells ⁵⁷. Knockdown of filamin in normal epithelial cells profoundly suppresses apical extrusion of the neighboring transformed cells. In addition, the Rho/Rho kinase pathway regulates filamin accumulation and filamin acts upstream of vimentin filaments that dynamically react against transformed cells (Fig. 22).

In this chapter, we examined whether EPLIN or Cav-1 regulates activity of these signaling pathways, or *vice versa*.

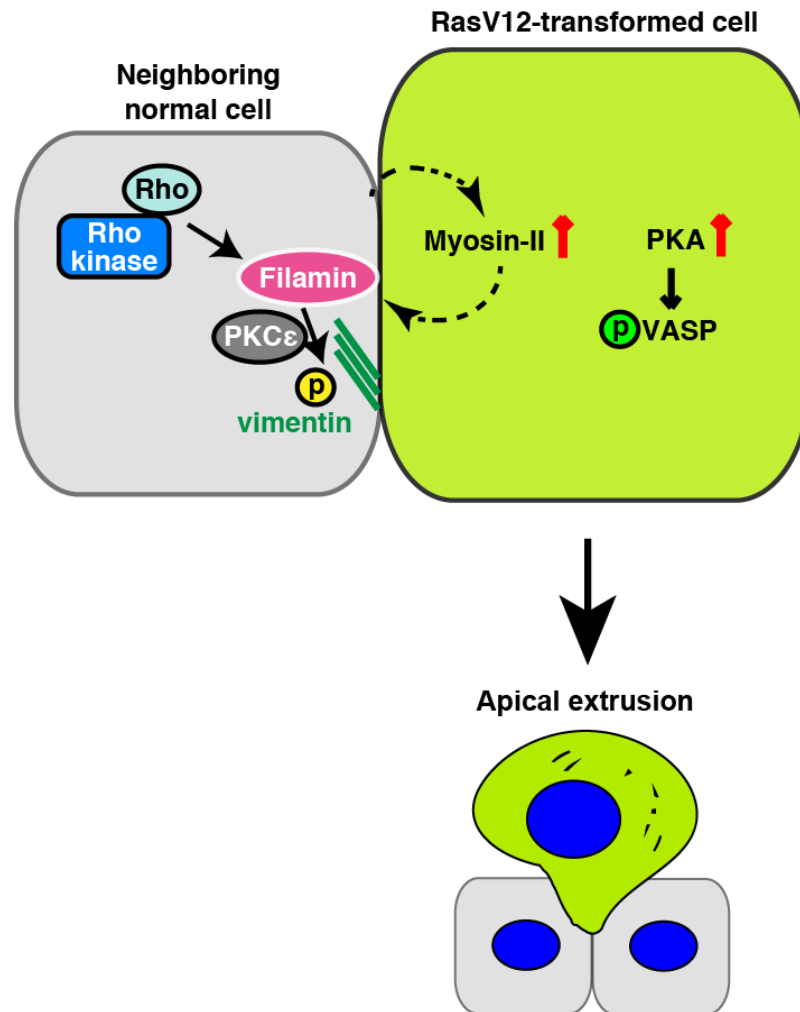


Fig. 22. A previous schematic model of the molecular regulations at the interface between transformed and the surrounding normal cells. When transformed cells are surrounded by normal cells, the activity of PKA and myosin-II are enhanced in the transformed cells, leading to filaminA accumulation at the interface between normal and transformed cells.

5.2. Results

5.2.1. EPLIN is a crucial regulator for the activation of myosin-II and PKA

We examined whether EPLIN or Cav-1 regulates activity of these signaling pathways, or *vice versa*. EPLIN knockdown in RasV12-transformed cells significantly suppressed activation of myosin-II (Fig. 23A,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 manner³. Consistently, we found that EPLIN knockdown significantly suppressed the height of RasV12 cells that were surrounded by normal cells (Fig. 23C). By contrast, Cav-1 knockdown did not affect myosin-II activity (Fig. 23D). In addition, expression of a dominant negative form of myosin light chain (MLCAA) did not influence EPLIN or Cav-1 enrichment (Fig. 23E,F). Similarly, activity of PKA was substantially repressed by EPLIN knockdown, but not by Cav-1 knockdown (Fig. 24A-C). Collectively, these data indicate that EPLIN plays a crucial role in the non-cell autonomous up-regulation of myosin-II and PKA, independently of Cav-1 (Fig. 26).

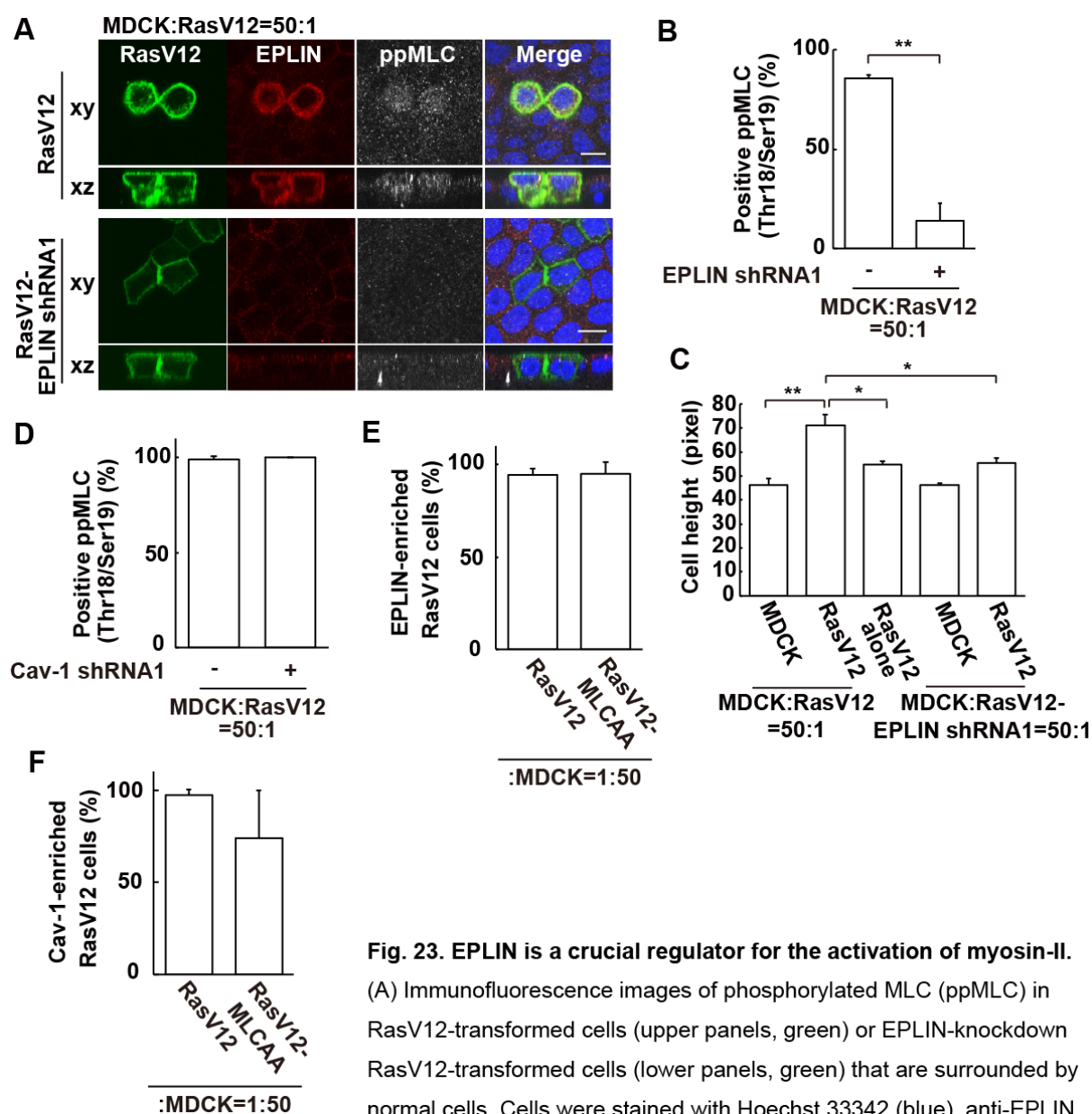


Fig. 23. EPLIN is a crucial regulator for the activation of myosin-II.

(A) Immunofluorescence images of phosphorylated MLC (ppMLC) in RasV12-transformed cells (upper panels, green) or EPLIN-knockdown RasV12-transformed cells (lower panels, green) that are surrounded by normal cells. Cells were stained with Hoechst 33342 (blue), anti-EPLIN (red), and anti-ppMLC antibodies. Scale bars: 10 μ m. (B) Quantification of phosphorylated MLC in RasV12-transformed cells or EPLIN-knockdown RasV12-transformed cells that are surrounded by normal cells. (C) 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 $\pm$ s.d. from three independent experiments. * P <0.05, ** P <0.005; n = 80, 62, 64, 96, and 82 cells. (D) Quantification of phosphorylated MLC in RasV12-transformed cells or Cav-1-knockdown RasV12-transformed cells that are surrounded by normal cells. Data are mean $\pm$ s.d. from three independent experiments. * P <0.05 and ** P <0.005; n =118 and 167 cells (B); 85 and 71 cells (D). (E,F) Quantification of EPLIN (E) or Cav-1 (F) 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 $\pm$ s.d. from three independent experiments. n =191 and 173 cells (E); 174 and 148 cells (F).

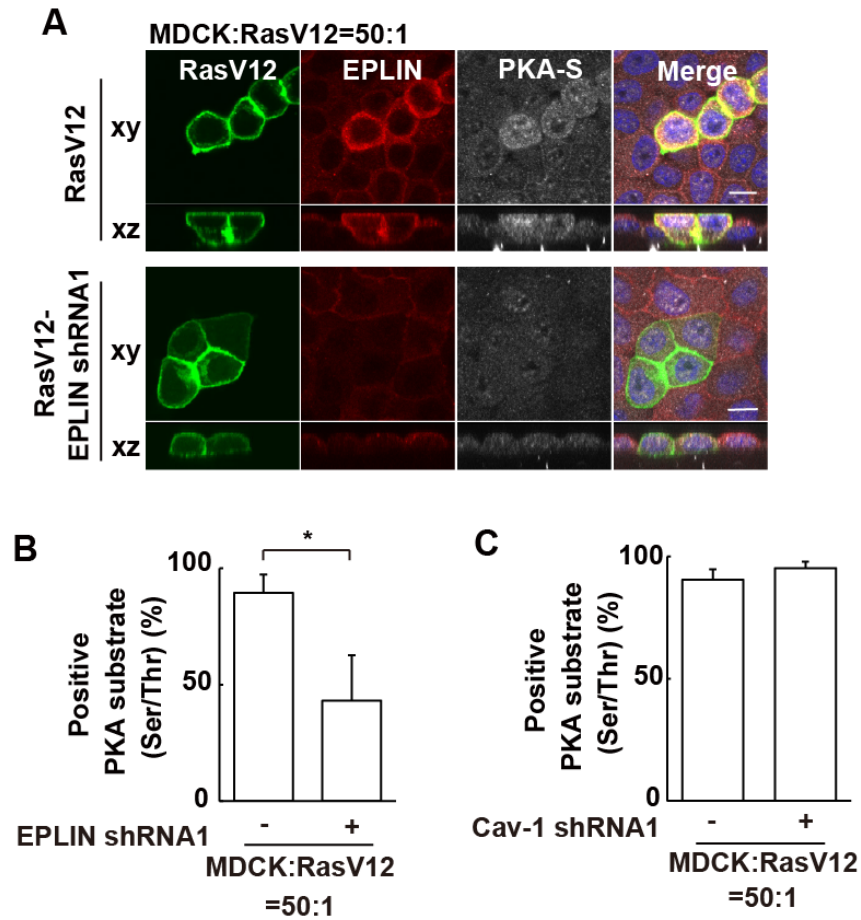


Fig. 24. EPLIN is a crucial regulator for the activation of PKA. (A) Immunofluorescence images of phosphorylated PKA substrate (PKA-S) in RasV12-transformed cells (upper panels, green) or EPLIN-knockdown RasV12-transformed cells (lower panels, green) that are surrounded by normal cells. Cells were stained with Hoechst 33342 (blue), anti-EPLIN (red), and anti-PKA-S (A, white) antibodies. Scale bars: 10 μ m. (B) Quantification of phosphorylated PKA substrate in RasV12-transformed cells or EPLIN-knockdown RasV12-transformed cells that are surrounded by normal cells. (C) Quantification of phosphorylated PKA substrate in RasV12-transformed cells or Cav-1-knockdown RasV12-transformed cells that are surrounded by normal cells. Data are mean $\pm$ s.d. from three independent experiments. * $P < 0.05$ and ** $P < 0.005$; $n = 85$ and 71 cells (B); 132 and 134 cells (C).

5.2.2. Mutual regulation between Cav-1 and EPLIN in RasV12 cells and filamin in the surrounding normal cells.

We have recently reported that filamin accumulates in normal cells at the interface with RasV12-transformed cells and plays a vital role in EDAC (Epithelial Defense Against Cancer) ⁵⁷. 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. 25A-D). 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. 25E-H). These data indicate that there exist mutual regulatory mechanisms between EPLIN-Cav-1 in RasV12-transformed cells and filamin in the surrounding normal cells (Fig. 26).

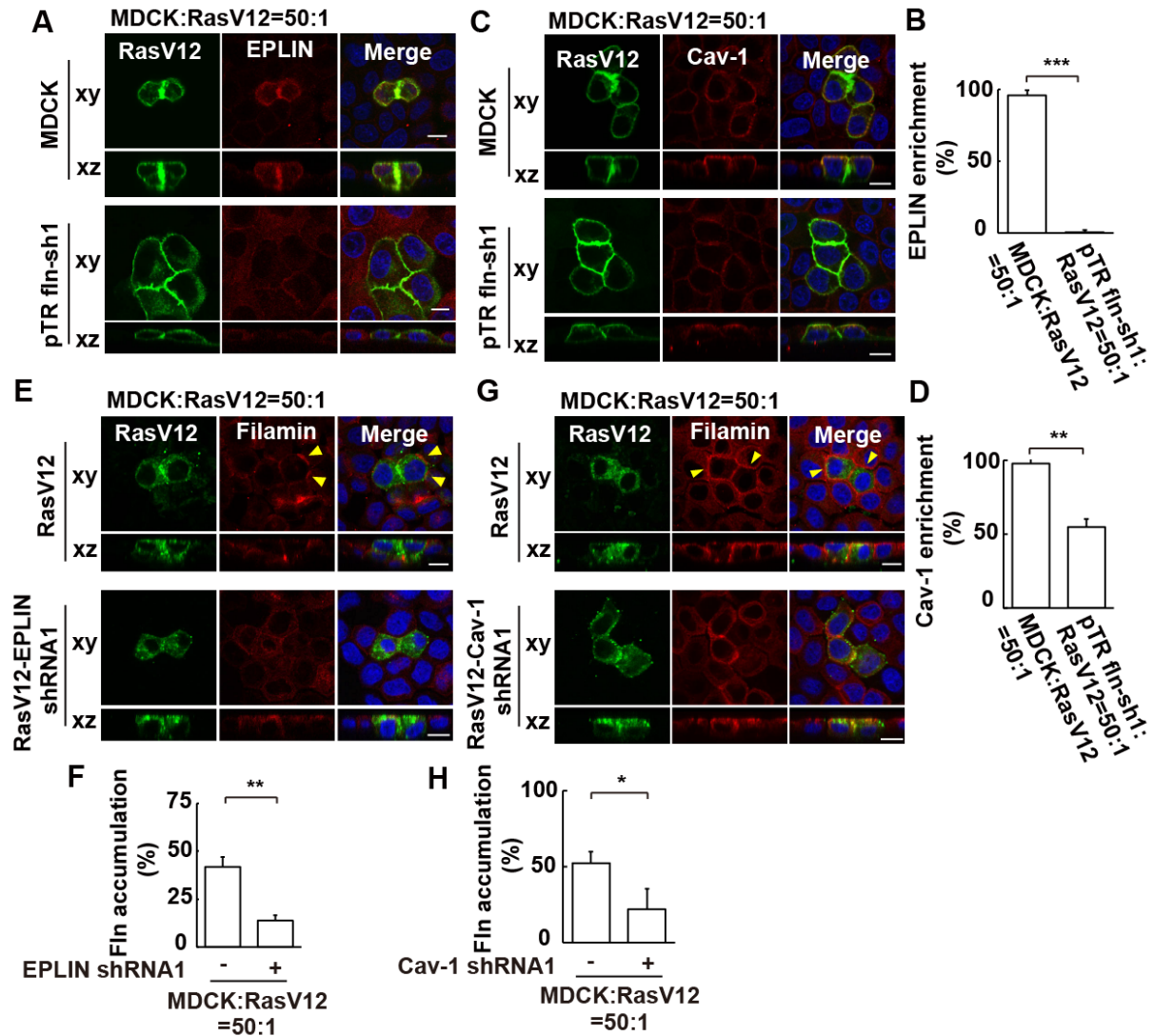


Fig. 25. Mutual regulatory mechanisms between EPLIN-Cav-1 in RasV12-transformed cells and filamin in the surrounding normal cells. (A,C) Immunofluorescence images of EPLIN (A) or Cav-1 (C) in RasV12-transformed cells (green) surrounded by normal MDCK cells (upper panels) or filamin A-knockdown MDCK cells (lower panels). Cells were stained with Hoechst 33342 (blue), anti-EPLIN (A, red) or anti-Cav-1 (C, red) antibody. Scale bars: 10 μ m. (B,D) Effect of knockdown of filamin A in the surrounding normal cells on the enrichment of EPLIN (B) or Cav-1 (D) in RasV12-transformed cells. (E,G) Immunofluorescence images of effect of knockdown of EPLIN (E) or Cav-1 (G) in RasV12-transformed cells (green) on the filamin accumulation in the neighboring normal cells. Cells were stained with Hoechst 33342 (blue), anti-filamin (red) antibody. Arrowheads indicate the filamin accumulation in the neighboring normal cells. Scale bars: 10 μ m. (F,H) Quantification of effect of knockdown of EPLIN (F) or Cav-1 (H) in RasV12-transformed cells on the filamin accumulation in the neighboring normal cells. Data are mean $\pm$ s.d. from three independent experiments. * P <0.05, ** P <0.005, and *** P <0.001; n =143 and 147 cells (B); 141 and 139 cells (D); 131 and 133 cells (F); 125 and 126 cells (H).

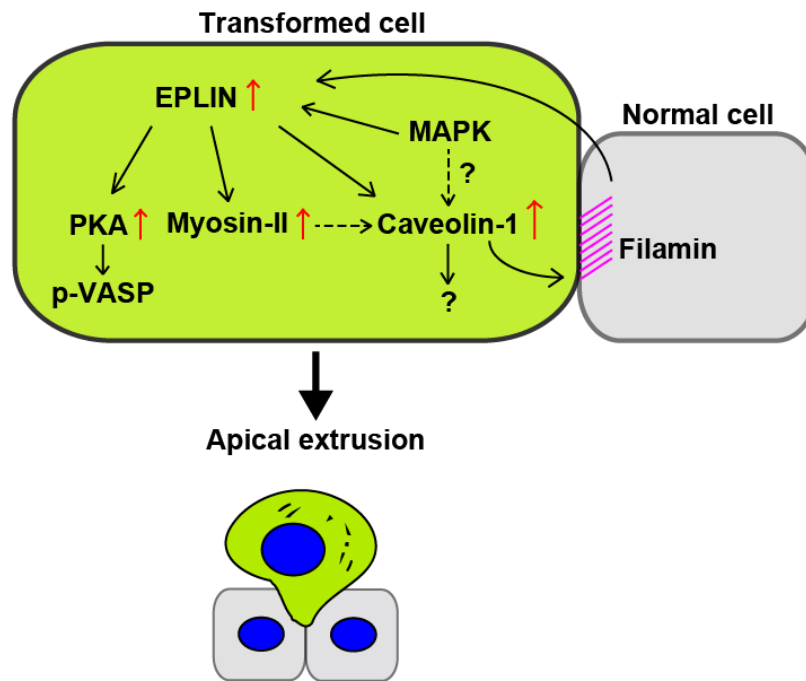


Fig. 26. A schematic model of the molecular regulations at the interface between transformed and the surrounding normal cells. When transformed cells are surrounded by normal cells, EPLIN is upregulated in the transformed cells, leading to Cav-1 accumulation and activation of myosin-II and PKA. EPLIN 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.

6. Discussion

6.1. Conclusion

In this study, we present several lines of evidence demonstrating that EPLIN is a crucial regulator for extrusion of RasV12-transformed cells (Fig. 26). 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 accumulation of filamin in the neighboring normal cells, and *vice versa*. In the previous studies, we have shown that myosin-II is activated in transformed cells when surrounded by normal cells and that enhanced myosin-II activity leads to increased cellular elasticity in the transformed cells, which induces accumulation of mechanosensor filamin in the neighboring normal cells^{3,4}. Collectively, our data suggest that EPLIN functions upstream of myosin-II in this process, thereby promoting apical extrusion via filamin.

In this future, we need to reveal several important points: (i) Which functions of Cav-1 is involved in enrichment of Cav-1 at the both apical and lateral membrane domains and apical extrusion of RasV12 cells? (ii) What is the functional significance of EPLIN accumulation in the cytosol? (iii) How

does Cav-1 interact with EPLIN in RasV12-transformed cells surrounded by normal cells? (iv) How do Cav-1-containing microdomains positively regulate the process of apical extrusion? (v) Is EPLIN or Cav-1 functionally involve in the initial stage of carcinogenesis?

6.2. (i) Which functions in Cav-1 is involved in enrichment of Cav-1 at the both apical and lateral membrane domains and apical extrusion of RasV12 cells?

Cav-1 recruits and regulates various signaling proteins through the interaction of its scaffolding domain. For instance, activity of EGF receptor ⁵⁸, NO synthase ⁵⁹, G proteins ⁶⁰, protein kinase C ⁶¹ and Src family proteins are negatively regulated by this interaction, whereas insulin receptor (IR) ⁶², Eph receptors ⁶³ or Estrogen Receptor ⁶⁴ are activated. We will clarify whether the interaction between CSD Cav-1 and these several proteins is involved in this study, and elucidate molecular mechanisms upstream or downstream of Cav-1. Furthermore, Cav-1 participates to the transmission of mechanical stimuli, such as mechanical stress, involved in cell reorganization and migration, through signaling regulation which modulates cells adhesion and cytoskeleton organization ^{65,66}. Endothelial cells undergoing shear stress display increased Cav-1 expression associated with ERK1/2 activation and caveolae formation ^{67,68}. Caveolae formation might occur at the luminal surface of endothelial cells resulting in local signaling via eNOS and G proteins ^{65-67,69}. Although Cav-1 expression is not changed under three different culture conditions: normal cells

alone, RasV12 cells alone, and the mixture of normal and RasV12 cells (Fig. 14B), we will examine the involvement of Cav-1 enrichment in activation of eNOS and G proteins at the interface between normal and transformed epithelial cells. A recent theoretical study has proposed that budded membrane domains like caveolae could play the role of membrane-mediated sensors and regulators of the plasma membrane tension ⁷⁰. Mechanical stress could cause changes in membrane elasticity ⁷¹. This is determined by the lipid composition of the bilayer. Membrane elasticity regulates the hydrophobic coupling between a membrane-spanning protein and the surrounding bilayer. Changes in cholesterol concentration have been shown to have a specific role in modifying the elastic properties of this coupling ⁷². Previously, it has been found that RasV12-transformed cells have higher membrane elasticity and cell viscosity than normal cells ³. Therefore, increase of Cav-1/caveolae at both the apical and lateral plasma membrane may involve in the elasticity of RasV12 transformed cells surrounded by normal cells.

6.3. (ii) What is the functional significance of EPLIN accumulation in the cytosol?

We have observed that the addition of MEK inhibitor U0126 induces dotted accumulation of EPLIN at the apical side of RasV12 cells (Fig. 21). This data show that activity of the MAPK pathway regulates non-cell autonomous enrichment of EPLIN. EPLIN contains a centrally located LIM domain that may mediate self-dimerization and N- and C-terminal actin-binding sites flanking the

LIM domain⁵¹. EPLIN cross-links and bundles actin filaments, thereby stabilizing actin stress fibers. Furthermore, EPLIN inhibits Arp2/3 complex-mediated branching nucleation of actin filaments. Thus, EPLIN controls actin filament dynamics by stabilizing actin filament networks⁵¹. Previously, EPLIN was identified as a novel substrate for ERK⁵². Furthermore, ERK-mediated phosphorylation of Ser360, Ser602, and Ser692 in EPLIN contributes to actin filament reorganization and enhanced cell motility. ERK-mediated phosphorylation of EPLIN may contribute to localization change from the lateral membrane domain to cytosol and actin filament reorganization. In addition, EPLIN interacts with α -catenin, and thus links the cadherin-catenin complex to F-actin⁵³. However, α -catenin is not co-localized with EPLIN at the apical membrane domain or in the cytosol, suggesting that the non-cell autonomous accumulation of EPLIN occurs by an α -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.

6.4. (iii) How does Cav-1 interact with EPLIN in RasV12-transformed cells surrounded by normal cells?

We found that EPLIN is co-immunoprecipitated with Cav-1 specifically under the mix culture condition (Fig. 14A). EPLIN binds to Cav-1 predominantly when normal and RasV12 cells interact with each other (Fig. 14B,C). In addition, in RasV12 cells surrounded by normal cells, EPLIN was accumulated

at both the apical and lateral membrane domains where Cav-1 was partially co-localized (Fig. 15A). Immunoprecipitation using anti-Cav-1 antibody showed that the band of actin is higher detected under the mix culture condition (Fig. 14A). Caveolae biology is intimately linked to the actin cytoskeleton^{33,73-75}, which is a key regulator of caveolae endocytosis^{76,77}. Disruption of the actin cytoskeleton using cytochalasin D strongly perturbs the enrichment of not only Cav-1 but also EPLIN (Fig. 13; Fig. 21). EPLIN is known as an actin-binding protein that regulates actin filament dynamics and cross-linking⁵¹. We need to be clarified whether Cav-1 directly interacts with EPLIN or indirectly via actin filament in future studies.

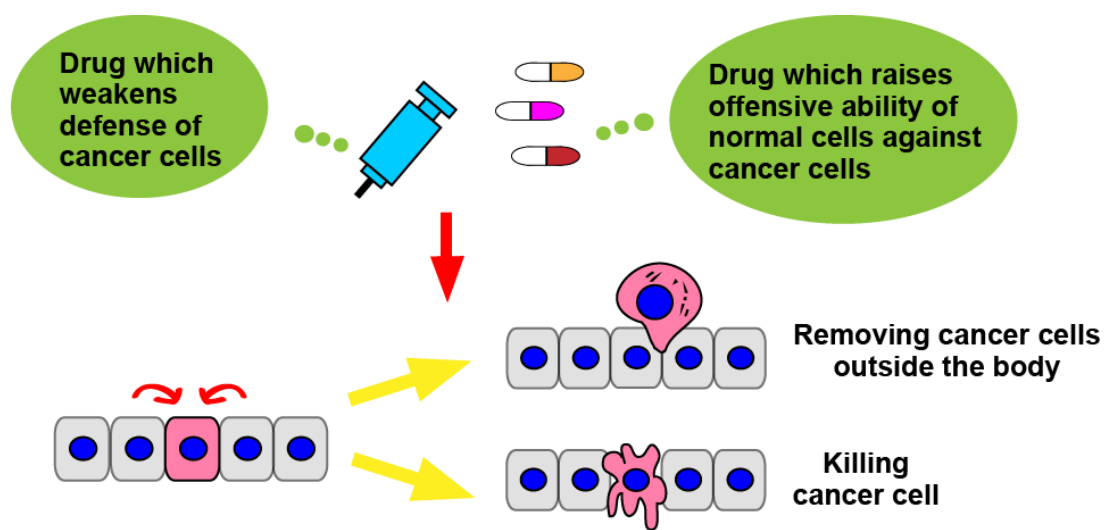
6.5. (iv) How do Cav-1-containing microdomains positively regulate the process of apical extrusion?

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 (Fig. 9A), and conversely, Cav-1 knockdown diminishes enrichment of Forssman antigen or lysenin (Fig. 11A,B). In addition, EPLIN knockdown perturbs accumulation of Cav-1 and lysenin (Fig. 19C,D; Fig. 20B). 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. 23D; Fig. 24C). The data shown in Fig. 14A 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.

6.6. (v) Is EPLIN or Cav-1 functionally involve in the initial stage of carcinogenesis?

EPLIN was originally identified as a tumor suppressor protein, and down-regulation of EPLIN expression was observed in various types of cancers⁴⁷⁻⁴⁹. In addition, expression of Cav-1 is often reduced in multiple cancers⁴⁴⁻⁴⁶. However, their roles in carcinogenesis still remain elusive. Our results demonstrate that knockdown of EPLIN or Cav-1 substantially suppresses apical extrusion of transformed cells, implying a notion that 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/Cav-1 would lead to establishment of novel types of cancer prevention and treatment: enhancing the ability of surrounding normal cells to fight against cancer cells or attenuating the defense of cancer cells against neighboring normal cells (Fig. 27).



*** Creating a novel type of cancer therapy**
 「Attacking cancer cells by the help of surrounding normal cells.」

Fig. 27. Novel types of cancer prevention or treatment. Removing or killing cancer cells by weakening defence of cancer cells or raising offensive ability in normal cells against cancer cells.

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