Study on the Role of the Paxillin-plectin-EPLIN Complex in Apical Extrusion of RasV12 Cells

（Paxillin-plectin-EPLIN 複合体による RasV12 変異細胞の管腔側への逸脱に対する役割の研究）

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1.Introduction

In 2012, more than 14 million people got cancer and more than 8 million people died of cancer [1]. To cure many people suffering from cancer, cancer therapy is required.

Previously a lot of researchers focused on the difference between normal and cancer cells and have been trying to cure the cancer patients. However, these trials often end in failure since the cancer cells gradually change their properties and become more malignant and resistant to anti-cancer drugs. In terms of cancer prevention, we therefore focus on the super initial stage of the carcinogenesis in which the cells obtain single mutation such as an oncogenic mutation on the gene *Ras*. We previously found that RasV12-transformed cells are outcompeted from an epithelial monolayer, this phenomenon is called ‘‘cell competition’’ occurs at the super initial stage of cancer[2-12]. The mechanism of these phenomena, however, still remain unknown. Therefore, to establish more effective therapies for cancer including cancer preventive treatment, we investigate the mechanisms of cell competition.

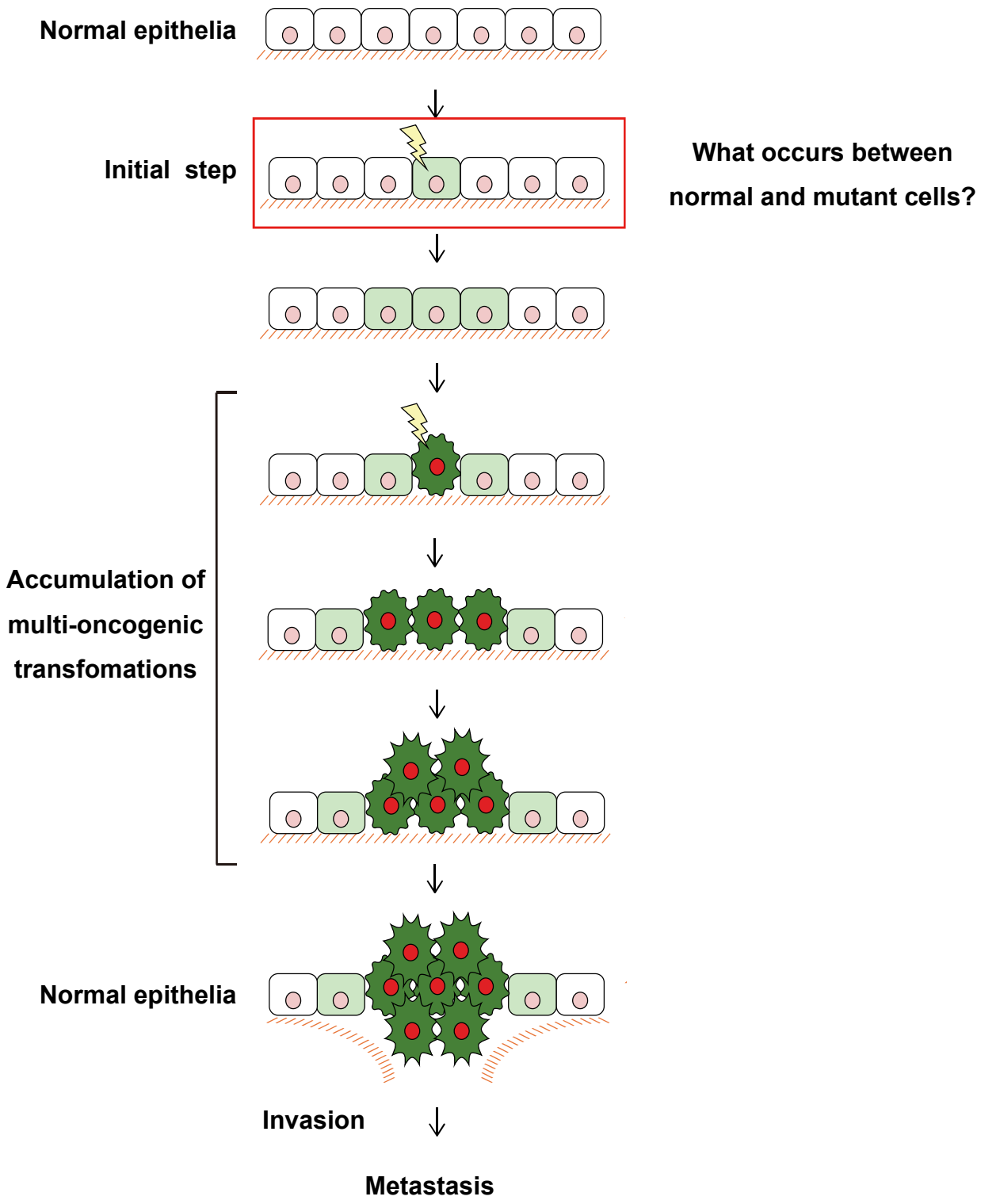

Figure 1-1. Multistep model of carcinogenesis.

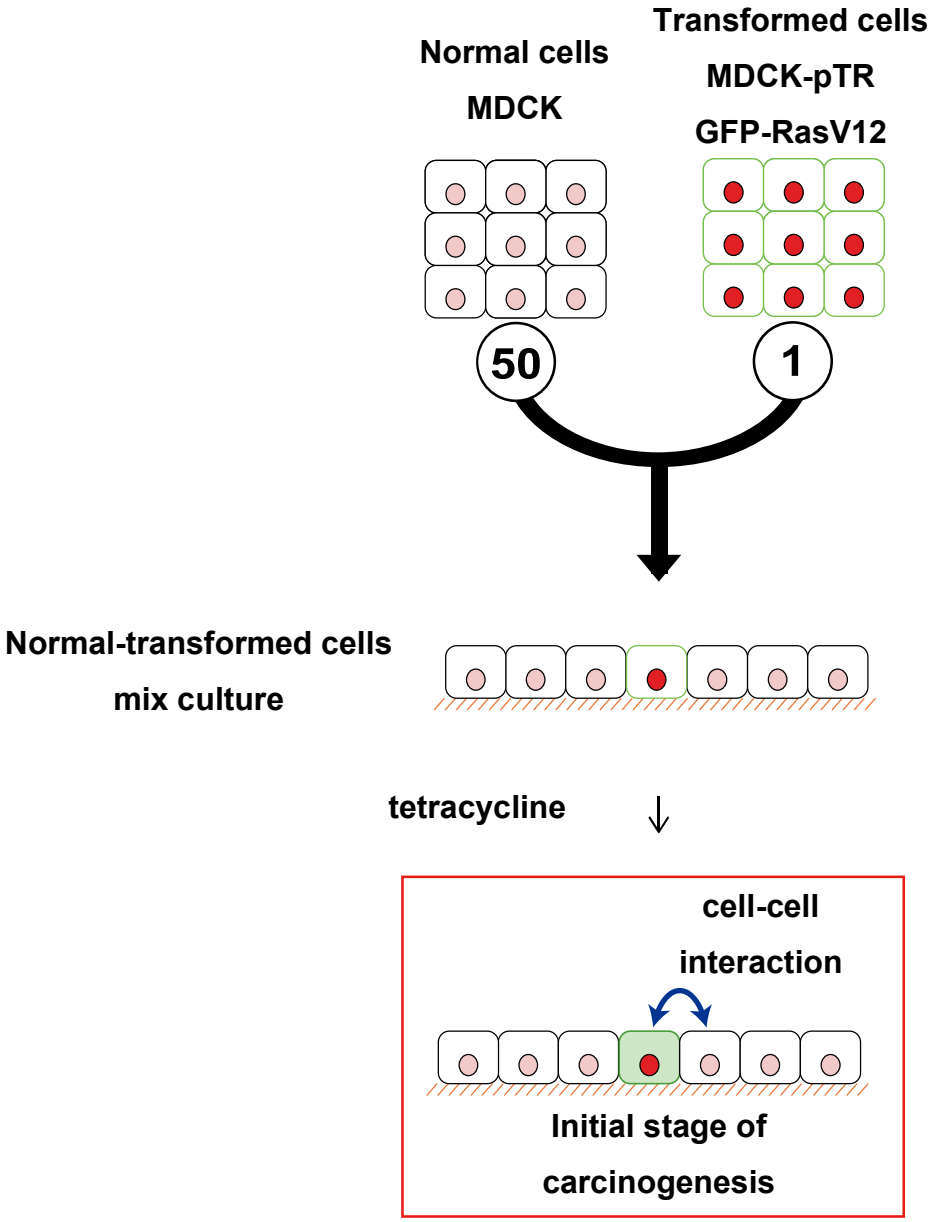

Figure 1-2. Cell competition *in vitro* model system

1.1. Cell competition in *Drosophila*

From previous studies of *Drosophila*, several events occur at the boundary region between normal and transformed cells. At first, Morata and Ripoll demonstrated that when cells which have a gene mutation are eliminated from epithelia[13]. On the other hand, the phenomena do not occur, when the mutated cells were cultured alone. Therefore, they considered that there are competitions at the interface between normal and transformed cells. Thus, they named the event ‘‘cell competition”. Mechanisms regulating this non-cell autonomous event remain elucidative.

1.1.1. *Minute*

Minute was the first reported gene involved in cell competition by Genes Morata and Pedro Ripoll[13] . It was known that Minute mutation is involved in the defects in ribosomal proteins [14]. *Drosophila* having homozygous *Minute* could not produce proteins, resulting in cell death. On the other hand, heterozygous *Minute* flies have the same sizes of normal flies although delay of growth rates or the smaller size of hair is observed. Morata *et al.* demonstrates that the normal size of heterozygous Minute flies is due to compensation by normal cells for vacant space opened by apoptotic elimination of Minute mutant cells by normal cells. This non-cell autonomous event does not occur in mutant flies which have only heterozygous cells. They therefore called the survived cells “winner’’ and dead cells were “loser’’.

1.2. Cell competition in mammals

In these days, we have reported that cell competition also occurs between mammalian epithelial cells when oncogenic mutations occur in epithelium[2]. I describe some oncogenes involved in mammalian cell competition in this section.

1.2.1. *Ras*

Ras is one of the small GTPase superfamily[15] which related to cell proliferation, viability, differentiation and motility. Additionally, many types of human cancer have mutation in the *Ras* gene (e.g. G12V (RasV12)) [16]. Previously, in order to investigate what happens at the interface between normal and transformed cells, we established MDCK cells which stably possess the cassette of tetracycline inducible RasV12 (MDCK-pTR GFP-RasV12, hereinafter referred to as RasV12 cells). Using this cell line, we observed interesting phenomenon at the boundary region of normal and transformed cells. To examine the interaction between normal and transformed cells, RasV12 cells mixed with normal MDCK cells at a ratio of 1:100, and the mixture of cells was seeded on collagen gel and cultured in the absence of tetracycline until seeded cells formed a monolayer. Then,

GFP-RasV12 expression was induced with tetracycline. After these steps, we observed cell behavior Ras12 cells surrounded by normal cells on collagen gels using a confocal microscopy. As a result, RasV12 cells surrounded by normal cells were apically extruded from the normal MDCK monolayer at 24 h after tetracycline addition. On the other hand, RasV12 cells which were cultured alone were not extruded, suggesting that the extrusion of RasV12 cells depends on the interaction with normal cells[17]. Thus, we concluded that cell competition also occurs in the mammalian epithelia. However, the mechanism of extrusion is unknown.

1.2.2. *Src*

Rous sarcoma virus *Src* gene (v-Src) was the first identified oncogene[18]. Furthermore, it has reported that v-Src and its cellular compartment Src (c-Src) are overexpressed and highly activated in various human cancers[19]. 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[20,21] . To investigate the cell-cell interaction between normal and transformed cells, MDCK cells expressing constitutively active v-Src in a temperature-dependent manner (Src cells). When the Src cells surrounded by normal cells, Src cells were apically extruded from epithelia[22]. Activity of myosin-Ⅱ, focal adhesion kinase (FAK) and mitogen-activated protein kinase (MAPK) is increased in Src cells that is surrounded by normal cells.

1.2.3. *Scribble*

Scribble was first identified in Drosophila as a tumor suppressor. In drosophila imaginal discs, cells which have scribble mutation are eliminated from epithelial monolayer by surrounding normal cells via JNK-pathway-mediated apoptosis[23]. Additionally, it was reported that Scribble could decrease the cancer progression in mice, and depletion of scribble was observed in human colon and breast cancers. Previously, Mark *et al.* reported that MDCK cells expressing Scribble shRNA was died and apically extruded from epithelia. Importantly, this phenomenon does not occur when scribble-knockdown cells were cultured alone, indicating that presence of surrounding normal cells induces this event. Moreover, p38 MAPK activation is required for the extrusion of Scribble-knockdown MDCK cells, suggesting there is different mechanism from *Drosophila.* But, the detailed mechanism is still unknown.

1.3. Proteins related to this thesis

In 2015, we demonstrated that Epithelial Protein Lost In Neoplasm (EPLIN) is a crucial regulator of apical extrusion of RasV12 cells surrounded by normal cells. Furthermore, we reported plectin forms the EPLIN-plectin complex and positively regulates apical extrusion in 2017. In my thesis, I show that paxillin also forms the complex and regulates apical extrusion through inhibition of HDAC6 activity. Therefore, I describe general function of these proteins (EPLIN, plectin, paxillin, HDAC6, IVNS1ABP) below.

1.3.1. EPLIN

EPLIN was originally identified as a protein, of which expression is often down-regulated or lost in various types of cancers[24-26]. *In vivo* studies also confirmed the down-regulation of EPLIN in a number of human epithelial cancer cells and tissues, suggesting that the loss of EPLIN could contribute to the transformed phenotype. This indicates that EPLIN may act as a tumor suppressor. There are two isoforms, EPLINα and EPLINβ. The difference of the sequences is the addition of a 160-amino acid extension at the N terminus of the α isoform. EPLIN is the family of LIM domain proteins which has a centrally located LIM domain known to form two closely packed zinc-binding subdomains. Moreover, EPLIN exhibits two functional actin binding sites, one on each side of the centrally located LIM domain. *In vitro*, EPLIN stabilizes actin filaments by inhibiting their depolymerization and suppresses the formation of branched filaments by blocking actin nucleation by Arp2/3. Based on these properties, EPLIN is related to different actin-related processes, such as cell motility and migration, cytokinesis, and intercellular junctions. Additionally, EPLIN binds to α-catenin associated with the cadherin-β-catenin complex. Through this interaction with α-catenin, EPLIN serves to link this complex to the actin fibers.

E-cadherin is stabilized at the cell surface by the link between E-cadherin and the actin cytoskeleton via β-catenin, α-catenin, and EPLIN. Thus, EPLIN plays a vital role in the establishment of adherens junctions

1.3.2. Plectin

Plectin is a member of the plakins family defined by plakin domain and/or plakin repeat domain[27]. Plectin is well known as a cytoskeletal cross-linker protein which link between three main components of cytoskeleton: actin, microtubules and intermediate filaments. Plectin is a giant protein, over than 500 kD with about 4000 amino acids, and is mainly expressed in mammalian tissue cells existing as numerous alternative splicing isoforms. This protein is regarded as a combination of multiple interacting domains. Moreover, plectin has also been proposed as a marker of pancreatic cancer.

1.3.3. Paxillin

Paxillin was identified as a tyrosine kinase substrates from Rous sarcoma virus-transformed cells[28]. Paxillin contains 4 LIM domains, a proline-rich domain containing a consensus SH3-binding site, and 3 potential SH2-binding sites. Functionally, paxillin is a cytoskeletal protein involved in actin-membrane attachment at sites of cell adhesion to the extracellular matrix (focal adhesion) and one of the key adaptor proteins in the integrin-based focal adhesion complex[29], forming complex with focal adhesion kinase (FAK) and integrin. In concert with these molecules, paxillin has a crucial role in cell adhesion.

1.3.4. HDAC6

In 2002, histone deacetylace 6 (HDAC6) was identified as a tubulin deacetylase [30]. It is known that tubulin acetylation is regulated by tubulin acetyltransfelases, HDAC6 and SIRT2. Then, HDAC6 is important for regulating acetylation of tubulin. Moreover, HDAC6 is localized in the cytoplasm, where it associates with microtubules and localizes with the microtubule motor complex. In cytoplasm, HDAC6 also regulates binding of tubulin with kinesin through tubulin acetylation. In addition to this, it is known that HDAC6 is related to vesicle transport, mitochondrial activity, cell migration.

1.3.5. IVNS1ABP

IVNS1ABP has Kelch repeats which is known to interacts with actin cytoskeleton and regulates the dynamic organization [31]. Additionally, IVNS1ABP is known to protect cells from cell death induced by actin destabilization. However, the function is almost unknown

1.4. The aims of this thesis

In this study, I have found that paxillin is a vital regulator of apical extrusion of RasV12-transformed cells by mechanically linking the plectin-EPLIN complex and acetylation of microtubules.

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2. The Role of Paxillin-plectin-EPLIN Complex in Apical Extrusion of RasV12 Cells

2.1. Abstract

Recent studies have revealed that newly emerging RasV12-transformed cells are often apically extruded from the epithelial layer. During this cancer preventive process, cytoskeletal proteins plectin and Epithelial Protein Lost In Neoplasm (EPLIN) are accumulated in RasV12 cells that are surrounded by normal cells, which positively regulate the apical elimination of transformed cells. However, the downstream regulators of the plectin-EPLIN complex remain to be identified. In this section, we have found that paxillin binds to EPLIN specifically in the mix culture of normal and RasV12-transformed cells. In addition, paxillin is accumulated in RasV12 cells surrounded by normal cells. Paxillin, plectin and EPLIN mutually influence their non-cell-autonomous accumulation, and paxillin plays a crucial role in apical extrusion of RasV12 cells. We also demonstrate that in RasV12 cells surrounded by normal cells, acetylated tubulin is accumulated. Furthermore, acetylation of tubulin is promoted by paxillin that suppresses the activity of histone deacetylase (HDAC) 6. Collectively, these results indicate that in concert with plectin and EPLIN, paxillin positively regulates apical extrusion of RasV12-transformed cells by promoting microtubule acetylation. This section shed light on the unexplored events occurring at the initial stage of

carcinogenesis and would potentially lead to a novel type of cancer preventive medicine.

2.2. Introduction

At the initial stage of carcinogenesis, an oncogenic mutation occurs in single cells within the epithelium. Recent studies have revealed that the newly emerging transformed cells and the surrounding normal epithelial cells often compete with each other for survival[1-10]. This phenomenon is called cell competition; the loser cells are eliminated from epithelial tissues, while the winner cells proliferate and fill the vacant spaces. By using Madin-Darby canine kidney (MDCK) epithelial cells stably expressing RasV12 in a tetracycline-inducible manner, we have demonstrated that when Ras-transformed cells appear within the epithelial monolayer, the transformed cells are extruded into the apical lumen of the epithelium in a cell death-independent fashion, a process called apical extrusion[11]. Together with other studies, it has become evident that normal epithelial cells can recognize and actively eliminate the neighboring transformed cells from epithelial tissues via cell competition. This cancer preventive mechanism is termed Epithelial Defense Against Cancer (EDAC)[12,13].

In the cell competition between normal and RasV12-transformed epithelial cells, the presence of normal cells profoundly influences various cellular processes and signaling pathways in the neighboring transformed cells, which

positively regulate their apical extrusion. In the previous studies, we have reported that cytoskeletal proteins plectin and Epithelial Protein Lost In Neoplasm (EPLIN) are accumulated in RasV12 cells when they are surrounded by normal cells[14,15]. The plectin-EPLIN complex then induces α-tubulin polymerization, leading to the accumulation of microtubule filaments. This process plays a crucial role in the apical extrusion of RasV12 cells, however the molecular mechanism of how plectin and EPLIN regulate the organization of microtubules remains unknown.

The structure and physical property of microtubule filaments are dynamically regulated by various mechanisms including acetylation of α-tubulin K40[16,17]. In addition, acetylation of tubulin can also influence a variety of cellular processes including vesicle transport, signaling pathways and cell migration[18,19]. Acetylation of tubulin is catalyzed by α-tubulin acetyltransferase (αTAT) 1[20,21], while deacetylation is mediated by histone deacetylase (HDAC) 6[22,23] and sirtuin (SIRT) 2[24]. The activity of HDAC6 can be regulated by multiple mechanisms such as suppression by paxillin[25]. Paxillin is one of the key adaptor proteins in the integrin-based focal adhesion complex[26]. But, additionally, paxillin localizes in the cytosol and can play other cellular functions[25].

In this study, we have found that paxillin is a vital regulator of apical extrusion of RasV12-transformed cells by mechanically linking the plectin-EPLIN complex and acetylation of microtubules.

2.3. Experimental procedures

2.3.1. Antibodies and Materials.

Mouse anti-acetylated tubulin (T6793) antibody was purchased from Sigma-Aldrich. Rat anti-α-tubulin (YOL1/34) antibody was from Abcam. Rabbit anti-paxillin (sc-5574) and mouse anti-EPLIN (sc-136399) antibodies were from Santa Cruz Biotechnology. Mouse anti-paxillin (clone 349) antibody was from BD Transduction Laboratories. Rabbit and mouse anti-paxillin antibodies were used in Fig.2-4, 6, 11, 12, respectively. Mouse anti-GAPDH (Clone 6C5) antibody was from Millipore. Rabbit polyclonal affinity-purified anti-plectin antibody was generated as previously described[14]. 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 described above were diluted in phosphate-buffered saline (PBS) containing 1% BSA at 1:100, except anti-α-tubulin antibody at 1:200, and

anti-paxillin and EPLIN antibodies 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 anti-α-GAPDH antibody at 1:2,000, and secondary antibodies were used at 1:1,000. The following inhibitors were used where indicated: tubacin (Sigma-Aldrich, 10 μM) and AGK2 (Sigma-Aldrich, 10 μM). DMSO (Sigma-Aldrich) was added as a control.

2.3.2. Cell Culture.

MDCK and MDCK-pTR GFP-RasV12 cells were cultured as previously described[11]. MDCK-pTR GFP-RasV12 cells stably expressing EPLIN-shRNA or plectin-shRNA were established as previously described[14,15]. MDCK-pTR GFP-RasV12 cells stably expressing paxillin-shRNA were established as follows:

Double-stranded DNA fragments coding paxillin-shRNA sequences (paxillin-shRNA1:

5’-GATCCCCGCCTACAGTCTGACCTGAATTCAAGAGATTCAGGTCAGACTGT AGGCTTTTTC-3’ and

5’-TCGAGAAAAAGCCTACAGTCTGACCTGAATCTCTTGAATTCAGGTCAGAC

TGTAGGCGGG-3’

or paxillin-shRNA2:

5’-GATCCCCGCTTACTGCCGGAAGGATTTTCAAGAGAAATCCTTCCGGCAG

TAAGCTTTTTC-3’ and

5’-TCGAGAAAAAGCTTACTGCCGGAAGGATTTCTCTTGAAAATCCTTCCGGC

AGTAAGCGGG-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

paxillin-shRNA1 or -shRNA2 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, the tetracycline-inducible MDCK-pTR

GFP-RasV12 cell lines were treated with 2 μg ml^{-1} tetracycline (Sigma-Aldrich).

For inhibitor treatment, the indicated inhibitors were simultaneously added

together with tetracycline, and then cells were further cultured for 16 h or 24 h.

For immunofluorescence, cells were seeded onto Type-I collagen-mounted

coverslips as described below in the section of immunofluorescence.

2.3.3. Immunofluorescence.

MDCK-pTR GFP-RasV12 cells were mixed with MDCK cells at a ratio of 1:50 and cultured on the collagen matrix as previously described[11]. 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 antibodies were incubated for 2 h or 1 h, respectively at ambient temperature. Immunofluorescence images were acquired by the Olympus FV1200 system and the Olympus FV10-ASW software. For quantification of immunofluorescence intensity, 30 transformed cells were analyzed for each experiment using the MetaMorph software (Molecular Devices). For analyses of apical extrusions, the samples were prepared as described above, except that cells were treated with tetracycline for 24 h (for Fig. 5d, apical extrusion was observed after 16 h of tetracycline). More than 95 cells were analyzed for each experiment, and apically extruded cells were quantified.

2.3.4. Immunoprecipitation and western blotting.

For immunoprecipitation, MDCK and MDCK-pTR GFP-RasV12 cells were seeded at the density of 1.2 x 10^7 cells in 14.5-cm dishes (two dishes for each experimental condition) (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 NP-40 lysis buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl and 1% NP-40) containing the following inhibitors: 1 mM Na_3VO_4, 0.1 mM Na_2MoO_4, 10 mM NaF, 5 μg ml^{-1} leupeptin, 1 mM phenylmethylsulfonyl fluoride and 7.2 trypsin inhibitor units of aprotinin. After centrifugation of the cell lysates at 21,500 g for 10 min, the supernatant was first pre-cleared with sepharose 4B (Sigma-Aldrich) at 4˚C for 30 min. This step was repeated three times. The pre-cleared cell lysates were then incubated with control IgG-conjugated Dynabeads protein G (Life Technologies) at 4˚C for 30 min and finally subjected to immunoprecipitation for 1 h with Dynabeads Protein G conjugated to rabbit anti-paxillin antibody (10 μg). Immunoprecipitated proteins were subjected to SDS-PAGE, followed by western blotting with the indicated antibodies. Western blotting data were acquired using ImageQuant™

LAS4010 (GE healthcare). To examine the efficiency of paxillin-knockdown, MDCK-pTR GFP-RasV12 cells stably expressing paxillin-shRNA were seeded onto 6-cm dishes (Greiner-Bio-One) at the density of 1 x 10^6 cells. After 24 h, 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.

2.3.5. Data Analyses.

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

2.4 Results

2.4.1. Paxillin plays a crucial role in apical elimination of RasV12-transformed cells.

EPLIN and plectin are accumulated in RasV12-transformed cells surrounded by normal cells and play a vital role in apical extrusion of the transformed cells[14,15]. In a previous study, EPLIN was shown to interact with paxillin[27]. We thus examined the interaction between EPLIN and paxillin in our *in vitro* cell competition model system[11]. Paxillin was co-immunoprecipitated with EPLIN, and the interaction was enhanced under the mix culture condition of normal and RasV12 cells (Fig. 2-1). In addition, by immunofluorescence, we demonstrated that paxillin was accumulated and partially co-localized with EPLIN in RasV12 cells that were surrounded by normal cells, but not in RasV12 cells cultured alone (Fig. 2-2, 4, 6).

To examine the functional role of paxillin, we have established RasV12-transformed cells stably expressing paxillin-shRNA1 or -shRNA2 (Fig. 2-3). Knockdown of paxillin substantially diminished the accumulation of EPLIN in RasV12 cells that were surrounded by normal cells (Fig. 2-4). In addition, paxillin-knockdown also suppressed accumulation of plectin in RasV12 cells surrounded by normal cells (Fig. 2-5). Conversely, knockdown of EPLIN (Fig. 2-6) or plectin (Fig. 2-7) significantly suppressed accumulation of paxillin.

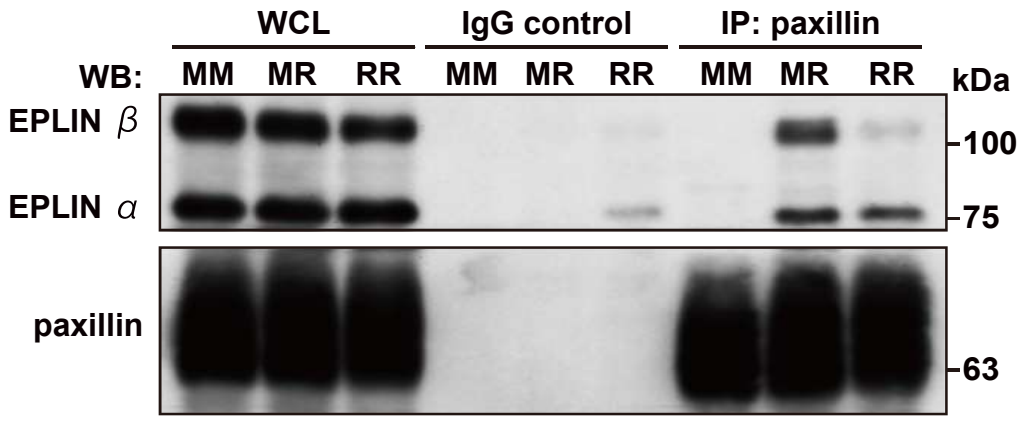

Figure 2-1. Paxillin increases the interaction with EPLIN under the mix culture condition of normal and RasV12-transformed cells. Co-immunoprecipitation of EPLIN with paxillin. MM, normal MDCK cells cultured alone; MR, 1:1 mix culture of normal MDCK and MDCK-pTR GFP-RasV12 cells; RR, MDCK-pTR GFP-RasV12 cells cultured alone.

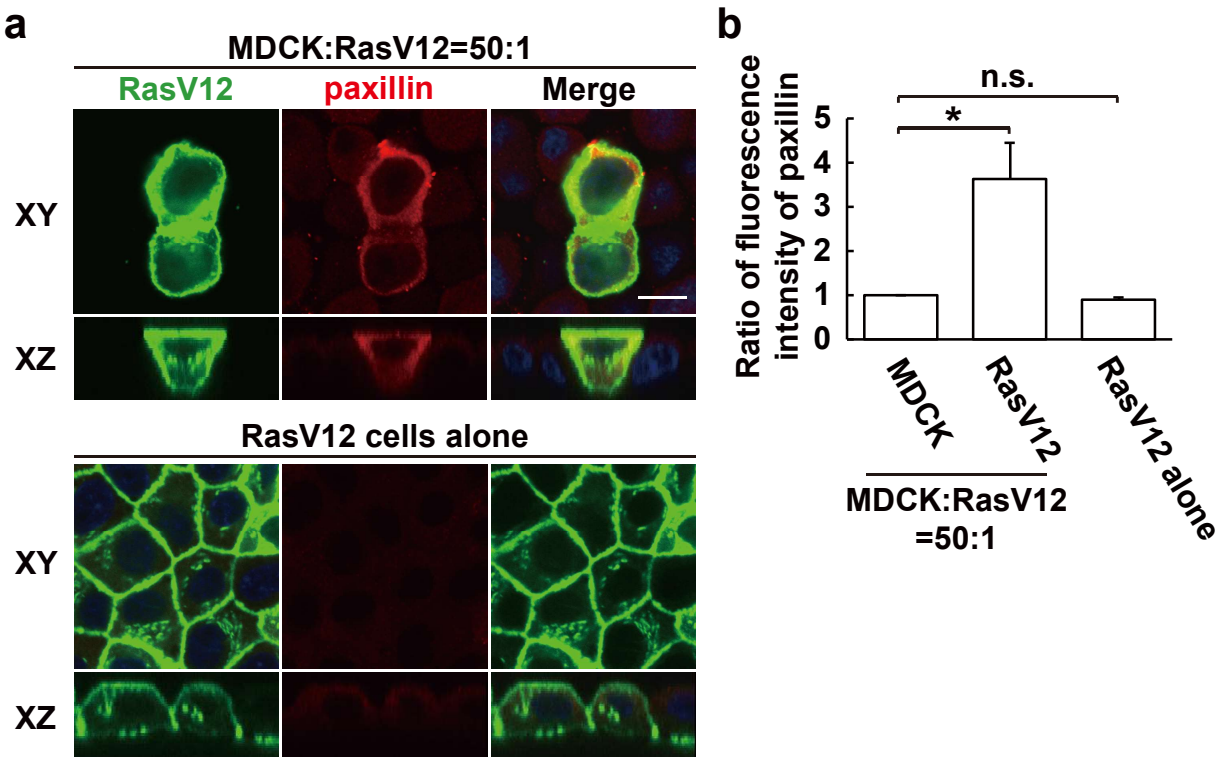

Figure 2-2. Paxillin is accumulated in RasV12-transformed cells that are surrounded by normal epithelial cells. (**a**) Immunofluorescence images of paxillin. MDCK-pTR GFP-RasV12 cells were mixed with normal MDCK cells or cultured alone on collagen gels. Cells were fixed after 16 h incubation with tetracycline and stained with anti-paxillin antibody (red) and Hoechst (blue). Scale bar, 10 μm. (**b**) Quantification of the fluorescence intensity of paxillin. Data are mean ± SD from three independent experiments. *P<0.05, n.s., not significant; n≥30 cells for each experimental condition. Values are expressed as a ratio relative to MDCK cells.

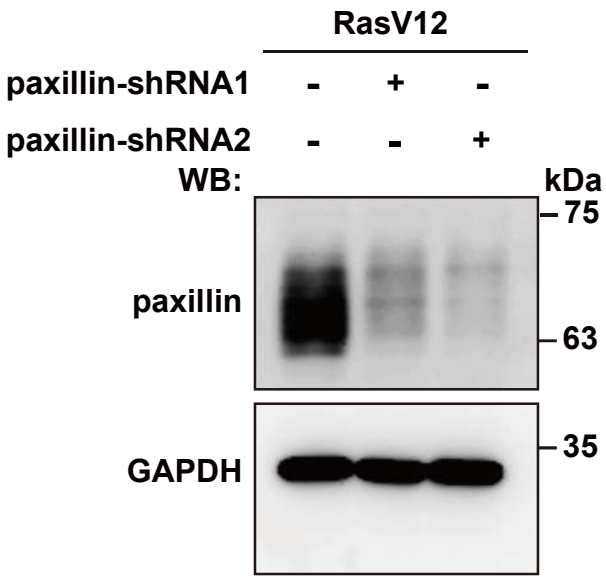

Figure 2-3 Establishment of Paxillin-knockdown RasV12-transformed cells. Expression of GFP-RasV12 was induced with tetracycline treatment, followed by western blotting with the indicated antibodies.

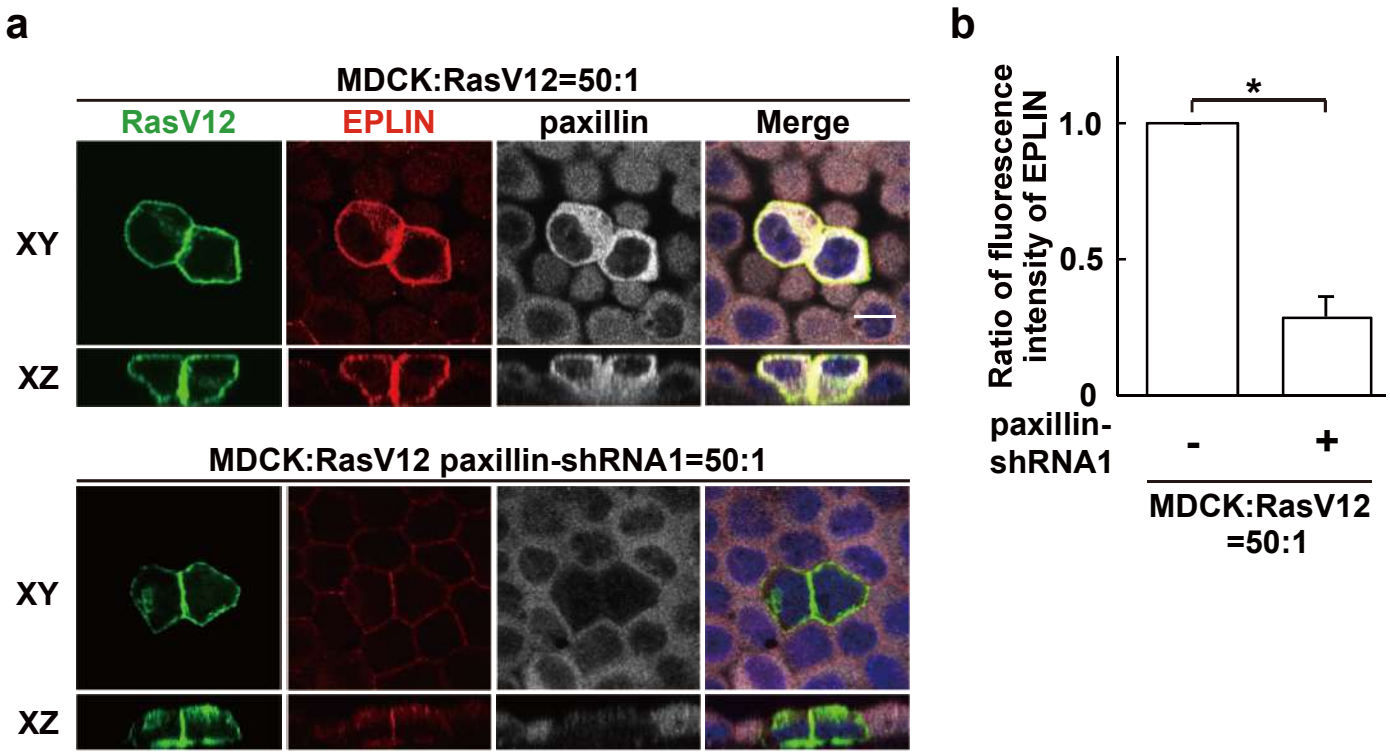

Figure 2-4. Paxillin regulates accumulation of EPLIN in RasV12-transformed cells that are surrounded by normal epithelial cells. (**a**, **b**) Effect of paxillin-knockdown on EPLIN accumulation. MDCK-pTR GFP-RasV12 cells or MDCK-pTR GFP-RasV12 paxillin-shRNA1 cells were mixed with normal MDCK cells on collagen gels. Cells were fixed after 16 h incubation with tetracycline and stained with anti-EPLIN (Red), anti-paxillin (white) antibodies and Hoechst (blue). Scale bars, 10 μm. (**b**) Quantification of (**a**). Data are mean ± SD from three independent experiments. (**b**) *P<0.005; n≥30 cells for each experimental condition. Values are expressed as a ratio relative to paxillin-shRNA1 (-).

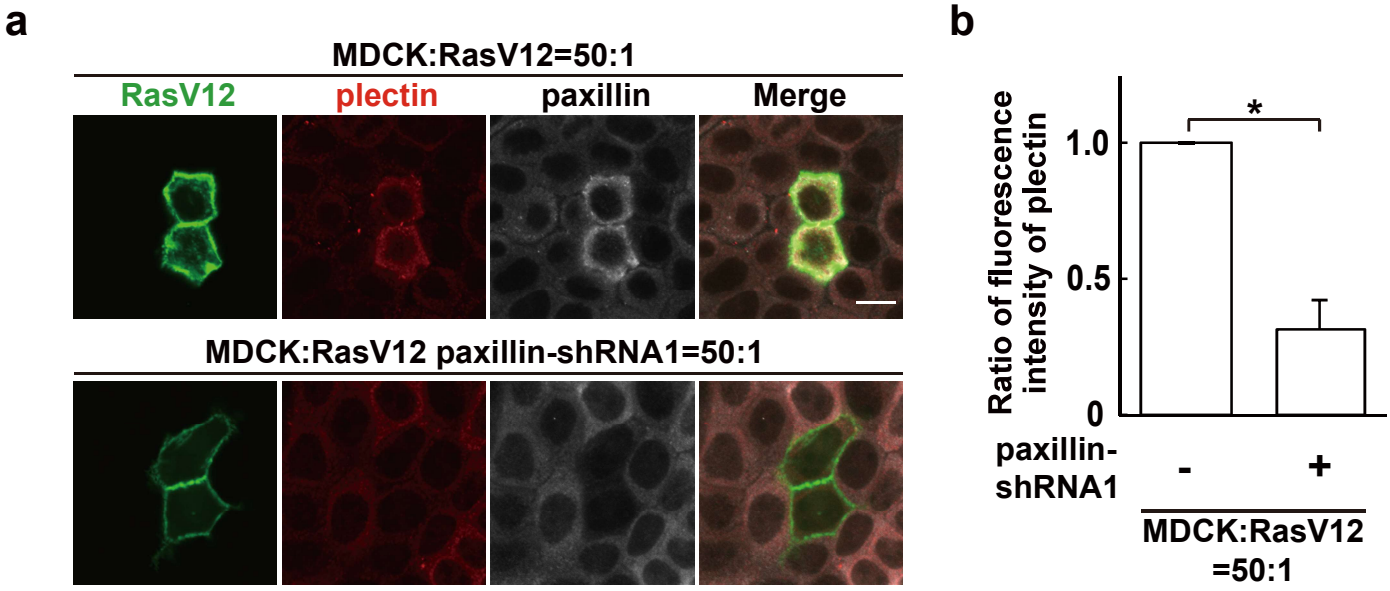

Figure 2-5. Paxillin regulates accumulation of plectin in RasV12-transformed cells that are surrounded by normal epithelial cells. (**a**, **b**) Effect of paxillin-knockdown on plectin accumulation. MDCK-pTR GFP-RasV12 cells or MDCK-pTR GFP-RasV12 paxillin-shRNA1 cells were mixed with normal MDCK cells on collagen gels. Cells were fixed after 16 h incubation with tetracycline and stained with anti-plectin (red) and anti-paxillin (white) antibodies and Hoechst (blue). Scale bars, 10 μm. (**b**) Quantification of (a). Data are mean ± SD from three independent experiments. (**b**) *P<0.001; n≥30 cells for each experimental condition. Values are expressed as a ratio relative to paxillin-shRNA1 (-).

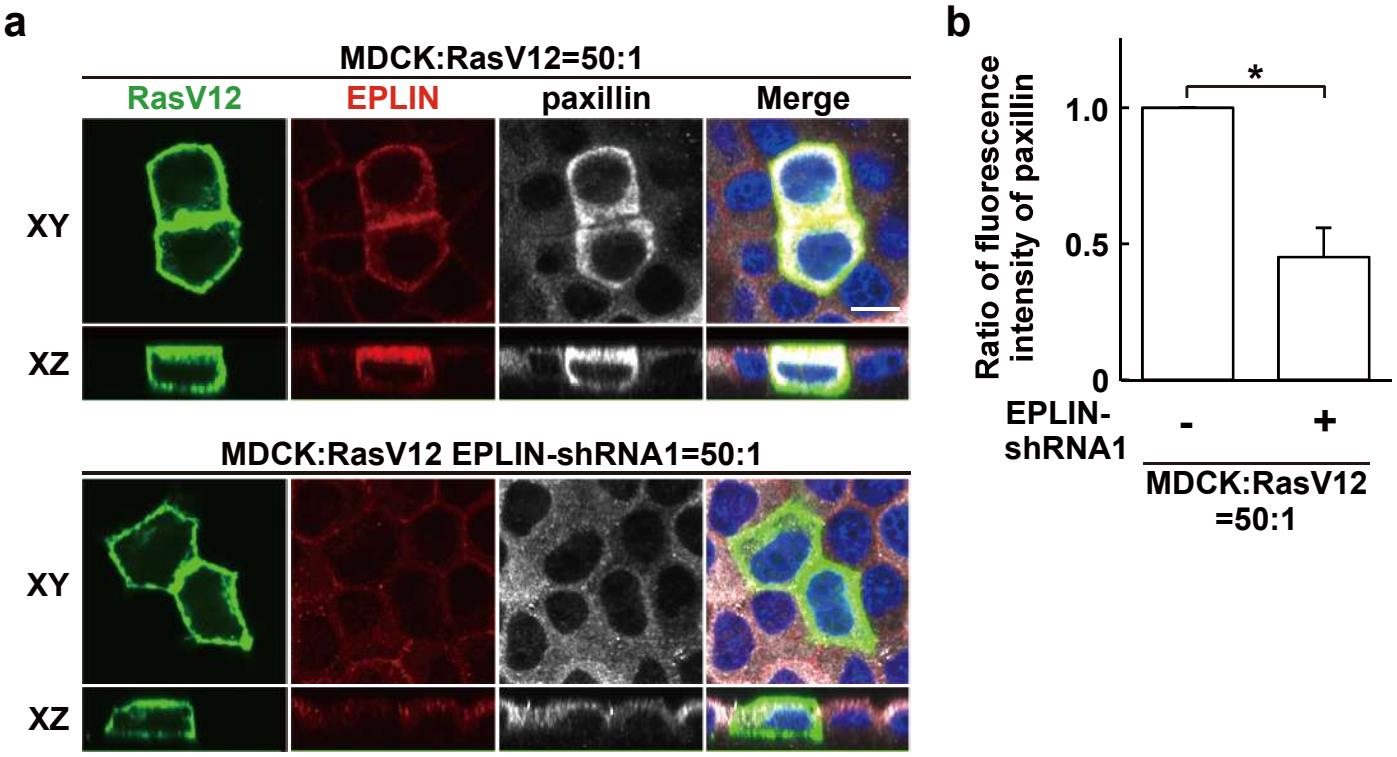

Figure 2-6. EPLIN regulates paxillin accumulation in RasV12-transformed cells surrounded by normal epithelial cells.

(a,b) Effect of EPLIN-knockdown on paxillin accumulation. MDCK-pTR GFP-RasV12 cells, MDCK-pTR GFP-RasV12 EPLIN-shRNA1 cells or MDCK-pTR GFP-RasV12 plectin-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 (a), and anti-paxillin (white) antibodies and Hoechst (blue). Scale bars, 10 μm. (b) Quantification of (a). Data are mean ± SD from three independent experiments. (b) *P<0.05, n≥28 cells for each experimental condition. Values are expressed as a ratio relative to EPLIN-shRNA1 (-).

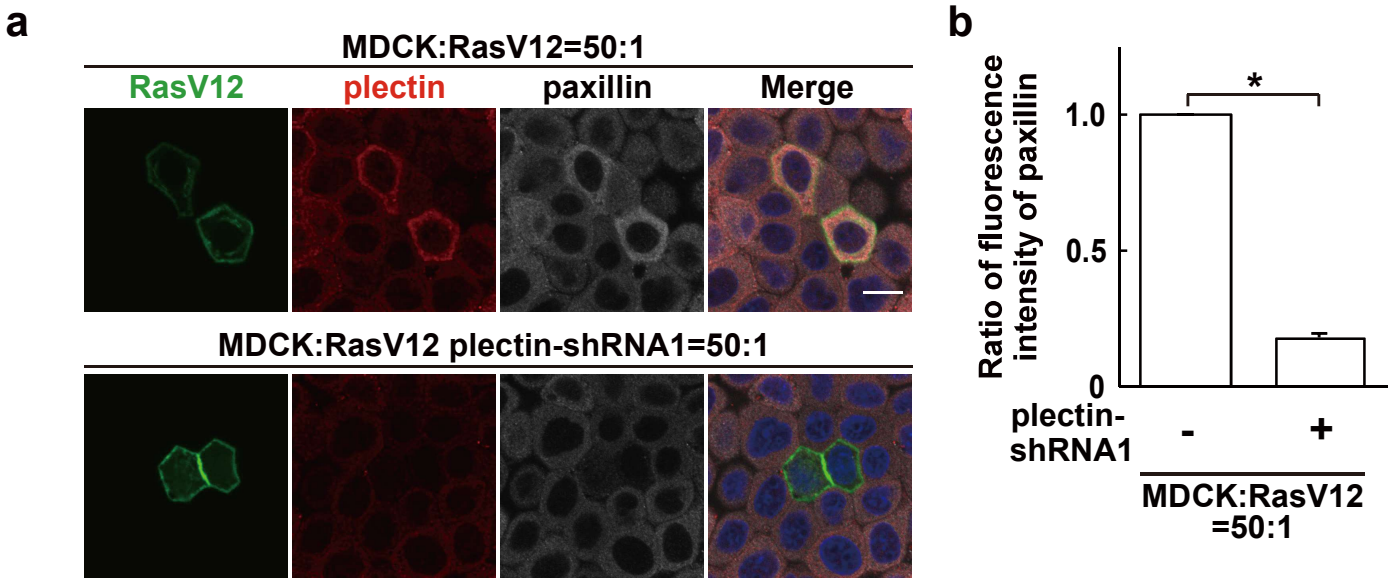

Figure 2-7. Plectin regulates paxillin accumulation in RasV12-transformed cells surrounded by normal epithelial cells. (a,b) Effect of plectin-knockdown on paxillin accumulation. MDCK-pTR GFP-RasV12 cells, MDCK-pTR GFP-RasV12 plectin-shRNA1 cells were mixed with normal MDCK cells on collagen gels. Cells were fixed after 16 h incubation with tetracycline and stained with anti-plectin (red) and anti-paxillin (white) antibodies and Hoechst (blue). Scale bars, 10 μm. (b) Quantification of (a). Data are mean ± SD from three independent experiments. (b)*P<0.0005; n≥28 cells for each experimental condition. Values are expressed as a ratio relative to plectin-shRNA1 (-).

Collectively, these results indicate that paxillin, plectin and EPLIN mutually influence their non-cell-autonomous accumulation in RasV12 cells. We next examined whether knockdown of paxillin affects the fate of RasV12-transformed cells upon cell competition with the surrounding normal cells. Knockdown of paxillin strongly suppressed apical extrusion of RasV12 cells, and most of paxillin-knockdown RasV12 cells remained within the epithelium (Fig. 2-8a, b), indicating that paxillin is a crucial regulator for the elimination of the transformed cells.

2.4.2. Acetylation of tubulin is enhanced in RasV12-transformed cells surrounded by normal cells.

In a previous study, we have reported that α-tubulin, a major component of microtubules, accumulates at the apical domain of RasV12-transformed cells that are surrounded by normal cells[14]. Plectin and EPLIN regulate the accumulation of α-tubulin, but the molecular linkage between the plectin-EPLIN complex and tubulin accumulation remains unclear. The organization of microtubule filaments is often regulated by tubulin acetylation[16,17]. We thus examined acetylation of α-tubulin by immunofluorescence. In RasV12-transformed cells, accumulation of acetylated tubulin was mainly

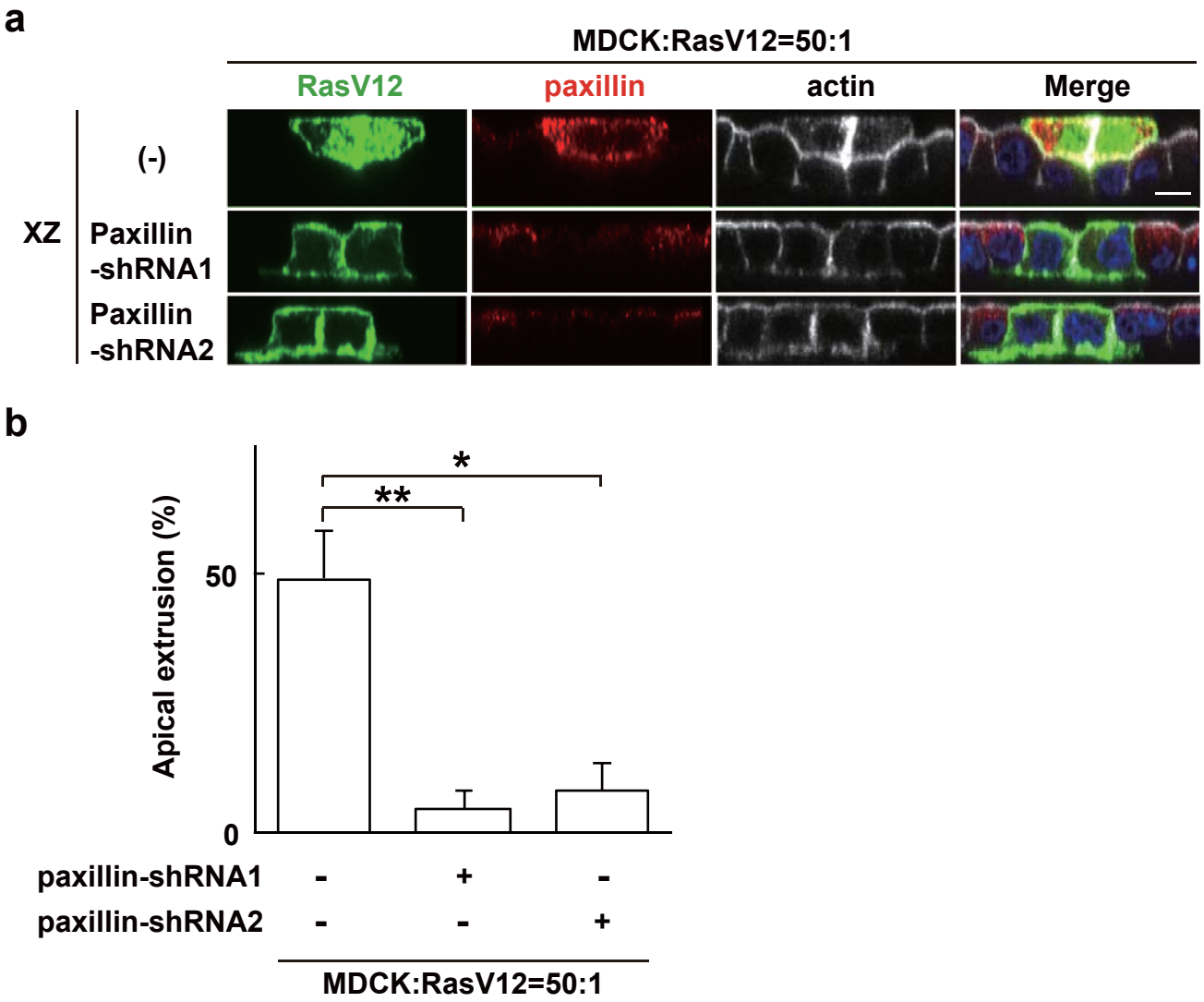

Figure 2-8. Paxillin plays a positive role in apical extrusion of RasV12-transformed cells. (a) XZ images of RasV12 cells or paxillin-knockdown RasV12 cells that were surrounded by normal epithelial cells. MDCK-pTR GFP-RasV12 cells, MDCK-pTR GFP-RasV12 paxillin-shRNA1 or -shRNA2 cells were mixed with normal MDCK cells. Cells were fixed after 24 h incubation with tetracycline and stained with anti-paxillin antibody (red), Alexa-Fluor-647-phalloidin (white) and Hoechst (blue). Scale bar, 10 μm. (b) Quantification of the effect of paxillin-knockdown on apical extrusion of RasV12 cells. Data are mean ± SD from four independent experiments. *P<0.005 and **P<0.001; n=95-135 cells for each experimental condition.

observed in the apical region, which overlapped with that of tubulin (Fig. 2-9). Moreover, acetylation of α-tubulin was substantially elevated when RasV12 cells were surrounded by normal cells, compared with that in RasV12 cells cultured alone (Fig. 2-9), indicating the non-cell-autonomous upregulation of tubulin acetylation in RasV12 cells. Acetylation of α-tubulin can be regulated by deacetylases: HDAC6 and SIRT2[14]. We then examined the effect of the inhibitor for HDAC6 (tubacin) or SIRT2 (AGK2). Acetylation of α-tubulin in RasV12 cells was strongly enhanced by tubacin, but not by AGK2 (Fig. 2-10a). Furthermore, treatment of tubacin, but not AGK2, significantly promoted apical extrusion of RasV12 cells (Fig. 2-10b) suggesting that acetylation of tubulin in RasV12 cells may be regulated by HDAC6, which plays a positive role in the elimination of transformed cells from epithelia.

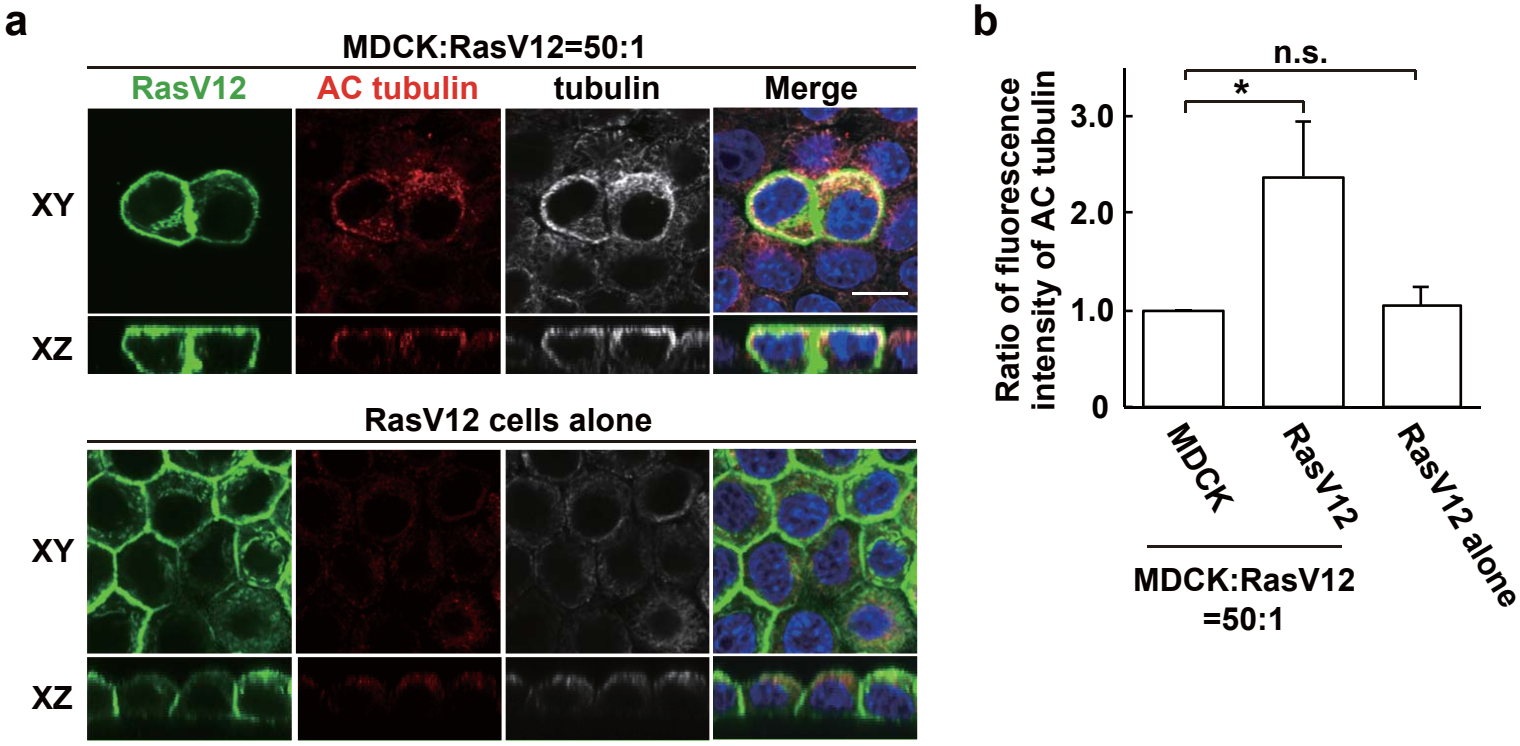

Figure 2-9. Accumulated tubulin is acetylated in RasV12-transformed cells surrounded by normal cells. (a) Immunofluorescence images of acetylated tubulin. (a) MDCK-pTR GFP-RasV12 cells were mix-cultured with normal MDCK cells or cultured alone on collagen gels in the presence or absence of tubacin or AGK2. Cells were fixed after 16 h incubation with tetracycline and stained with anti-acetylated tubulin antibody (red) with anti-tubulin antibody (white) and Hoechst (blue). Scale bars, 10 μm. (b) Quantification of fluorescence intensity of acetylated tubulin. Data are mean ± SD from three independent experiments. *P<0.05; n≥30 cells for each experimental condition. n.s., not significant.

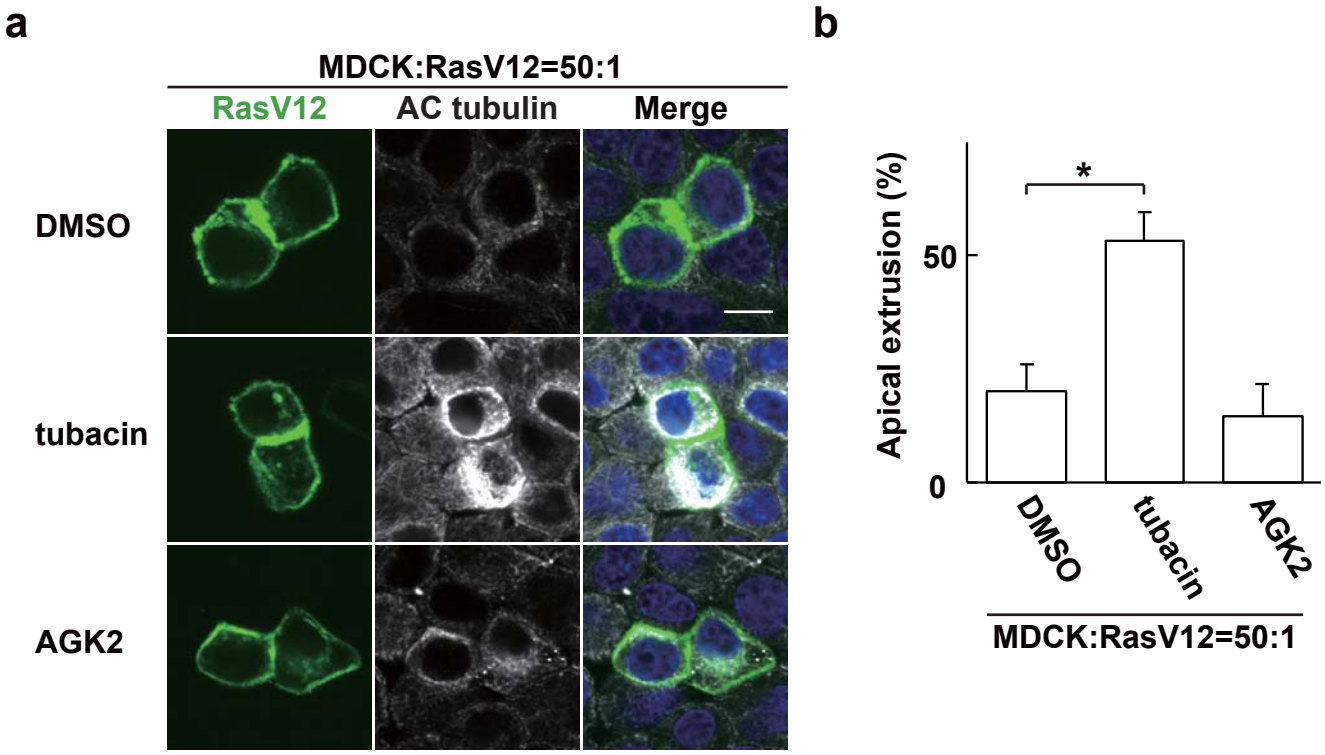

Figure 2-10. HDAC6-modulated tubulin acetylation regulates apical extrusion of RasV12-transformed cells. (a) Effect of tubacin on tubulin acetylation in RasV12 cells. (a) MDCK-pTR GFP-RasV12 cells were mix-cultured with normal MDCK cells or cultured alone on collagen gels in the presence or absence of tubacin or AGK2. Cells were fixed after 16 h incubation with tetracycline and stained with anti-acetylated tubulin antibody (white) and Hoechst (blue). Scale bars, 10 μm. (b) Quantification of the effect of tubacin or AGK2 on apical extrusion of RasV12-transformed cells. Data are mean ± SD from three (DMSO, tubacin) or two (AGK2) independent experiments. *P<0.01; n=101-176 cells for each experimental condition.

2.4.3. Paxillin regulates tubulin acetylation thereby promoting apical extrusion of RasV12-transformed cells.

A previous study demonstrated that paxillin positively regulates acetylation of α-tubulin by suppressing HDAC6[25]. We found that paxillin was partially colocalized with acetylated α-tubulin in RasV12 cells that were surrounded by normal cells (Fig. 2-11a). In addition, knockdown of paxillin profoundly diminished the accumulation of acetylated tubulin (Fig. 2-11b), demonstrating that paxillin is a crucial upstream regulator of tubulin acetylation upon cell competition between normal and RasV12 cells. Knockdown of EPLIN or plectin also significantly suppressed the accumulation of acetylated tubulin. Furthermore, tubacin restored accumulation of acetylated tubulin in paxillin-knockdown cells (Fig. 2-12a), suggesting that paxillin regulates tubulin acetylation by suppressing HDAC6. Moreover, tubacin partially rescued the inhibitory effect of paxillin-knockdown on the apical extrusion of RasV12 cells (Fig. 2-12b). Collectively, these data indicate that HDAC6-regulated tubulin acetylation is one of the downstream effectors of the paxillin-plectin-EPLIN complex in the apical elimination of transformed cells (Fig. 2-13).

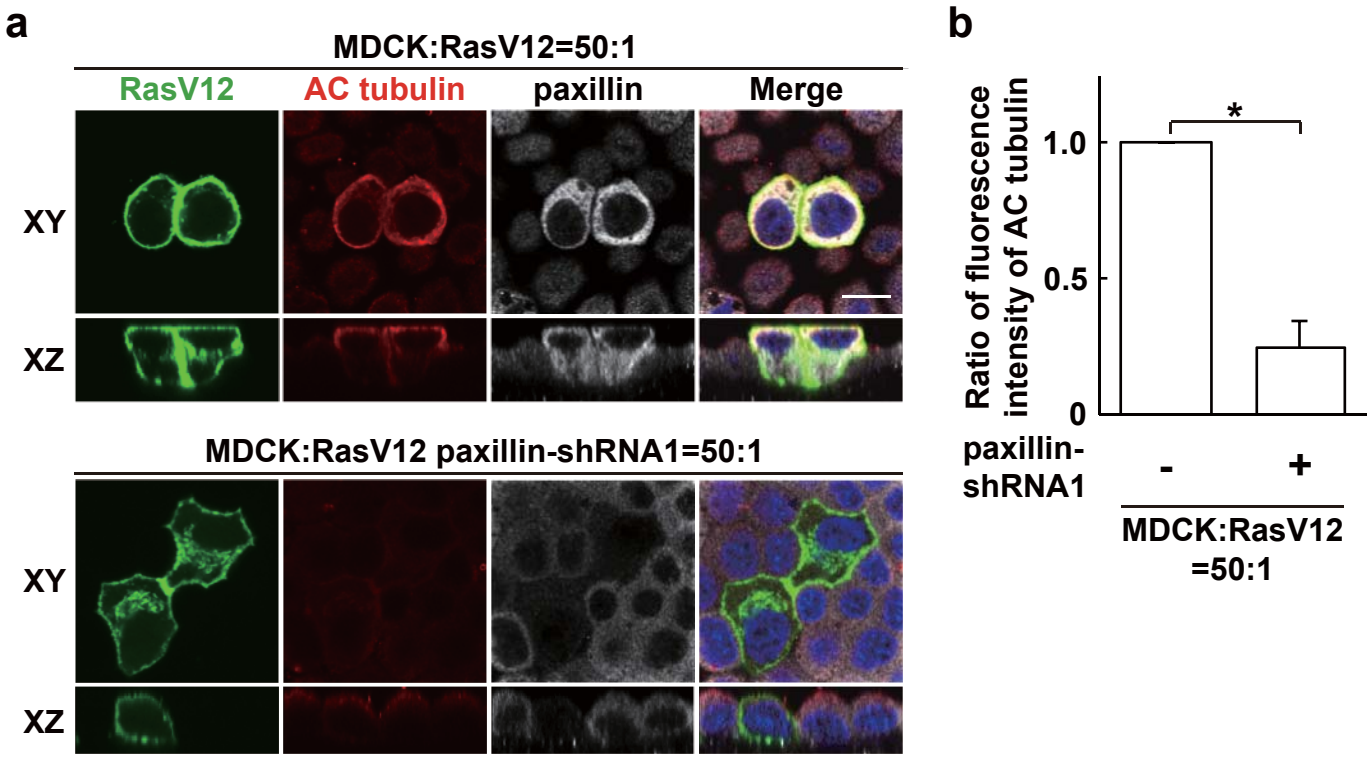

Figure 2-11. Paxillin regulates accumulation of acetylated tubulin in RasV12-transformed cells surrounded by normal cells. (a) Effect of paxillin-knockdown on accumulation of acetylated tubulin. MDCK-pTR GFP-RasV12 cells or MDCK-pTR GFP-RasV12 paxillin-shRNA1 cells were mixed with normal MDCK cells on collagen gels. Cells were fixed after 16 h incubation with tetracycline and stained with anti-acetylated tubulin (red) and anti-paxillin (white) antibodies and Hoechst (blue). Scale bar, 10 μm. (b) Quantification of (a). 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 paxillin-shRNA1 (-)

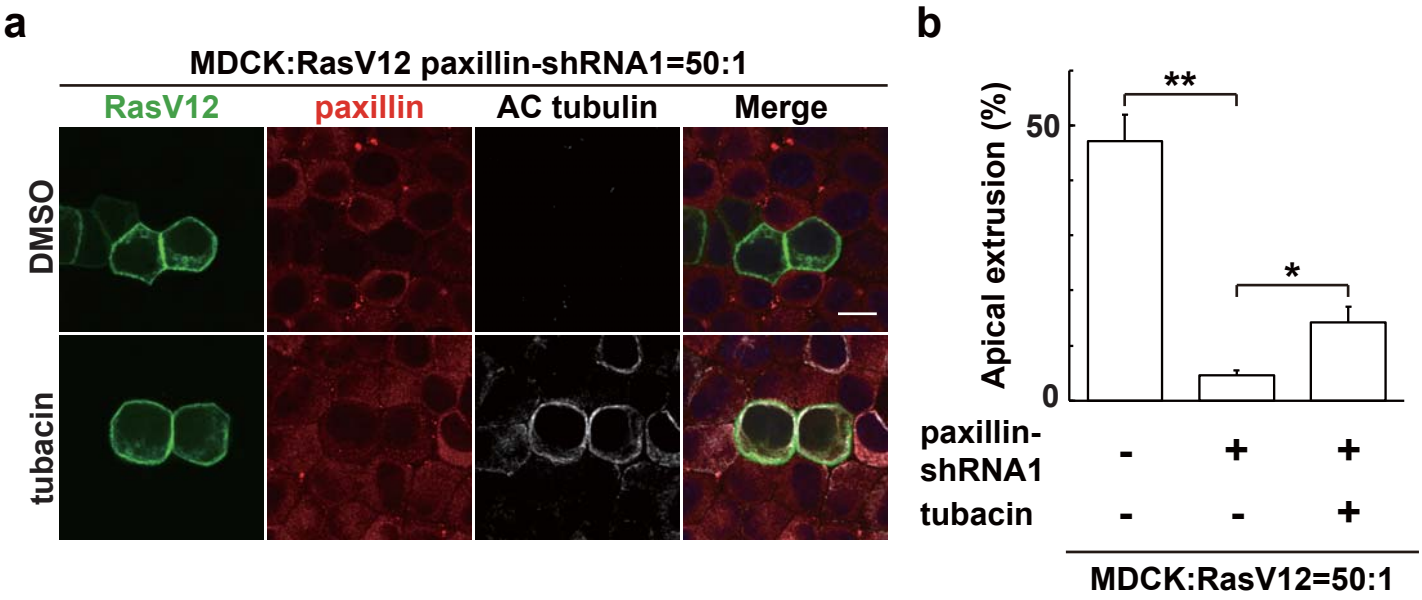

Figure 2-12. Inhibition of HDAC6 partially rescues the paxillin-knockdown phenotype. (a) Effect of tubacin on accumulation of acetylated tubulin in paxillin-knockdown RasV12-transformed cells. MDCK-pTR GFP-RasV12 paxillin-shRNA1 cells were mixed with normal MDCK cells on collagen gels. Cells were fixed after 24 h incubation with tetracycline in the presence or absence of tubacin and stained with anti-paxillin (red) and anti-acetylated tubulin (white) antibodies and Hoechst (blue). Scale bar, 10 μm. (b) Quantification of the effect of tubacin on apical extrusion of RasV12 paxillin-shRNA1 cells. Data are mean ± SD from three independent experiments. *P<0.05 and **P<0.005; n≥100 cells for each experimental condition.

2.5. Discussion

In this chapter, I have demonstrated that paxillin is a novel regulator for the elimination of RasV12-transformed cells from the epithelium. Paxillin regulates the accumulation of other regulators plectin and EPLIN, and *vice versa*. In addition, paxillin, in concert with plectin and EPLIN, induces acetylation of α-tubulin, leading to reorganization of microtubule filaments, at least partly, via HDAC6 (Fig. 2-13). Plectin and paxillin bind to microtubules and/or HDAC6; thus the paxillin-plectin-EPLIN complex could act as a scaffolding-platform that efficiently induces the HDAC6-mediated acetylation of tubulin. However, suppression of the HDAC6 activity only partially rescued the inhibitory effect of paxillin-knockdown on the apical extrusion of RasV12 cells, suggesting that other molecules may also function downstream of the paxillin-plectin-EPLIN complex.

It still remains unclear how the accumulation of the paxillin-plectin-EPLIN complex is regulated and how tubulin acetylation positively regulates apical extrusion of transformed cells. We have previously reported that at the boundary between normal and transformed epithelial cells, various non-cell autonomous changes occur in both cells. For example, Rab5-mediated endocytosis is enhanced in RasV12 cells when they are surrounded by normal cells[28]. In

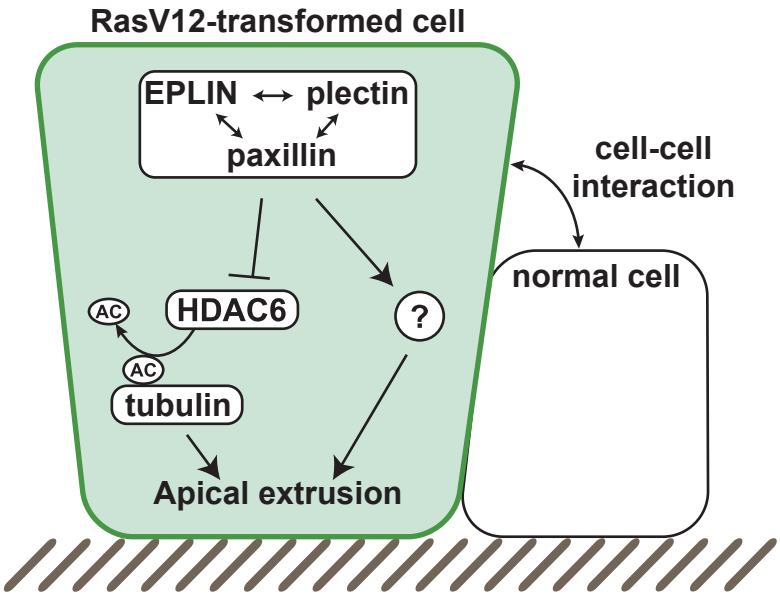

Figure 2-13. The schematic of non-cell-autonomous changes in RasV12-transformed cells neighboring normal epithelial cells.

addition, Warburg effect-like metabolic changes, increased glycolysis and decreased mitochondrial activity, occur in RasV12 cells neighboring normal cells[29]. Furthermore, cytoskeletal proteins filamin and vimentin accumulate in normal cells at the interface with transformed cells, providing physical forces for apical extrusion[12]. In future studies, it needs to be elucidated whether and how these processes function upstream or downstream of the paxillin-plectin-EPLIN complex.

2.6. References

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3.SILAC Screening

3.1. Abstract

In the previous chapter, I demonstrated that the paxillin-plectin-EPLIN complex is involved in apical extrusion through HDAC6-mediated acetylation. However, the upstream and downstream molecules are still unknown. To identify the up/down stream molecules, using SILAC screening which is a powerful proteomics method, I searched for novel proteins related to cell competition. In the screening, I have identified IVNS1ABP as a positive regulator for the apical extrusion in this section.

3.2. Introduction

In the previous chapter, I showed the relationship between some molecules (paxillin, HDAC6, and tubulin) and cell competition. However, the mechanism of cell competition is still unclear. In this chapter, to identify new molecules which involved in cell competition, we performed SILAC screening. As a result, Influenza virus NS1A binding protein (IVNS1ABP) was identified as one of molecules which is phosphorylated and interacted with EPLIN under the mix culture condition. Therefore, I investigated whether this protein affected apical extrusion of RasV12 cells surrounded by normal cells.

3.3. Experimental procedures

3.3.1. Antibodies and Materials.

Mouse anti-EPLIN (sc-136399) antibodies was from Santa Cruz Biotechnology. Rabbit IVNS1ABP (ab101278) was from Abcam. Mouse anti-GAPDH (Clone 6C5) antibody was from Millipore. Rabbit polyclonal affinity-purified anti-phospho(S338)-IVNS1ABP antibody was generated using the following peptide as an antigen: SLSFEMQPDELIEKPMSPMQYA (Ab mart Co., Ltd). 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 described above were diluted in phosphate-buffered saline (PBS) containing 1% BSA at 1:100 and EPLIN antibodies 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 anti-α-GAPDH antibody at 1:2,000, and secondary antibodies were used at 1:1,000.

3.3.2. Cell Culture.

MDCK and MDCK-pTR GFP-RasV12 cells were cultured as previously described[1]. MDCK-pTR GFP-RasV12 cells stably expressing EPLIN-shRNA [2,3]. MDCK-pTR GFP-RasV12 cells stably expressing IVNS1ABP-shRNA were established as follows:

Double-stranded DNA fragments coding IVNS1ABP-shRNA sequences

(IVNS1ABP-shRNA1:

5’-GATCCCCGCTGCTTGATGGGAACCTATTCAAGAGATAGGTTCCCATCAAG

CAGCTTTTTC -3’ and

5’-TCGAGAAAAAGCTGCTTGATGGGAACCTATCTCTTGAATAGGTTTCCCAT

CAAGCAGCGGG-3

or IVNS1ABP-shRNA2:

5’-GATCCCGGTGGTGGCTTTGATGGTTTTCAAGAGAAACCATCAAAGCCAC

CACCTTTTTC -3’ and

5’-TCGAGAAAAAGGTGGTGGCTTTGATGGTTTCTCTTGAAAACCATCAAAGC

CACCACCGGG-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

IVNS1ABP-shRNA1 or -shRNA2 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, the tetracycline-inducible MDCK-pTR GFP-RasV12 cell lines were treated with 2 μg ml^{-1} tetracycline (Sigma-Aldrich). For immunofluorescence, cells were seeded onto Type-I collagen-mounted coverslips as described below in the section of immunofluorescence.

3.3.3. Immunofluorescence.

MDCK-pTR GFP-RasV12 cells were mixed with MDCK cells at a ratio of 1:50 and cultured on the collagen matrix as previously described[1]. 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 antibodies were incubated for 2 h or 1 h, respectively at ambient temperature. Immunofluorescence images were acquired by the Olympus FV1200 system

and the Olympus FV10-ASW software. For quantification of immunofluorescence intensity, 30 transformed cells were analyzed for each experiment using the MetaMorph software (Molecular Devices). For analyses of apical extrusions, the samples were prepared as described above, except that cells were treated with tetracycline for 24 h. More than 100 cells were analyzed for each experiment, and apically extruded cells were quantified.

3.3.4. Immunoprecipitation and western blotting.

For immunoprecipitation, MDCK and MDCK-pTR GFP-RasV12 cells were seeded at the density of 1.6 x 10^7 cells in 14.5-cm dishes (two dishes for each experimental condition) (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 RIPA lysis buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl and 1% NP-40, 0.5% deoxycholate, 0.1% SDS) containing the following inhibitors: 1 mM Na_3VO_4, 0.1 mM Na_2MoO_4, 10 mM NaF, 5 μg ml^{-1} leupeptin, 1 mM phenylmethylsulfonyl fluoride and 7.2 trypsin inhibitor units of aprotinin. After centrifugation of the cell lysates at 21,500 g for

10 min, the supernatant was incubated with control IgG-conjugated Dynabeads protein G (Life Technologies) at 4˚C for 30 min and finally subjected to immunoprecipitation for 1 h with Dynabeads Protein G or A conjugated to rabbit anti-IVNS1ABP antibody (10 μg) or mouse anti-EPLIN antibody (10 μg). Immunoprecipitated proteins were subjected to SDS-PAGE, followed by western blotting with the indicated antibodies. Western blotting data were acquired using ImageQuant^{TM} LAS4010 (GE healthcare). To examine the efficiency of IVNS1ABP-knockdown, MDCK-pTR GFP-RasV12 cells stably expressing IVNS1ABP-shRNA were seeded onto 6-cm dishes (Greiner-Bio-One) at the density of 1 x 10^6 cells. After 24 h, 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.

3.3.5. Stable isotope labeling with amino acids in cell culture.

For SILAC labeling, cells were maintained in DMEM minus L-Lysine and L-Arginine (Cambridge Isotope Laboratories) supplemented with 10% dialyzed

FCS and 1% penicillin/streptomycin (Gibco). 50 μg ml^{-1} of U-^{13}C6-^{15}N2 -L-lysine (Lys8) and 50 μg ml^{-1} of U-^{13}C6-^{15}N4-L-arginine (Arg10) were added for "heavy" labeling, 50 μg ml^{-1} of U-^{2}H4-L-lysine (Lys4) and 50 μg ml^{-1} of U-^{13}C6-L-arginine (Arg6) were added for "medium" labeling, and 50 μg ml^{-1} of lysine-0 (unlabeled) and 50 μg ml^{-1} of arginine-0 (unlabeled) for "light" labeling. In addition, 200 μg ml^{-1} of L-proline (Sigma-Aldrich) was added to the culture medium to prevent arginine to proline conversion. In phospho-SILAC screening, after two weeks of SILAC labeling, 1.0 x 10^7 heavy-labeled MDCK cells and 1.0 x 10^7 heavy-labeled MDCK-pTR GFP-RasV12 cells were mixed and co-cultured in a 145-mm dish. On the other hand, medium-labeled MDCK or MDCK-pTR GFP-RasV12 cells were separately cultured in a 145-mm dish. The SILAC labeled cells were cultured without tetracycline for 8-12 h, and then treated with 2 μg ml^{-1} tetracycline for 6 or 10 h. After culture with tetracycline, the cells were collected and analyzed by LC-MS/MS. In SILAC screening for identifying EPLIN interacting proteins, After two weeks of SILAC labeling, 1.0 x 10^7 light-labeled MDCK cells and 1.0 x 10^7 medium-labeled MDCK-pTR GFP-RasV12 cells expressing EPLIN-flag were mixed and co-cultured in a 145-mm dish. On the other hand, medium-labeled MDCK-pTR GFP-RasV12 cells and e MDCK-pTR

GFP-RasV12 cells expressing EPLIN-flag were mixed and cocultured in a 145-mm dish. The SILAC labeled cells were cultured without tetracycline for 8-12 h, and then treated with 2 μg ml^{-1} tetracycline for 16 h. After culture with tetracycline, the cells were collected and analyzed by LC-MS/MS.

3.3.6. LC-MS/MS analysis.

For SILAC quantification, medium/heavy ratio (M:H) was calculated by Proteome Discoverer 1.4, and proteins that contain more than 5 SILAC peptides were listed.

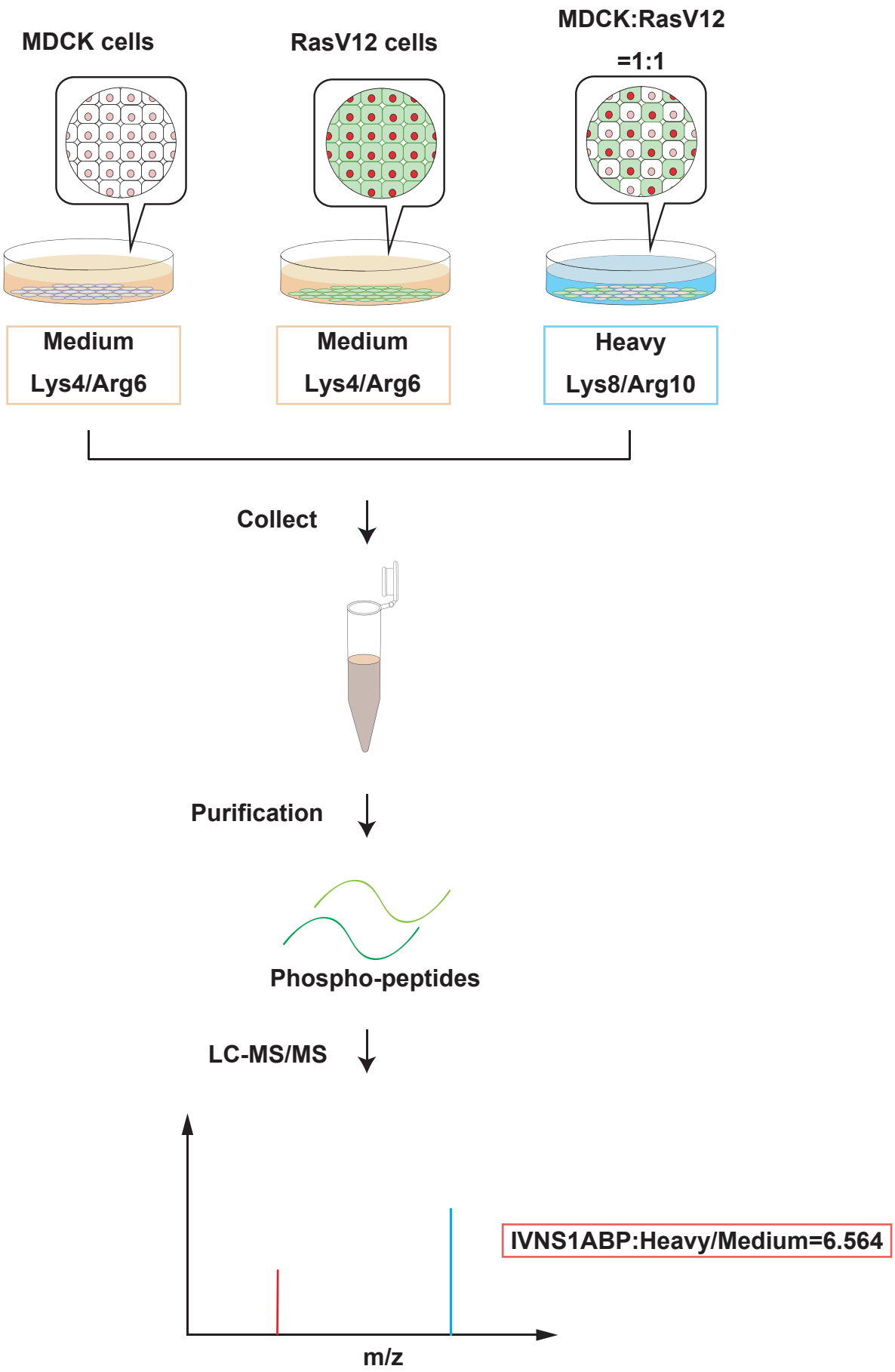
3.3.7. Data Analyses.

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

3.4. Results

3.4.1. Phosphorylation of IVNS1ABP at Ser338 is increased in mix culture condition.

In the phospho-SILAC screening, we found that the phosphorylation level of influenza virus NS-1 binding protein (IVNS1ABP) at serine 338 was upregulated (Fig. 3-1). To validate this upregulation of phosphorylation, I generated p-IVNS1ABP (S338) antibody. Using this antibody, I examined the phosphorylation level of IVNS1ABP and, confirmed this phosphorylation was enhanced under the mix culture condition(Fig. 3-2). Moreover, in SILIC screening of EPLIN interacting proteins. IVNS1ABP was co-immunoprecipitated with EPLIN which is a crucial regulator of apical extrusion [3], and the interaction was enhanced under the mix culture condition (Fig. 3-3). IVNS1ABP has Kelch repeats (Fig. 3-4) which is known to interacts with actin cytoskeleton and regulates the dynamic organization [4]. Additionally, IVNS1ABP is known to protect cells from cell death induced by actin destabilization. However, the function is almost unknown including the relevance of phosphorylation identified using our phospho-SILAC screening. Therefore, I investigated whether IVNS1ABP is involved in cell competition.



Firure 3-1. Schematic representation of the SILAC screen for phosphorylated peptides. MDCK and RasV12 cells labbeled were plated at three conditions:MDCK cells alone, RasV12 cells alone, MDCK cells with RasV12 cells After 8 h tetracycline was added. Following a subsequent incubation for 6 h, cells were lysed, lysates were combined, and indicated biochemiccal steps were peformed.

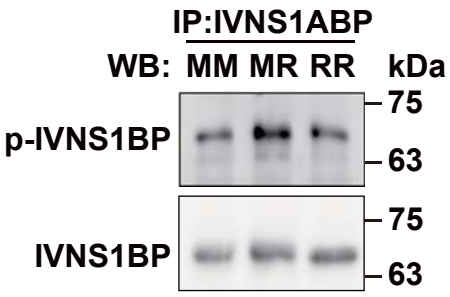
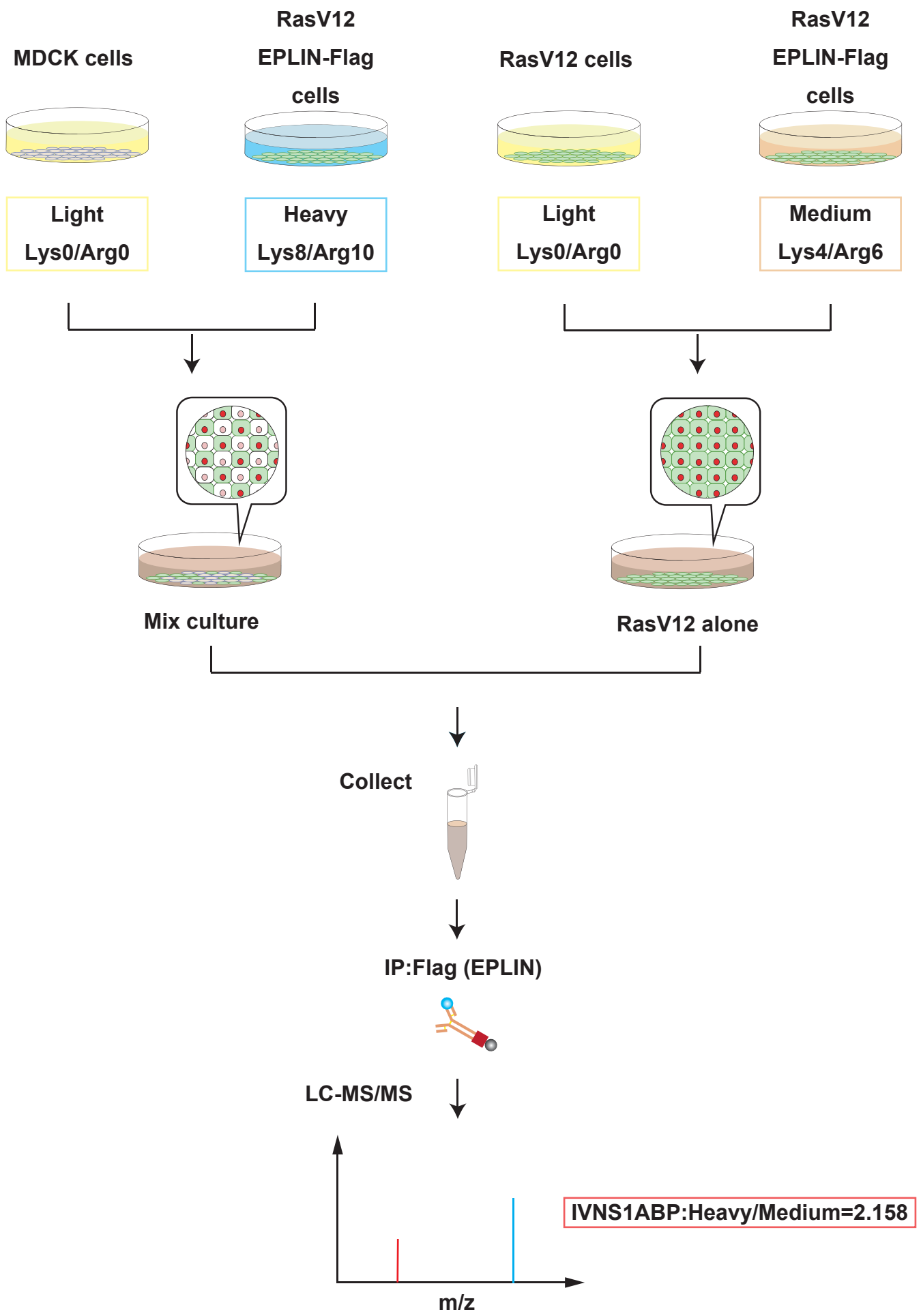
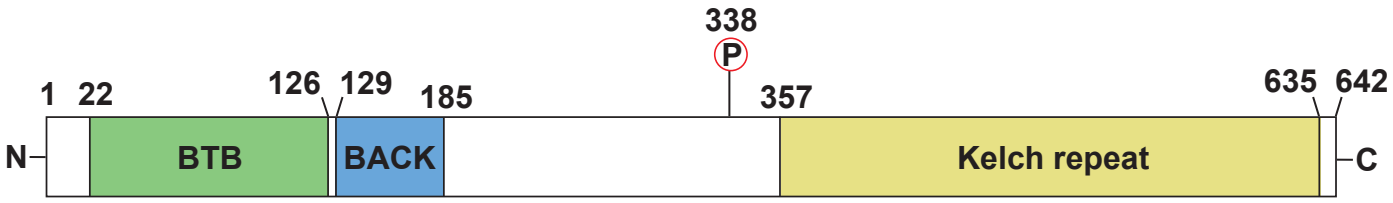


Figure 3-2 Validation of the SILAC screen.

Western blotting of phospho-IVNS1ABP. 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. Western blotting was performed with the indicated antibodies.



Firure 3-3. Schematic representation of the SILAC screen for EPLIN-interacting peptides. MDCK and RasV12 cells labbeled were plated at two conditions:RasV12 cells expessing EPLIN-Flag mixed with MDCK cells or RasV12 cells. After 8 h tetracycline was added. Following a subsequent incubation for 16 h, cells were lysed, lysates were combined, and indicated biochemiccal steps were peformed.



Firure 3-4. Domain structure of IVNS1ABP

3.4.2. IVNS1ABP positively regulates apical extrusion of RasV12 cells surrounded by normal cells.

To investigate whether IVNS1ABP is related to apical extrusion or not, I have established RasV12-transformed cell lines stably expressing IVNS1ABP-shRNA1 or -shRNA2 (Fig. 3-5). Using these cell lines, I demonstrated that knockdown of IVNS1ABP significantly suppressed apical extrusion (Fig. 3-6), indicating that IVNS1ABP positively regulates apical extrusion of RasV12 cells surrounded by normal cells. Furthermore, I investigated whether accumulation of EPLIN were affected by IVNS1ABP. Knockdown of IVNS1ABP does not affect the EPLIN accumulation in RasV12 cells surrounded by normal cells (Fig. 3-7). Taken together, these results suggest that IVNS1ABP plays a positive role in apical extrusion and may be one of downstream molecules of EPLIN.

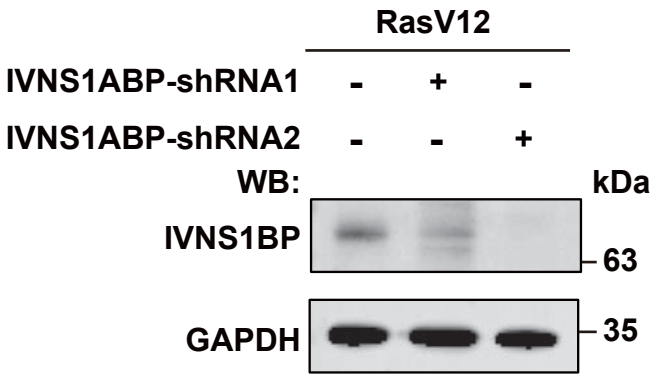


Figure 3-5 Establishment of IVNS1ABP-knockdown RasV12-transformed cells. Expression of GFP-RasV12 was induced with tetracycline treatment, followed by western blotting with the indicated antibodies.

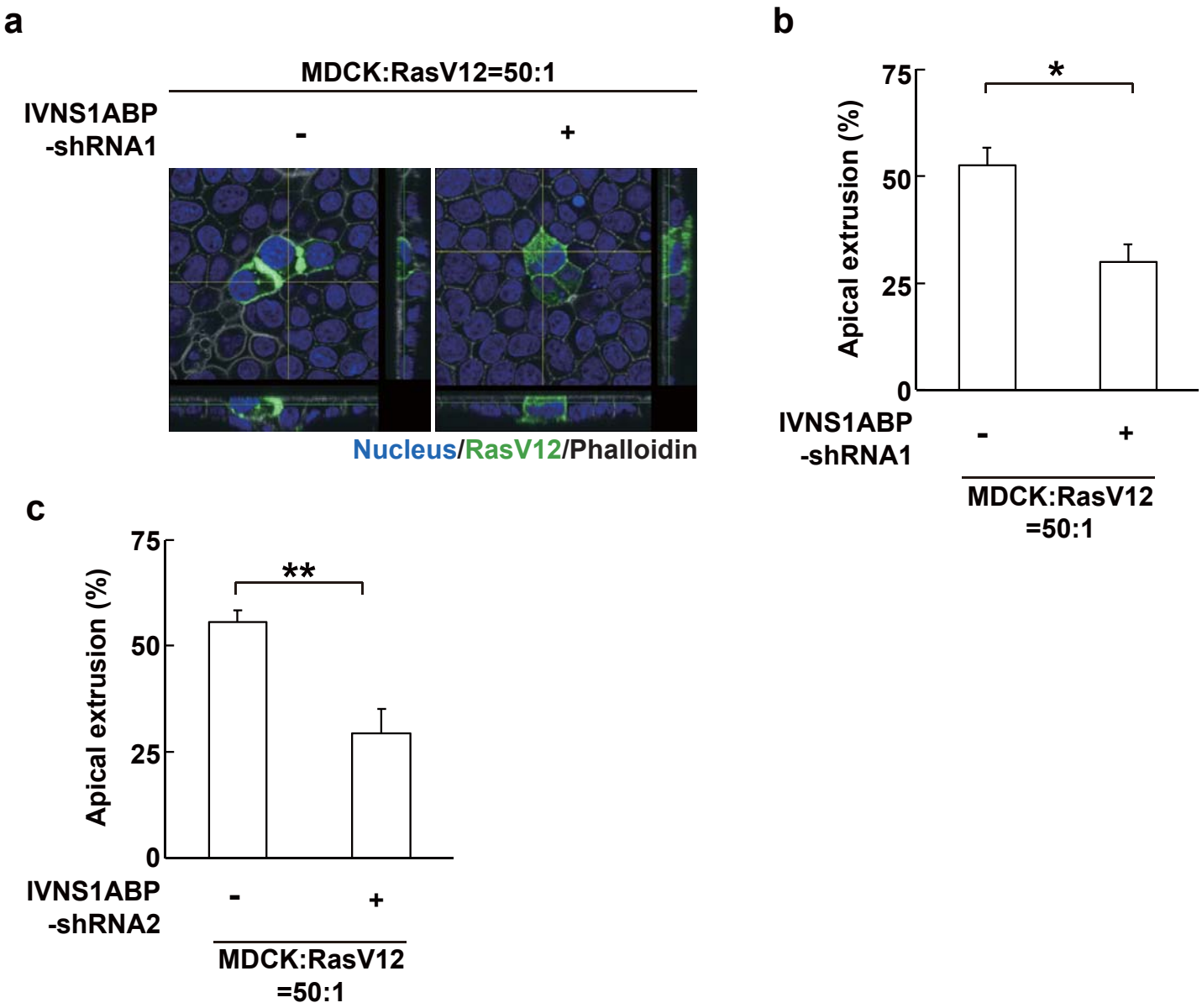


Figure 3-6. IVNS1ABP plays a positive role in apical extrusion of RasV12-transformed cells. (a) Immunofluorescent images of RasV12 cells or IVNS1ABP-knockdown RasV12 cells that were surrounded by normal epithelial cells. MDCK-pTR GFP-RasV12 cells or MDCK-pTR GFP-RasV12 IVNS1ABP-shRNA1 cells were mixed with normal MDCK cells. Cells were fixed after 24 h incubation with tetracycline and stained with Alexa-Fluor-647-phalloidin (white) and Hoechst (blue). Scale bar, 10 μm. (b) Quantification of the effect of IVNS1ABP-knockdown on apical extrusion of RasV12 cells. Data are mean ± SD from four independent experiments. **P*<0.05 and ***P*<0.01; n=100 cells for each experimental condition.

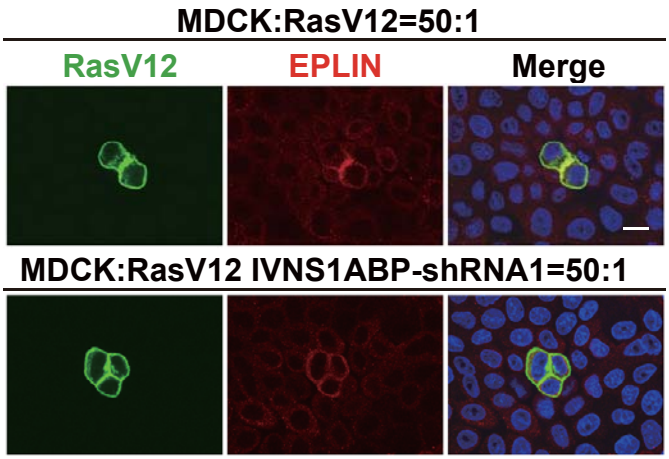


Figure 3-7. EPLIN may function upstream of IVNS1ABP.

(a,b) Effect of IVNS1ABP-knockdown on EPLIN accumulation. MDCK-pTR GFP-RasV12 cells, MDCK-pTR GFP-RasV12 IVNS1ABP-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) antibody and Hoechst (blue). Scale bars, 10 μm.

3.5. Discussion

In this section, I have demonstrated that IVNS1ABP is also a positive regulator for the apical extrusion. IVNS1ABP was phosphorylated, and the phosphorylation was enhanced under the mix culture condition. In addition, IVNS1ABP might be interact with EPLIN under the mix culture condition. However, knockdown of IVNS1ABBP does not affect the accumulation of EPLIN in RasV12 cells surrounded by normal cells. Then, IVNS1ABP may function downstream of EPLIN in RasV12 cells that is surrounded by normal cells.

In future studies, it needs to be elucidated whether and how the paxillin-plectin-EPLIN complex affect function or phosphorylation of IVNS1ABP. To address this question, it should be examined whether localization of the complex change when IVNS1ABP is knocked down. It should be also investigated whether phosphorylation of IVNS1ABP affect the IVNS1ABP localization.

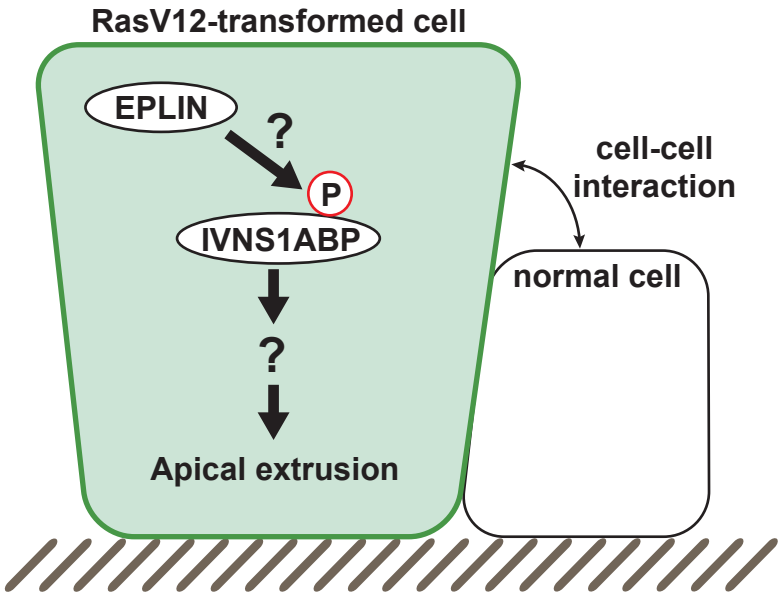


Figure 3-8. The schematic of non-cell-autonomous changes in RasV12-transformed cells neighboring normal epithelial cells.

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4. Conclusion

In chapter 2, I have demonstrated that paxillin is a novel regulator for the elimination of RasV12-transformed cells from the epithelium. However, the downstream and upstream molecules of paxillin-plectin-EPLIN complex are unknown. In chapter 3, I have demonstrated that IVNS1ABP is positive regulator of apical extrusion, and phosphorylated under mix culture condition. However, I could not investigate whether IVNS1ABP is related to cell competition through the paxillin-plectin-EPLIN complex. In the future study, it should be elucidated. Furthermore, apical extrusion of transformed cells can be observed *in vivo* as well, and the extruded transformed cells disappear from the tissues[1], implying that apical extrusion is a cancer preventive phenomenon. Therefore, the molecules governing this process could be potential therapeutic targets for cancer preventive medicine. Expression of HDAC6 is upregulated in various cancer cells[2,3]. In addition, inhibition of the HDAC6 activity can suppress tumorigenesis and diminish tumor cell migration[4,5]. Thus, HDAC6 currently attracts substantial attention as one of the potential drug targets for cancer treatment[6-8]. Our data suggest that HDAC6 inhibitor could facilitate the eradication of potentially precancerous cells at the initial stage of

carcinogenesis, implying that HDAC6 inhibitor can be applied not only to cancer treatment, but also cancer prevention. Further understanding of cytoskeletal organization machineries in apical extrusion would represent an interesting challenge for biological fields and cancer preventive medicine.

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