学　位　論　文

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規制御因子である）

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A) List of publications and presentations

Publication

Ailijiang Kadeer, Takeshi Maruyama, Mihoko Kajita, Tomoko Morita, Ayana Sasaki, Atsuko Ohoka, Susumu Ishikawa, and Yasuyuki Fujita: Plectin is a novel regulator for apical extrusion of RasV12-transformed cells. *Sci. Rep.*, accepted on February 7th 2017.

Posters and Oral Presentations

(International conference)

Ailijiang Kadeer, Takeshi Maruyama, Yasuyuki Fujita, "A role of plectin in the interaction between normal and transformed cells", 2nd Cell Competition International Symposium (April 12, 2016, Kyoto, Japan). (Oral presentation)

(National conference)

1） ○Ailijiang Kadeer (アリジャン　カデル)、 梶田美穂子、 藤田恭之 :「変異細胞と正常上皮細胞の境界における plectin の役割」、第２回細胞競合コロキウム、2013 年３月、札幌。（ポスター発表、査読有）

2） ○Ailijiang Kadeer (アリジャン　カデル)、 梶田美穂子、 藤田恭之 :「変異細胞と正常上皮細胞の境界における plectin の役割」、第 3 回細胞競合コロキウム、2014 年３月、札幌。（ポスター発表、査読有）

3） ○Ailijiang Kadeer (アリジャン　カデル)、 梶田美穂子、 藤田恭之 :「変異細胞と正常上皮細胞の境界における plectin の役割」、第４回細胞競合コロキウム、2015 年３月、札幌。（口頭発表、査読有）

4） ○Ailijiang Kadeer (アリジャン　カデル)、 梶田美穂子、 藤田恭之 :「変異細胞と正常上皮細胞の境界における plectin の役割」、第５回細胞競合コロキウム、2016 年３月、札幌。（口頭発表、査読有）

B) Introduction

1 A new field in the cancer research: cell competition at the initial stage of carcinogenesis

Scientists make great efforts to improve the medical treatments through basic research, since cancers are ever-growing life-threatening diseases for human being. However, poor prognosis of cancer patients has not been changed significantly. Late detection of cancer is an important reason of treatment failure, because cancer with multi-oncogenic mutations at the late stage shows complicated biological behaviors, such as anti-cancer drug resistance or invasion to surrounding tissues and metastasis to other parts of the body. To overcome these, researchers reviewed mechanisms of carcinogenesis and focused on the super initial stage of this process defined as an occurrence of single oncogenic transformation within the epithelium. Many questions have yet to be answered in relation with the super initial state of carcinogenesis. For example, when a newly transformed cell disrupted the integrity of normal epithelial layer, what was the response of the neighboring normal cells to this stranger (Fig. 1)? Facing to the surrounding normal cells, does this transformed cell keep growing, proliferating and developing to malignant cancer without any restriction (Fig. 1)? By studying the early stages of carcinogenesis, is it possible to find an ideal drug target for the cancer treatment?

In order to answer these questions, the *in vitro* and *in vivo* initial stage of carcinogenesis has been tested by us and others. Early studies in *Drosophila* show that when a single transformation occurs within the epithelium, normal cells could recognize and remove the transformed cell(s) to maintain the whole tissues integrity[1]. In our

laboratory, we have used the mammalian cell lines *in vitro* model system and shown that oncogenic Ras- or Src-transformed cells are extruded apically by surrounding normal cells[2,3]. In addition, depletion of scribble in a cell within the normal epithelium result in apoptosis prior to apical extrusion[4]. Interestingly, in both *Drosophila* and mammalian cell lines, transformed cells are not eliminated when they are cultured alone, suggesting that elimination of transformed cells needs contact with surrounding normal cells. This non-cell autonomous interaction between the normal and transformed cells to survive within the epithelium is called cell competition (described in section 2)[1]. At the initial stage of carcinogenesis, cell competition can be regarded as a cancer protective process. But molecular mechanism of this process still remains unknown. To develop an effective cancer treatment, underlying mechanisms of cell competition need to be clearly understood. In this thesis, using biochemical and cell biology approaches I aimed to identify the molecules specifically functioning at the interface between normal and transformed cells and to clarify their role in cell competition.

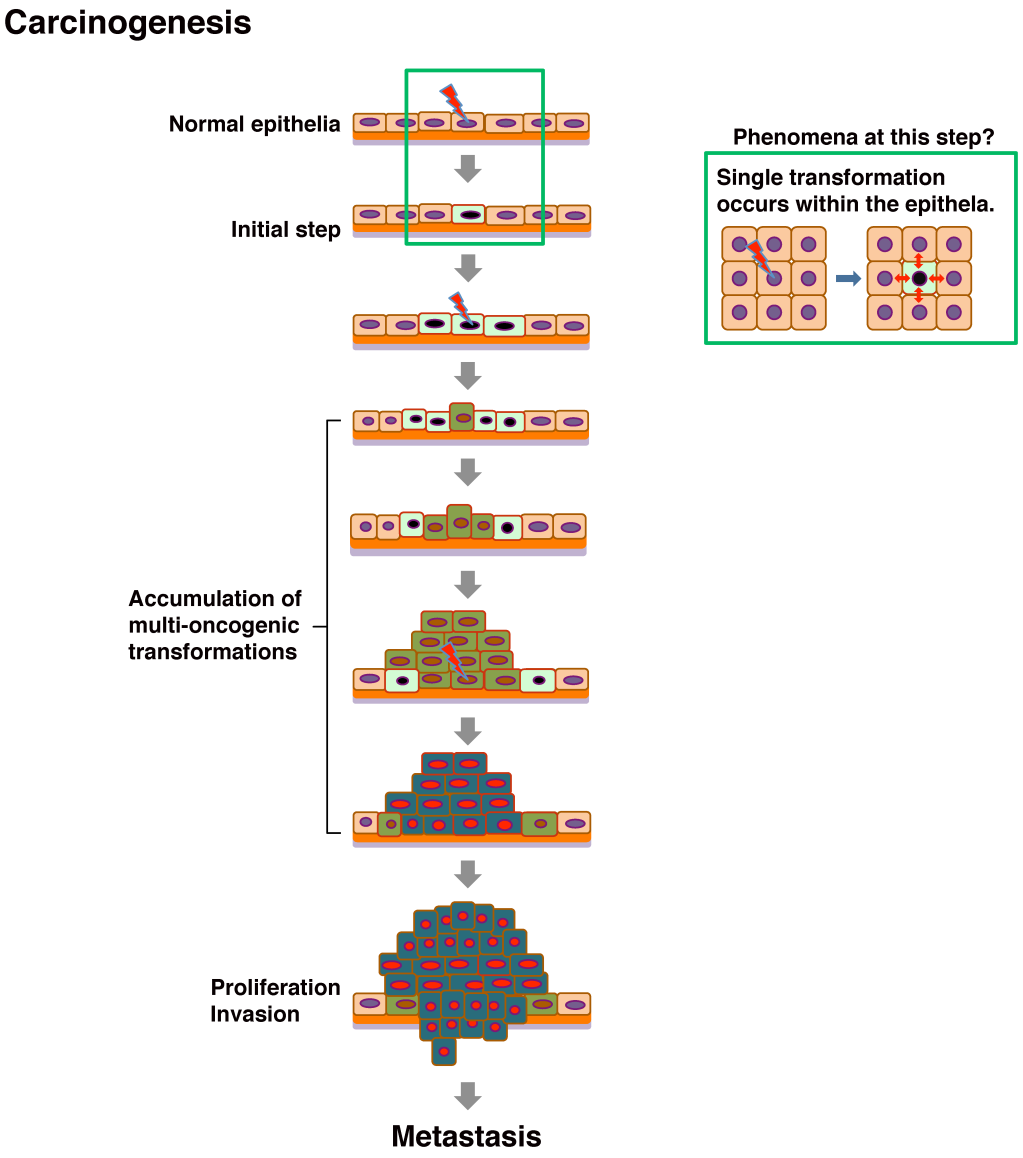

Figure 1. Multistep model of carcinogenesis. At the initial stage of carcinogenesis, transformation occurs in a single cell within a normal epithelial monolayer, and the transformed cell(s) grows while being surrounded by normal epithelial calls

2 Cell competitions in *Drosophila*

Drosophila melanogaster has become a popular model system to study cancers[5-10] and the interaction between transformed cells and normal cells.

Minute

Cell competition was described by Genes Morata and Pedro Ripoll for the first time in their study on *minute* mutations in *Drosophila* in 1975[1]. *Minute* mutations were identified as the defects in ribosomal protein genes[9]. Cell populations with *minute* mutation (hereafter called *minute* cells) could not have a “peaceful life" when they co-existed with normal epithelial cells. *Minute* cells were characterized by slower cell proliferation compared with normal cells. This made *minute* cells difficult to adapt to the epithelium when they faced to the faster proliferating normal cells. Then *Minute* cells were eliminated from the imaginal discs of mosaic *Drosophila* embryo following the rule “survival of the fittest”. Interestingly, this “natural selection” of *minute* cells did not occur without surrounding normal cells. The survived cells were called “winner”, and the failed cells were called “loser”. Based on these findings, the new term “cell competition” appeared in the biology. Regarding the processes of elimination, the most important point is that cell competition only occurs at the interface of normal and mutant cells, indicating cell competition is a non-cell-autonomous phenomenon.

Myc

Myc is a proto-oncogene regulating expression of genes that are involved in various cellular functions, such as proliferation, apoptosis, metabolism and protein synthesis[11]. In the cell competition, myc in *Drosophila* (dMyc) shows a reversible phenomenon,

meaning that expression level of myc in two adjacent cells determines which cell is "winner" or "loser"[12]. In *Drosophila* embryo, populations expressing low level of dMyc were eliminated by surrounding wild type normal cells through induction of apoptosis dependent on Hid[10]. On the contrary, populations expressing high level of dMyc changed the fate of cell competition and wild type "winners" became "losers"[12]. dMyc overexpressing cells overgrew towards surrounding wild-type tissue until they build up the whole compartment. During this condition, surrounding normal cells were eliminated by apoptosis that was induced through DPP deprivation, JNK activation and induction of Hid in the loser cells. Therefore, dMyc was called a super-competitor. *Minute* mutation suppressed this super-competitor phenotype of dMyc overexpression, suggesting that dMyc cell competition could be genetically related to *minute*[12].

3 Cell competitions in mammals

Elaborately established cell lines using mammalian epithelium enable us to observe cell competition *in vitro*[2-4,13-15]. Our laboratory has reported several studies in which Madin-Darby canine kidney cells were used for experimental cell culture system in which a Tet-on system was used[13]. Here, I briefly introduced several phenomena at the interface between normal and transformed cells.

Ras

Ras proteins are the members of small GTPase superfamily. Ras regulates diverse cellular behaviors, such as cell proliferation, differentiation, viability and motility, and oncogenic mutations of Ras were observed in various types of human cancers[16]. We mimic the initial stage of carcinogenesis where oncogenic Ras occurred in a single cell

in vitro within the epithelium[2] (Fig. 2). We established Madin-Darby canine kidney (MDCK) cell lines expressing constitutively active oncogenic Ras (RasV12) in a tetracycline-inducible manner (MDCK-pTR GFP-RasV12 cells, hereafter referred as RasV12 cells)[2] (Fig. 2). Firstly, mixture of normal and RasV12 cells (100:1) were plated and cultured on collagen gel until a monolayer was formed. Subsequently, tetracycline was added to induce expression of GFP-RasV12. Under this condition, 80% of RasV12 cells were apically extruded from the epithelial monolayer after 24 hours with tetracycline treatment. Interestingly, these extruded RasV12 cells were still alive, continually proliferated and formed multicellular aggregations. On the other hand, 20% RasV12 cells still remained within the epithelial monolayer, even formed dynamic protrusions at the basal side and invaded surrounding normal cells basal matrix. Through the analysis of interaction between normal and RasV12 cells, a number of molecules were specifically identified in RasV12 cells surrounded by normal cells. These molecules included Cdc42 and myosin-II. Among them, suppression of Cdc42 could decrease the frequency of apical extrusion and increase the formation of basal protrusions suggesting that the apical extrusion or basal protrusion of RasV12 cells was related to active or inactive state of Cdc42. In addition, Epithelial Protein Lost in Neoplasm (EPLIN) is accumulated at the apical and lateral membrane domains in RasV12 cells surrounded by normal cells, thereby regulating the downstream molecules including protein kinase A (PKA) and caveolin-1 (Cav-1), and leading to apical extrusion of transformed cells[17]. Interestingly, without surrounding normal cells, RasV12 cells in the single culture were not extruded apically. This finding suggests that activation of autonomous Ras signaling pathway alone is not sufficient to induce apical extrusion of RasV12 cells, and that the interaction with surrounding normal cells is

required.

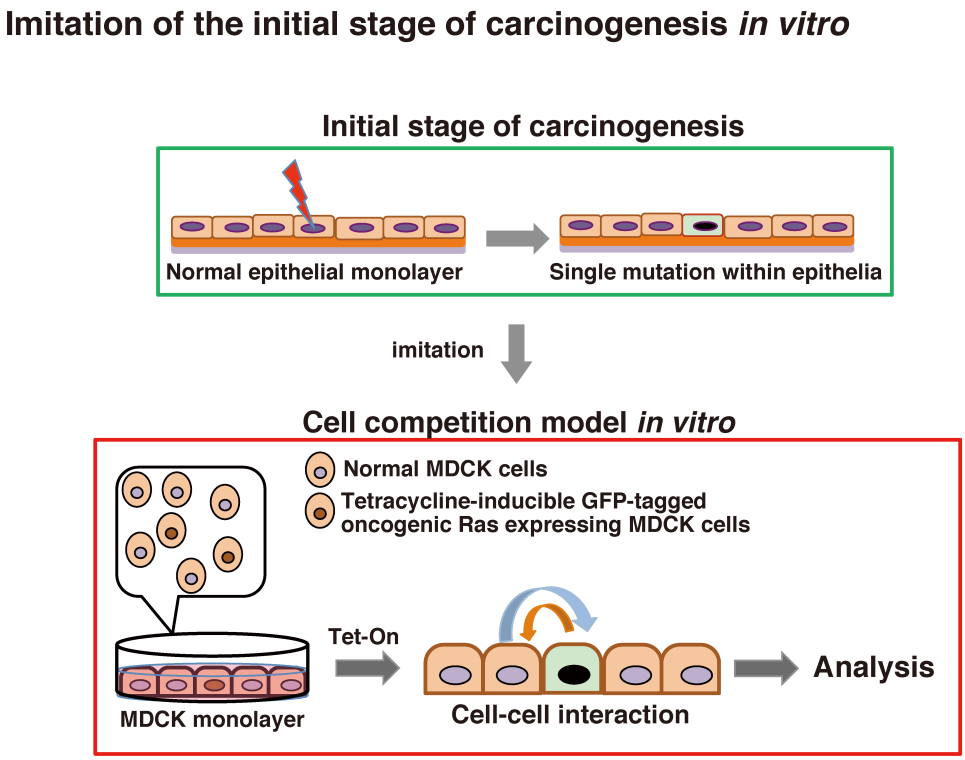

Figure 2. ***In vitro*** **model of cell competition between normal and RasV12-transformed cells.**

Src

Rous sarcoma virus Src gene (v-Src) was the first identified oncogene[18]. V-Src and its cellular compartment Src (c-Src) are overexpressed and highly activated in various human cancers[19,20]. v-Src and c-Src are non-receptor tyrosine kinases that phosphorylate multiple proteins on tyrosine residues and thereby regulate the actin cytoskeleton , cell adhesion, cell proliferation and other cellular processes[21]. To observe the phenomena occurring at the interface between normal and Src-transformed epithelial cells, Src-transformation in MDCK cells was designed in a temperature-sensitive inducible manner[3]. Activation of Src is induced by shifting temperature from 40.5°C to 35°C. 80% of Src-transformed cells were apically extruded by surrounded normal cells after Src activation for 24 hours. In Src cells surrounded by normal cells, myocin-II and FAK were identified as the positive regulator for apical extrusion. In the other hand, we identified filamin, actin cross-linker, in the normal cells neighboring to Src cells. Filamin in surrounding normal cells organizing and accumulating vimentin at the interface played a key role in the defense against Src cells[22].

Scribble

Scribble was originally identified in Drosophila as a tumor suppressor[23]. Evidence show that Scribble could reduce the cancer progression in mice[24], and depletion of scribble was observed in human colon and breast cancers[25,26]. Scribble mutation in drosophila imaginal discs leaded to cell competition in which scribble mutant cell(s) were eliminated from epithelial monolayer by surrounding normal cells through JNK-pathway-mediated apoptosis[27]. Mark et al. established MDCK cell lines that stably express Scribble shRNA in a tetracycline-inducible manner (MDCK-pTR Scribble

shRNA)[4]. Using this cell lines, they tested the involvement of Scribble in cell competition. When Scribble-knockdown cells were surrounded by normal cells, cell death occurred in Scribble-knockdown cell populations prior to apical extrusion from the epithelia. Interestingly, similar phenomena were not observed when Scribble-knockdown cells were cultured alone, indicating that the presence of surrounding normal cells induces death of scribble deficient cells.

4 Proteins investigated in this thesis

In this thesis, I focused on plectin and Epithelial Protein Lost in Neoplasm (EPLIN), one of the plectin-interacting proteins.

Plectin

Plectin is a member of the plakins family defined by plakin domain and/or plakin repeat domain[28,29]. Plakins are cytoskeletal cross-linkers associating with cytoskeletal elements and junctional complexes[28-31]. Plectin is a giant protein, over than 500 kD with about 4000 amino acids[32,33]. Plectin is mainly expressed in mammalian tissue cells existing as numerous alternative splicing isoforms[30,33-35]. This protein is regarded as a combination of multiple interacting domains[28-30]. The domain structure of plectin from N- to C-termini has been described as below[28-30].

Actin-binding domain (ABD)[36,37] this is located in N-terminal. ABD containing two calponin homology domains enables plectin to bind actin filaments[38-40]. In vitro, wild type plectin or plectin fragments containing ABD could bind to G-actin in a PIP2-dependent manner[39]. Variable splicing forms of this domain modulate plectin's subcellular localization leading to interact with different partners. In addition, ABD is a

multifunctional domain; it cooperates with adjacent plakin domain to bind to integrin β_4[41-43]. Other interacting partners of plectin via ABD are vimentin, nesprin-3 and etcetera.

Plakin domain plakin domain is next to ABD[44]**.** Plakin domain comprises nine spectrin repeats inserting a SH3 domain into the 5th spectrin repeat and harbors binding sites for junctional proteins. Plectin interacts with integrin β4 subunits, BPAG2, β-dystroglycan and β-synemin via plakin domain. SH3 domain in plectin 1c interacts with MAPs to destabilize microtubules[45].

Coiled-coil rod domain this domain with a length of 200 nm flanked between globular N-terminal head and C-terminal tail is formed by several heptad repeats. Coiled-coil rod domain mediated plectin to form dimer or multimer in parallel and in register, thereby facilitating the crosslinking role of plectin. Mutations in coiled-coil rod domain decreased stabilization of hemidesmosome, and lead to catastrophic changes in skin and muscle tissues[46].

Plakin repeat domain (PRD) & Linker-subdomain the former, the fourth domain of plectin, is consisted by 6 plakin repeats. Linker-subdomain interacting with keratin and vimentin is localized between plakin repeat 5th and 6th. With PRD and linker-subdomain, plectin can bind with all types of intermediate filaments[47-49]. Additionally, plakin repeats associate with junctional proteins or other cytoskeletal proteins.

Glycine-serine-arginine (GSR) domain GSR domain, also known as microtubule-binding domain, locates in the C-termini of plectin. In vitro, through GSR domain plectin could form microtubules bundle[50].

Based on the multi-functional domains, plectin is not only a cytoskeleton cross-linker but also a noble scaffolding protein. In signaling transductions, plectin

recruits energy-controlling AMP-activated protein kinase (AMPK), non-receptor tyrosine kinase FER, receptor for activated C kinase 1 (RACK1) to the cytoskeletons[51].

Several studies reported that phosphorylation of plectin modulates plectin-intermediate filament interactions. Phosphorylation of plectin by protein kinase C (PKC) leads to dissociate plectin from vimentin, whereas phosphorylation by cAMP-dependent protein kinase (PKA) enhances plectin to bind to vimentin. Either phosphorylation by PKA or PKC decrease plectin's association with lamin B. Mitosis-specific phosphorylation of plectin at threonine 4542 locating C-terminal plakin repeat 6th domain by p34^{cdc2} in vitro decreases plectin's association with vimentin filaments during the cell cycle[52].

In some diseases, plectin deficiency or mutations could cause catastrophic changes, such as skin blistering and muscle dystrophy (EB-MD)[46,53,54]. Plectin knockout mouse pups died within several days after birth. Abnormalities of plectin were observed in human pancreatic cancer, hepatocellular carcinoma, and head and neck squamous cell carcinoma[55].

There are no reports showing how plectin organizes cytoskeletons and functions in signaling transductions in cell competition.

EPLIN

Epithelial Protein Lost in Neoplasm (EPLIN) was originally called as LIM domain and actin binding 1 or Lima-1. In some cancers Lima-1 was decreased or lost, and then it was named EPLIN[56-58]. EPLIN is generated from one gene and alternatively spliced into two isoforms, α and β, the latter is different from the former by the N-terminal extension of 160 amino acids[57,59]. EPLIN is a member of LIM domain proteins

superfamily composed of two closely located zinc finger domains[60-62]. Apart from mediating protein-protein interaction in cytoskeleton organization, LIM domain may mediate EPLIN self-dimerization. Besides LIM domain, EPLIN has actin binding sites locating two sides of LIM domain. Based on these structural properties, EPLIN cross-links the actin filaments to form bundles[62]. In vitro, EPLIN prevents actin depolymerization and suppresses the formation of branched actin filaments by inhibiting the Arp 2/3-mediated actin nucleation to stabilize actin filaments[62]. In vitro and in vivo, EPLIN is phosphorylated at Ser360, Ser602 and Ser692 by extracellular signal-regulated kinase (ERK), a member of the MAPK family, to dynamically reorganize actin networks by decreasing the actin-binding affinity of EPLIN, thereby facilitating the cell motility and migration. Studies reported that EPLIN-mediated actin networks play an important role in different cellular processes, such as cell motility and migration[63,64]. In cancer research, down-regulation or loss of EPLINα caused cancer progression; subsequent overexpression of EPLINα decreased the tumor growth, indicating that EPLIN may function as tumor suppressor[58].

In addition, EPLIN anchors actin filaments to the cadherin-catenin complex by interacting with α-catenin[65]. Connection of intracellular actin cytoskeleton to E-cadherin by bridging EPLIN-α-catenin-β-catenin stabilizes E-cadherin at the cell surface. Thus, EPLIN plays a crucial role in the establishment of adherence junctions[66]. Also studies show that EPLIN deficiency could affect successful cytokinesis by compromising actin organization and myosin dynamics throughout the cell cycle, increasing their tendency to form cancer[63].

5 Aim of this thesis

Studying the interaction between transformed cells and surrounding normal cells may lead us to find a new way to effective cancer treatment. Enhancing the power of normal cells to kill cancer cells or attenuating the defensive ability of cancer cells might be possible after finding the ideal drug targets. Existence of crucial molecules regulating the elimination of cancer cells has been reported in our previous studies. In this study, I detected plectin at the interface between normal and transformed cells *in vitro*. Here, I tried to demonstrate a role of plectin for apical extrusion in transformed cells.

C) List of Abbreviations

ABD	Actin binding domain
Ab	Antibody
AMPK	Adenosine monophosphate-activated protein kinase
Arp 2/3	Actin-Related Proteins 2/3
BAPG2	Bullous pemphigoid antigen 1
Cav-1	Caveolin-1
Cdc42	Cell division control protein 42 homolog
C-terminus	Carboxyl-terminus
dMyc	*Drosophila* myc
DPP	Decapentaplegic
EB-MD	Epidermolysis bullosa and muscular dystrophy
EDAC	Epithelial defense against cancer
EPLIN	EPLIN Epithelial Protein Lost in Neoplasm
ERK	Extracellularr signal-regulated kinase
FAK	Focal adhesion kinase
FER	Feline encephalitis virus-related
GFP	Green fluorescence protein
GTPase	Guanosine triphosphatase
GSR	Glycine-serine-arginine
Hid	Head involution defect
IP	Immunoprecipitation
JNK	Jun kinase
LIM	Lin 11, Isl-1 and Mec-3

Lima-1	LIM domain and actin-binding 1
MAPK	Mitogen associated protein kinase
MAPs	Microtubule associated proteins
MDCK	Madin-Darby-Canine Kidney
N-terminus	Amino-terminus
p34^{cdc2}	Cyclin-dependent kinase 1
PBS	Phosphate buffered saline
PKA	Protein kinase A
PKA-S	Protein kinase A-substrates
PRD	Plakin repeat domain
pY	Phospho-tyrosine
RACK1	Receptor for activated C kinase 1
Ras	Rat sarcoma
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SH3	Src homology 3
shRNA	Short hairpin ribonucleic acid
Src	Sarcoma
Tet-on	Tetracycline-on
ts-Src	Temperature sensitive Sarcoma
v-Src	Virus sarcoma
WB	Western blotting

D) Materials and Methods

Antibodies and inhibitors

Rabbit polyclonal affinity-purified anti-plectin antibody was generated using the following peptide as an antigen: CNKDTHDQLSEPSEVRSY (Medical & Biological Laboratories Co., Ltd) and validated by using mouse anti-plectin antibody (10F6, sc-33649, Santa Cruz) as a control (Fig. 3). Rabbit anti-phospho-(Ser/Thr) PKA substrate and mouse anti-phospho-tyrosine (pY110, 9411S) antibodies were purchased from Cell Signaling Technology. Mouse anti-phospho-tyrosine (4G10, Cat. # 0501050) and mouse anti-α-actin (clone 4, Cat. # MAB1501R) antibodies were from Millipore. Mouse anti-GFP (a combination of two clones, 7.1 and 13.1, Cat. # 11814460001) antibody was from Roche Diagnostics. Mouse anti-keratin K5/K8 (65131) antibody was from PROGEN Bio-technik GmbH. Rat monoclonal anti-α-tubulin (YOL1/34) and rabbit anti-caveolin (610059) antibodies were obtained from Abcam. and anti-EPLIN antibody (sc-136399) were from Santa Cruz Biotechnology. Mouse anti-FLAG antibody (M2) was from Sigma-Aldrich. Alexa-Fluor-568- and -647-conjugated secondary antibodies were from ThermoFisher Scientific. Hoechst 33342 (Life Technologies) was used at a dilution of 1:5,000. For immunofluorescence, the primary antibodies describe above were diluted in PBS containing 1% BSA at 1:100, except rat anti-α-tubulin antibody at 1:200 and mouse anti-EPLIN antibody at 1:50. All secondary antibodies were used at 1:200. Alexa-Fluor-647-conjugated phalloidin (Life Technologies) was used at 1.0 U ml^{-1}. For western blotting, primary antibodies were used at 1:1,000 except mouse anti-α-actin antibody that was used at 1:2,000, and secondary antibodies were used at 1:1,000.

The following inhibitors were used where indicated: Cytochalasin D (Sigma-Aldrich,

100 nM) and nocodazole (Sigma-Aldrich, 200 ng ml^{-1}). DMSO (Sigma-Aldrich) was added as a control.

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% CO_2. Stably expressing GFP-RasV12 MDCK cell line was established by using a Tet-On expression system. A brief description was given here. Firstly, pcDNA/TR was transfected into MDCK cells by using Lipofectamine 2000 (Life Technologies). Blasticidin (Invivogen, California, USA) was used at 5 μg ml^{-1} in medium for colonies selection. pcDNA4/TO/GFP-RasV12 was then used for the second transfection. Followed by second transfection, 5 μg ml^{-1} of blasticidin and 400 μg ml^{-1} of zeocin (Invivigen) were added in 10% FBS medium for colonies selection. MDCK-pTR GFP-RasV12 cells stably expressing EPLIN-shRNA and MDCK-pTR GFP-cSrcY527F cells were kindly provided by former lab member Atsuko Ohoka, and cultured in medium containing 5 μg ml^{-1} blasticidin (InvivoGen), 400 μg ml^{-1} zeocin (InvivoGen) and 800 μg ml^{-1} G418 (Life Technologies). MDCK-pTR GFP-RasV12 cells stably expressing plectin-shRNA were established as follows:

Double-stranded DNA fragments coding plectin-shRNA sequences (plectin-shRNA1: 5'-GATCCCCGCTTCAACTGGTCACGTATTTCAAGAGAATACGTGACCAGTTG AAGCTTTTTC-3' and 5'-TCGAGAAAAAGCTTCAACTGGTCACGTATTCTCTTGAAATACGTGACCAG TTGAAGCGGG-3' or plectin-shRNA2: 5'-GATCCCCGCATGATCATCATCATCATTTCAAGAGAATGATGATGATGATC

ATGCTTTTTC-3' and

5'-TCGAGAAAAAGCATGATCATCATCATCATTCTCTTGAAATGATGATGATG

ATCATGCGGG-3')

were inserted into the *Bgl*II and *Xho*I site of pSUPER.neo+gfp (Oligoengine). MDCK-pTR GFP-RasV12 cells were transfected with pSUPER.neo+gfp plectin-shRNA using Lipofectamine 2000 (Invitrogen), followed by antibiotic selection in the medium containing 5 μg ml^{-1} blasticidin (InvivoGen), 400 μg ml^{-1} zeocin (InvivoGen) and 800 μg ml^{-1} G418 (Life Technologies).

To induce the expression of GFP-RasV12 or GFP-cSrcY527F in the tetracycline-inducible MDCK-pTR GFP-RasV12 or MDCK-pTR GFP-cSrcY527F cell lines, 2 μg ml^{-1} tetracycline (Sigma-Aldrich) was added. To transiently express EPLIN-FLAG, pcDNA3-EPLIN-FLAG was constructed as follows. The cDNA of mouse EPLINβ was inserted into the EcoRV/HindIII site, and then an oligo fragment of FLAG was inserted into the SalI site. MDCK cells or MDCK-pTR GFP-RasV12 cells were transfected with pcDNA3-EPLIN-FLAG. At 6 h, media were changed to fresh media containing tetracycline, and at 24 h, cells were fixed and analyzed. For the inhibitor treatment, the indicated inhibitor was added together with tetracycline, and then cells were cultured with them for 16 h or 24 h. For immunofluorescence, cells were seeded onto Type-I collagen-mounted coverslips as previously described.

Immunofluorescence

MDCK-pTR GFP-RasV12 or MDCK-pTR GFP-cSrcY527F cells were mixed with MDCK cells at a ratio 1:50 and cultured on the collagen matrix as previously described [67]. For immunofluorescence analyses, the mixture of cells was incubated for 8-12 h until

they formed a monolayer, followed by tetracycline treatment for 16 h. Cells were fixed with 4% paraformaldehyde in PBS and permeabilized with 0.5% Triton X-100 in PBS, followed by blocking with 1% BSA in PBS. Primary or secondary antibody was incubated for 2 h or 1h respectively at ambient temperature. After incubation with antibodies, immunofluorescence images were acquired using the Olympus FV1000 system and Olympus FV10-ASW software. For Fig. 19a-c, Alexa-Fluor-568-conjugated anti-Cav-1 or anti-PKA substrate antibody was prepared using Zenon® Rabbit IgG Labeling Kit (Life Technologies) according to the manufacturer's instructions. For analyses of apical extrusions, the samples were analyzed after 24 h of tetracycline treatment. More than 100 cells were analyzed for each experiment, and apically extruded cells were quantified. For quantification of immunofluorescence intensity, 30 transformed cells were analyzed for each experiment using the MetaMorph software (Molecular Devices).

Immunoprecipitation and western blotting

MDCK and MDCK-pTR GFP-RasV12 cells were seeded at the density of 1.2 x 10^7 cells in 14.5-cm dishes (Greiner-Bio-One), and cultured at 37˚C for 6-8 h until a monolayer was formed. Tetracycline was then added to induce RasV12 expression. After 16 h culture with tetracycline, cells were washed with ice-cold PBS containing 1 mM Na_3VO_4, and lysed for 30 min in Triton X-100 lysis buffer or RIPA lysis buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) containing the following inhibitors: 1 mM Na_3VO_4, 10 mM NaF, 5 μg ml^{-1} leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 7.2 trypsin inhibitor units of aprotinin. For immunoprecipitation, 0.1 mM Na_2MoO_4 was also added in Triton X-100

lysis buffer. The former and latter buffers were used for immunoprecipitation with anti-phospho-tyrosine and anti-EPLIN antibodies, respectively. After centrifugation of the cell lysates at 21,500 g for 10 min, the supernatant was first pre-cleared with sepherose protein G beads (GE Healthcare) for 30 min. The pre-cleared cell lysates were then incubated with control IgG-conjugated Dynabeads protein G (Life Technologies) for 30 min, and finally subjected to immunoprecipitation for 1 h with Dynabeads Protein G conjugated to anti-phospho-tyrosine antibodies (10 μg: a combination of 4G10 and pY110 at 1:1) or anti-EPLIN antibody (10 μg). Immunoprecipitated proteins were subjected to 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 Fig. 3a, the combination of immobilized phospho-tyrosine antibodies beads (antibodies were covalently coupled to the beads) was used (Cell Signaling #9419 and Millipore #16-638). Stained gels and western blotting data were acquired using ImageQuant^{TM} LAS4010 (GE healthcare, Chalfont St Giles, UK). Selected protein bands were identified by mass spectrometry as described in next section. To validate the customized plectin antibody and to examine the plectin-knockdown efficiency, MDCK-pTR GFP-RasV12 cells stably expressing plectin-shRNA were seeded onto 6-cm dishes (Greiner-Bio-One) at 1 x 10^6 cells. After 8 h, the cells were further incubated for 16 h in the absence or presence of tetracycline. The incubated cells were lysed with Triton X-100 lysis buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, and 1% Triton X-100) containing protease inhibitors (5 μg ml^{-1} leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 7.2 trypsin inhibitor units of aprotinin) and directly boiled with SDS-PAGE sample buffer. Western blotting was

performed as described[67]. For antibody validation, SDS samples prepared in the absence of tetracycline were used (Fig. 3).

Protein Identification by Mass-Spectrometry

Protein bands were excised from the gel, and the protein was identified by peptide mass fingerprinting (PMF) and post-source decay (PSD) MS/MS analysis. Briefly, protein bands were digested in-gel by trypsin gold (Promega) after reductive S-alkylation with dithiothreitol and iodoacetamide. After incubation at 37ºC for 12 h, tryptic peptides were purified by ZipTip mC18 (Millipore). High-purity alpha-cyano-4-hydroxycinnamic acid (Shimadzu GLC) was used as MALDI matrix. Mass spectrometric analysis was performed by AXIMA Performance MALDI-TOF/TOF MS (Shimadzu Corporation). The acquisition mass range was 700-4000 Da. Mass spectrometer was first externally calibrated by angiotensin II (*m/z* 1046.54) and ACTH fragment 18-39 (*m/z* 2465.20), and each result was internally calibrated by tryptic autolysis fragment (*m/z* 842.51 and 2211.10). The peak processing of peptide mass spectra and MS/MS ion selection were analysed by Launchpad version 2.9.1 (Shimadzu), and protein was identified against NCBI non-redundant canis familiaris database (2011_11, 16,245,521 sequence entries) on Mascot Server version 2.3 (Matrix Science), up to one missed cleavage, peptide tolerance of 0.2 Da, fixed modifications of cysteine carbamydomethylation, and variable modifications of methionine oxidation. MOWSE scores of PMF above 56 were considered significant ($P < 0.05$) and ion scores of MS/MS above 28 were indicated identity or extensive homology ($P < 0.05$).

Data Analyses

Two-tailed Student's *t*-tests were used to determine *P*-values for statistical analyses.

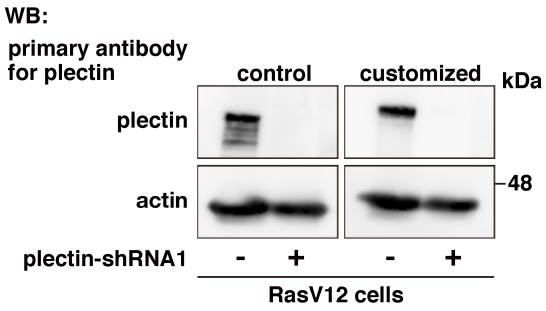

Figure 3. Validation of customized rabbit polyclonal anti-plectin antibody. For validation of customized anti-plectin antibody, commercial mouse anti-plectin antibody (10F6, sc-33649, Santa Cruz) was used as a control. MDCK-pTR GFP-RasV12 cells stably expressing plectin-shRNA1 were cultured without tetracycline, followed by western blotting with the indicated antibodies. Equal protein amount was validated by blotting α-actin.

E) Results

1 Identification of plectin at the interface between normal and RasV12-transformed cells

As I described in the beginning of this thesis, Ras- or Src-transformed cells and surrounding normal cells interacted each other and showed various phenomena, but underlying molecular mechanisms remain largely unknown.

I hypothesized that in signaling pathways for apical extrusion numerous proteins function at the interface between normal and transformed cells, and activity of these proteins are regulated through several modifications, such as phosphorylation, methylation, acetylation and ubiquitination. It has already been known that tyrosine phosphorylation is one of the important processes involving in various intracellular signaling transductions and its deregulation was observed in many types of cancers[69-78]. In 1979, tyrosine phosphorylation was identified as related to v-Src kinase activity; later v-Src was confirmed as the first tyrosine kinase[78]. Further studies show Ras-MAPK signaling pathways involved in activation of various tyrosine kinases including Src[76]. So far, we are expecting to identify tyrosine-phosphorylated proteins involved in signaling transduction during cell competition between normal and Ras- or Src-transformed cells. I thus decided to screen tyrosine-phosphorylated proteins at the interface between normal and Ras-transformed cells.

Firstly, under the three different cell culture conditions (normal MDCK cells alone, RasV12 cells alone and their mixture at 1:1 ratio), I used anti-phospho-tyrosine antibody and performed immunoprecipitation. Immunoprecipitated proteins were subjected to SDS-PAGE, followed by RUBY protein staining to visualize the protein

bands. Then I found that several protein bands averagely increased under the mixture of normal and RasV12 cells, compared with normal or RasV12 cells cultured alone (Fig. 2a). Using mass spectrometry, I confirmed the identity of proteins. Plectin was one of the identified proteins (Fig. 4a,b). Up to now, there are no reports about pelctin in cell competition. I thus focused on plectin. By western blotting with anti-phospho-tyrosine antibody I could not detect tyrosine-phosphorylation of plectin *per se* (Fig. 4b), suggesting that plectin binds to unidentified, tyrosine-phosphorylated protein(s).

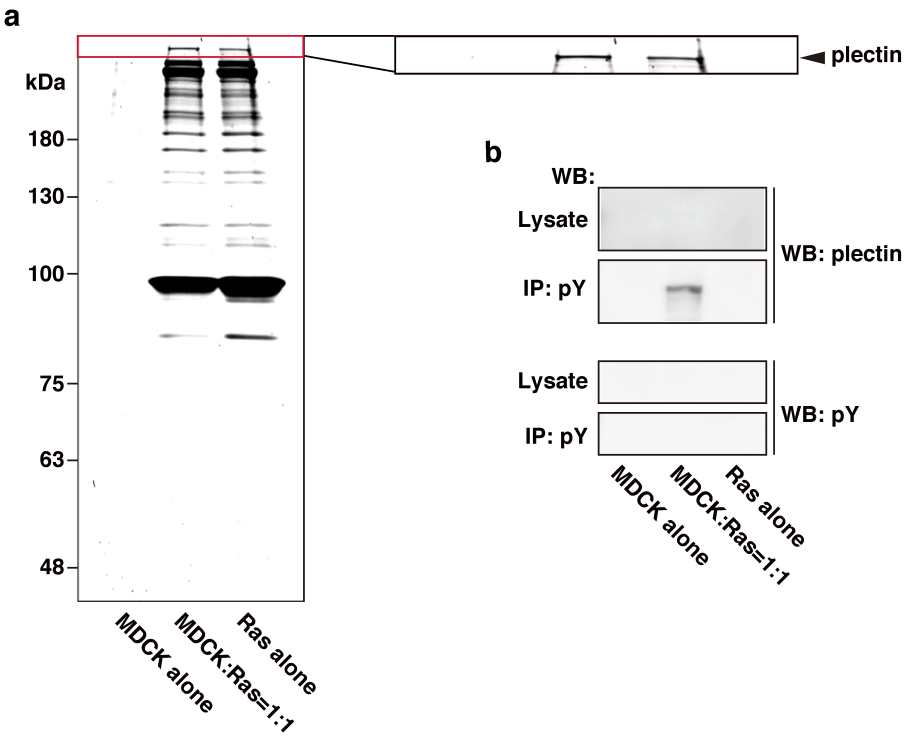

Figure 4. Identification of plectin at the interface between normal and RasV12-transformed cells. (**a**) SYPRO ruby staining (9% SDS-PAGE) of immunoprecipitated proteins with a mixture of anti-phospho-tyrosine antibodies. Cells were cultured under three different conditions: i) normal MDCK cells alone, ii) 1:1 mix culture of normal and RasV12-transformed MDCK cells, and iii) RasV12-transformed MDCK cells alone. The cell lysates were collected after 16 h incubation with tetracycline, followed by immunoprecipitation. The area in the red box is shown at higher magnification in the right panel. The arrowhead indicates the band for plectin. (**b**) Validation of the band for plectin by western blotting. Immunoprecipitated samples were examined by western blotting with anti-plectin or anti-phospho-tyrosine antibody.

2 Plectin is accumulated in RasV12-transformed cells surrounded by normal cells in a non-cell-autonomous manner

I then examined subcellular localization of plectin by immunofluorescence analysis. When RasV12-transformed cells were surrounded by normal epithelial cells, plectin was substantially accumulated in the cytoplasm within the transformed cells (Fig. 5a,b). The accumulation of plectin was observed in both apically extruding cells and already extruded cells (Fig. 5a and 8a), suggesting that plectin are accumulated in the transformed cells during the process of apical extrusion. In contrast, plectin accumulation did not occur when RasV12 cells alone were present (Fig. 5a,b). Similarly, plectin was accumulated in Src-transformed cells, when they were surrounded by normal cells (Fig. 6). These data suggest that the presence of surrounding normal cells induces the accumulation of plectin in the transformed cells in a non-cell-autonomous fashion.

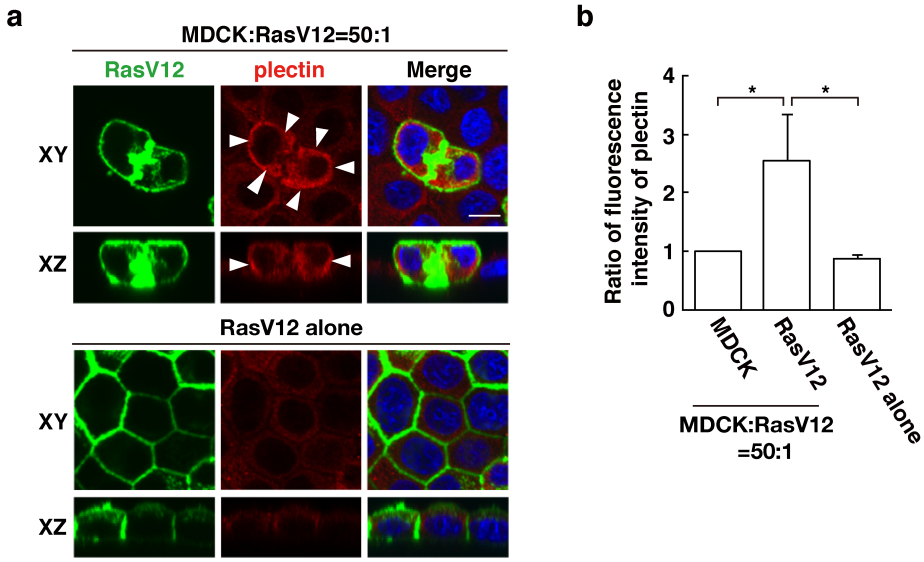

Figure 5. Plectin is accumulated in RasV12-transformed cells that are surrounded by normal epithelial cells. (**a**) Immunofluorescence images of plectin (red) in the mix culture of normal and RasV12-transformed MDCK cells. MDCK-pTR GFP-RasV12 cells were mixed with normal MDCK cells or cultured alone on collagen gels. Cells were fixed after 16 h incubation with tetracycline and stained with anti-plectin antibody (red) and Hoechst (blue). Scale bar, 10 μm. (**b**) Quantification of the fluorescence intensity of plectin for **a**. Data are mean ± SD from three independent experiments. **P*<0.05; n=30 cells for each experimental condition. Values are expressed as a ratio relative to MDCK cells. White triangle indicates accumulated plectin in transformed cell.

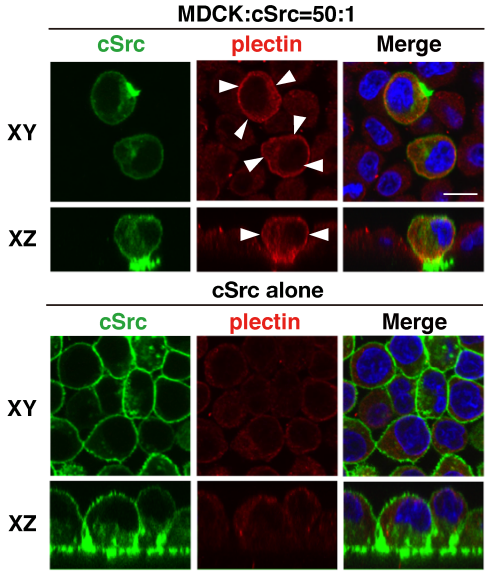

Figure 6. Plectin is accumulated in Src-transformed cells when they are surrounded by normal epithelial cells. Immunofluorescence images of plectin (red) in the mix culture of normal and Src-transformed MDCK cells. MDCK-pTR GFP-cSrcY527F cells were mixed with normal MDCK cells or cultured alone on collagen gels. Cells were fixed after 16 h incubation with tetracycline and stained with anti-plectin antibody (red) and Hoechst (blue). Scale bar, 10 μm. White triangle indicates accumulated plectin in transformed cell.

3 Plectin is a novel regulator for apical extrusion of RasV12-transformed epithelial cells

To understand the functional significance of the plectin accumulation, I established RasV12-transformed MDCK cells stably expressing plectin-shRNA (Fig. 7a,b). I demonstrated that knockdown of plectin profoundly suppressed apical extrusion of RasV12-transformed cells (Fig. 8a,b, and c), indicating that plectin plays a positive role in the apical elimination of transformed cells. Despite of several trials, I was unable to establish Src-transformed cells stably expressing plectin-shRNA, thus hereafter mainly focused on RasV12 cells for functional analyses.

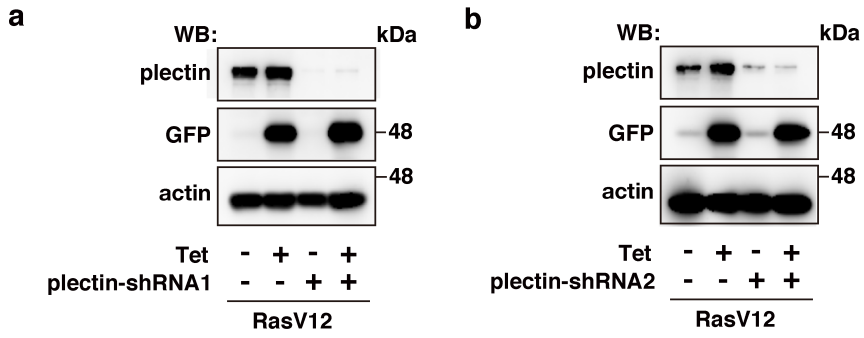

Figure 7. Establishment of plectin-knockdown RasV12 cells. (**a,b**) Establishment of MDCK-pTR GFP-RasV12 cells stably expressing plectin-shRNA1. Expression of GFP-RasV12 was induced with tetracycline treatment, followed by western blotting with the indicated antibodies. Equal protein amount was validated by blotting α-actin.

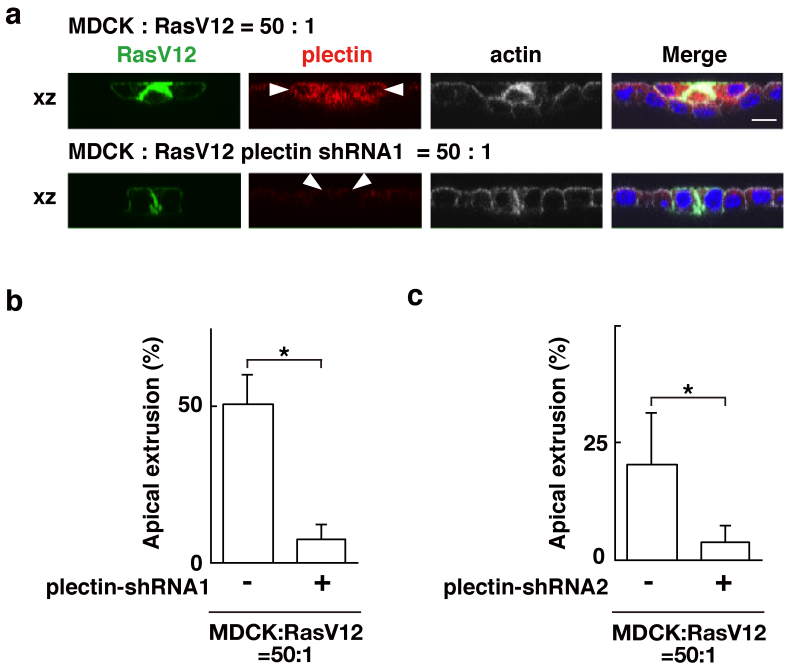

Figure 8. Plectin plays an active role in apical extrusion of RasV12-transformed cells. (**a**) XZ images of RasV12 cells or plectin-knockdown RasV12 cells that were surrounded by normal epithelial cells. MDCK-pTR GFP-RasV12 cells or MDCK-pTR GFP-RasV12 plectin-shRNA1 cells were mixed with normal MDCK cells. Cells were stained with anti-plectin antibody (red), Alexa-Fluor-648-phalloidin (white), and Hoechst (blue). Scale bar, 10 μm. (**b**) Quantification of the effect of plectin-shRNA1 on apical extrusion of RasV12 cells. Data are mean ± SD from four independent experiments. **P* < 0.005; n=100-170 cells for each experimental condition. (**c**) Quantification of the effect of plectin-shRNA2 on apical extrusion of RasV12 cells. Data are mean ± SD from three independent experiments. **P* < 0.05; n=190-290 cells for each experimental condition. White triangle indicates accumulated or depleted plectin in transformed cells.

4 Microtubules and plectin mutually regulate their accumulation and promote apical extrusion of RasV12-transformed cells

Plectin crosslinks microtubules and intermediate filaments and regulates their dynamics[45,79]. Therefore, I next examined functional relationship between plectin and those cytoskeletal proteins. By immunofluorescence, I found that tubulin, a major component of microtubules, was accumulated in the apical region of RasV12-transformed cells that were surrounded by normal cells, which was partially co-localized with plectin (Fig. 9). When RasV12 cells alone were present, accumulation of tubulin did not occur (Fig. 9). Comparable non-cell-autonomous accumulation of tubulin was observed in Src-transformed cells (Fig. 10). In addition, intermediate filament protein keratin5+8 were also accumulated in the apical region of RasV12 cells that were surrounded by normal cells (Fig. 16a). Next, I examined the effect of nocodazole, an inhibitor of microtubule polymerization. Nocodazole strongly disrupted the structure of microtubule filaments (Fig. 11a). Nocodazole also attenuated the frequency of plectin accumulation in RasV12- or Src-transformed cells surrounded by normal cells, whereas cytochalasin D, an inhibitor of actin polymerization, had no significant effect (Fig. 11a,b and Fig. 13a,b). In addition, nocodazole diminished accumulation of keratin5+8 (Fig. 10), suggesting the coordinated regulation of these cytoskeletons. Furthermore, treatment of nocodazole substantially suppressed apical extrusion of RasV12 or Src cells (Fig. 11c and Fig. 13c), suggesting a crucial role of microtubules in this process. Moreover, we showed that knockdown of plectin profoundly diminished accumulation of tubulin and keratin in RasV12-transformed cells surrounded by normal cells (Fig. 14a,b and Fig. 16a,b). It is widely accepted that microtubules dynamic stability was achieved by acetylation at lysine 40 of α-tubulin.

Finally, I found that RasV12 cells surrounded by normal cells increased the acetylation of α-tubulin in microtubules and plectin-knockdown suppressed this modification of tubulins (Fig. 15a,b). Collectively, these data demonstrate that plectin and microtubules mutually affect their accumulation, thereby promoting elimination of transformed cells from epithelia.

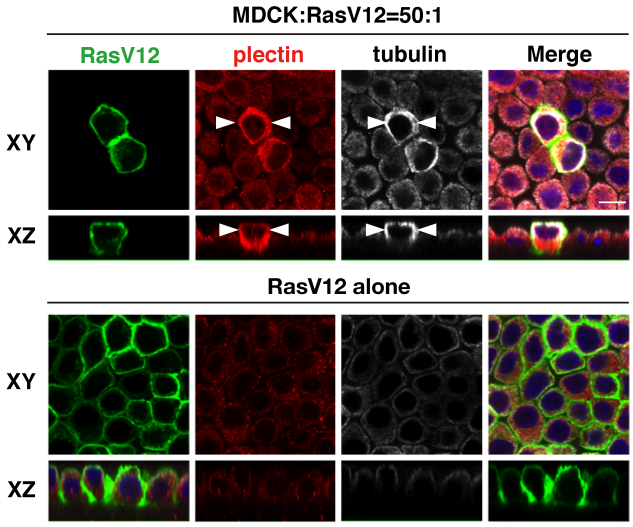

Figure 9. Microtubules non-cell-autonomously assemble in RasV12-transformed cells surrounded by normal cells and partially co-localize with plectin. Accumulation of plectin and tubulin in RasV12-transformed cells surrounded by normal epithelial cells. MDCK-pTR GFP-RasV12 cells were mixed with normal MDCK cells or cultured alone on collagen gels. Cells were fixed after 16 h incubation with tetracycline and stained with anti-plectin (red) and anti-tubulin (white) antibodies and Hoechst (blue). Scale bar, 10 μm. White triangle indicates accumulated plectin or tubulin in transformed cells.

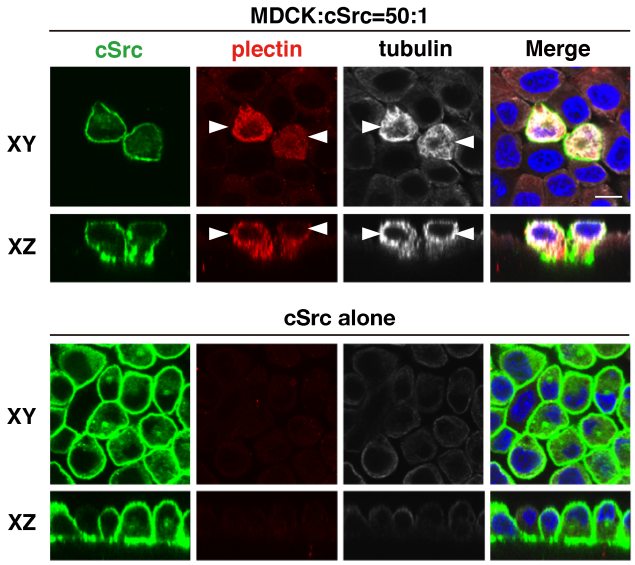

Figure 10. Microtubules non-cell-autonomously assemble in Src-transformed cells surrounded by normal cells and partially co-localize with plectin. Accumulation of plectin and tubulin in Src-transformed cells surrounded by normal epithelial cells. MDCK-pTR GFP-cSrcY537F cells were mixed with normal MDCK cells or cultured alone on collagen gels. Cells were fixed after 16 h incubation with tetracycline and stained with anti-plectin (red) and anti-tubulin (white) antibodies and Hoechst (blue). Scale bar, 10 μm. White triangle indicates accumulated plectin or tubulin in transformed cells.

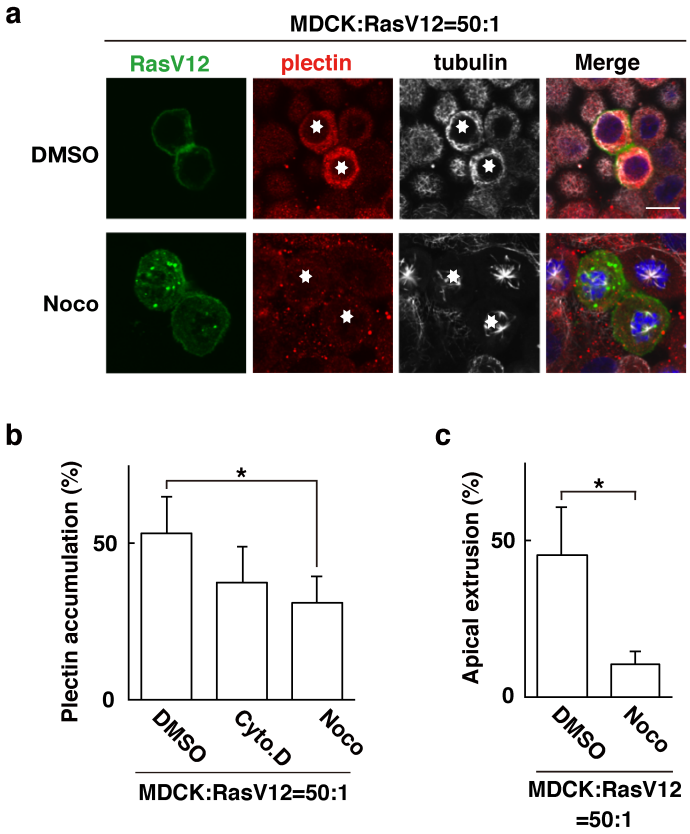

Figure 11. Microtubules and plectin mutually regulate their accumulation and promote apical extrusion of RasV12-transformed cells. (**a**) Immunofluorescence image for nocodazole treatment showing plectin and microtubules were treated with DMSO (upper panel) or nocodazole (lower panel). MDCK-pTR GFP-RasV12 cells were mixed with normal MDCK cells or cultured alone on collagen gels. Cells were fixed after 16 h incubation with tetracycline and inhibitors and stained with anti-plectin (red) and anti-tubulin (white) antibodies and Hoechst (blue). White star indicates transformed cell. (**b**) Effect of nocodazole or cytochalasin D on the accumulation of plectin and tubulin. Data are mean ± SD from four independent experiments. **P*<0.05; n=140-180 cells for each experimental condition. (**c**) The effect of nocodazole on apical extrusion of RasV12-transformed cells. Data are mean ± SD from three independent experiments. **P*<0.05; n=100-130 cells for each experimental condition.

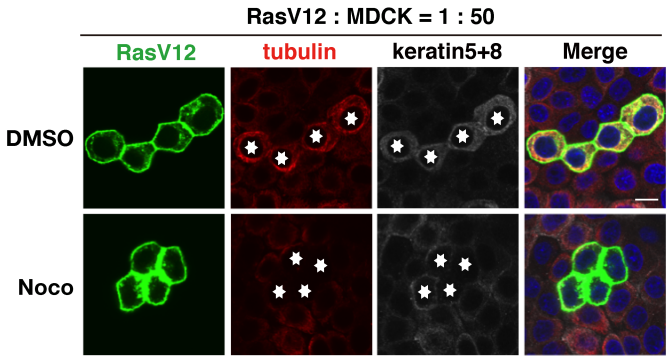

Figure 12. Microtubules regulate accumulation of keratin5+8 in RasV12-transformed cells surrounded by normal cells. Immunofluorescence image showing microtubules and keratin5+8 were treated with DMSO (upper panel) or nocodazole (lower panel). MDCK-pTR GFP-RasV12 cells were mixed with normal MDCK cells and cultured on collagen gels. Cells were fixed after 16 h incubation with tetracycline and inhibitors and stained with anti-plectin (red) and anti-keratin5+8 (white) antibodies and Hoechst (blue). Scale bar, 10 μm. White star indicates transformed cell.

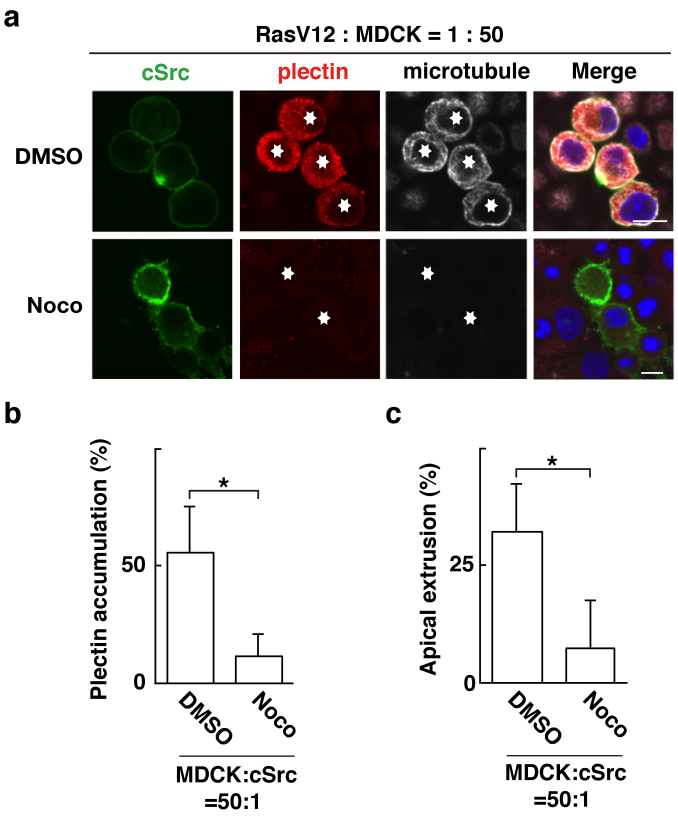

Figure 13. Microtubules and plectin mutually regulate their accumulation and promote apical extrusion of Src-transformed cells. (**a**) Accumulation of plectin and tubulin in Src-transformed cells surrounded by normal epithelial cells. MDCK-pTR GFP-cSrcY527F cells were mixed with normal MDCK cells and cultured on collagen gels. Cells were fixed after 16 h incubation with tetracycline and stained with anti-plectin (red) and anti-tubulin (white) antibodies and Hoechst (blue). White star indicates transformed cell. (**b**) Effect of nocodazole on the accumulation of plectin and tubulin. Data are mean ± SD from three independent experiments. **P*<0.05; n=150-180 cells for each experimental condition. (**c**) The effect of nocodazole on apical extrusion of Src-transformed cells. Data are mean ± SD from three independent experiments. **P*<0.05; n=60-120 cells for each experimental condition.

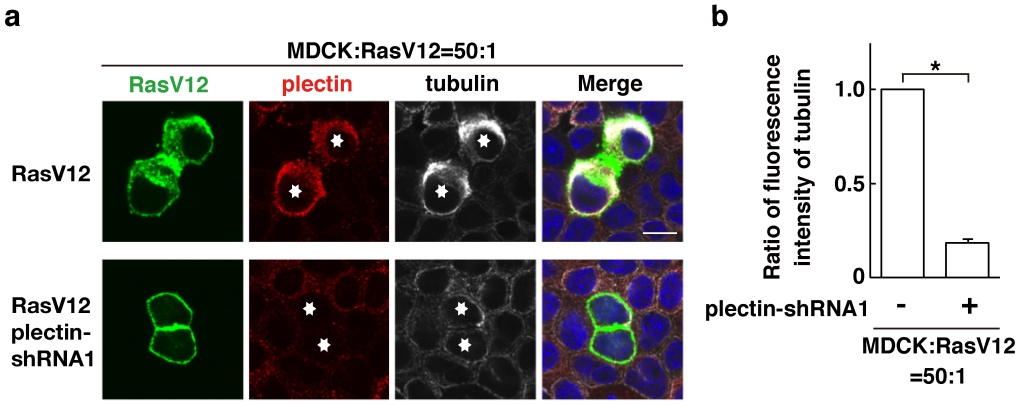

Figure 14. Plectin-knockdown suppresses microtubules accumulation in RasV12-transformed cells surrounded by normal cells. (**a)** Immunofluorescence images of microtubules in plectin-knockdown RasV12 cells mixed with normal MDCK cells at 1:50 (lower panel) and RasV12 cells mixed with normal MDCK cells at 1:50 as a control (upper panel). Cells were fixed after 16 h incubation with tetracycline and stained with anti-acetylatedα-plectin (red) and anti-tubulin (white) antibodies and Hoechst (blue). Scale bar, 10 μm. White star indicates transformed cell. (**b**) Data are mean ± SD from three independent experiments. **P*<0.0001; n=30 cells for each experimental condition. Values are expressed as a ratio relative to plectin-shRNA1 (-).

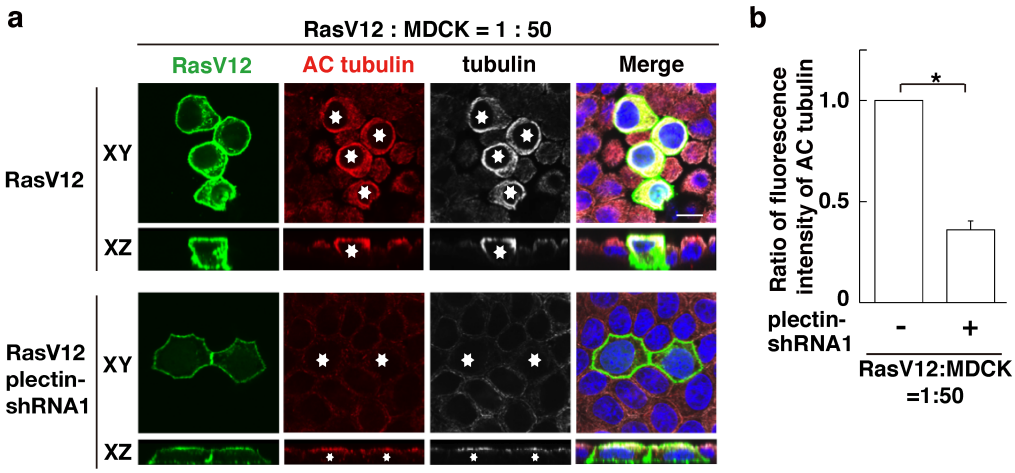

Figure 15. plectin in RasV12 cells surrounded by normal cells contributes to acetylation of α-tubulin for dynamic modulation of microtubules. (**a**) Effect of plectin-knockdown on the acetylation of α-tubulin in microtubules. MDCK-pTR GFP-RasV12 cells or MDCK-pTR GFP-RasV12 plectin-shRNA1 cells were mixed with normal MDCK cells. Cells were fixed after 16 h incubation with tetracycline and stained with anti-acetylatedα-tubulin (red) and anti-tubulin (white) antibodies and Hoechst (blue). Scale bar, 10 μm. (**b**) Data are mean ± SD from three independent experiments. **P*<0.001; n=30 cells for each experimental condition. Values are expressed as a ratio relative to plectin-shRNA1 (-). White star indicates transformed cell.

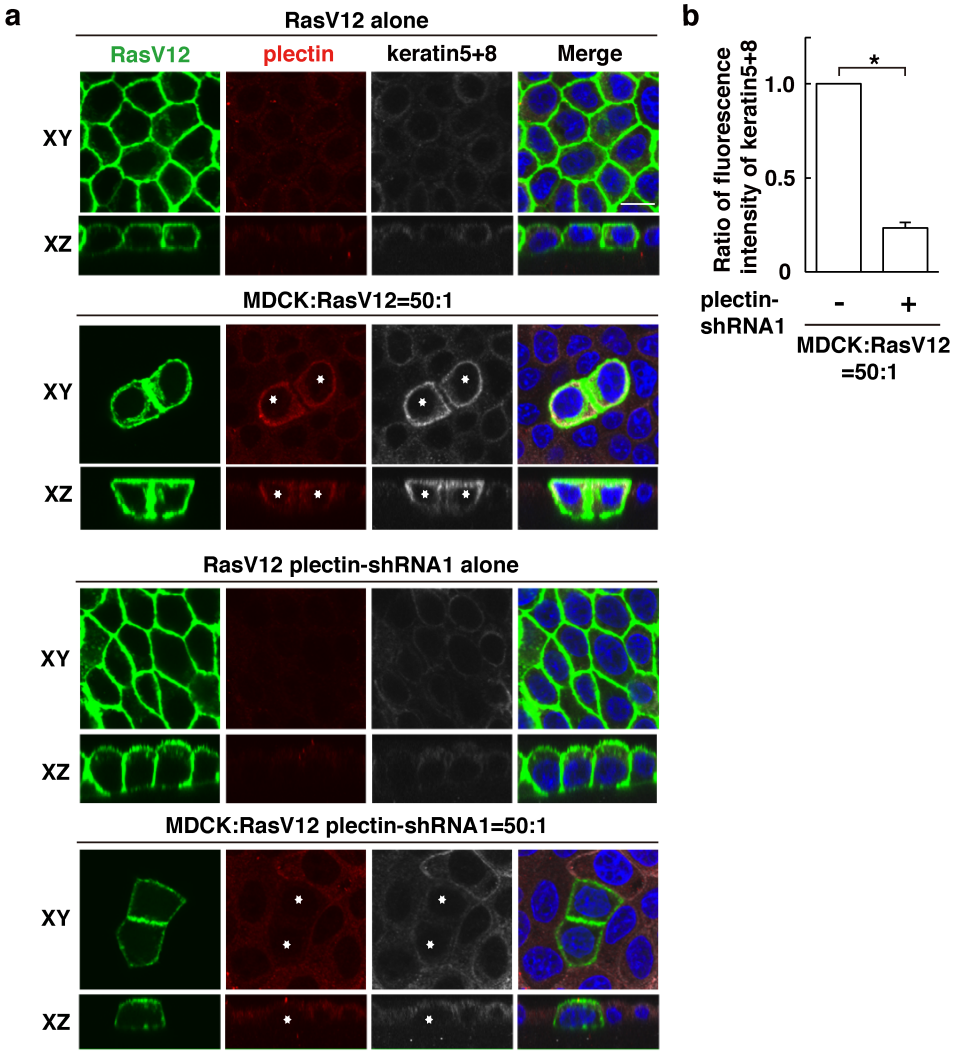

Figure 16. Plectin regulates accumulation of keratin intermediate filaments in RasV12-transformed cells surrounded by normal epithelial cells. (**a**) Accumulation of plectin and keratin5+8 in RasV12-transformed cells surrounded by normal epithelial cells. MDCK-pTR GFP-RasV12 cells or MDCK-pTR GFP-RasV12 plectin-shRNA1 cells were mixed with normal MDCK cells or cultured alone on collagen gels. Cells were fixed after 16 h incubation with tetracycline and stained with anti-plectin (red) and anti-keratin5+8 (white) antibodies and Hoechst (blue). (**b**) Effect of plectin-knockdown on accumulation of keratin5+8. Data are mean ± SD from three independent experiments. **P*<0.001; n=30 cells for each experimental condition. Values are expressed as a ratio relative to plectin-shRNA1 (-). White star indicates transformed cell.

5 Plectin positively regulates apical elimination of RasV12-transformed cells in concert with EPLIN and microtubules.

We have previously reported that Epithelial Protein Lost in Neoplasm (EPLIN) is accumulated in RasV12-transformed cells surrounded by normal cells and plays a vital role in the apical extrusion of the transformed cells[17]. I thus examined the functional relevance between EPLIN and plectin. By immunoprecipitation, I first examined the interaction between EPLIN and plectin using normal and RasV12-transformed MDCK cells. Plectin was co-immunoprecipitated with EPLIN under the mix culture condition of normal and RasV12 cells (Fig. 17). In addition, when plectin was depleted in RasV12-transformed cells, EPLIN accumulation was substantially suppressed (Fig. 18a,b). Conversely, knockdown of EPLIN in RasV12 cells significantly attenuated the accumulation of plectin (Fig. 18c,d). Similarly, EPLIN-knockdown suppressed microtubule accumulation and acetylation of α-tubulin (Fig. 20), and treatment of nocodazole diminished the EPLIN accumulation (Fig. 19a-d). When EPLIN was exogenously overexpressed within a monolayer of normal or RasV12 cells in a mosaic manner, accumulation of plectin or microtubules was not observed (Fig. 21a,b). Collectively, these data indicate that plectin, EPLIN, and microtubules are co-accumulated in an interdependent manner in RasV12 cells surrounded by normal cells and that the interaction between normal and transformed cells is required for this process.

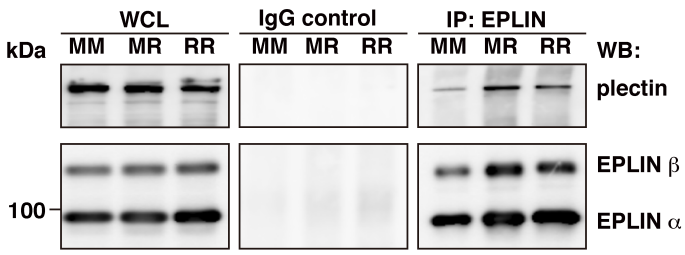

Figure 17. Plectin increases the interaction with EPLIN under the mix culture of normal cells and RasV12-transformed cells Co-immunoprecipitation of EPLIN and plectin with anti-EPLIN antibody. MM, normal MDCK cells cultured alone; MR, 1:1 mix culture of normal MDCK and MDCK-pTR GFP-RasV12 cells; RR, MDCK-pTR GFP-RasV12 cells cultured alone.

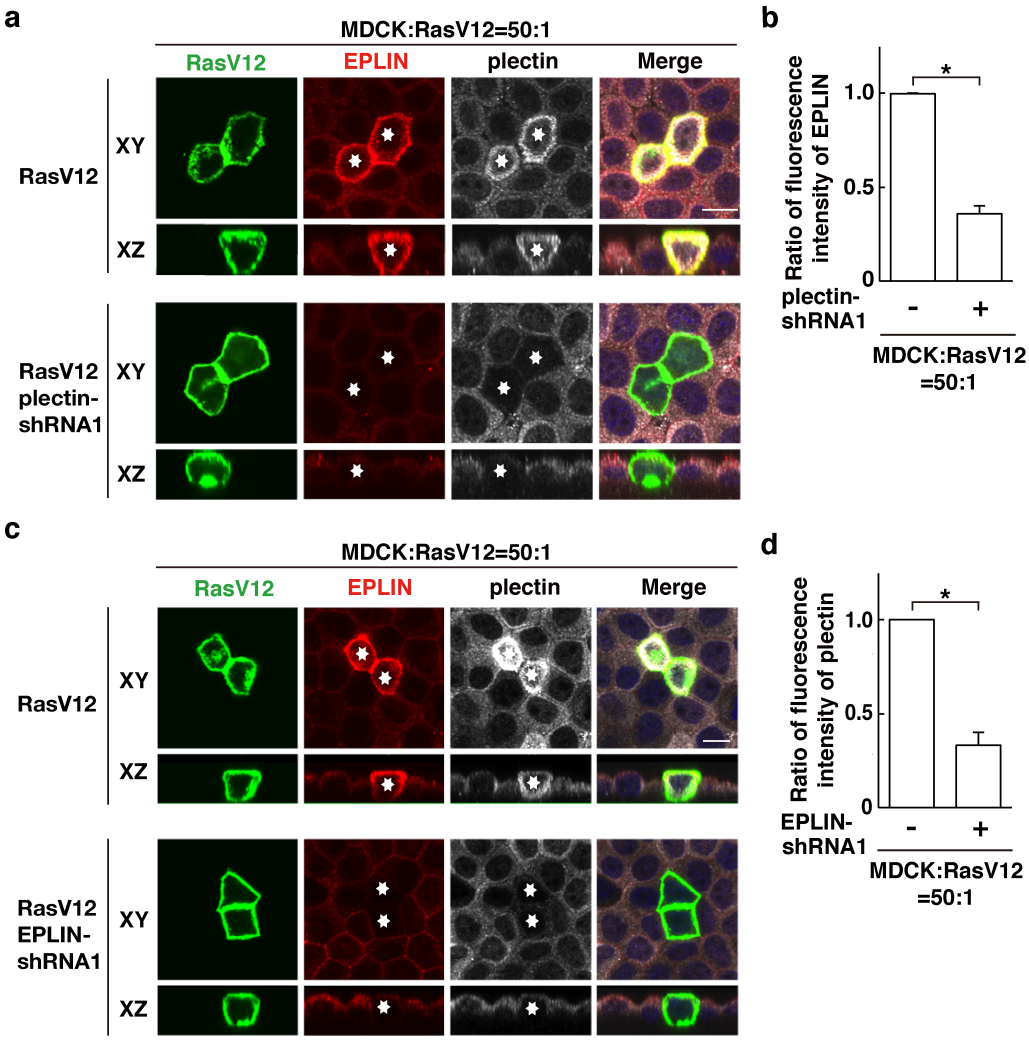

Figure 18. Plectin and EPLIN mutually regulate their accumulation in RasV12-transformed cells surrounded by normal epithelial cells. (**a**-**d**) Effect of plectin-knockdown on accumulation of EPLIN (**a**, **b**) or effect of EPLIN-knockdown on accumulation of plectin (**c**, **d**). MDCK-pTR GFP-RasV12 cells, MDCK-pTR GFP-RasV12 plectin-shRNA1 cells, or MDCK-pTR GFP-RasV12 EPLIN-shRNA1 cells were mixed with normal MDCK cells on collagen gels. Cells were fixed after 16 h incubation with tetracycline and stained with anti-EPLIN (red) and anti-plectin (white) antibodies and Hoechst (blue). (**a**, **c**) Scale bars, 10 μm. (**b**, **d**) Data are mean ± SD from three independent experiments. **P*<0.005; n=30 cells for each experimental condition. Values are expressed as a ratio relative to plectin-shRNA1 (-) (**b**) or EPLIN-shRNA1 (-) (**d**). White star indicates transformed cell.

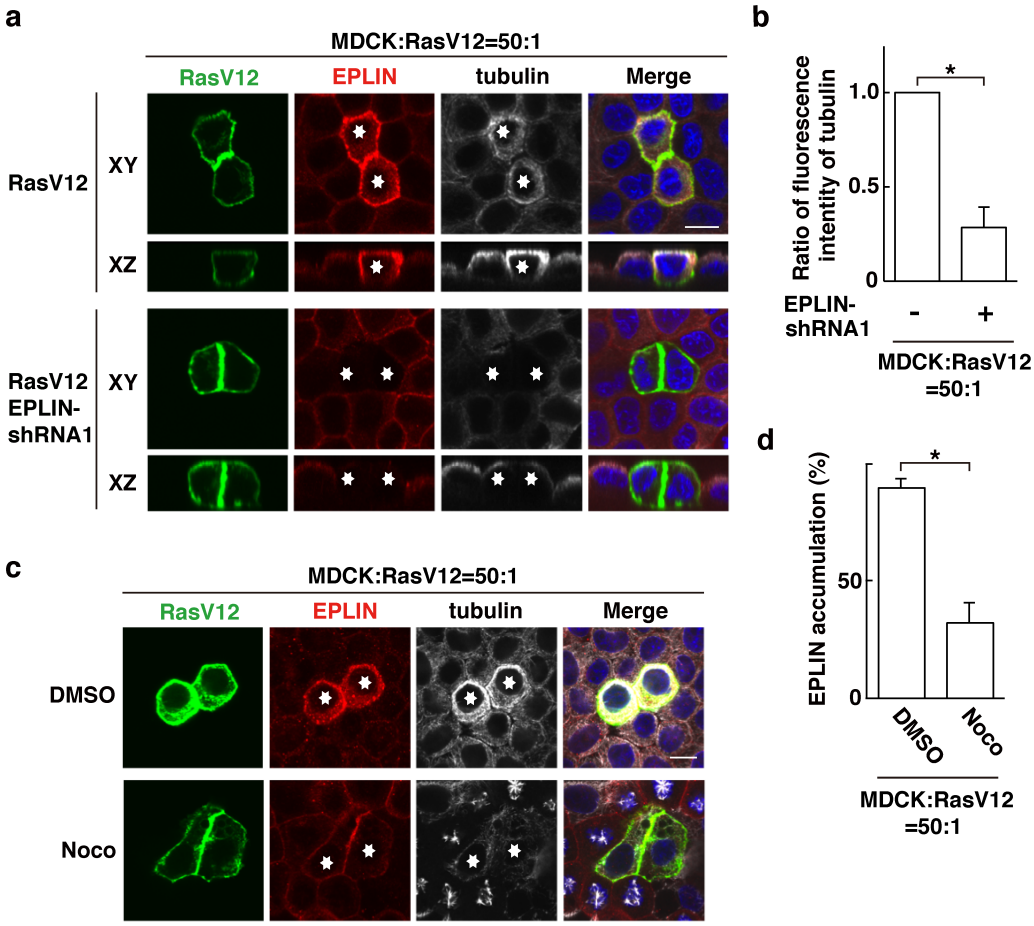

Figure 19. EPLIN and microtubules mutually regulate their accumulation in RasV12-transformed cells surrounded by normal epithelial cells. Effect of EPLIN-knockdown on accumulation of tubulin (**a**, **b**) or effect of nocodazole on accumulation of EPLIN (**c**, **d**). MDCK-pTR GFP-RasV12 cells or MDCK-pTR GFP-RasV12 EPLIN-shRNA1 cells were mixed with normal MDCK cells on collagen gels. Cells were fixed after 16 h incubation with tetracycline and stained with anti-EPLIN (red) and anti-tubulin (white) antibodies and Hoechst (blue). (**a**, **c**) Scale bar, 10 μm. (**b**) Data are mean ± SD from three independent experiments. *P<0.01; n=30 cells for each experimental condition. Values are expressed as a ratio relative to EPLIN-shRNA1 (-). (**d**) Data are mean ± SD from three independent experiments. *P<0.0005; n=100-110 cells for each experimental condition. White star indicates transformed cell.

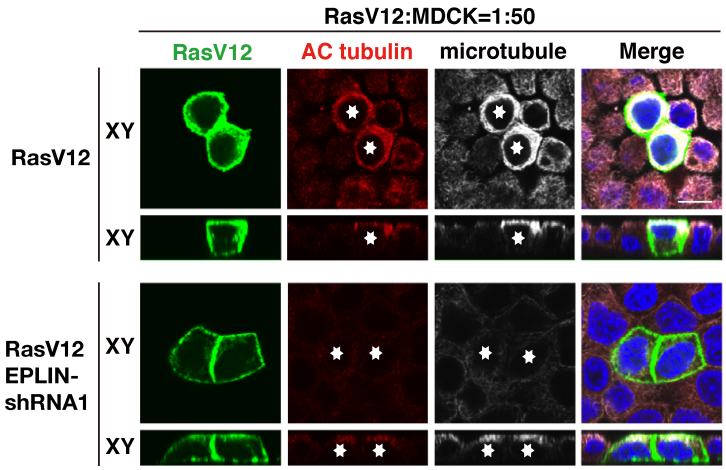

Figure 20. EPLIN in RasV12 cells surrounded by normal cells is involved in the acetylation of α-tubulin for dynamic stabilization of microtubules. Effect of EPLIN-knockdown on the acetylation of α-tubulin in microtubules. MDCK-pTR GFP-RasV12 cells or MDCK-pTR GFP-RasV12 EPLIN-shRNA1 cells were mixed with normal MDCK cells. Cells were fixed after 16 h incubation with tetracycline and stained with anti-acetylatedα-tubulin (red) and anti-tubulin (white) antibodies and Hoechst (blue). Scale bar, 10 μm. White star indicates transformed cell.

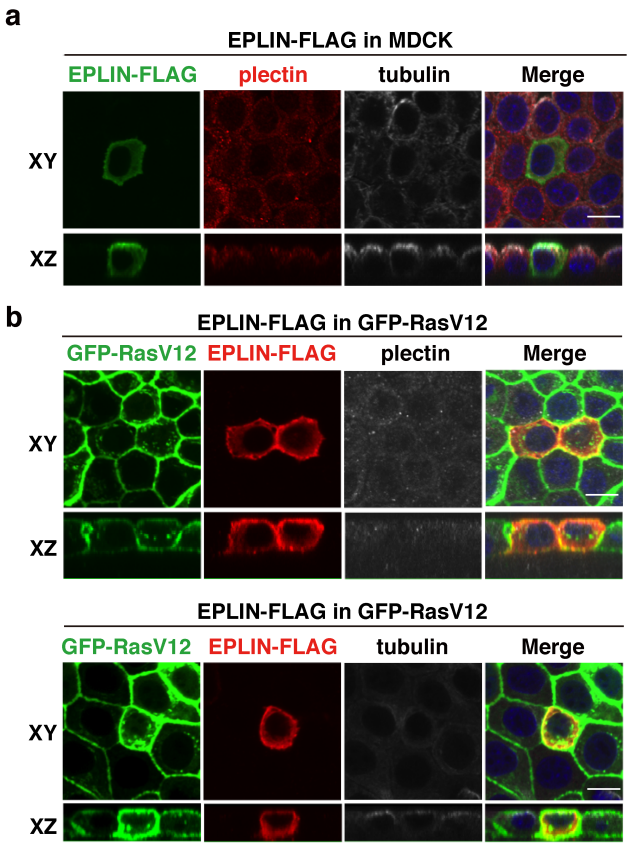

Figure 21. Overexpression of EPLIN alone is not sufficient to cause accumulation of plectin or microtubules. EPLIN-FLAG was transiently expressed within a monolayer of MDCK cells (**a**) or MDCK-pTR GFP-RasV12 cells (**b**) in a mosaic manner. Cells were fixed after 24 h of transfection and stained with anti-FLAG, anti-plectin, and/or anti-tubulin antibodies and Hoechst. Scale bars, 10 μm.

6 The plectin-EPLIN-microtubule complex regulates various downstream proteins to eliminate RasV12-transformed cells from the epithelium

In the process of apical extrusion of RasV12-transformed cells, EPLIN functions upstream of Caveolin-1 (Cav-1), a crucial component of caveolae, and regulates its enrichment in RasV12-transformed cells that are surrounded by normal cells. In addition, EPLIN regulates activation of protein kinase A (PKA) that specifically occurs in RasV12 cells when they are surrounded by normal cells. Plectin-knockdown diminished Cav-1 accumulation in RasV12-transformed cells surrounded by normal cells, whereas knockdown of Cav-1 did not affect the accumulation of plectin (Fig. 22a,b). Furthermore, plectin-knockdown profoundly suppressed the non-cell-autonomous activation of PKA (Fig. 22c). Collectively, these data indicate that in concert with EPLIN and mictotubules, plectin regulates these downstream regulators for apical extrusion of RasV12-transformed cells (Fig. 23).

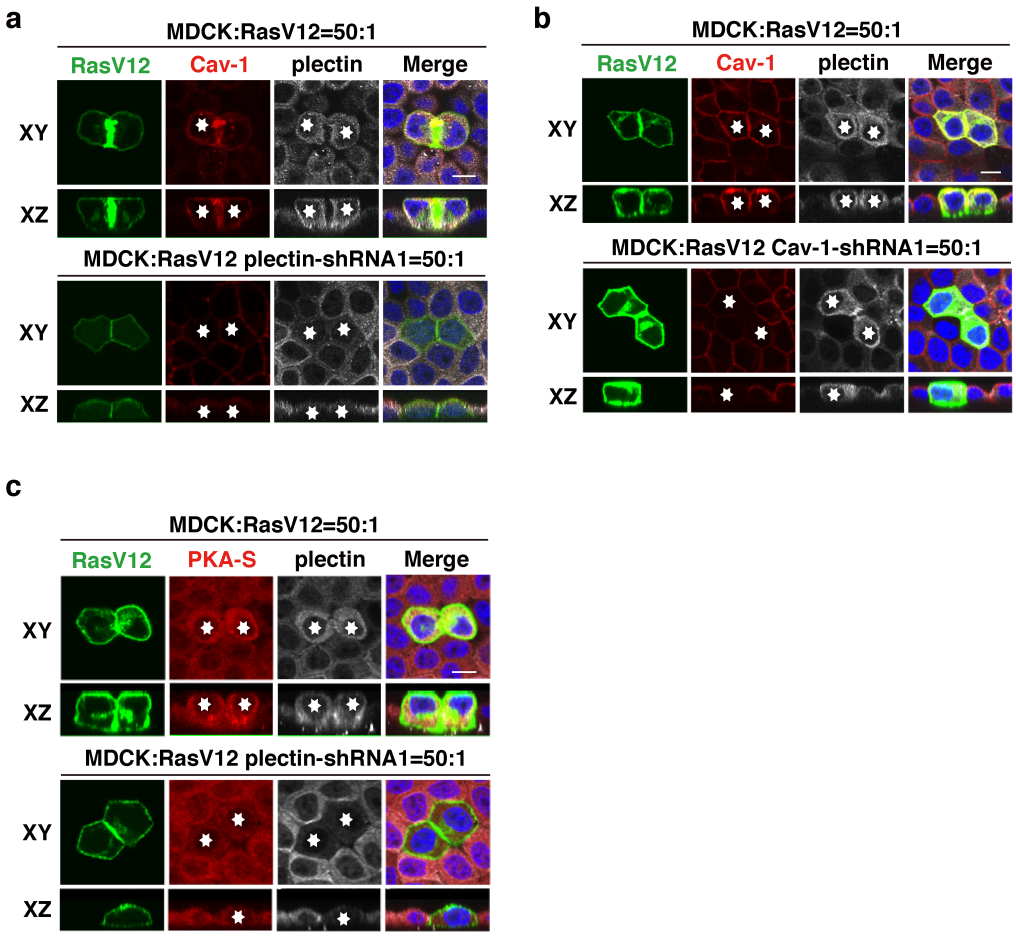

Figure 22. Plectin functions upstream of Cav-1 and PKA in RasV12-transformed cells surrounded by normal epithelial cells. (**a**, **b**) Effect of plectin-knockdown on accumulation of Cav-1 (**a**) or effect of Cav-1-knockdown on accumulation of plectin (**b**). (**c**) Effect of plectin-knockdown on activity of PKA. (**a-c**) MDCK-pTR GFP-RasV12 cells, MDCK-pTR GFP-RasV12 plectin-shRNA1 cells, or MDCK-pTR GFP-RasV12 Cav-1-shRNA1 cells were mixed with normal MDCK cells on collagen gels. Cells were fixed after 16 h incubation with tetracycline and stained with anti-Cav-1 (red), anti-plectin (white), or anti-PKA-substrate (red) antibody and Hoechst (blue). Scale bars, 10 μm. White star indicates transformed cell.

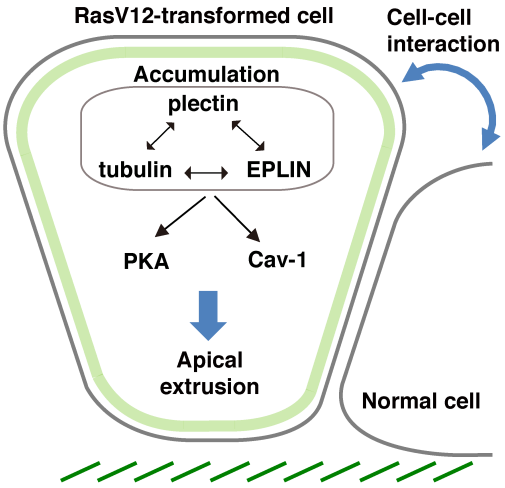

Figure 23. Schematic for plectin-EPLIN-microtubules complex in apical extrusion of RasV12-transformed cells surrounded by normal cells.

F) Discussion

In this study, I have revealed that plectin is a new player in apical elimination of RasV12-transformed cells from the epithelium. In RasV12 cells surrounded by normal cells plectin is co-accumulated with EPLIN, microtubules, and intermediate filaments and regulates downstream signaling molecules Cav-1 and PKA, thereby promoting apical extrusion (Fig. 23). Plectin has been reported to interact with a variety of cytoskeletal proteins including three cytoskeletal filaments: actin, microtubule and intermediate filament. Indeed, plectin is co-accumulated with tubulin and keratin in RasV12 cells surrounded by normal cells and is probably involved in the formation of integrated cytoskeletal webs. Previous reports have shown that plectin deficiency enhances the stability of microtubule filaments, suggesting that plectin mediates dynamic properties of microtubules[45,79]. As tubulin accumulation predominantly occurs at the apical membrane domain, it is plausible that accumulated plectin induces dynamic modulation of microtubules, which might provide physical forces and/or influence various cellular processes such as vesicle transport that are required for apical extrusion. I have not observed the comparable accumulation of actin filaments[67], suggesting that actin is not, at least, a major component within the cytoskeletal complex. EPLIN is also a crucial molecule in this complex, as knockdown of EPLIN substantially diminishes accumulation of plectin and microtubules. In this multiple-molecular complex, plectin seems to act as a key adaptor that interlinks these cytoskeletal proteins.

There still remains a question to be addressed: what are the upstream and downstream regulators of the plectin-EPLIN-microtubules complex? The accumulation of these molecules is mutually regulated; knockdown or disruption of one component

diminishes the accumulation of the other. Thus, there should be a coordinated molecular machinery that induces the simultaneous assembly of these molecules in a concerted fashion. It has been shown that accumulation of EPLIN in RasV12-transformed cells is triggered by EDAC; knockdown of filamin in the neighboring normal cells suppresses EPLIN accumulation[80]. The mechanical link between filamin in normal cells and the plectin-EPLIN-microtubules complex in transformed cells needs to be elucidated in future studies. A previous study has demonstrated that filamin accumulates in normal epithelial cells at the interface with the neighboring transformed cells and that accumulated filamin positively promotes apical extrusion of the transformed cells[13]. Filamin crosslinks actin filament to form orthogonal actin-meshworks[21] and acts as mechanosensor/transducer[22]. Indeed, plectin can positively regulate cell stiffness and traction forces[23]. Thus, it is plausible that accumulated plectin modulates the physical properties of transformed cells, which then induces the recruitment of filamin in the neighboring normal cells. This is compatible with the recent reports demonstrating the involvement of physical forces in cell competition[24-26]. The mechanical link between filamin in normal cells and the plectin-EPLIN-microtubules complex in transformed cells needs to be elucidated in future studies. Recently, it has become clear that plectin, in addition to its role in cytoskeletal regulation, acts as a scaffold for signaling pathways by interacting with a number of signaling molecules including PIP2, Fer, RACK1 and AMPK[51,81-83]. Thus, it is plausible that plectin functions as a hub for the downstream signaling pathways in cell competition. In addition, the immunoprecipitation results in Figure 1a, b and Supplementary Figure S1a suggest that there are unidentified, tyrosine-phosphorylated plectin-binding protein(s). To understand the missing link between the plectin complex and downstream molecules Cav-1 and PKA, I will need to

identify binding protein(s) to the plectin-EPLIN-microtubules complex under the mix culture of normal and transformed cells.

G) FINAL CONCLUSION

In this study, I have revealed plectin is a new player in apical extrusion of RasV12-transformed cells. I have provided a model in which plectin regulates apical extrusion of RasV12-transformed cells in concert with EPLIN and microtubules.

Activation of various tyrosine kinases would be involved in Ras-MAPK signaling pathways. To address the molecular mechanism of apical elimination of RasV12-transformed cells, I screened tyrosine-phosphorylated proteins using three different cell culture conditions (normal or RasV12 cells alone and their mixture at 1:1). I found that plectin increased at the interface between normal and transformed cells by immunoprecipitation with anti-phosphor-tyrosine antibody. I then demonstrated that plectin was not tyrosine-phosphorylated, suggesting that plectin interacted with unidentified, tyrosine-phosphorylated protein(s). I show evidence that in RasV12 cells surrounded by normal cells plectin was accumulated in a non-cell-autonomous manner and knockdown of plectin substantially suppressed apical extrusion of RasV12 cells, suggesting that plectin is a positive regulator for apical extrusion.

Plectin is a versatile cytoskeleton cross-linker, organizing intermediate filament networks and linking them to microtubules, actin filaments. Here, I provided evidence that the relevance between plectin and several cytoskeleton proteins. Plectin was originally identified with intermediate filaments, bridging intermediate filaments to form networks. As I expected, plectin regulated keratin accumulation in RasV12-transformed cells surrounded by normal cells. Plectin interacts with actin and microtubules via its N-terminal ABD and C-terminal GSR domain, respectively. However, I did not examine the role of these domains in the regulation of actin and

microtubules. Plectin is more than cross-linker. Recent studies show evidence that plectin regulates dynamic modulation of actin and microtubules. I demonstrated that accumulation of plectin enhanced accumulation of tubulins at the apical region of RasV12-transformed cells surrounded by normal cells, and also observed acetylation of α-tubulin increased and overlapped with accumulated tubulins. Plectin-knockdown suppressed accumulation of tubulin and its acetylation, suggesting that plectin regulates dynamic stability of microtubules. Conversely, disruption of microtubules by nocodazole suppressed plectin accumulation and apical extrusion. Collectively, these findings indicated plectin and microtubules mutually affect their accumulation to co-regulate apical extrusion of RasV12-transformed cells surrounded by normal cells. I did not observe co-accumulation of plectin and actin filaments, and disruption of actin filaments did not affect plectin accumulation, indicating that actin filaments may not be included in plectin-mediated cytoskeletal components.

In our previous work we provided evidence that EPLIN in transformed cells and filamin in surrounding normal cells mutually affect their accumulation in EDAC to promote apical elimination of transformed cells. I examined the relevance between EPLIN and plectin. By immunoprecipitation with EPLIN antibody, I found that plectin and EPLIN increased the interaction under the mix culture condition. I then demonstrated that when plectin knocked down or EPLIN knocked down, accumulation of EPLIN or plectin was substantially suppressed, suggesting that plectin and EPLIN mutually regulate their accumulation in RasV12-transformed cells. Similarly, EPLIN-knockdown or nocodazole treatment suppressed accumulation of tubulin or EPLIN. In addition, acetylation of α-tubulin was suppressed by EPLIN-knockdown. These findings indicate that plectin, EPLIN, and microtubules form a complex in

RasV12 cells.

Plectin not only is a cross-linker, but also a noble scaffolding protein in signaling transduction. To reinforce the cytoskeleton structures, plectin recruits signaling molecules and proteins to the cytoskeletons, by positioning them at specific sites within the cells. Examples are the signaling molecule PIP2, Fer, RACK1, AMPK and otherwise. Previous studies show that caveolin-1 and PKA function as downstream of EPLIN in RasV12 cells that are surrounded by normal cells during the process of EDAC. Here we show that plectin-knockdown suppressed accumulation of caveolin-1 and activation of PKA, and caveolin-1-knockdown did not affect accumulation of plectin, suggesting that plectin regulates these molecules as an upstream.

Taken together, I have illustrated that plectin-EPLIN-microtubules complex regulates downstream proteins, PKA and caveoilin-1, to promote apical extrusion. But, I did not find the answer to these questions: how is plectin-EPLIN-microtubule complex regulated in apical extrusion? What are the missing links between this complex and the downstream proteins (Cav-1 and PKA)? And what is the significance of unidentified, plectin-interacting tyrosine-phosphorylated protein(s) in apical extrusion? However, apical extrusion of the transformed cells can be regarded as a cancer preventive process, which is supposed to occur at the initial stage of carcinogenesis. Thus, the plectin-EPLIN-microtubules complex could be a potential drug target; the activation of the plectin complex is expected to enhance the eradication of newly emerging and/or remaining transformed cells from epithelia. Further development of this study would open a new avenue for cancer preventive medicine.

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