Study on the Effect of Sequential Oncogenic Mutations on Cell Competition

（多段階発がんにおける細胞競合の関与の研究）

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Abbreviations

EDAC	Epithelial defense against cancer
MDCK	Madin-Darby canine kidney
GFP	Green fluorescent protein
PKC	Protein kinase C
BSA	bovine serum albumin
DMSO	dimethyl sulfoxide
DMEM	Dulbecco's modified Eagle's medium
EGFP	enhanced green fluorescent protein
PCR	polymerase chain reaction
Ras	rat sarcoma
ROS	reactive oxygen species
SDS-PAGE	sodium dodecyl labeling with amino acids in cell culture
Src	Rous sarcoma oncogene
Tet	tetracycline
Dox	doxycycline
TMRM	tetramethylrhodamine methyl ester
CCCP	carbonyl cyanide m-chlorophenyl hydrazone

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1. Introduction

1.1. The Concept Behind This Study

In human, more than 80% of cancers arise from the activation of oncoproteins and/or inactivation of tumor suppressor proteins. In most cancers, transformation occurs in a single cell within an epithelial cell sheet (Figure 1-1). However, it is unknown what happens at the interface between normal and transformed cells once the initial transformation has occurred. To address this question, previously Yasuyuki Fujita group has established a mammalian cell culture system. Using this cell culture system, they demonstrated that when transformed cells arise from a monolayer of normal epithelial cells, the newly emerging transformed cells are apically eliminated. Interestingly, when transformed cells are cultured alone, they remain within epithelia, suggesting that during this process the interaction between normal and transformed cells is required. This phenomenon is called cell competition.

1.2. Oncogene Dealt with in This Study

1.2.1. Ras

Ras genes were first identified in retroviruses in 1964 (Cox and Der, 2010). The three Ras genes have been identified in humans. The Harvey sarcoma virus-associated oncogene was named *HRAS* (Harvey, 1964), the Kirsten sarcoma virus-associated

oncogene was *KRAS* (Kirsten and Mayer, 1967). Another prominent member of the *Ras* family, *NRAS* was cloned from neuroblastoma and leukemia cell lines (Hall et al., 1983; Shimizu et al., 1983).

Ras genes are the most frequently mutated oncogene family in human cancers. Missense mutations of Ras are mostly located at codons 12, 13 or 61. But the missense mutation frequency at each position varies widely between isoforms. G12 mutations are the most common in *K-Ras* and *H-Ras*, while Q61 mutations are the most common in *N-Ras*. In human cancers, *K-Ras* mutations are far more frequently observed in the three genes. Most common malignancies are lung, intestines, and pancreas (Table 1-1). *H-Ras* mutations are found in cancers arising from the skin, brain, and reproductive organs, and *N-Ras* mutations are found in cancers originating from the skin, thyroid, and blood (Table1-1).

All Ras proteins belong to small GTPase family that functions as GDP/GTP-regulated molecular switches. Ras GTPases cycle between an active and inactive GDP binding state. They are inactivated by GTPase activating protein (GAP) and activated by guanine nucleotide exchange factor (GEF) (Figure 1-2A). Ras GTPase plays an important role in the regulation of cell growth, cell differentiation, cell migration, and lipid vesicle trafficking (Figure 1-2B). Oncogenic potential of Ras is enhanced by point

mutations (as shown in 1.2.1.1), which not only impair the intrinsic GTPase activity but also render Ras insensitive to the GAP action, resulting in the constitutive activation of the downstream effectors (Figure 1-2B).

1.2.2. Scribble

Scribble is a neoplastic tumor suppressor gene that was identified in *Drosophila*. In epithelia of *scrib* homozygous mutant larvae, apicobasal cell polarity and proliferative control are lost, leading to multilayered amorphous tumor formation (Bilder et al., 2000). Scribble is a LAP (leucine-rich repeats and PDZ) protein that contains 16 leucine-rich repeat (LRR) and four PDZ [PSD95, Discs large and Zonula adherens-1 (ZO-1)] domains (Bilder and Perrimon, 2000) and is localized at the basolateral membrane in *Drosophila* and mammalian epithelial cells.

Alterations in Scribble expression levels are observed in a variety of tumor types including breast, cervical and prostate (Gardiol et al., 2006; Kamei et al., 2007; Navarro et al., 2005; Pearson et al., 2011; Zhan et al., 2008; Zhuo et al., 2010). Data from cell lines and primary tissue samples suggest that altered expression and/or mislocalization of cell polarity proteins play a causal role in tumorigenesis and mislocalization of Scribble in human prostate cancer correlates with poor survival (Pearson et al., 2011).

Although the molecular mechanisms behind Scribble function are not well understood, Scribble has been implicated in numerous cellular processes including proliferation, differentiation, apoptosis, stem-cell maintenance, migration and vesicle trafficking (Elsum et al., 2012; Humbert et al., 2008). Studies in *Drosophila* have demonstrated that loss of *scrib* cooperates with activated Ras to increase tumor growth and metastasis (Brumby and Richardson, 2003; Pagliarini and Xu, 2003). This cooperative relationship is conserved in mammals as Scribble loss cooperates with activated Ras in the non-transformed human breast cell line MCF10A to promote invasion, and in the prostate to accelerate tumorigenesis in mice (Dow et al., 2008; Pearson et al., 2011).

1.3. Cell Competition

1.3.1. Cell competition

The interaction between normal and transformed cells during differentiation are studied well by using *Drosophila Melanogaster*. *Drosophila Melanogaster* is a reasonable model organism because it is easy to generate transgenic and mutant flies and a lot of mutants that we want could be got from fly bank.

In the early of the 1970's, using wing discs in *Drosophila Melanogaster*, Morata and Ripoll reported an interesting cell competition phenomenon that occurs at the interface

between normal and *Minute* mutant cells (Morata and Ripoll, 1975). *Minute* is a genetic mutant that arises in ribosomal genes. Homozygous *Minute* flies (*Minute*$^{-/-}$) are lethal. Although heterozygous *Minute* (*Minute*$^{+/-}$) flies develop slowly and have smaller bristles, they are able to glow and develop to normal adult. Morata and Ripoll observed that when *Minute*$^{+/-}$ cells are surrounded by normal cells, the *Minute*$^{+/-}$ cells are eliminated by apoptosis and surrounding normal cells grow to compensate eliminated cell space. They thought that this phenomenon is a result of competition for their survival and they called this phenomenon "cell competition" (Figure 1-3). Importantly, when *Minute*$^{+/-}$ cells alone are present, cell competition does not occur. Therefore, cell competition is cell-context-dependent manner.

Myc is also an important cell competition regulator. In 2004, two groups independently demonstrated that the difference of the Myc expression level triggers cell competition and higher level of dMyc comes to be "winner" and eliminate neighboring "loser" cells with lower level of dMyc (de la Cova et al., 2004; Moreno and Basler, 2004). Myc is a well conserved transcription factor that regulates multiple downstream targets involved in cell growth and ribosome biogenesis (de la Cova and Johnston, 2006).

It has become clear what molecules regulate dMyc-driven cell competition (Amoyel and Bach, 2014). When dMyc lower expressing cells sense their low fitness, they express "loser marker". Flower is a loser marker and its loser isoforms (*fwe*^{LoseA} and *fwe*^{LoseB}) are expressed in loser cells and loser isoform expression is key process to eliminate them by apoptosis (Rhiner et al., 2010). At the downstream of cell competition, Hid expression and JNK activation also occur in winner cells and lead to loser cell engulfment by winner cells (Li and Baker, 2007; Ohsawa et al., 2011). It is bit controversial but interesting finding was reported that loser cells express dSPARC that is the *Drosophila* homolog of the SPARC/Osteonectin protein family, and dSPARC acts as a specific inhibitor for cell competition-induced apoptosis (Portela et al., 2010). This finding suggests that not only positive regulators but also negative regulators are expressed and/or activated during cell competition, and cell competition is a result in the balance of these factor.

In *Drosophila Melanogaster*, other genes which induce cell competition are identified (Table 1-2). Not only mutations in genes related to cell growth, but also mutations in tumor suppressor genes involved in cell polarity (*Scrib, Lgl*) or endocytosis (Vps25) lead to cell death.

In normal culture systems, when two different types of cells are cultured at a mix condition, cell sorting occurs and the same type of cells form colonies (Foty and Steinberg, 2005; Krieg et al., 2008; Steinberg and Takeichi, 1994). Therefore, it is difficult to form mosaic pattern when normal and transformed cells are co-cultured. To prevent the cell sorting phenomenon, using Madin-Darby canine kidney (MDCK) cell lines, Yasuyuki Fujita group has established a cell culture system, where transformed cells arise from a monolayer of normal epithelial cells in a tetracycline-inducible manner (Hogan et al., 2009). In this system, normal MDCK cells and tetracycline-inducible transformed MDCK cells are mixed at a ratio 50:1. After formation of monolayer, we add tetracycline to induce transformation. Using this system, we successfully mimic the initial stage of carcinogenesis, newly emerging transformed cells are surrounded by normal epithelial cells (Figure 1-4).

Recently, we also reported apical extrusion of RasV12-transformed cells using mouse model system (Kon et al., 2017; Sasaki et al., 2018). In this model, Cre-Lox recombination system has been applied to generate RasV12-transformed cells in a mosaic manner. CreERT2 is an advanced Cre which can be activated by tamoxifen and activated CreERT2 can recognize the DNA sequence of “loxP” and induce recombination at that site. We used offspring of CK19-CreERT2 mice and CAG-LSL-

RasV12-IRES-eYFP mice. In this mouse, CreERT2 is expressed in only intestinal epithelia because “CK19” is a specific promotor of epithelial tissue. CreERT2 can kick out the STOP codon sequence in front of RasV12 stochastically in presence of tamoxifen, thereby RasV12-transformed cells are generated in a mosaic manner at various epithelial tissue. Administration of a low dose of tamoxifen induced recombination events less frequently, resulting in mosaic expression of RasV12 within various epithelial tissues. The apical extrusion of RasV12-transformed cells are also observed in mouse small intestine, pancreas and lung in this model mouse (Sasaki et al., 2018).

1.3.2. Cell competition at the initial stage of carcinogenesis

Using MDCK cells expressing constitutively active form of Ras in a tetracycline-inducible manner (MDCK-pTR GFP-RasV12), our group reported that when RasV12-transformed cells are surrounded by normal epithelial cells, the majority of RasV12 cells are apically eliminated from epithelial monolayer in an apoptosis-independent manner (Hogan et al., 2009). We also demonstrated that MAPK pathway, Cdc42, and ROCK activity in RasV12 cells are involved in this process. Importantly, when RasV12 cells are cultured alone, apical extrusion does not occur, indicating that the activation of

downstream signaling pathways of Ras itself is not sufficient to induce apical extrusion, and the presence of surrounding normal cells is also required. Similar to RasV12 cells, when oncoprotein v-Src-transformed cells are surrounded by normal epithelial cells, v-Src cells are also apically extruded from a monolayer of normal epithelial cells (Kajita et al., 2010) (Table 1-3). Apical extrusion of RasV12 cells is observed in mouse small intestine *in vivo* cell competition mouse model (Kon et al., 2017), and apical extrusion of v-Src expressing cells is observed in the enveloping layer (EVL) of zebrafish embryos (Kajita et al., 2010) as well. These findings suggest that apical extrusion occurs *in vivo* and is an evolutionarily conserved process in vertebrates for elimination of transformed cells from the epithelium (Table 1-4). Moreover, we reported that when cells with mutation in tumor suppressor genes such as Scribble or p53 are surrounded by normal cells, these transformed cells are eliminated by apoptosis or necroptosis (Norman et al., 2012; Watanabe et al., 2018). These results indicate that cell competition has an important role in protection against cancer (Table 1-3).

1.3.3. Epithelial defense against cancer (EDAC)

Apical extrusion of transformed cells does not occur when transformed cells are cultured alone, suggesting that the neighboring normal cells have ability to sense and

actively eliminate the transformed cells. To reveal this, Kajita et al. performed a biochemical screening, and showed that filamin and vimentin are specifically modulated under the mix culture condition of normal and Src- or RasV12-transformed epithelial cells (Kajita et al., 2014). Immunofluorescence analysis demonstrated that both filamin and vimentin are strongly accumulated in the neighboring normal cells at the interface with transformed cells, and positively regulate apical extrusion of transformed cells. Furthermore, we demonstrated that the Rho/Rho-kinase pathway regulates filamin accumulation and filamin acts upstream of vimentin in the apical extrusion of transformed cells. These findings suggest that normal epithelial cells recognize and actively eliminate neighboring transformed cells and that filamin is a key mediator in the interaction between normal and transformed epithelial cells (Figure 1-5).

1.4. Accumulation of Mutations in Cancer

Cancer development generally involves accumulation of a series of oncogenic mutations, it is plausible that the order of mutations affects the consequence of cell competition (Watanabe et al., 2018). At the initial stage of carcinogenesis, cells with a single oncogenic mutation somehow grow and spread into a focal lesion, a process termed field cancerization. When an additional mutation occurs in a single cell within

the precancerous cell group, the newly emerging double-mutant cell may outcompete the surrounding normal or single-mutant cells (Figure 1-6). A series of recent studies using next-generation sequencing have revealed that in various epithelial tissues of the adult human there are a number of precancerous lesions with a single or double oncogenic mutations that apparently look normal (Anglesio et al., 2017; Martincorena et al., 2018; Martincorena et al., 2015; Murai et al., 2018; Yokoyama et al., 2019).

1.5. The Aim of This Study

At the initial step of carcinogenesis, transformation occurs in single cells within the normal epithelium. A series of studies has demonstrated that the newly emerging transformed cells and surrounding normal cells often compete with each other for survival, a process called cell competition (Amoyel and Bach, 2014; Baker, 2011; Claveria and Torres, 2016; Di Gregorio et al., 2016; Johnston, 2009; Maruyama and Fujita, 2017; Merino et al., 2016; Morata and Ballesteros-Arias, 2015; Vincent et al., 2013; Wagstaff et al., 2013). Cell competition was originally found in *Drosophila(Morata and Ripoll, 1975)*, but recent studies have demonstrated that cell competition between normal and transformed cells can also occur in mammals (Maruyama and Fujita, 2017). For example, when oncoprotein RasV12- or v-Src-expressing cells are surrounded by normal epithelial cells, the transformed cells are apically eliminated from epithelial tissues (Hogan et al., 2009; Kajita et al., 2010; Kon et al., 2017; Wu et al., 2014). In addition, when tumor suppressor protein Scribble- or

Lethal giant larvae (Lgl)-knockdown/mutant cells are surrounded by normal cells, the transformed cells undergo apoptosis and are removed from epithelia (Norman et al., 2012; Tamori et al., 2010). It has been also revealed that normal epithelial cells are able to recognize and actively eliminate the neighboring transformed cells, implying that normal epithelia have intrinsic anti-tumor activity that does not involve immune cells. This homeostatic machinery is termed EDAC (Epithelial Defense Against Cancer) (Kajita et al., 2014).

However, the strength of EDAC varies depending on epithelial tissues, and multiple environmental factors such as inflammation and obesity would diminish EDAC; under these environmental conditions, a certain fraction of transformed cells remains within epithelia and forms potentially precancerous lesions (Sasaki et al., 2018; Sato et al., 2020). Indeed, recent studies using next-generation sequencing have revealed that in the human adult body there exist clonally expanded cells with a single oncogenic mutation (Martincorena et al., 2018; Martincorena et al., 2015; Yizhak et al., 2019). However, it remains enigmatic whether and how these lesions eventually develop into malignant tumors. For cancer progression, additional oncogenic mutations are supposed to accumulate within such transformed clones, acquiring more malignant phenotypes. For example, in *Drosophila*, mutant clones bearing a single mutation for the *Ras* or *Scribble* gene in an epithelial tissue contributes to tumorigenesis, but does not present metastatic behavior (Pagliarini and Xu, 2003). Instead, cells with double mutations in *Ras* and *Scribble* genes are highly invasive and metastasize into distant organs (Igaki et al., 2006; Pagliarini and Xu, 2003). At the second step of carcinogenesis, the second mutation occurs within pre-existing single-transformed clones; double-transformed cells would then expand their territory while being surrounded by single-transformed cells.

However, it remains unknown what occurs at the boundary between these heterogenous transformed clones.

In this study, I demonstrate that sequential oncogenic mutations can induce cell competition between single- and double-transformed cells and that the fate of transformed cells can be converted from loser to winner depending on the preceding mutant background.

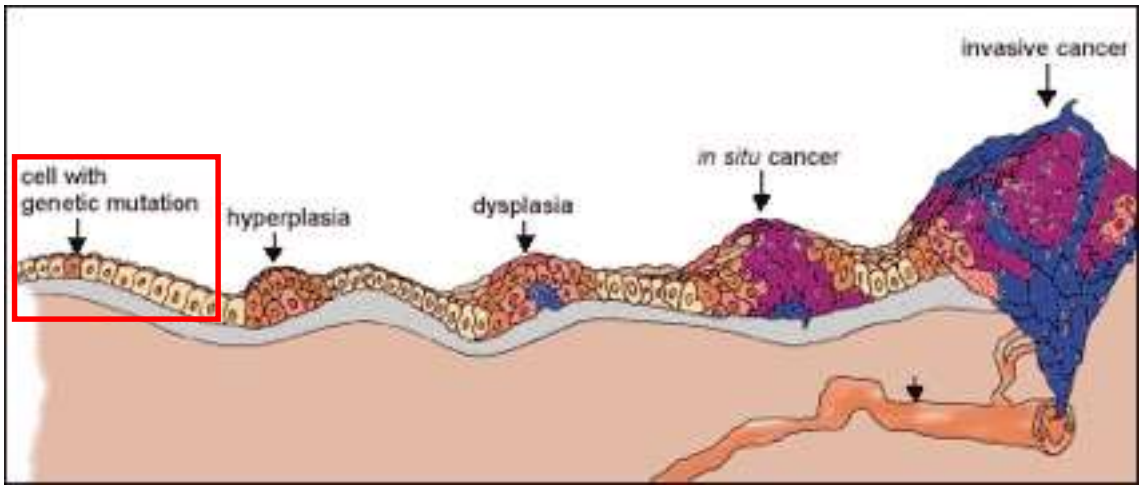

Figure 1-1. Stage of cancer development adopted from understanding cancer, teacher's guide NIH 2017

Tumor development begins when a cell sustains an oncogenic mutation. We focus on this stage: the cells that have just one or two mutation that is difficult to detect in current clinical diagnosis as shown in red circle.

https://science.education.nih.gov/supplements/webversions/cellBiology/guide/understanding1.html

Cancer	% *KRAS*	% *NRAS*	% *HRAS*	% All RAS
Pancreatic ductal adenocarcinoma	97.7	0.0	0.0	97.7
Colorectal adenocarcinoma	44.7	7.5	0.0	52.2
Multiple myeloma	22.8	19.9	0.0	42.6
Lung adenocarcinoma	30.9	0.9	0.3	32.2
Skin cutaneous melanoma	0.8	27.6	1.0	29.1
Uterine corpus endometrioid carcinoma	21.4	3.6	0.4	24.6
Uterine carcinosarcoma	12.3	1.8	0.0	14.3
Thyroid carcinoma	1.0	8.5	3.5	12.5
Acute myeloid leukaemia	3.1	6.7	1.6	11.4
Bladder urothelial carcinoma	3.1	1.4	5.9	10.6
Gastric adenocarcinoma	11.4	0.9	0.0	10.0
Cervical adenocarcinoma	8.3	0.0	0.0	8.3
Head and neck squamous cell carcinoma	0.5	0.3	4.7	5.5
Diffuse large B cell lymphoma	5.2	0.0	0.0	5.2
Oesophageal adenocarcinoma	3.8	0.0	0.6	4.4
Chronic lymphocytic leukaemia	1.9	2.5	0.0	4.4
Lung squamous cell carcinoma	2.2	0.0	2.2	4.4
Small cell lung carcinoma	1.4	0.0	1.4	2.8
Renal papillary cell carcinoma	1.2	0.6	0.6	2.4
Adenoid cystic carcinoma	0.0	0.0	1.7	1.7
Chromophobe renal cell carcinoma	0.0	1.5	0.0	1.5
Hepatocellular carcinoma	0.8	0.4	0.0	1.4
Breast invasive carcinoma	0.7	0.4	0.3	1.4
Cervical squamous cell carcinoma	1.3	0.0	0.0	1.3
Ovarian serous adenocarcinoma	0.6	0.6	0.0	1.3
Adrenocortical carcinoma	1.1	0.0	0.0	1.1
Prostate adenocarcinoma	0.3	0.0	0.3	0.9
Neuroblastoma	0.0	0.8	0.0	0.8
Brain lower grade glioma	0.5	0.5	0.0	0.8
Glioblastoma	0.7	0.0	0.0	0.7
Medulloblastoma	0.0	0.4	0.0	0.4
Kidney renal clear cell carcinoma	0.2	0.0	0.2	0.4
Oesophageal squamous cell carcinoma	0.0	0.0	0.0	0.0
Osteosarcoma (paediatric)	0.0	0.0	0.0	0.0
Ovarian small cell carcinoma	0.0	0.0	0.0	0.0
Rhabdoid tumours	0.0	0.0	0.0	0.0
Sarcoma	0.0	0.0	0.0	0.0
Small intestine neuroendocrine tumours	0.0	0.0	0.0	0.0
T cell prolymphocytic leukaemia	0.0	0.0	0.0	0.0

Table 1-1. Frequency of RAS mutations in human cancers (Cox and Der, 2010)

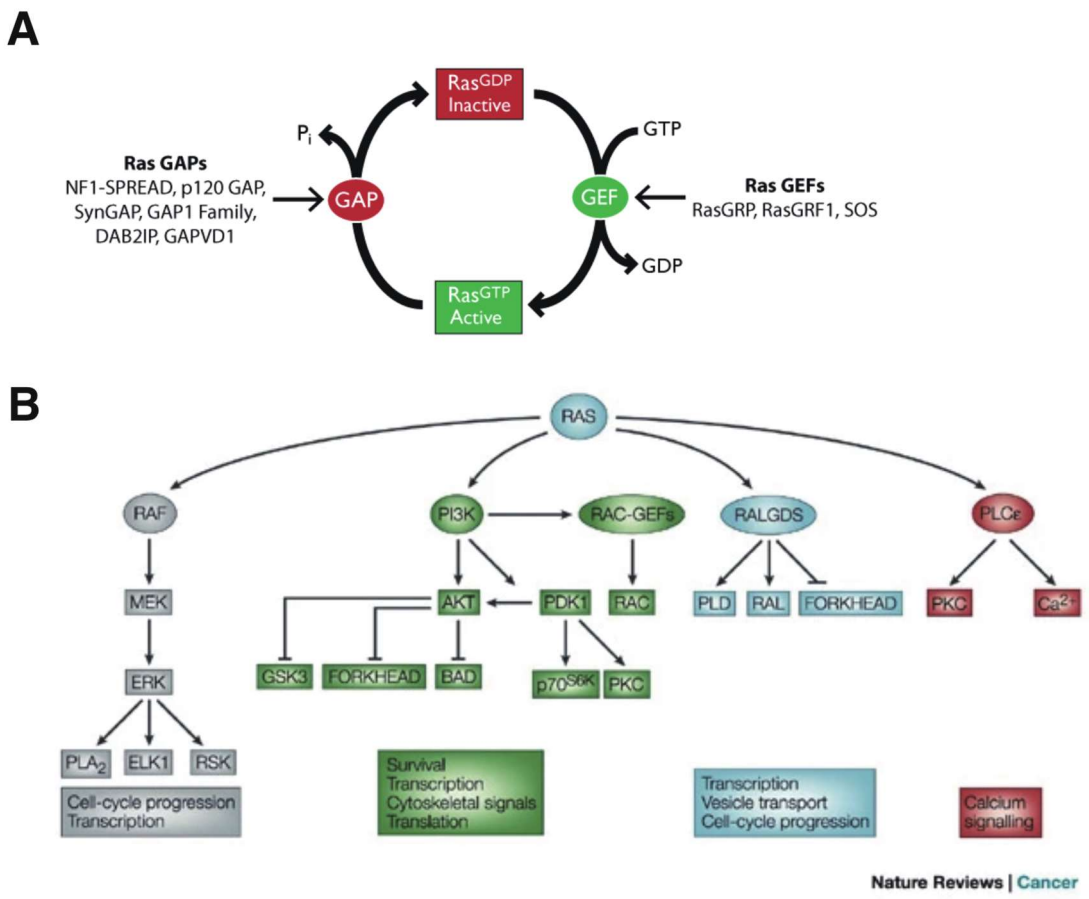

Figure 1-2. Ras activation and downstream effectors

(A) Ras proteins function as molecular switches, cycling between the GDP-bound ("off") and the GTP-bound ("on") states and exist predominantly in the GDP-bound states.

(https://www.cytoskeleton.com/ras-cancer-therapeutic-targets)

(B) A key pathway that signals downstream from activated Ras (Downward, 2003).

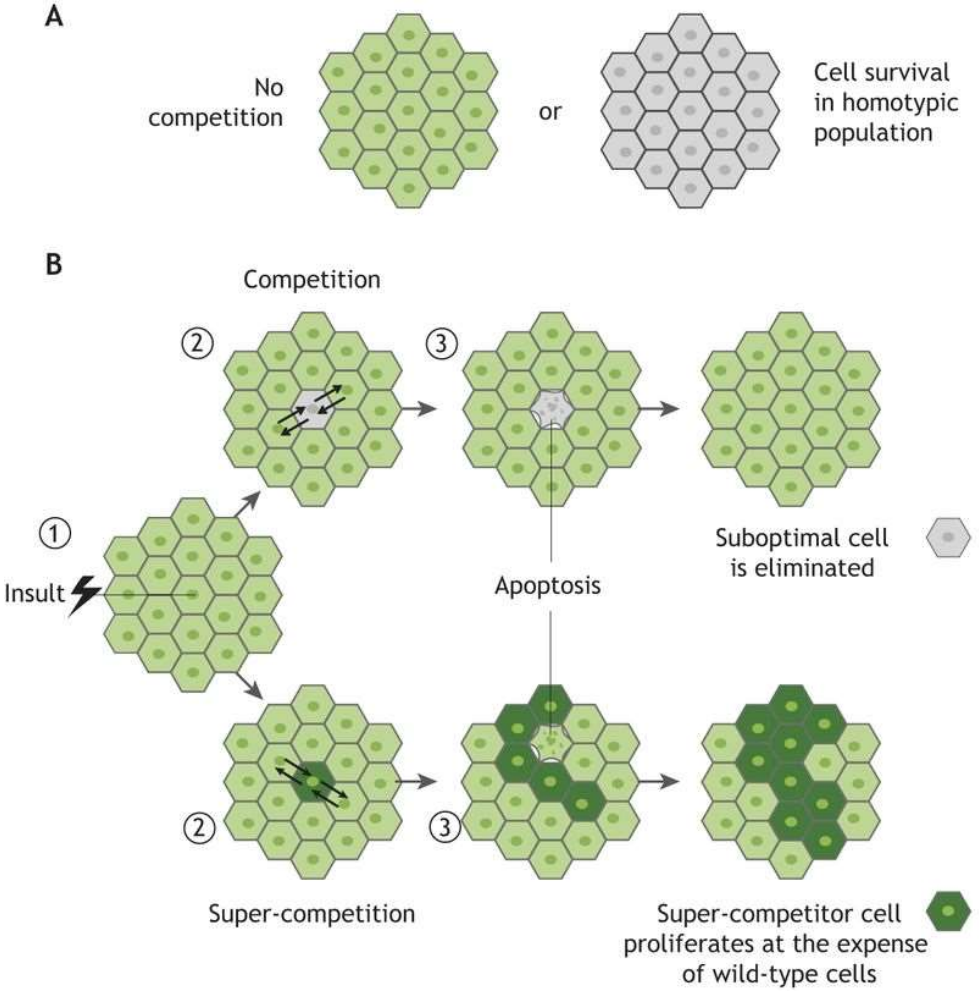

Figure 1-3. Conceptual model of "Cell competition" (Coelho and Moreno, 2019).
(A) In homotypic cell population composed of winner cells (light green) or loser cells (gray), there are no relative fitness differences between cells. Competition is not initiated, and all cells curvive.
(B) An initial insult or mutation can induce competition. In classical cell competition, suboptimal cells (gray) are eliminated from tissue by wild-type cells (light green), which proliferate to maintain organ size. In super-competition, mutant cells (dark green) become super-competitors.

Mutation/Genes	Phenotype	References
Minute	Apoptosis of *Minute*$^{+/-}$ cells	(Morata and Ripoll, 1975)
Scribble	Apoptosis of Scribble-knockdown cells	(Brumby and Richardson, 2003)
dMyc	Apoptosis of dMyc-lower expressing cells	(Moreno and Basler, 2004) (De La Cova et al., 2004)
Vps25	Apoptosis of Vps25 mutant cells	(Thompson et al., 2005)
Csk	Apoptosis and basal exclusion of Csk-deficient boundary cells	(Vidal et al., 2006)
Ras	Apoptosis of Ras^{N17} cells Apical or basal extrusion of Ras-transformed cells	(Prober and Edgar, 2000) (Hogan et al., 2009)
Lgl/Mahjong	Apoptosis of Lgl/Mahjong-knockdown cells	(Grzeschik et al., 2010) (Tamori et al., 2010)

Table 1-2. Triggers of cell competition in *Drosophila* Melanogaster.

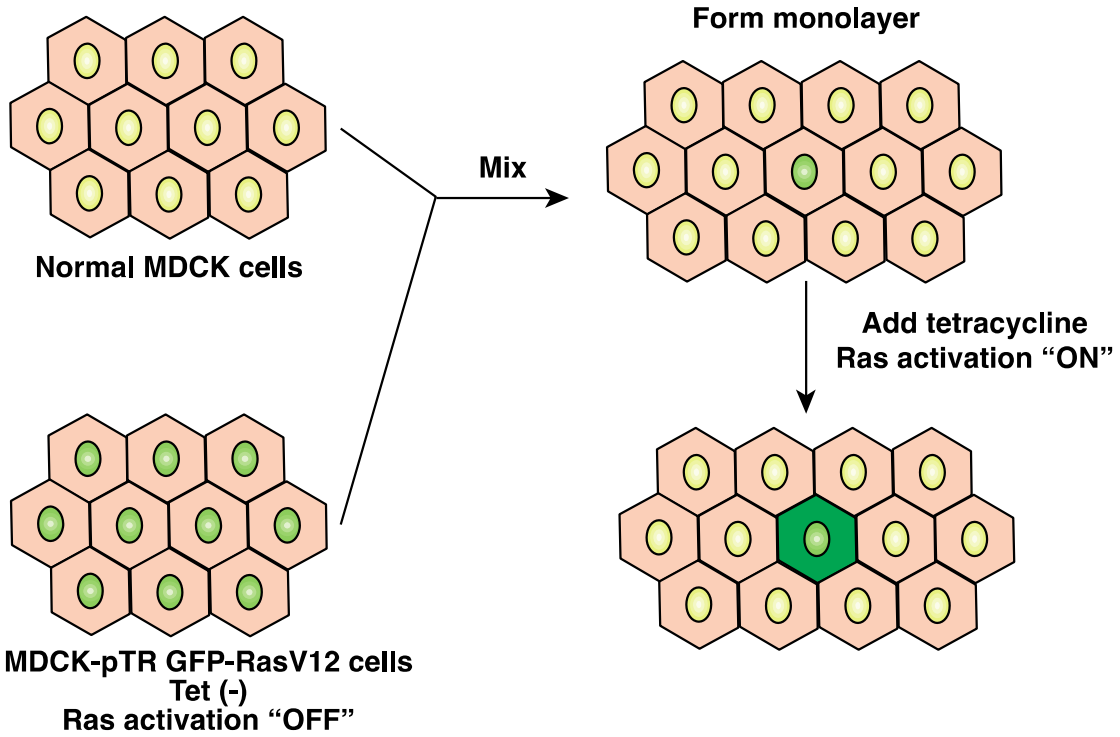

Figure 1-4. Experimental design

First, MDCK-pTR GFP-RasV12 cells are trypsinised, and mixed with trypsinised normal MDCK cells. Then, we can obtain the experimental condition where a MDCK-pTR GFP-RasV12 cell is surrounded by normal cells. After cell-cell contacts are established, GFP-RasV12 expression is induced by tetracycline addition.

Mutation	Phenotype	References
Ras	Apical extrusion or basal protrusion of Ras-transformed cells	(Hogan et al., 2009)
Src	Apical extrusion of Src-transformed cells	(Kajita et al., 2010)
Mahjong	Apoptosis of Mahjong-knockdown cells	(Tamori et al., 2010)
Scribble	Apoptosis of Scribble-knockdown cells	(Norman et al., 2012)
ErbB2	Translocation and clonal expansion of ErbB2-overexpressing cells	(Leung and Brugge, 2012)
p53	Necroptosis of constitutively active p53 expressing cells	(Watanabe et al., 2018)
Cdc42	Apical extrusion of constitutively active Cdc42-expressing cells	(Grieve and Rabouille, 2014)
Yap	Apical extrusion of constitutively active YAP expressing cells	(Chiba et al., 2016)

Table 1-3. Cell competition in mammalian cell culture systems.

Mutation	Phenotype	References
Mouse		
Minute	Elimination of Minute-knockout cells in the liver	(Oliver et al., 2004)
p53	Loss of wild-type cells by senescence-like phenotype in the hematopoietic system	(Bondar and Medzhitov, 2010)
Myc	Cell death of low myc-expressing cells in the epiblast and myocardium	(Clavería et al., 2013) (Villa et al., 2014) (Sancho et al., 2013)
Ras	Apical extrusion of Ras-transformed cells	(Kon et al., 2017) (Sasaki et al., 2018)
Zebrafish		
Src	Apical extrusion of Src-transformed cells	(Kajita et al., 2010) (Kajita et al., 2014)

Table 1-4. Cell competition *in vivo* system (vertebrate).

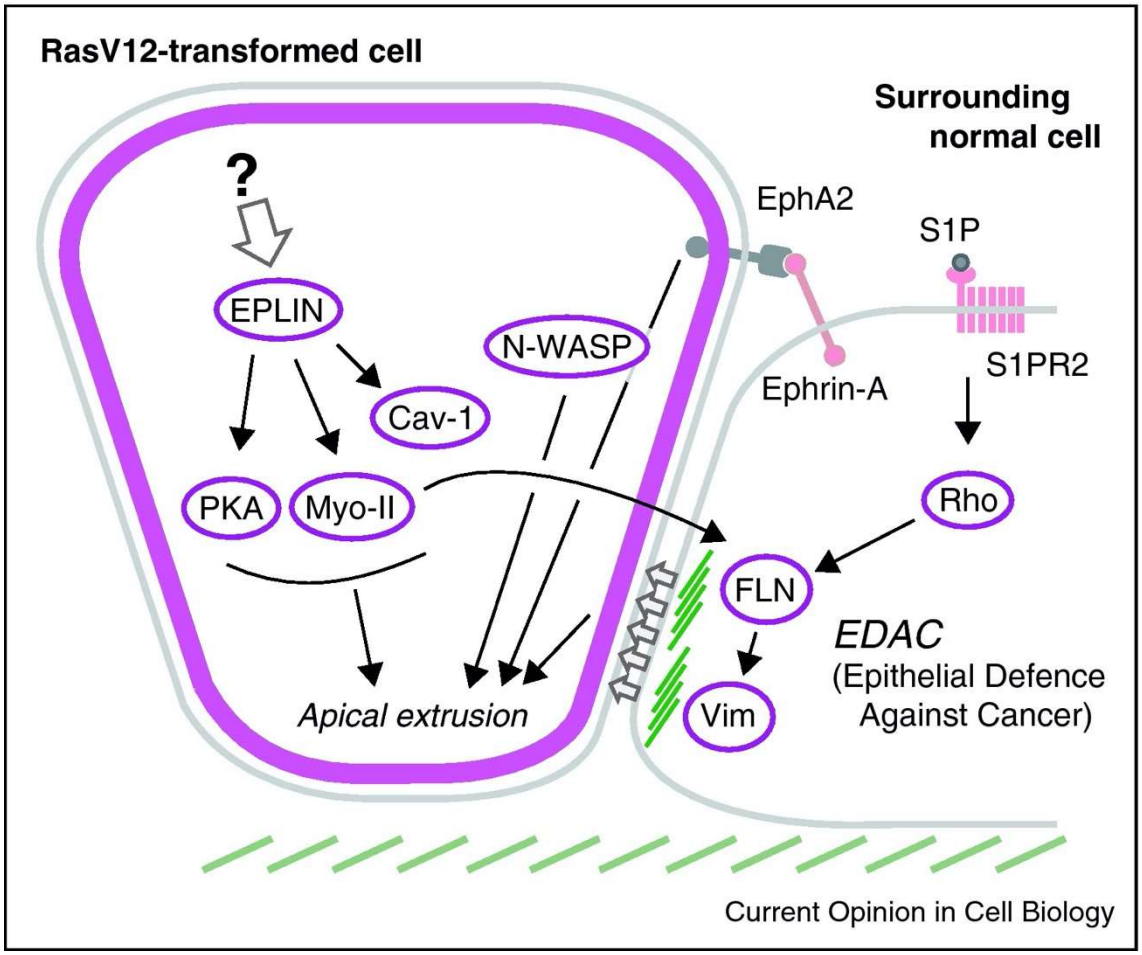

Figure 1-5. Molecular mechanisms of cell competition between normal and RasV12-transformed cells (Maruyama and Fujita, 2017).

At the interface between normal and RasV12-transformed cells, a variety of non-cell-autonomous changes occur in both cells.

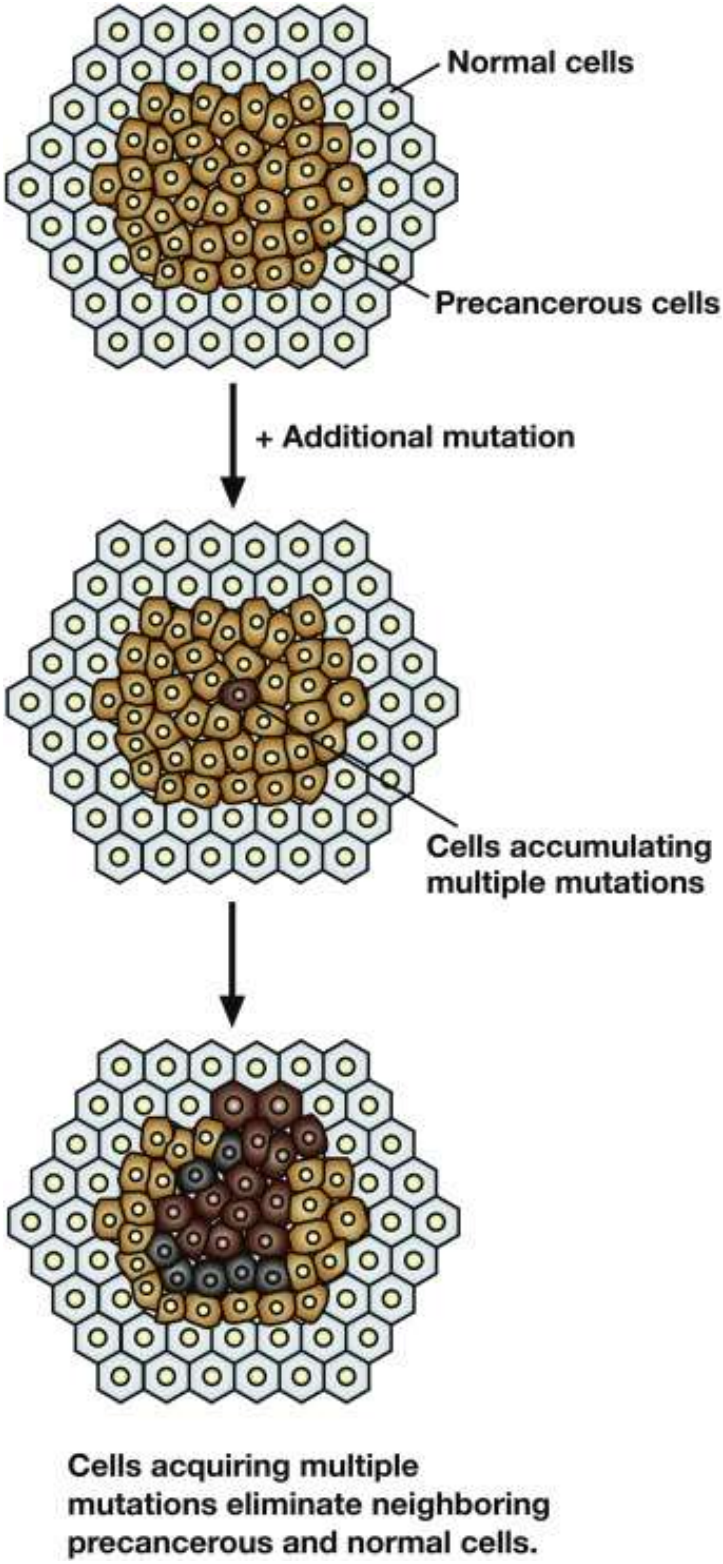

Figure 1-6. A schematic model for cancer progression driven by multiple oncogenic mutations (Tanimura and Fujita, 2020).

1.6. References

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2. Influences of sequential oncogenic mutations on cell competition

2.1. Experimental procedures

2.1.1. Antibodies, plasmids, and materials

The following primary antibodies were used in this study: rabbit anti-Cleaved Caspase-3 (Asp175) (#9661), rabbit anti-Phospho-mTOR (Ser2448) (#5536), and rabbit anti-phospho-Myosin Light Chain (Thr18/Ser19) (#3674) antibodies from Cell Signaling Technology, mouse anti-Myc-tag (05-724) and mouse anti-Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) (MAB374) antibodies from Merck Millipore, goat anti-Scribble (C-20) (sc-11049) antibody from Santa Cruz Biotechnology, rabbit anti-CD68 (25747-1-AP) antibody from Protein tech, rabbit anti-phospho-JNK (pTPpY) (V793A) antibody from Promega. Alexa-Fluor-488-, -568-, and -647-conjugated phalloidin (Life Technologies) were used at 1.0 U ml^{-1}. Alexa-Fluor-488-, -568-, and -647-conjugated secondary antibodies were from Life Technologies. Hoechst 33342 (Life Technologies) was used at a dilution of 1:5,000 for immunofluorescence of cultured cells. The inhibitors Z-VAD-FMK (100 μM), SB202190 (5 μM), Bisindolylmaleimide (BIM)-I (10 μM), CK666 (100 μM), SMIFH2 (25 μM), Y27632 (20 μM) were from Calbiochem. SP600125 (10 μM), Ferrostatin (Fer)-1 (20 μM), CCCP (5 μM), and

Rapamycin (0.1 μM) were from Sigma-Aldrich. Trolox (1 mM) was from Cayman Chemical. sc-356160 (40 μM), CAPE (10 μg ml^{-1}), and Necrostatin-1 (40 μM) were from Santa Cruz Biotechnology. Blebbistatin (30 μM) was from Millipore. Type I collagen (Cellmatrix® Type I-A) was obtained from Nitta Gelatin and was neutralized on ice to a final concentration of 2 mg ml^{-1} according to the manufacturer's instructions. The CellTracker dyes CMTPX (red) and Deep Red (Life Technologies) were used according to the manufacturer's instructions.

2.1.2. Cell lines

MDCK cell lines were used in this study. The parental MDCK cells were a gift from W. Birchmeier. Mycoplasma contamination was regularly tested for all cell lines in use using a commercially available kit (MycoAlert, Lonza). MDCK cells stably expressing GFP-RasV12 (MDCK-pTR GFP-RasV12) or Scribble-short hairpin RNA (shRNA) (MDCK-pTR Scribble-shRNA1) in a tetracycline-inducible manner were established and cultured as previously described (Hogan et al., 2009; Norman et al., 2012). MDCK-pTR Scribble-shRNA2 cells were established similarly for MDCK-pTR Scribble-shRNA1, except that Scribble-shRNA2 oligonucleotide were used (Scribble-shRNA2: 5'- GATCCCCGAGGTGACACTGTGCAGCATTCAAGAGATGCTGCACAGT GTCACCTCTTTTTTGGAAA-3' and 5'-

TTTCCAAAAAAGAGGTGACACTGTGCAGCATCTCTTGAATGCTGCACAGTGT CACCTCGGGGATC-3')(Qin et al., 2005). MDCK-pTRE3G Myc-RasV12 cells were established and cultured as previously described (Kon et al., 2017). MDCK-pTR GFP-RasV12 or MDCK-pTRE3G Myc-RasV12 cells stably expressing Scribble-shRNA were established as follows; Scribble-shRNA oligonucleotide (Scribble-shRNA1: 5'-GATCCCCCAGATGGTCCTCAGCAAGTTTCAAGAGAACTTGCTGAGGAC-CATCTGTTTTTC-3' and 5'-TCGAGAAAAACAGATGGTCCTCAGCAAGTT-CTCTTGAAACTTGCTGAGGACCATCTGGGG-3') (Qin et al., 2005) were cloned into the BglII/XhoI sites of pSUPER.neo+gfp (Oligoengine). MDCK-pTR GFP-RasV12 cells were transfected with pSUPER.neo+gfp Scribble-shRNA using Lipofectamine 2000, followed by selection in the medium containing 5 μg ml^{-1} blasticidin, 400 μg ml^{-1} Zeocin, and 800 μg ml^{-1} G418. MDCK-pTRE3G Myc-RasV12 cells were transfected with pSUPER.neo+gfp Scribble-shRNA using Lipofectamine 2000, followed by selection in the medium containing 5 μg ml^{-1} blasticidin and 800 μg ml^{-1} G418. For tetracycline-inducible MDCK cell lines, 2 μg ml^{-1} of tetracycline (Sigma-Aldrich) was used to induce expression of proteins or shRNAs except for MDCK-pTRE3G Myc-RasV12 cells, for which 1 μg ml^{-1} of doxycycline (Sigma-Aldrich) was used.

2.1.3. Immunofluorescence and western blotting

MDCK-pTR GFP-RasV12 Scribble-shRNA or MDCK-pTRE3G Myc-RasV12 Scribble-shRNA cells were mixed with MDCK or MDCK-pTR Scribble-shRNA cells at a ratio of 1:50 and plated onto collagen-coated coverslips as previously described (Hogan et al., 2009). MDCK-pTR Scribble-shRNA cells were incubated with tetracycline for 48 h to induce sufficient Scribble knockdown prior to co-incubation. The mixture of cells was incubated for 8-12 h, followed by tetracycline or doxycycline treatment for 24 h, except for analysis of p-mTOR that was examined after 16 h of tetracycline or doxycycline addition. After incubation for 24 h (or 16 h), cells were fixed with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) and permeabilized as previously described(Kajita et al., 2010). All primary antibodies were used at 1:100, except for mouse anti-Myc-tag and anti-Phospho-mTOR antibodies at 1:500. All secondary antibodies were used at 1:200. Immunofluorescence images were analyzed with the Olympus FV1000 or FV1200 and Olympus FV10-ASW software. Images were quantified with the MetaMorph software (Molecular Devices) and ImageJ. For transient expression of mCherry, RasV12, v-Src, Rac1WT, Rac1V12, or Rac1N17, MDCK-pTR Scribble-shRNA cells were first seeded on collagen-coated coverslips in 12-well culture dishes (Nunc) at a density of 4.5 x 10^5 cells per well in the absence of

tetracycline or 4.3 x 10^5 cells per well in the presence of tetracycline. For the latter case, to induce sufficient knockdown, MDCK-pTR Scribble-shRNA cells were incubated with tetracycline for 24 h prior to seeding. On the following day, cells were transiently transfected with pmCherryN1, pCS2-EGFP-RasV12, pRK5-Myc-RasV12, pcDNA3-v-Src-mCherry, pBOS-Myc-Rac1WT, pBOS-Myc-Rac1V12, or pBOS-Myc-Rac1N17 using Lipofectamine™ LTX or Lipofectamine™ 3000 (Life Technologies). After 24 h of transfection, cells were fixed and immunostained as described above. For quantification of engulfment in mCherry-, RasV12-, or v-Src-expressing cells, more than fifty cells were analyzed from three independent experiments. When these cells contain dead or live cells intracellularly, they are counted as engulfment-positive. For quantification of the occurrence of apoptosis, more than fifty mCherry-, RasV12-, or v-Src-expressing cells were analyzed from three independent experiments. When more than one cells were stained with cleaved caspase-3 antibody among the cells adjacent to mCherry-, RasV12-, or v-Src-expressing cells, I defined as 'cleaved caspase-3-positive surrounding'. For analyses of effect of inhibitors on apoptosis and engulfment of surrounding Scribble-knockdown cells, MDCK-pTR Scribble-shRNA cells transiently transfected with the indicated constructs were incubated with or without the indicated inhibitor for 20 h at 4 h after transfection. To monitor the intracellular ROS, cells were

cultured for 24 h after doxycycline addition and then loaded with 5 μM CellROX (Thermo Fisher Scientific) according to the manufacturer's instructions. After labeling, twelve randomly selected fields (2,048 x 2,048 pixels) were photographed (x60) under a phase-contrast microscope. To monitor the mitochondrial membrane potential or superoxide production, cells were loaded with 50 nM TMRM (Thermo Fisher Scientific) or 5 μM MitoSOX (Thermo Fisher Scientific) respectively for 30 min, followed by microscopic observation as previously described(Kon et al., 2017). For the analysis of nascent protein synthesis, Click-iT™ Plus (O-propargyl-puromycin) OPP Alexa Fluor™ 647 Protein Synthesis Assay Kit (Thermo Fisher Scientific) was used. OPP intake (2 μM) was performed for 30 min and the signal was detected with Alexa 647 picolyl azide. Western blotting was performed as previously described(Hogan et al., 2004). Primary antibodies were used at 1:1,000. Western blotting data were analyzed using ImageQuant™ LAS4010 (GE healthcare).

2.1.4. Time-lapse observation of cultured cells

For live imaging, cells were incubated in Leibovitz's medium (L-15) (Gibco) containing 10% fetal bovine serum (Sigma-Aldrich). For the observation of protrusion, cell death, or engulfment, 8.0 x 10^5 MDCK-pTR Scribble-shRNA cells were seeded on the collagen-coated 35-mm glass bottom dish (Matsunami), followed by incubation with

tetracycline for 24 h to induce sufficient knockdown prior to transfection. The cells were then co-transfected with pmCheeryN1 and pRK5-Myc-RasV12 using Lipofectamine™ 3000. After 6-10 h of transfection, time-lapse images were captured and analyzed by Nikon confocal microscopy (A1 HD25) with the NIS-Elements software (Nikon).

2.2.5. Statistics analysis

For data analyses, paired two-tailed Student's *t*-test, unpaired two-tailed Student's *t*-test, or one-way ANOVA with Tukey's test was used to determine P-values. P-values less than 0.05 were considered to be significant. No statistical method was used to predetermine sample size.

2.2. Results

2.2.1. RasV12-Transformation within the Scribble-Knockdown Epithelium Induces Cell Competition between Scribble-Knockdown/RasV12 (Double-Mutant) Cells and the Surrounding Scribble-Knockdown (Single-Mutant) Cells

To investigate whether and how sequential oncogenic mutations affect cell competition, our group established cell culture systems to examine the consecutive mutations of oncoprotein Ras and tumor suppressor protein Scribble, as the concurrent mutations in the *Ras* and *Scribble* genes can cause malignant phenotypes in *Drosophila* and mice (Brumby and Richardson, 2003; Dow et al., 2008; Elsum et al., 2014; Igaki et al., 2006; Pagliarini and Xu, 2003; Pearson et al., 2011), and mutations in these two genes are frequently found in human cancers such as colon, lung, kidney, prostate, and breast cancers (Dhillon et al., 2007; Elsum et al., 2014; Gardiol et al., 2006; Navarro et al., 2005; Pearson et al., 2011; Pylayeva-Gupta et al., 2011). For Scribble-transformed cells, I used Madin-Darby canine kidney (MDCK) epithelial cells stably expressing Scribble-shRNA in a tetracycline-inducible manner (MDCK-pTR Scribble-shRNA cells) (Norman et al., 2012). When MDCK-pTR Scribble-shRNA cells are cultured in the absence of tetracycline, knockdown of Scribble does not occur (Norman et al., 2012). Under such condition, when RasV12 expression was induced in a mosaic manner, RasV12-expressing cells were often apically extruded from the epithelial monolayer (Figure 2-1) (Hogan et al., 2009). In contrast, in the presence of tetracycline where RasV12 expression was induced under the Scribble-knockdown background,

apical extrusion of Scribble-knockdown/RasV12 double-mutant cells (hereafter referred to as Scribble-KD/RasV12 cells) was profoundly diminished and, at the same time, they often formed the basal protrusions underneath the neighboring Scribble-knockdown cells (hereafter referred to as Scribble-KD cells) (Figure 2-1). When mCherry was expressed in a mosaic manner as negative control, mCherry-expressing cells just remained within the epithelium in the absence or presence of tetracycline.

I also realized that around Scribble-KD/RasV12 cells, some of the surrounding Scribble-KD cells represented a cell death phenotype with fragmented nuclei and that the double mutant cells often engulfed them (Figure 2-2). Most of the engulfed Scribble-KD cells were dead with fragmented nuclei, and immunofluorescence with cleaved caspase-3 antibody showed that apoptosis occurred in both the surrounding and engulfed Scribble-KD cells (Figure 2-3). The similar apoptosis and engulfment phenotypes were also observed under the comparable experimental condition using Scribble-knockdown MDCK cells stably expressing RasV12 (Figure 2-4). I also found that the small fraction (<5%) of the engulfed cells had intact nuclei, suggesting that Scribble-KD/RasV12 double-mutant cells can engulf Scribble-KD single-mutant cells via entosis, live cell engulfment (Figure 2-2) (Overholtzer et al., 2007). Dead Scribble-KD cells were often found along or above protrusions from the Scribble-KD/RasV12 cells (Figure 2-2; arrow and yellow arrowheads). Indeed, time-lapse analysis revealed that a protrusion from a Scribble-KD/RasV12 cell, upon touching, induced cell death of a Scribble-KD cell: 'death touch' (Figure 2-5). Rac1 is one of the major regulators for the formation of protrusions (Couto et al., 2017). Expression of the constitutively inactive (dominant-negative) form of Rac1 (Rac1N17), but not of the wild-type (Rac1WT) or constitutively active (dominant-active) form of Rac1 (Rac1V12), in

Scribble-KD/RasV12 cells suppressed the basal protrusion formation. In addition, expression of Rac1N17 substantially diminished the frequency of apoptosis or engulfment of the neighboring Scribble-KD cells, suggesting that Rac1-mediated protrusion formation may play a certain role in those processes (Figure 2-6). In contrast with these phenotypes, when Scribble-KD/RasV12 double-mutant cells were surrounded by RasV12-transformed cells, the double mutant cells were apically extruded, and neither apoptosis nor engulfment was observed (Figure 2-7). Similarly, when Scribble-KD/RasV12 double-mutant cells were surrounded by non-mutant (normal) cells, apoptosis or engulfment rarely occurred (Figure 2-7) (Menendez et al., 2010). These results imply that sequential oncogenic mutations can induce cell competition and that the order of mutations substantially affects cell competition phenotypes.

Previous studies have demonstrated that Src-transformed cells are apically eliminated from an epithelial monolayer, similarly for RasV12-transformed cells (Figure 2-8) (Kajita et al., 2010). When v-Src expression was mosaically induced within the Scribble-knockdown epithelium, apical extrusion of Src-activated cells rarely occurred and instead frequently extended basal protrusions (Figures 2-8). As observed for RasV12, expression of v-Src also induced apoptosis in the directly contacting Scribble-knockdown cells, which were engulfed by the Src/Scribble-KD double-mutant cells (Figure 2-8). The phenotypes of apoptosis and engulfment were more frequently observed for RasV12, thus in the following experiments I focused on the cell competition between Scribble-KD/RasV12 and Scribble-KD cells.

2.2.2. Scribble-Knockdown/RasV12 Double-Mutant Cells Outcompete the Surrounding Scribble-Knockdown Single-Mutant Cells by Inducing JNK-Dependent Apoptosis and Engulfment

I then further examined the molecular mechanism of cell competition. p38 mitogen-activated protein kinase (p38 MAPK) or Jun N-terminal kinase (JNK) can be involved in cell death of loser cells in cell competition in mammals (Norman et al., 2012; Tamori et al., 2010; Wagstaff et al., 2016). I found that the JNK inhibitor SP600125 significantly suppressed both apoptosis and engulfment (Figure 2-9). Indeed, JNK activation was often observed in Scribble-KD cells that were touched by protrusions of Scribble-KD/RasV12 cells (Figure 2-9). These results indicate that the double-mutant cells induce JNK-mediated apoptosis in the neighboring single-mutant cells and eradicate them by engulfment. A previous study demonstrated that Scribble-KD cells were outcompeted by the surrounding normal cells by compaction-mediated physical forces, a phenomenon called mechanical cell competition (Wagstaff et al., 2016). This process is mediated by the p38 MAPK pathway. But, the p38 MAPK inhibitor SB202190 did not affect apoptosis or engulfment (Figure 2-9). Although Scribble-KD

cells are outcompeted by either normal or Scribble-KD/RasV12 cells by cell competition, these data suggest that distinct molecular mechanisms are involved, at least partially, in these two competitive interactions.

2.2.3. Scribble-Knockdown/RasV12 Cells Engulf Both Dead and Live Neighboring Scribble-Knockdown Cells

I next examined the phagocytic activity of Scribble-KD/RasV12 double-mutant cells using phagocytic marker CD68 (Travaglione et al., 2002). When Scribble-KD/RasV12 cells were surrounded by Scribble-KD cells, CD68 expression was promoted in Scribble-KD/RasV12 cells (Figure 2-10). In contrast, when RasV12 cells were surrounded by normal cells or when mCherry-expressing cells were surrounded by Scribble-KD cells, the CD68 expression level remained low (Figure 2-10). In addition, when Scribble-KD/RasV12 double-mutant cells alone were present, the elevation of CD68 expression was not observed (Figure 2-10), indicating that the presence of the surrounding Scribble-KD cells promotes the phagocytic activity in Scribble-KD/RasV12 cells in a non-cell-autonomous manner. The treatment with the caspase inhibitor Z-VAD-FMK strongly suppressed the occurrence of apoptosis, but did not affect the engulfment of Scribble-KD cells (Figure 2-11). Indeed, I found that in the

presence of Z-VAD-FMK, Scribble-KD/RasV12 cells frequently engulfed the surrounding Scribble-KD cells with intact nuclei by entosis, demonstrating the profound phagocytic activity of Scribble-KD/RasV12 double-mutant cells, mediating both apoptotic and live cell engulfment.

2.2.4. Mitochondrial Reactive Oxygen Species (ROS) Positively Regulate Apoptosis and Engulfment of the Neighboring Scribble-Knockdown Cells

To further understand the competitive interaction between Scribble-KD/RasV12 double-mutant and Scribble-KD single-mutant cells, I analyzed the effect of various inhibitors on apoptosis and engulfment (Table 1). Among the tested inhibitors, the ROS scavenger Trolox significantly suppressed both apoptosis and engulfment (Figure 2-12). The intracellular ROS level was then examined by CellROX Orange, a cumulative fluorescence probe of intracellular ROS. Compared with Scribble-KD cells, the CellROX fluorescence intensity was high in Scribble-KD/RasV12 cells in single culture (Figure 2-12). The CellROX intensity was further elevated when Scribble-KD/RasV12 cells were surrounded by Scribble-KD cells (Figure 2-12), which was diminished by Trolox treatment (Figure 2-12). In addition, the elevation of CellROX fluorescence was

observed in some of the surrounding Scribble-KD cells in mix culture as well (Figure 2-12; arrows, Figure 2-12; red bar).

Mitochondria are one of the major sources for ROS production. Using MitoSOX, an indicator for mitochondrial superoxide production, I demonstrated that under the single culture condition, the MitoSOX fluorescence intensity was profoundly elevated in Scribble-KD/RasV12 cells compared with that in Scribble-KD cells (Figure 2-13). The MitoSOX intensity was further increased when Scribble-KD/RasV12 cells were surrounded by Scribble-KD cells (Figure 2-13). To examine the functional involvement of mitochondrial ROS, I examined the effect of Mitoquinone (MitoQ), a mitochondria-targeted antioxidant (Figure 2-14). The MitoQ treatment substantially diminished both apoptosis and engulfment (Figure 2-14), suggesting that the elevated ROS production in mitochondria positively regulates these processes. TMRM (tetramethylrhodamine methyl ester) is a positively charged red fluorescent dye that is incorporated into mitochondria according to the membrane potential gradient across their inner membranes. I found that the TMRM fluorescence was higher in Scribble-KD/RasV12 cells than Scribble-KD cells in single culture, which was further enhanced in Scribble-KD/RasV12 cells when surrounded by Scribble-KD cells (Figure 2-15). CCCP (carbonyl cyanide m-chlorophenyl hydrazone) is a mitochondrial oxidative

phosphorylation uncoupler, which reduces mitochondrial membrane potential (Figure 2-16). The CCCP treatment diminished the MitoSOX intensity in Scribble-KD/RasV12 cells surrounded by Scribble-KD cells (Figure 2-17) and, moreover, suppressed the frequency of apoptosis and engulfment (Figure 2-17). Collectively, these results indicate that the mitochondrial ROS production plays a crucial role in cell competition induced by sequential oncogenic mutations.

2.2.5. The mTOR Pathway Is a Crucial Downstream Regulator for Apoptosis and Engulfment

Previous studies have revealed that the mTOR pathway can be involved in cell competition (Bowling et al., 2018; Sanaki et al., 2020). I found that phosphorylation of mTOR was enhanced in Scribble-KD/RasV12 cells that were surrounded by Scribble-KD cells (Figure 2-18), which was suppressed by the mTOR inhibitor Rapamycin (Figure 2-19). When Scribble-KD/RasV12 cells alone were present, increased phosphorylation of mTOR was not observed (Figure 2-18), demonstrating that the presence of the surrounding Scribble-KD cells promotes the mTOR activity. Treatment with Trolox or CCCP strongly suppressed the phosphorylation of mTOR in Scribble-KD/RasV12 cells surrounded by Scribble-KD cells (Figure 2-19). In contrast, addition

of Rapamycin did not affect the MitoSOX or TMRM fluorescent intensity (Figure 2-20). Furthermore, Rapamycin treatment significantly suppressed apoptosis, CD68 accumulation, and engulfment (Figure 2-21). Collectively, these results indicate that the mTOR pathway is a crucial regulator for cell competition, acting downstream of the mitochondrial ROS production.

Moreover, I checked protein synthesis ability. Protein synthesis is one of the main downstream targets of mTOR pathway. I found that protein synthesis ability of Scribble-KD/RasV12 cells is higher than that of Scribble-KD cells (Figure 2-22). And, Treatment with Rapamycin or CCCP suppressed protein synthesis ability in Scribble-KD/RasV12 cells (Figure 2-23). However, although phosphorylation of mTOR is upregulated in Scribble-KD/RasV12 cells in a non-cell autonomous manner, protein synthesis ability is enhanced in cell-autonomous manner. These data suggest that mTOR-independent protein synthesis is involved, at least partially, in this upregulation of protein synthesis ability.

2.2.6. Rho pathway Is Upregulated in the Surrounding Scribble-Knockdown cells

As described above, I found that phagocytic marker CD68 is upregulated in Scribble-

KD/Ras cells surrounded by Scribble-KD cells. Previous studies have revealed that Rho activation varied the intercellular localization of CD68 and Rho-activated epithelial cells behaved as professional phagocytes. I found that treatment with Y27632 or Blebbistatin strongly suppressed apoptosis of surrounding Scribble-KD cells and engulfment by Scribble-KD/RasV12 cells (Figure 2-24). Surprisingly, phosphorylation of Myosin light chain is upregulated in the surrounding Scribble-KD cells, but not Scribble-KD/RasV12 cells (Figure 2-25). Furthermore, Trolox treatment significantly suppressed phosphorylation of Myosin light chain in the surrounding Scribble-KD cells (Figure 2-26). Collectively, these results indicate that the Rho pathway is a crucial regulator for cell competition, acting downstream of the ROS production in the surrounding Scribble-KD cells.

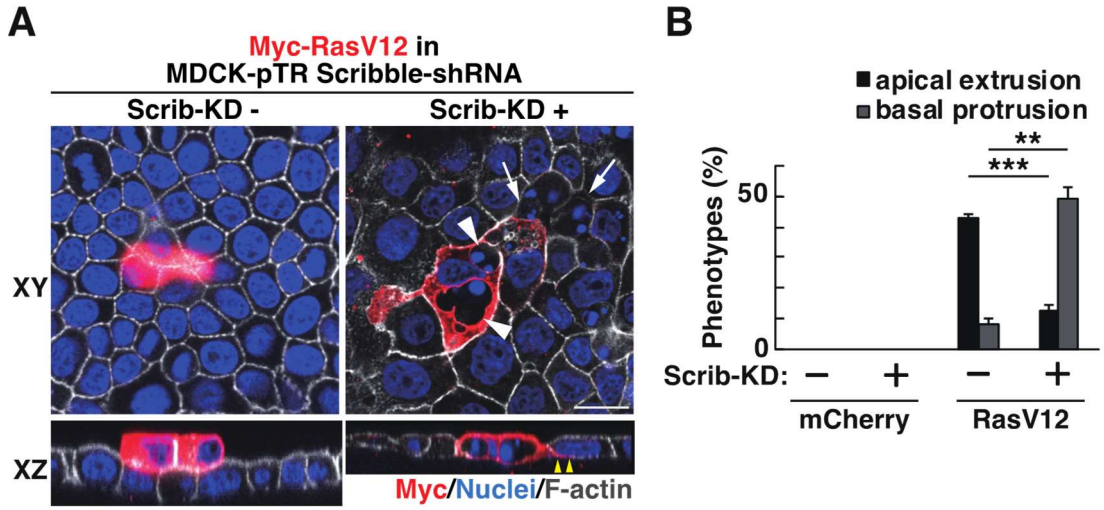

Figure 2-1. Phenotypes of RasV12-transformed cells in Scribble-knockdown background

(A) Immunofluorescence images of RasV12-transformed cells in normal or Scribble-knockdown epithelia. Myc-RasV12 was transiently expressed in a mosaic manner within the monolayer of tetracycline-inducible MDCK-pTR Scribble-shRNA1 cells. Cells were cultured in the absence or presence of tetracycline and analyzed by immunofluorescence with anti-Myc-tag antibody (red), Alexa-Fluor-647-phalloidin (grey), and Hoechst (blue). White arrows and white arrowheads indicate dead surrounding Scribble-KD cells with fragmented nuclei and Scribble-KD cells engulfed by Scribble-KD/RasV12 cells, respectively. The yellow arrowheads show the protrusion from a Scribble-KD/RasV12 cell. **(B)** Quantification of apical extrusion or basal protrusion. Myc-RasV12 was transiently expressed in a mosaic manner within the monolayer of MDCK-pTR Scribble-shRNA1 cells. Cells were cultured in the absence or presence of tetracycline. Data are mean ± s.d. from three independent experiments. **P<0.01, ***P<0.001 (unpaired two-tailed Student's *t*-test); n=50, 50, 50, 50 cells. Note that neither apical extrusion nor basal protrusion was observed in mCherry-expressing cells. **(A)** Scale bar, 20 μm.

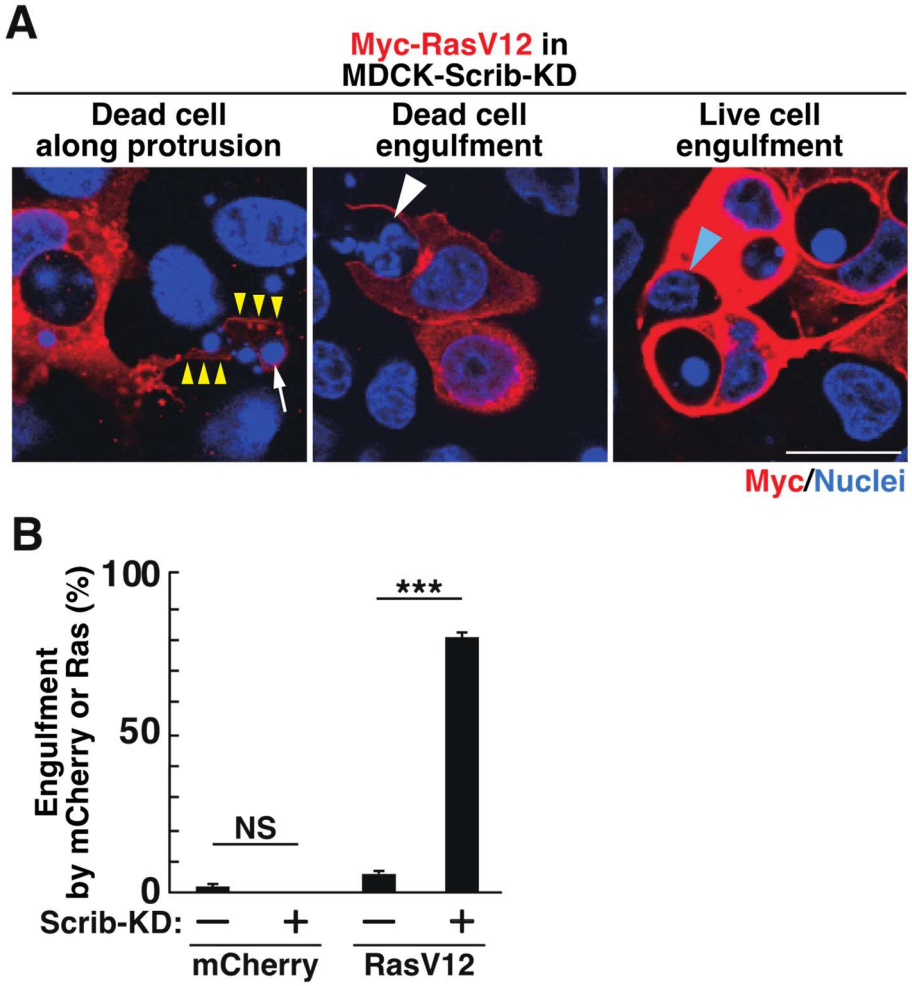

Figure 2-2. Sequential oncogenic mutations induce three characteristic phenotypes
(A) Magnified immunofluorescence images of RasV12-transformed cells in Scribble-knockdown epithelia. The yellow arrowheads and white arrow show the protrusion from a Scribble-KD/RasV12 cell and a dead Scribble-KD cell, respectively. The white and blue arrowheads indicate dead and live surrounding Scribble-KD cells engulfed by Scribble-KD/RasV12 cells, respectively. **(B)** Quantification of engulfment by mCherry- or RasV12-expressing Scribble-KD cells. Data are mean ± s.d. from three independent experiments. ***P<0.001 (unpaired two-tailed Student's *t*-test); n=81, 67, 78, 93 cells. NS: not significant. **(A)** Scale bar, 20 μm.

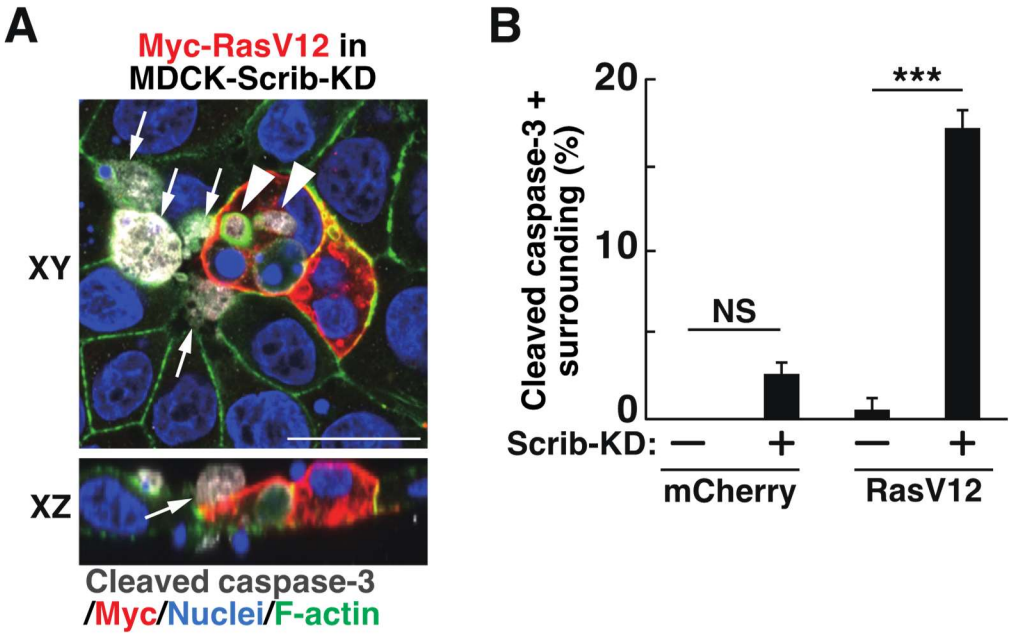

Figure 2-3. Apoptosis occurred in both the surrounding and engulfed Scribble-knockdown cells

(A) Immunofluorescence images of apoptotic Scribble-knockdown cells neighboring or inside Scribble-KD/RasV12 cells. Cells were analyzed by immunofluorescence with anti-cleaved caspase-3 antibody (grey), anti-Myc-tag antibody (red), Alexa-Fluor-488-phalloidin (green), and Hoechst (blue). White arrows and arrowheads indicate apoptotic, surrounding and engulfed Scribble-KD cells, respectively. (B) Quantification of apoptosis of the surrounding cells. Data are mean ± s.d. from three independent experiments. ***P<0.001 (unpaired two-tailed Student's *t*-test); n=250, 250, 250, 250 cells. NS: not significant. (A) Scale bars, 20 μm.

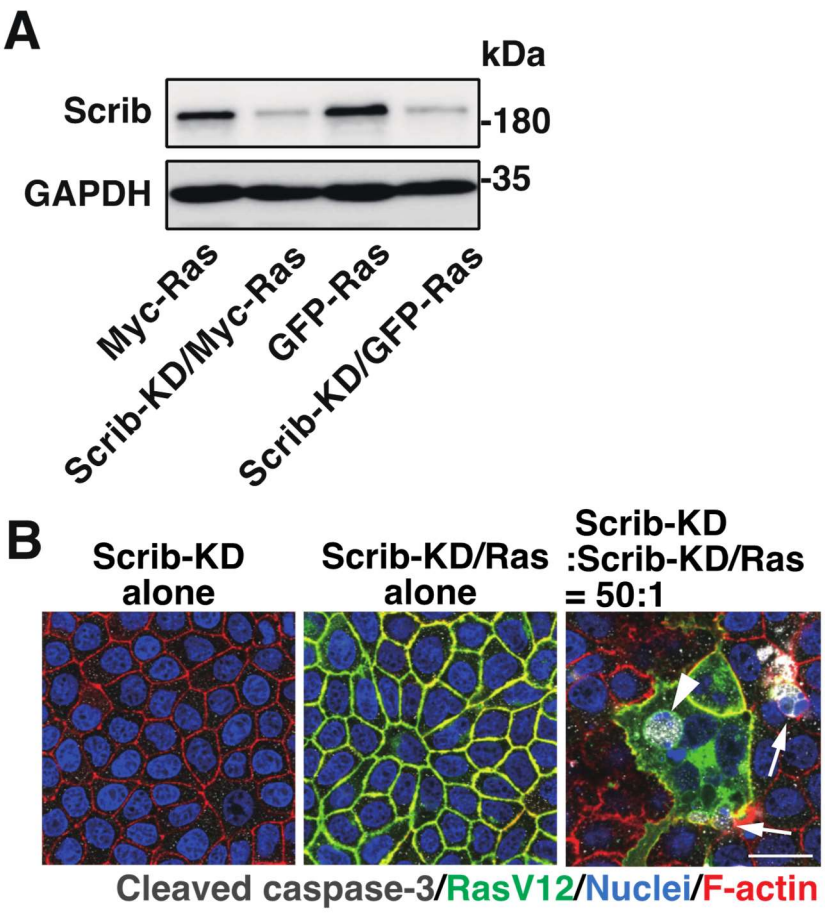

Figure 2-4. Establishment of Scribble-knockdown MDCK cells stably expressing Myc-RasV12 or GFP-RasV12.

(A) Cell lysates from MDCK-pTRE3G Myc-RasV12, MDCK-pTRE3G Myc-RasV12 Scribble-shRNA1, MDCK-pTR GFP-RasV12, or MDCK-pTR GFP-RasV12 Scribble-shRNA1 cells were examined by western blotting with anti-Scribble and GAPDH antibodies. (B) Comparable apoptotic and engulfment phenotypes under another experimental condition using Scribble-knockdown MDCK cells stably expressing RasV12. MDCK-pTR Scribble-shRNA1 cells and MDCK-pTR GFP-RasV12 Scribble-shRNA1 cells were cultured alone or co-cultured at a ratio of 50:1, and were stained with anti-cleaved caspase-3 antibody (grey), Alexa-Fluor-568-phalloidin (red), and Hoechst (blue). The white arrows and arrowhead indicate apoptotic, surrounding and engulfed Scribble-KD cells, respectively. (B) Scale bars, 20 μm.

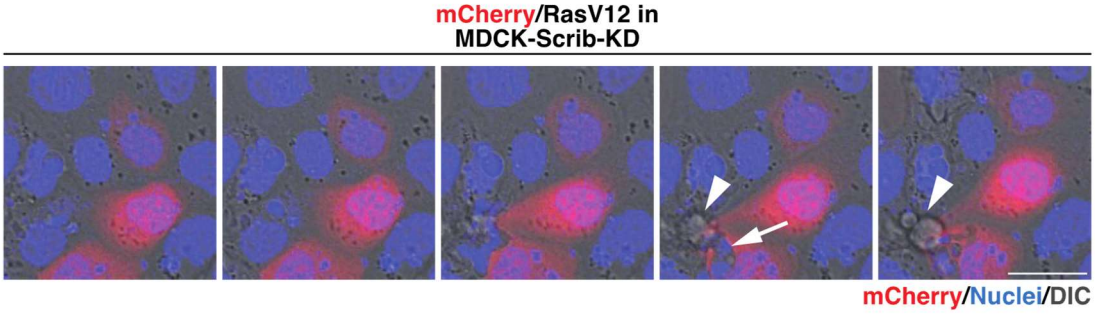

Figure 2-5. Death touch; a neighboring Scribble-knockdown cell dies at the tip of a protrusion from a Scribble-KD/RasV12 cells

Cell death induced by a dynamic protrusion from a Scribble-KD/RasV12 cell. Images were extracted from a representative time-lapse analysis. mCherry and RasV12 were transiently co-expressed in a mosaic manner within the monolayer of MDCK-pTR Scribble-shRNA1 cells. The white arrowheads indicate a dead surrounding Scribble-KD cell at the tip of protrusion of a Scribble-KD/RasV12 cell. The white arrow shows engulfed Scribble-KD cells. Scale bars, 20 μm.

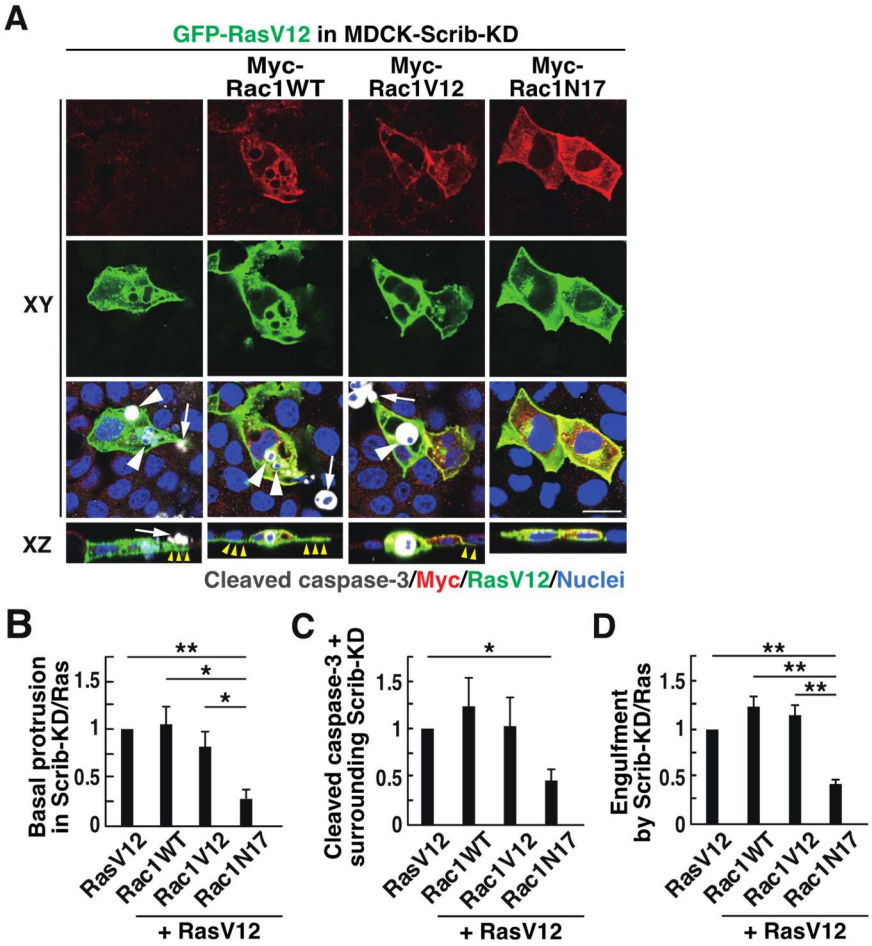

Figure 2-6. Effect of expression of a constitutively inactive form of Rac1 on behavior and fate of RasV12-expressing cells in Scribble-knockdown epithelia. (A) Immunofluorescence images of cells co-expressing RasV12 and a mutant form of Rac1 in Scribble-knockdown epithelia. GFP-RasV12 was transiently co-expressed with Myc-Rac1WT, Myc-Rac1V12, or Myc-Rac1N17 in a mosaic manner within the monolayer of MDCK-pTR Scribble-shRNA1 cells. Cells were stained with anti-cleaved caspase-3 antibody (grey), anti-Myc-tag antibody (red), and Hoechst (blue). White arrows and arrowheads indicate apoptotic, surrounding and engulfed Scribble-KD cells, respectively. The yellow arrowheads show basal protrusion from a Scribble-KD/RasV12 cell. (B-D) Quantification of basal protrusion of Scribble-KD/RasV12 cells (B), apoptosis of the surrounding Scribble-KD cells (C), or engulfment by Scribble-KD/RasV12 cells (D). Values are expressed as a ratio relative to RasV12. Data are mean ± s.d. from three independent experiments. *P<0.05, **P<0.01 (paired two-tailed Student's *t*-test); n=185, 112, 169, 149 cells. (A) Scale bar, 20 μm.

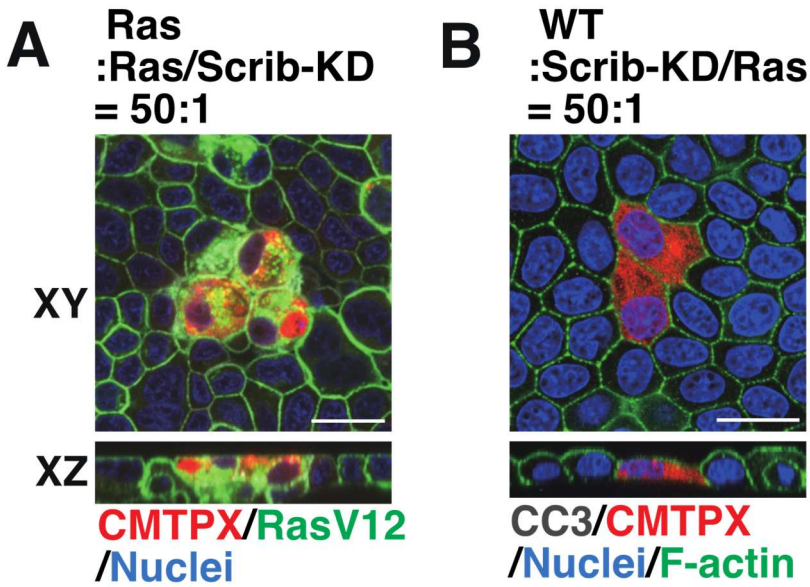

Figure 2-7. Phenotypes of RasV12/Scribble-knockdown cells surrounded by RasV12 cells or Scribble-knockdown/RasV12 cells surrounded by normal cells

(A) Immunofluorescence images of RasV12/Scribble-knockdown cells surrounded by RasV12-transformed cells. MDCK-pTR GFP-RasV12 cells were co-cultured with MDCK-pTR GFP-RasV12 Scribble-shRNA1 cells stained with CMTPX (red) at a ratio of 50:1, followed by immunofluorescence with Hoechst (blue). (B) Immunofluorescence images of Scribble-KD/RasV12 cells surrounded by normal cells. Normal MDCK cells were co-cultured with MDCK-pTRE3G Myc-RasV12 Scribble-shRNA1 cells stained with CMTPX (red) at a ratio of 50:1, followed by immunofluorescence with anti-cleaved caspase-3 (CC3) antibody (grey), Alexa-Fluor-488-phalloidin (green), and Hoechst (blue). (A and B) Scale bars, 20 μm.

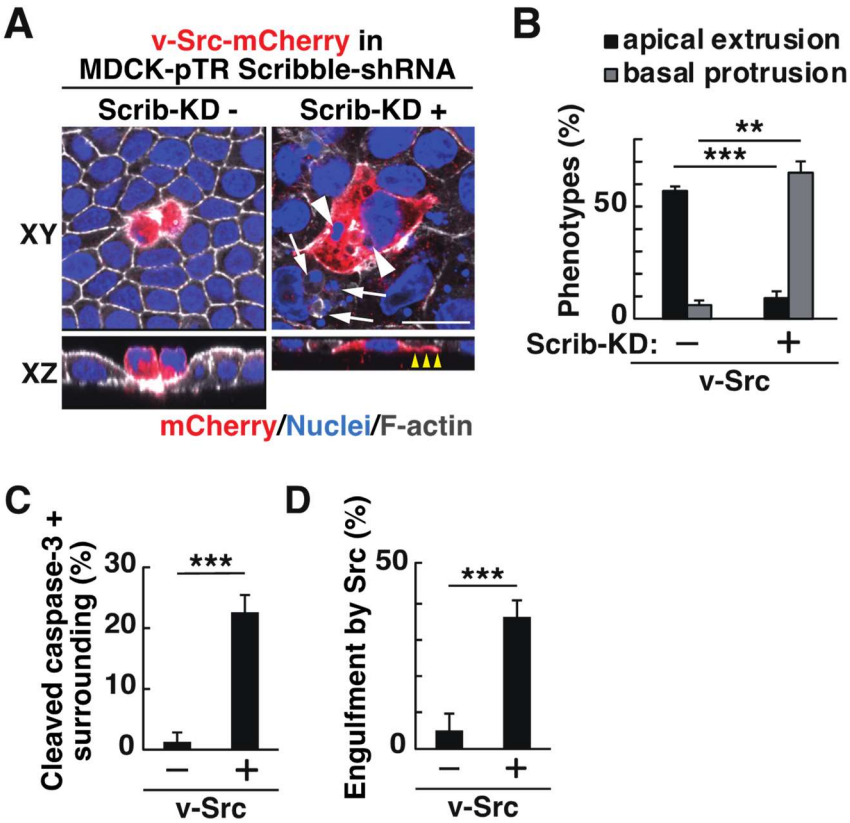

Figure 2-8. Phenotypes of v-Src-transformed cells in Scribble-knockdown background

(A-D) Behavior and fate of v-Src-transformed cells in normal or Scribble-knockdown epithelia. v-Src-mCherry was transiently expressed in a mosaic manner within the monolayer of MDCK-pTR Scribble-shRNA1 cells in the absence or presence of tetracycline. (A) Immunofluorescence images of v-Src-mCherry-expressing cells in normal or Scribble-knockdown epithelia. Cells were stained with Alexa-Fluor-647-phalloidin (grey) and Hoechst (blue). White arrows and white arrowheads indicate dead surrounding Scribble-KD cells with fragmented nuclei and Scribble-KD cells engulfed by Scribble-KD/v-Src cells, respectively. The yellow arrowheads show the basal protrusion from a Scribble-KD/v-Src cell. (B-D) Quantification of apical extrusion and basal protrusion of Scribble-KD/v-Src cells (B), apoptosis of the surrounding Scribble-KD cells (C), and engulfment by Scribble-KD/v-Src cells (D). Data are mean ± s.d. from three independent experiments. **P<0.01, ***P<0.001 (unpaired two-tailed Student's *t*-test); n=50, 50, 50, 50 cells (L), n=50, 50 cells (M and N). (A) Scale bar, 20 μm.

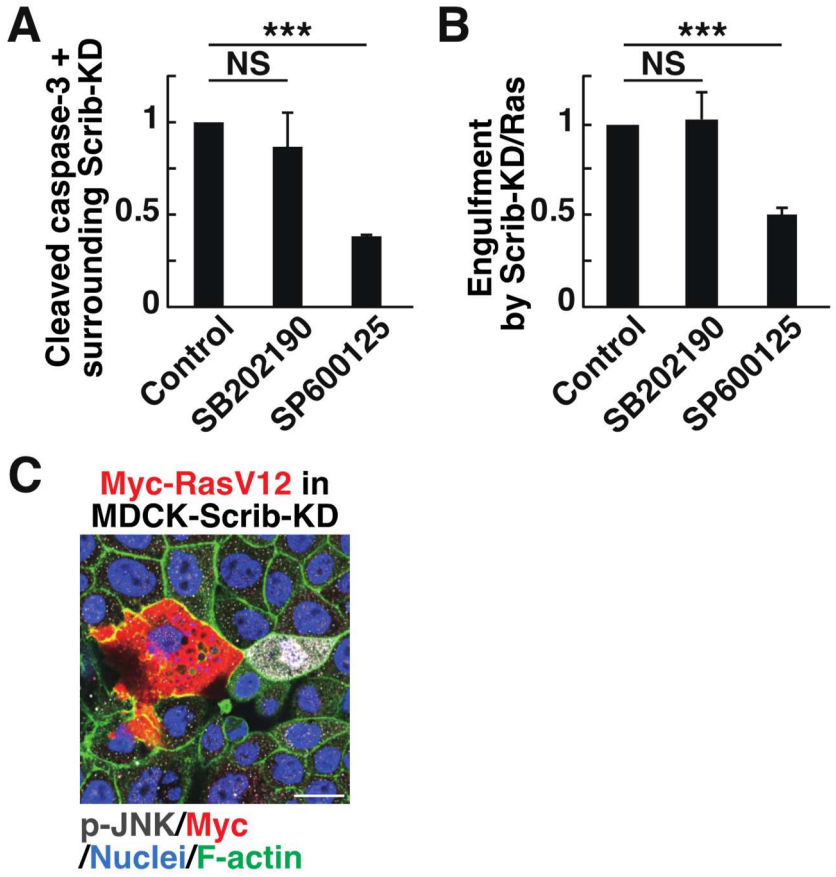

Figure 2-9. Effect of the p38 MAPK inhibitor SB202190 or JNK inhibitor SP600125 on apoptosis or engulfment of the surrounding Scribble-knockdown cells.

(A and B) Quantification data of apoptosis (A) or engulfment (B). Myc-RasV12 was transiently expressed in a mosaic manner within the monolayer of MDCK-pTR Scribble-shRNA1 cells. Values are expressed as a ratio relative to control. Data are mean ± s.d. from three independent experiments. ***P<0.001 (paired two-tailed Student's *t*-test); n=50, 250, 250 cells. NS: not significant. (C) Immunofluorescence image of phospho-JNK antibody staining. The caspase inhibitor Z-VAD-FMK was added to prevent apoptosis-mediated cell extrusion. Cells were stained with anti-phospho-JNK antibody (grey), anti-Myc-tag antibody (red), Alexa-Fluor-488-phalloidin (green), and Hoechst (blue). (C) Scale bar, 20 μm.

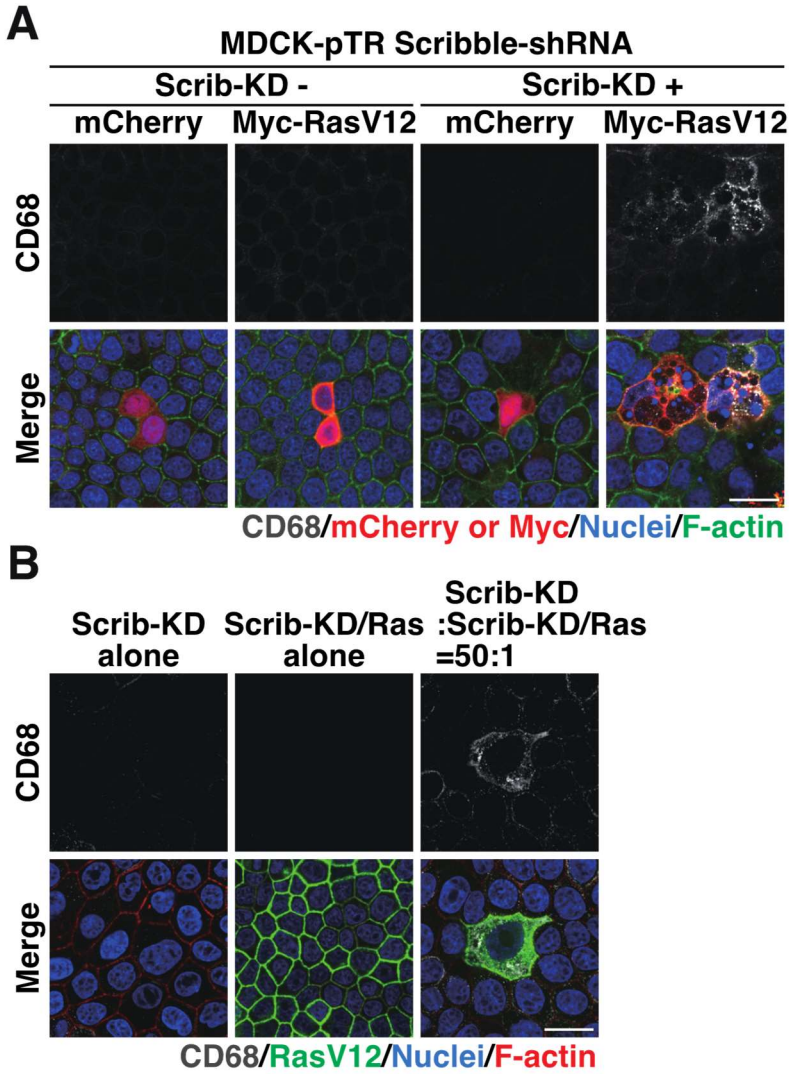

Figure 2-10. Phagocytic marker CD68 is upregulated in Scribble-knockdown/RasV12 cells surrounded by Scribble-knockdown cells.

(A and B) Immunofluorescence images of CD68 antibody staining. (A) mCherry or Myc-RasV12 was transiently expressed in a mosaic manner within the monolayer of MDCK-pTR Scribble-shRNA1 cells in the absence or presence of tetracycline, followed by immunofluorescence with anti-CD68 antibody (grey), anti-Myc-tag antibody (red), Alexa-Fluor-488-phalloidin (green), and Hoechst (blue). (B) MDCK-pTR Scribble-shRNA1 cells and MDCK-pTR GFP-RasV12 Scribble-shRNA1 cells were cultured alone or co-cultured at a ratio of 50:1. Cells were stained with anti-CD68 antibody (grey), Alexa-Fluor-568-phalloidin (red), and Hoechst (blue). (A and B) Scale bars, 20 μm.

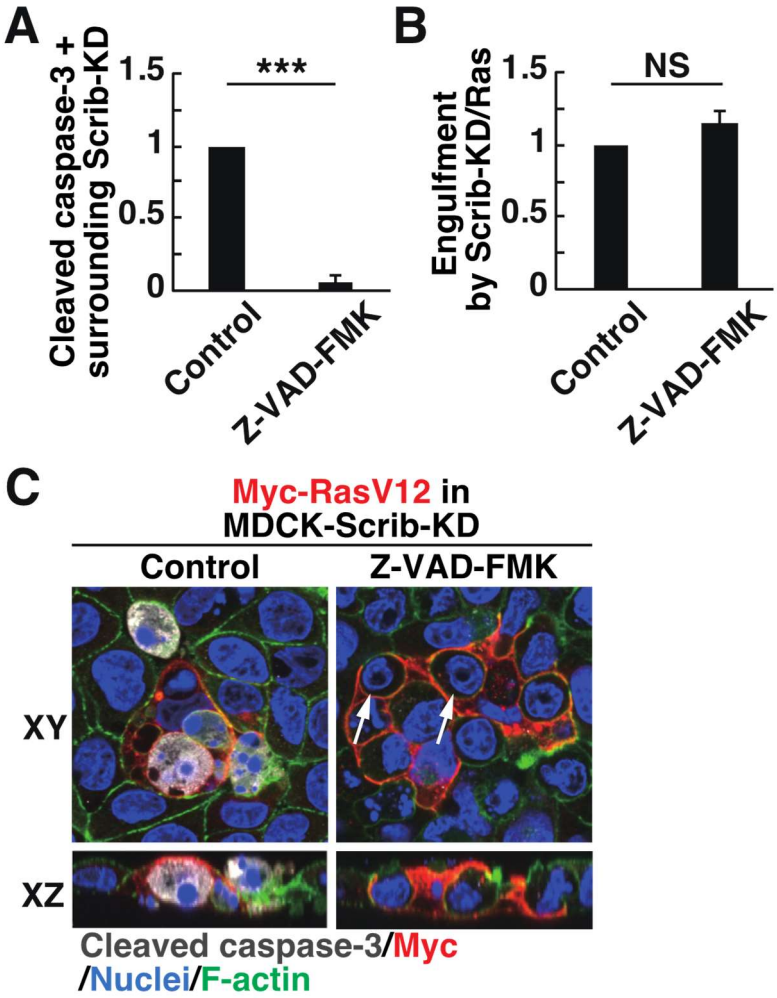

Figure 2-11. Effect of caspase inhibitor Z-VAD-FMK on apoptosis or engulfment of the surrounding Scribble-knockdown cells

Myc-RasV12 was transiently expressed in a mosaic manner within the monolayer of MDCK-pTR Scribble-shRNA1 cells. (A and B) Quantification of the apoptosis (A) or engulfment (B). Values are expressed as a ratio relative to control. Data are mean ± s.d. from three independent experiments. ***P<0.001 (paired two-tailed Student's *t*-test); n=161, 120 cells (A and B). NS: not significant. (C) Immunofluorescence images of engulfed, live Scribble-knockdown cells under the treatment with Z-VAD-FMK. Cells were stained with anti-cleaved caspase-3 antibody (grey), anti-Myc-tag antibody (red), Alexa-Fluor-488-phalloidin (green), and Hoechst (blue). The white arrows indicate live Scribble-KD cells with intact nuclei engulfed by Scribble-KD/RasV12 cells. (C) Scale bar, 20 μm.

Inhibitors	Target	Apoptosis	Engulfment
Trolox	ROS	↓*	↓*
BIM-Ⅰ	PKC	No effect	↓*
CK666	Arp2/3	No effect	↓*
Smifh2	Formin	↓*	No effect
sc-356160	TNF-α	No effect	No effect
CAPE	NF-κB	No effect	No effect
Ferrostatin-1	Ferroptosis	No effect	No effect
Necrostatin-1	RIPK1 or RIPK3	No effect	No effect

Table 1. Effect of Various Inhibitors on Apoptosis or Engulfment of the Surrounding Scribble-Knockdown Cells

Myc-RasV12 was transiently expressed in a mosaic manner within the monolayer of MDCK-pTR Scribble-shRNA1 cells in the presence of the indicated inhibitor.

*: statistically significant (paired two-tailed Student's *t*-test)

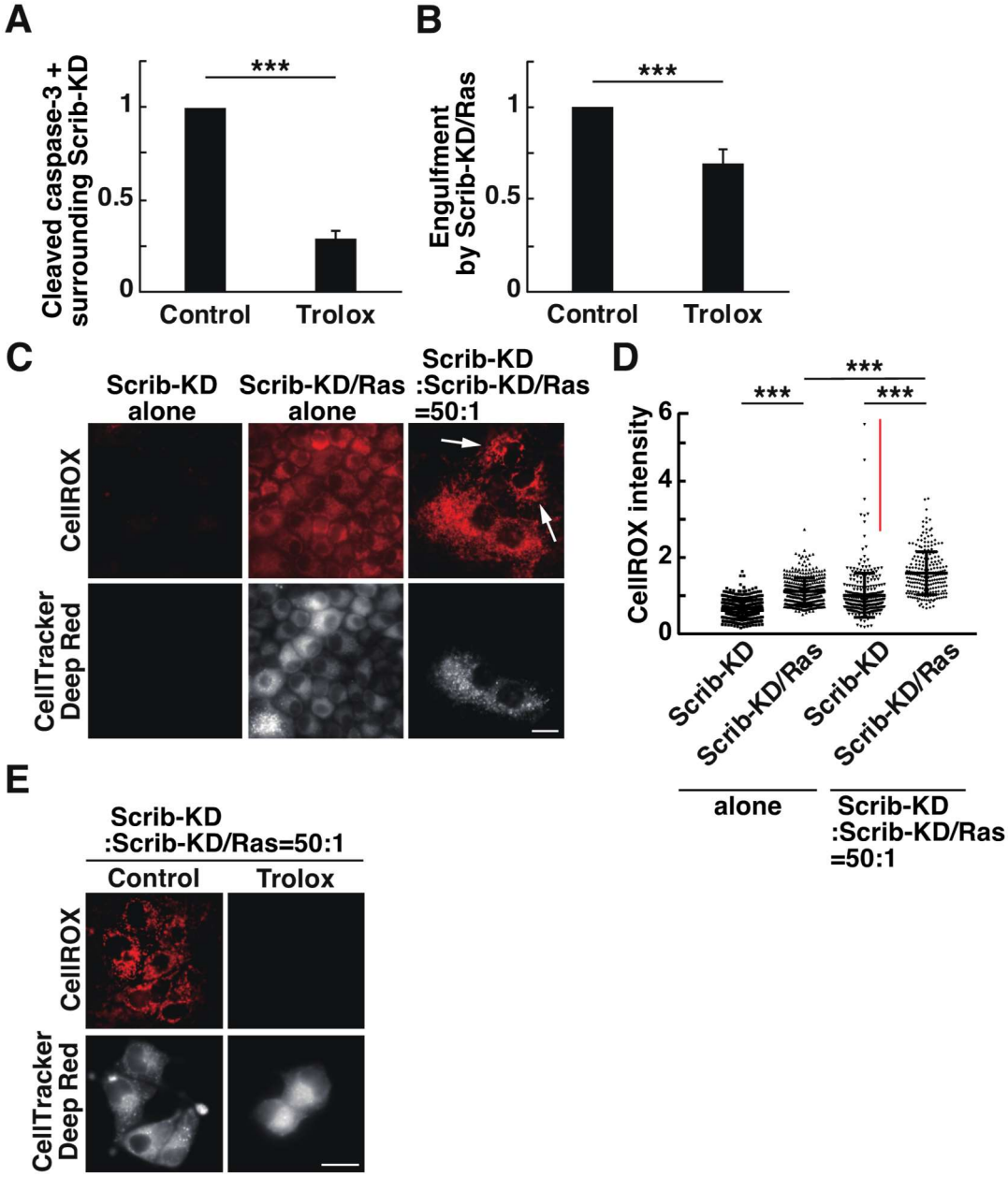
A
Cleaved caspase-3 + surrounding Scrib-KD
***
1
0.5
0
Control
Trolox
B
Engulfment by Scrib-KD/Ras
***
1
0.5
0
Control
Trolox
C
Scrib-KD alone
Scrib-KD/Ras alone
Scrib-KD :Scrib-KD/Ras =50:1
CellROX
CellTracker Deep Red
D
CellROX intensity
***
***
***
6
4
2
0
Scrib-KD
Scrib-KD/Ras
Scrib-KD
Scrib-KD/Ras
alone
Scrib-KD :Scrib-KD/Ras =50:1
E
Scrib-KD :Scrib-KD/Ras=50:1
Control
Trolox
CellROX
CellTracker Deep Red

Figure 2-12. Involvement of ROS in apoptosis or engulfment of the surrounding Scribble-knockdown cells

(A and B) Effect of the ROS scavenger Trolox on apoptosis (A) or engulfment (B) of the surrounding Scribble-knockdown cells. Myc-RasV12 was transiently expressed in a mosaic manner within the monolayer of MDCK-pTR Scribble-shRNA1 cells, and cells were incubated in the absence or presence of Trolox, followed by immunofluorescence with anti-cleaved caspase-3 antibody. Values are expressed as a ratio relative to control. Data are mean ± s.d. from four independent experiments. ***P<0.001 (paired two-tailed Student's *t*-test); n=213, 235 cells. (C and D) The intracellular ROS level in Scribble-KD/RasV12 cells surrounded by Scribble-KD cells. (C) The fluorescence images of CellROX. MDCK-pTR Scribble-shRNA1 cells and MDCK-pTRE3G Myc-RasV12 Scribble-shRNA1 cells (stained with CellTracker Deep Red Dye (grey)) were cultured alone or co-cultured at a ratio of 50:1, followed by incubation with CellROX (red). Arrows indicate the surrounding Scribble-KD cells with the high level of CellROX fluorescence. (D) Quantification of the fluorescence intensity of CellROX. Red bar indicates the surrounding Scribble-KD cells with the high level of CellROX fluorescence. Values are expressed as a ratio relative to normal cells. Data are mean ± s.d. from three independent experiments. ***P<0.001 (one-way ANOVA with Tukey's test); n=360, 358, 302, 232 cells. (E) Effect of Trolox on the intracellular ROS level. MDCK-pTR Scribble-shRNA1 cells were co-cultured with MDCK-pTRE3G Myc-RasV12 Scribble-shRNA1 cells stained with CellTracker Deep Red Dye (grey) at a ratio of 50:1 and were incubated in the absence or presence of Trolox, followed by loading with CellROX (red). (C and E) Scale bars, 20 μm.

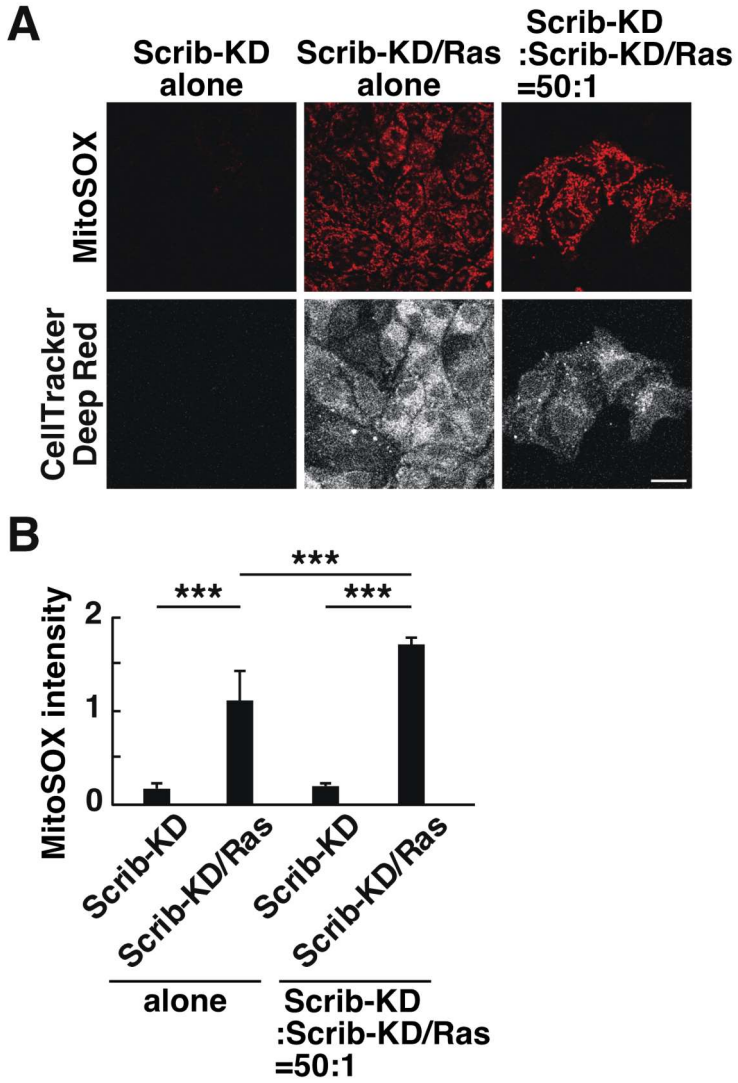

Figure 2-13. Mitochondrial superoxide indicator MitoSOX is profoundly elevated in Scribble-knockdown/RasV12 cells compared with that in Scribble-knockdown cells.

(A and B) Incorporation of MitoSOX in Scribble-knockdown/RasV12 cells surrounded by Scribble-knockdown cells. (A) Fluorescence images of MitoSOX. MDCK-pTR Scribble-shRNA1 cells and MDCK-pTRE3G Myc-RasV12 Scribble-shRNA1 cells (stained with CellTracker Deep Red Dye (grey)) were cultured alone or co-cultured at a ratio of 50:1. Cells were loaded with MitoSOX. (B) Quantification of the fluorescence intensity of MitoSOX. Values are expressed as a ratio relative to normal cells. Data are mean ± s.d. from three independent experiments. ***P<0.001 (one-way ANOVA with Tukey's test); n=300, 299, 261, 224 cells. (A) Scale bar, 20 μm.

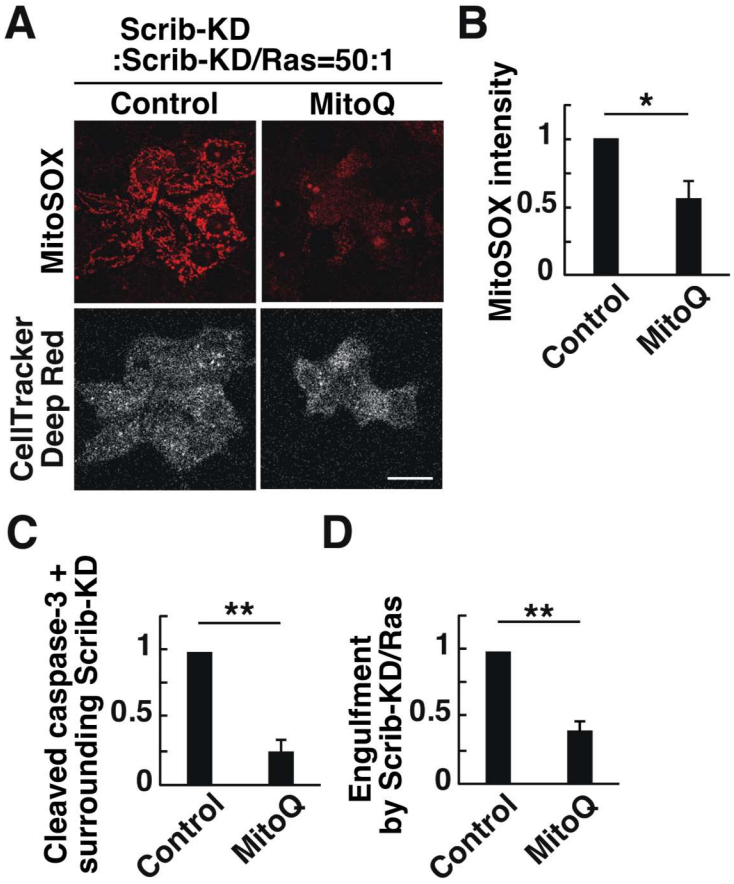

Figure 2-14. Effect of the mitochondrial ROS scavenger Mitoquinone (MitoQ) on MitoSOX, apoptosis or engulfment

(A) Fluorescence images of MitoSOX. MDCK-pTR Scribble-shRNA1 cells were co-cultured with MDCK-pTRE3G Myc-RasV12 Scribble-shRNA1 cells stained with CellTracker Deep Red Dye (grey) at a ratio of 50:1 in the absence or presence of MitoQ, followed by loading with MitoSOX (red). (B) Quantification of the fluorescence intensity of MitoSOX. Values are expressed as a ratio relative to control. Data are mean ± s.d. from three independent experiments. *P<0.05 (paired two-tailed Student's *t*-test); n= 122, 116 cells. (C and D) Effect of MitoQ on apoptosis (C) or engulfment (D) of the surrounding Scribble-knockdown cells. Myc-RasV12 was transiently expressed in a mosaic manner within the monolayer of MDCK-pTR Scribble-shRNA1 cells in the absence or presence of MitoQ. Values are expressed as a ratio relative to control. Data are mean ± s.d. from three independent experiments. **P<0.001 (paired two-tailed Student's *t*-test); n=147, 149 cells. (A) Scale bar, 20 μm.

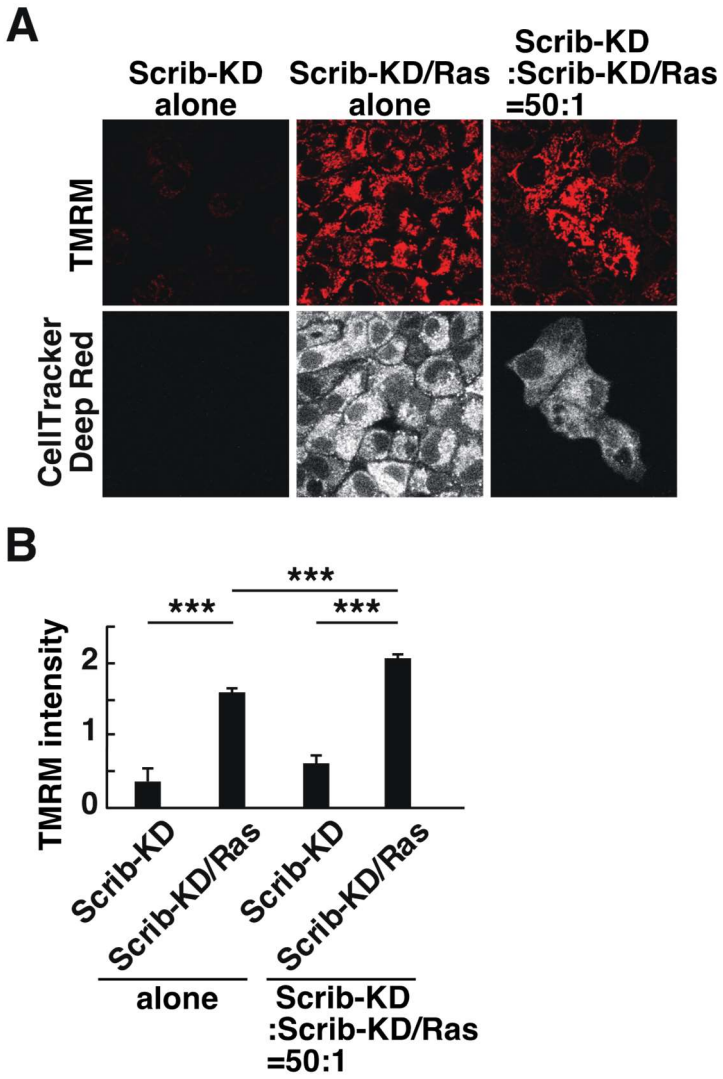

Figure 2-15. Mitochondrial membrane potential is profoundly elevated in Scribble-knockdown/RasV12 cells compared with that in Scribble-knockdown cells.

(A and B) Incorporation of TMRM in Scribble-knockdown/RasV12 cells surrounded by Scribble-knockdown cells. (A) Fluorescence images of TMRM. MDCK-pTR Scribble-shRNA1 cells and MDCK-pTRE3G Myc-RasV12 Scribble-shRNA1 cells (stained with CellTracker Deep Red Dye (grey)) were cultured alone or co-cultured at a ratio of 50:1. Cells were loaded with TMRM (red). (D) Quantification of the fluorescence intensity of TMRM. Values are expressed as a ratio relative to normal cells. Data are mean ± s.d. from three independent experiments. ***P<0.001 (one-way ANOVA with Tukey's test); n=360, 360, 383, 233 cells (D).

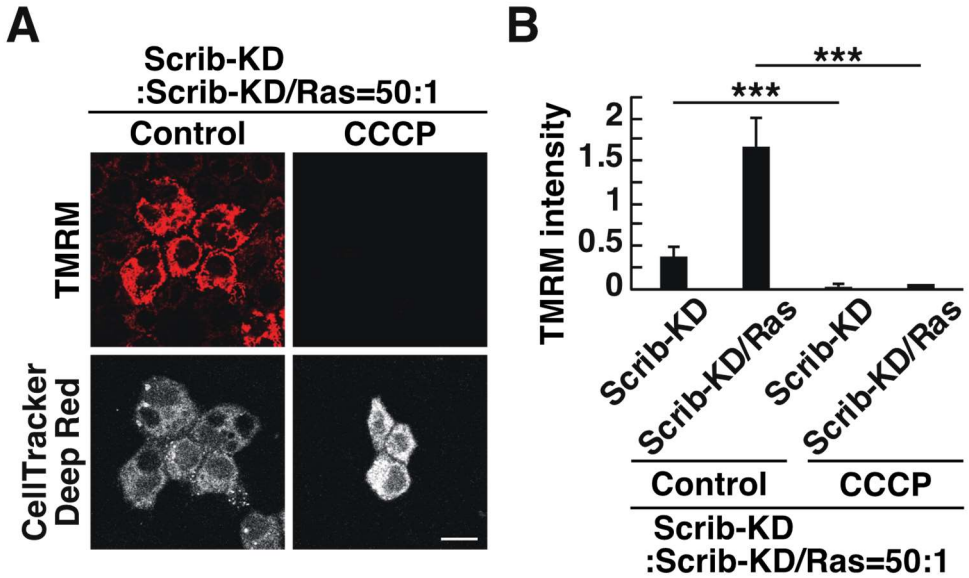

Figure 2-16. Effect of oxidative phosphorylation uncoupler CCCP on TMRM

(A and B) Effect of CCCP on TMRM. (A) Fluorescence images of TMRM. MDCK-pTR Scribble-shRNA1 cells were co-cultured with MDCK-pTRE3G Myc-RasV12 Scribble-shRNA1 cells stained with CellTracker Deep Red Dye (grey) at a ratio of 50:1 in the absence or presence of CCCP, followed by loading with TMRM (red). (B) Quantification of the fluorescence intensity of TMRM. Values are expressed as a ratio relative to normal cells. Data are mean ± s.d. from two independent experiments. ***P<0.001 (one-way ANOVA with Tukey's test); n=137, 142, 119, 91 cells.

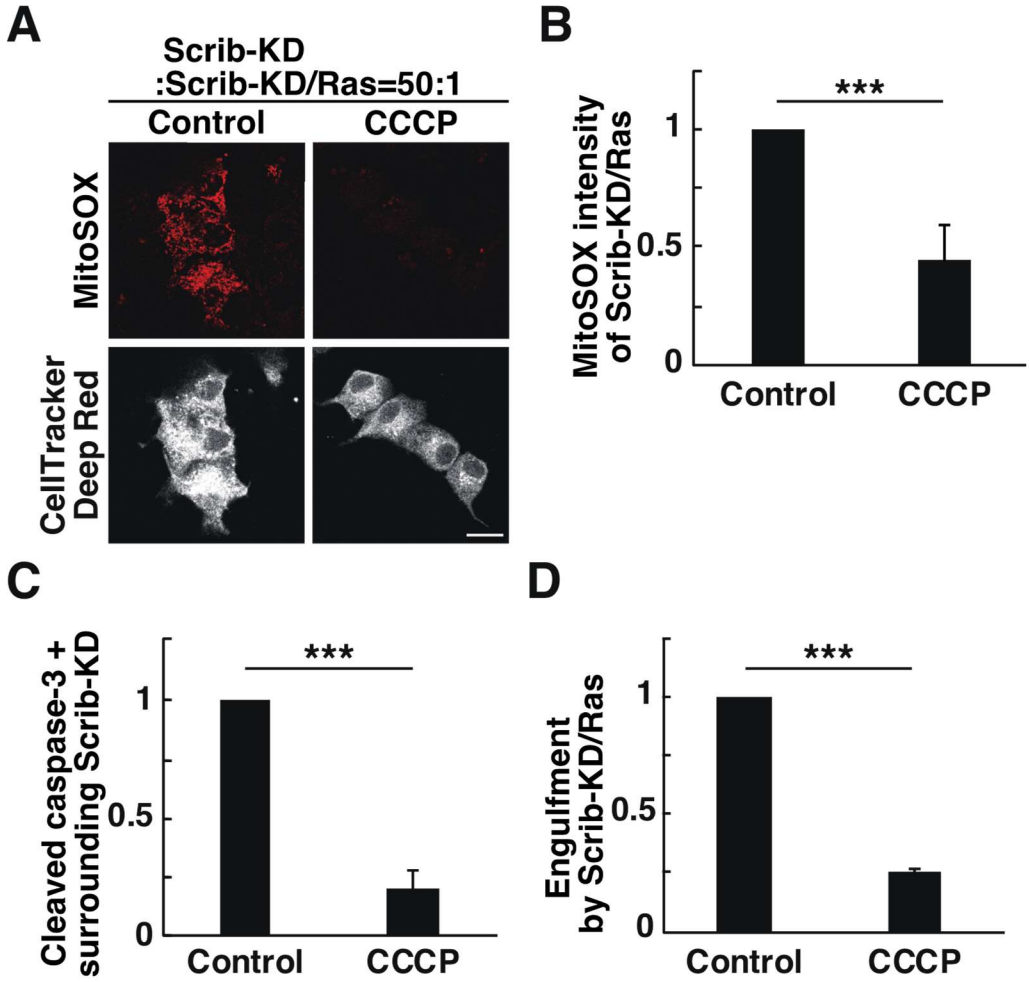

Figure 2-17. Effect of CCCP on MitoSOX, apoptosis or engulfment

(A and B) Effect of the CCCP on MitoSOX. (A) Fluorescence images of MitoSOX. MDCK-pTRE3G Myc-RasV12 Scribble-shRNA1 cells stained with CellTracker Deep Red Dye (grey) were co-cultured with MDCK-pTR Scribble-shRNA1 cells in the absence or presence of CCCP, followed by loading with MitoSOX (red). (B) Quantification of the fluorescence intensity of MitoSOX. Values are expressed as a ratio relative to control. Data are mean ± s.d. from three independent experiments. ***P<0.001 (paired two-tailed Student's *t*-test); n=198, 139 cells. (C and D) Effect of CCCP on apoptosis (C) or engulfment (D) of the surrounding Scribble-knockdown cells. Myc-RasV12 was transiently expressed in a mosaic manner within the monolayer of MDCK-pTR Scribble-shRNA1 cells in the absence or presence of CCCP, followed by immunofluorescence with anti-cleaved caspase-3 antibody. Values are expressed as a ratio relative to control. Data are mean ± s.d. from three independent experiments. ***P<0.001 (paired two-tailed Student's *t*-test); n=150, 149 cells. (A) Scale bar, 20 μm.

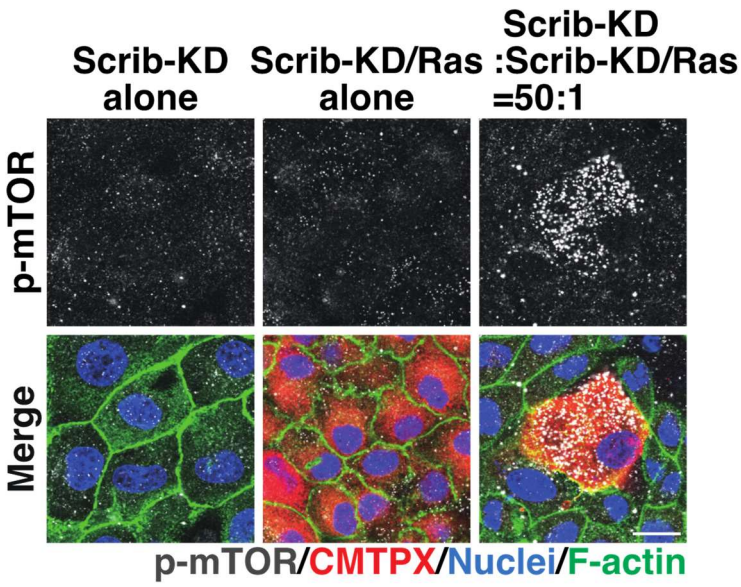

Figure 2-18. Phosphorylation of mTOR is upregulated in Scribble-knockdown/RasV12 cells surrounded by Scribble-knockdown cells.
Immunofluorescence images of phospho-mTOR antibody staining. (A) MDCK-pTR Scribble-shRNA1 cells and MDCK-pTRE3G Myc-RasV12 Scribble-shRNA1 cells (stained with CMTPX (red)) were cultured alone or co-cultured at a ratio of 50:1, followed by immunofluorescence with anti-phospho-mTOR antibody (grey), Alexa-Fluor-488-phalloidin (green), and Hoechst (blue). Scale bar, 20 μm.

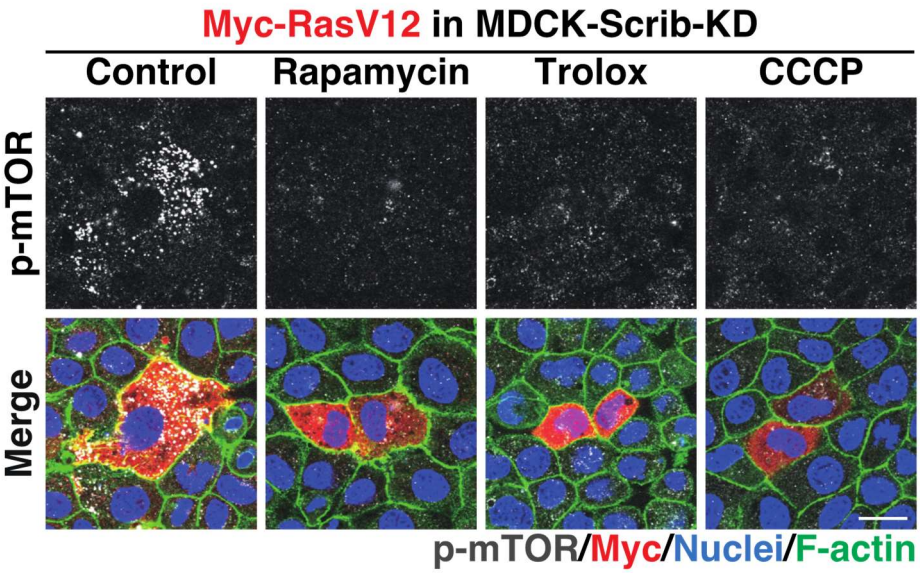

Figure 2-19. Effect of mTOR inhibitor Rapamycin, Trolox or CCCP on phosphorylation of mTOR

Immunofluorescence images of phospho-mTOR antibody staining. Effect of Rapamycin, Trolox, or CCCP on phosphorylation of mTOR. Myc-RasV12 was transiently expressed in a mosaic manner within the monolayer of MDCK-pTR Scribble-shRNA1 cells in the absence or presence of Rapamycin, Trolox, or CCCP, followed by immunofluorescence with anti-phospho-mTOR antibody (grey), anti-Myc-tag antibody (red), Alexa-Fluor-488-phalloidin (green), and Hoechst (blue). Scale bar, 20 μm.

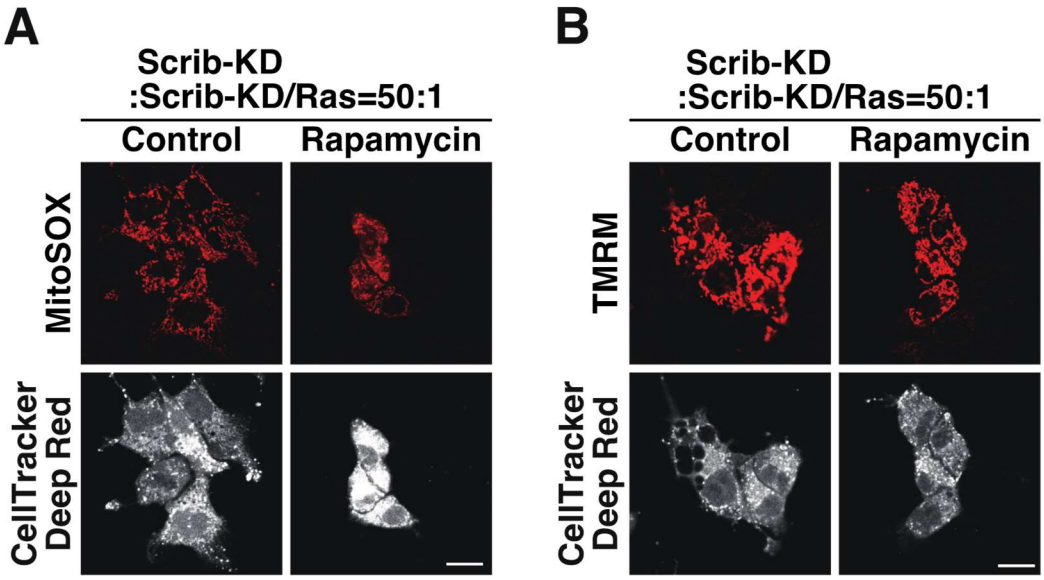

Figure 2-20. The relationship between mitochondrial membrane potential and mTOR pathway

(A and B) Effect of Rapamycin on MitoSOX (A) or TMRM (B). MDCK-pTR Scribble-shRNA1 cells were co-cultured with MDCK-pTRE3G Myc-RasV12 Scribble-shRNA1 cells stained with CellTracker Deep Red Dye (grey) at a ratio of 50:1. Cells were loaded with MitoSOX (red) (A) or TMRM (red) (B). (A and B) Scale bars, 20 μm.

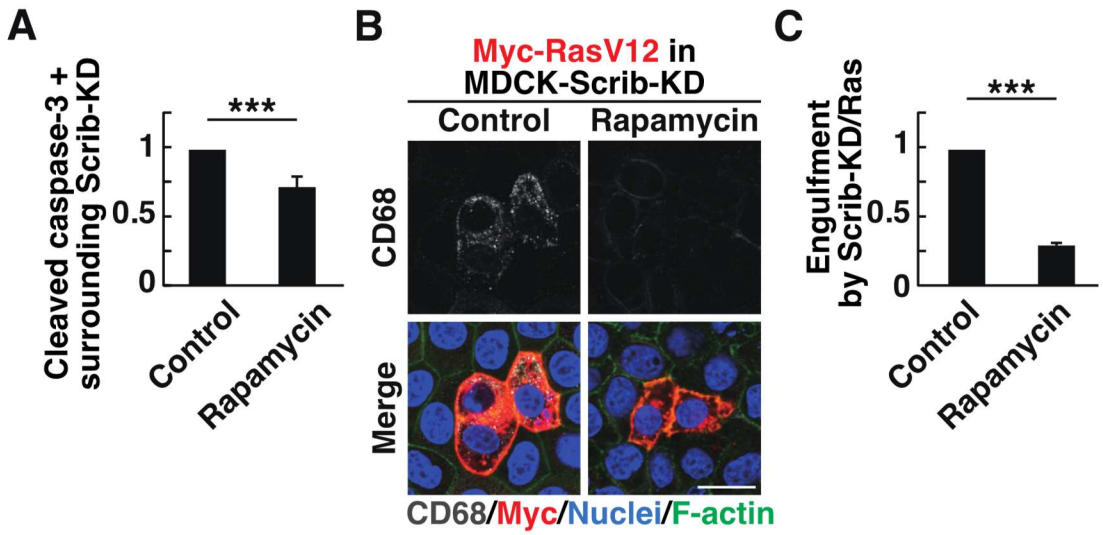

Figure 2-21. Effect of Rapamycin on apoptosis of the surrounding Scribble-knockdown cells, CD68 expression in Scribble-knockdown/Ras cells, or engulfment by Scribble-knockdown/RasV12 cells.

(A and C) Quantification data of apoptosis (A) or engulfment of the surrounding Scribble-knockdown cells (C). Values are expressed as a ratio relative to control. Data are mean ± s.d. from three independent experiments. ***P<0.001 (paired two-tailed Student's *t*-test); n=152, 173 cells. (B) Immunofluorescence images of CD68 antibody staining. Cells were stained with anti-CD68 antibody (grey), anti-Myc-tag antibody (red), Alexa-Fluor-488-phalloidin (green), and Hoechst (blue). (B) Scale bar, 20 μm.

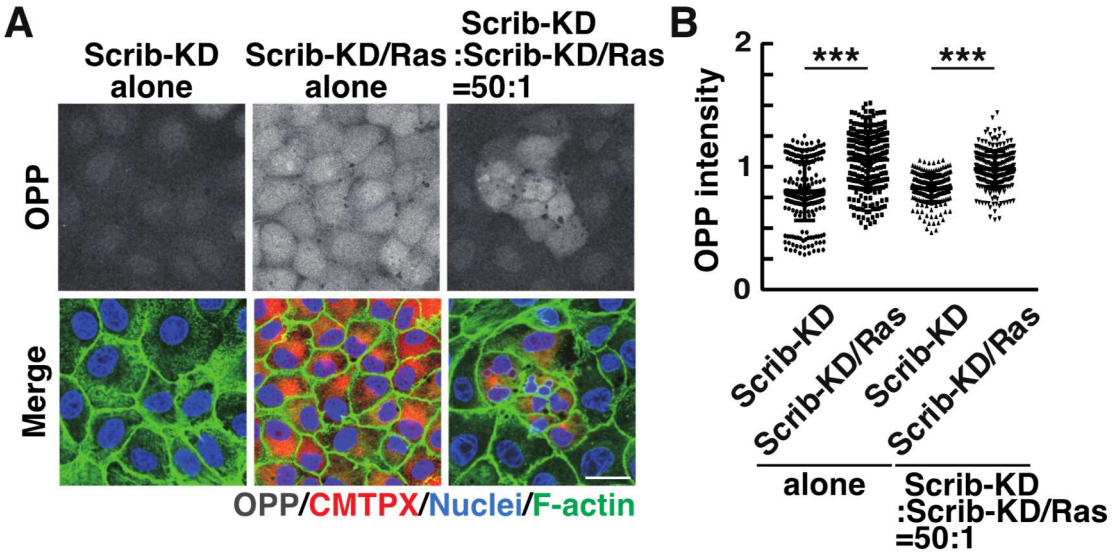

Figure 2-22. Protein synthesis ability is elevated in Scribble-knockdown/RasV12 cells compared with that in Scribble-knockdown cells.

(A) Immunofluorescence images of OPP protein synthesis assay. MDCK-pTR Scribble-shRNA1 cells and MDCK-pTRE3G Myc-RasV12 Scribble-shRNA1 cells (stained with CMTPX (red)) were cultured alone or co-cultured at a ratio of 50:1, and were stained with OPP (grey), Alexa-Fluor-488-phalloidin (green), and Hoechst (blue). (B) Quantification of the fluorescence intensity of OPP. Values are expressed as a ratio relative to normal cells. Data are mean ± s.d. from two independent experiments. ***P<0.001 (one-way ANOVA with Tukey's test); n=259, 271, 237, 277 cells. (B) Scale bar, 20 μm.

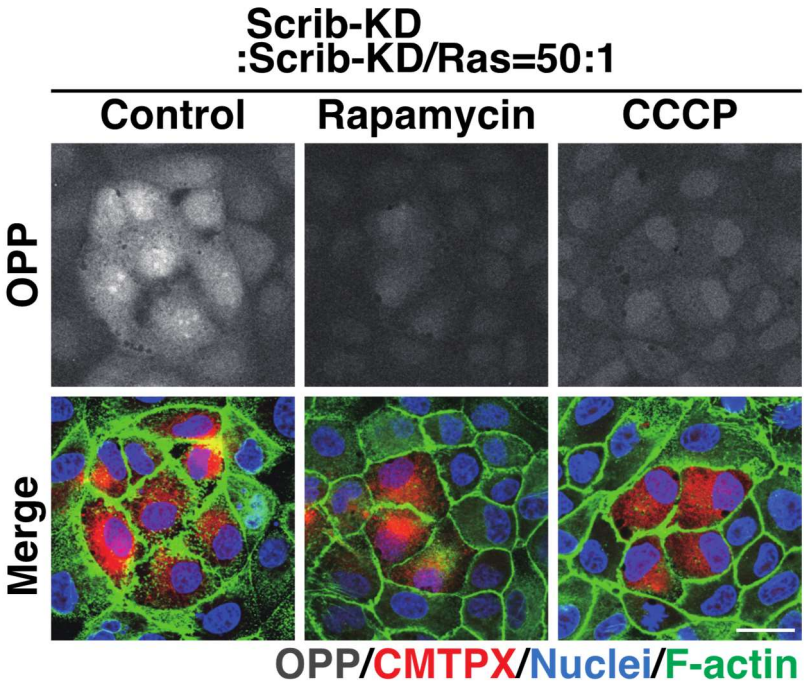

Figure 2-23. Effect of Rapamycin and CCCP on protein synthesis ability

Immunofluorescence images of OPP protein synthesis assay. MDCK-pTR Scribble-shRNA1 cells and MDCK-pTRE3G Myc-RasV12 Scribble-shRNA1 cells (stained with CMTPX (red)) were cultured alone or co-cultured at a ratio of 50:1, and were stained with OPP (grey), Alexa-Fluor-488-phalloidin (green), and Hoechst (blue).

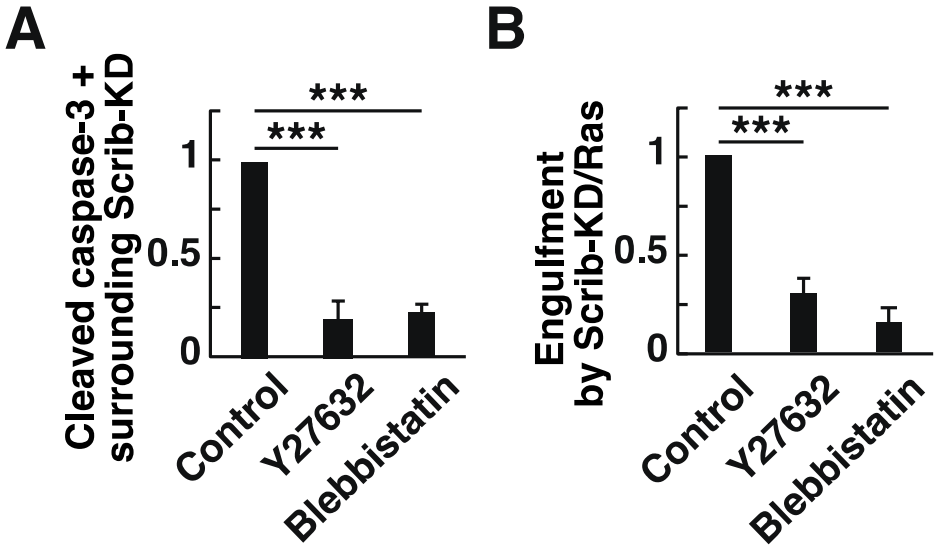

Figure 2-24. Effect of Rho-kinase inhibitor Y27632 and Myosin-Ⅱ inhibitor on apoptosis of the surrounding Scribble-knockdown cells or engulfment by Scribble-knockdown/RasV12 cells.

(A and B) Quantification data of apoptosis (A) or engulfment of the surrounding Scribble-knockdown cells (B). Values are expressed as a ratio relative to control. Data are mean ± s.d. from three independent experiments. ***P<0.001 (paired two-tailed Student's *t*-test); n=164, 149, 164 cells.

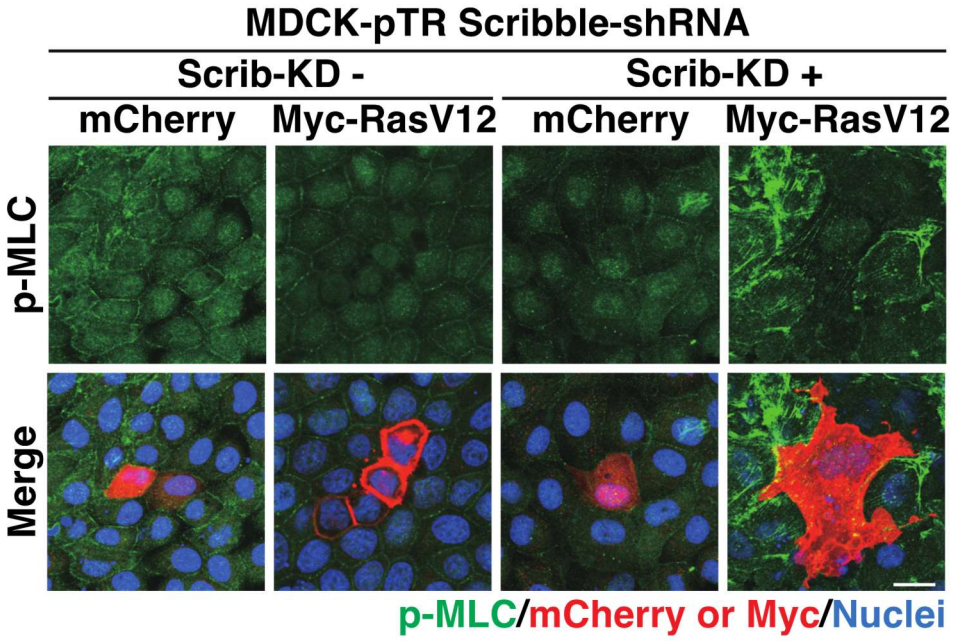

Figure 2-25. Phosphorylation of myosin light chain is profoundly elevated in the surrounding Scribble-knockdown cells.

Immunofluorescence images of phospho-myosin light chain 2 antibody staining. mCherry or Myc-RasV12 was transiently expressed in a mosaic manner within the monolayer of MDCK-pTR Scribble-shRNA1 cells in the absence or presence of tetracycline, followed by immunofluorescence with anti-phosphor-myosin light chain 2 antibody (green), anti-Myc-tag antibody (red), and Hoechst (blue). Scale bar, 20 μm.

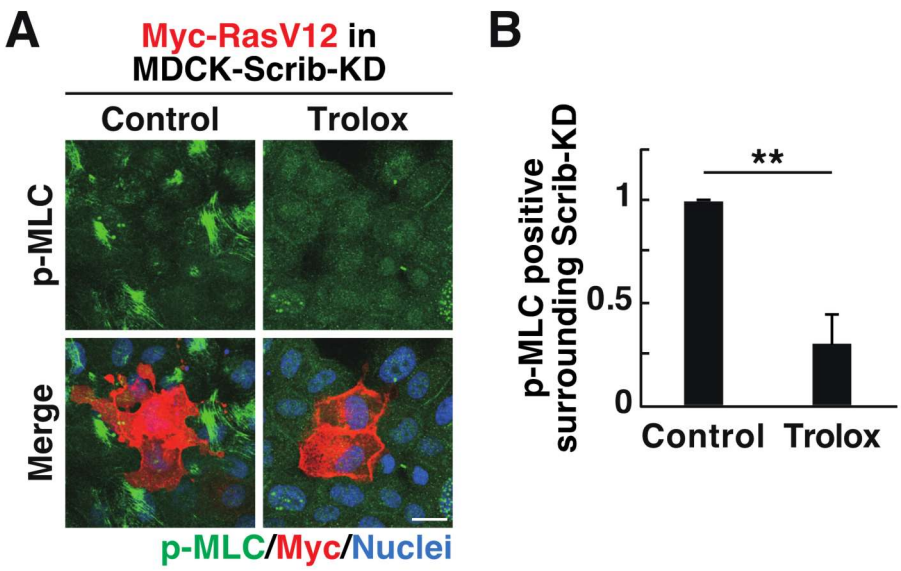

Figure 2-26. Effect of Trolox on phosphorylation of myosin light chain in the surrounding Scribble-knockdown cells

Effect of Trolox on phosphorylation of myosin light chain 2. (A) Immunofluorescence images of phosphor-myosin light chain 2 antibody staining. Myc-RasV12 was transiently expressed in a mosaic manner within the monolayer of MDCK-pTR Scribble-shRNA1 cells in the absence or presence of Trolox, followed by immunofluorescence with anti-phospho-myosin light chain 2 antibody (green), anti-Myc-tag antibody (red), and Hoechst (blue). (B) Quantification data of phospho-myosin light chain 2 positive surrounding Scribble-knockdown cells. Values are expressed as a ratio relative to control. Data are mean ± s.d. from three independent experiments. **P<0.001 (paired two-tailed Student's *t*-test); n=352, 307 cells. (A) Scale bar, 20 μm

2.3. Discussion

A wealth of studies in *Drosophila* and mammals has shown that cell competition can occur between normal and various types of transformed cells at the initial stage of carcinogenesis (Amoyel and Bach, 2014; Baker, 2011; Claveria and Torres, 2016; Di Gregorio et al., 2016; Johnston, 2009; Maruyama and Fujita, 2017; Merino et al., 2016; Morata and Ballesteros-Arias, 2015; Wagstaff et al., 2013). At the interface with normal cells, transformed cells are often eliminated from epithelia via apical extrusion or apoptosis. On the other hand, certain types of transformed cells, such as Myc or Yes-associated protein (YAP)/Yorkie, outcompete normal cells and fill the vacant spaces, a process called super-competition (de la Cova et al., 2004; Mamada et al., 2015; Moreno and Basler, 2004; Neto-Silva et al., 2010; Ziosi et al., 2010). Recent studies have demonstrated that several environmental factors can affect the occurrence of cell competition such as obesity, inflammation, and calorie restriction (Bruens et al., 2020; Sasaki et al., 2018; Sato et al., 2020). Here, I demonstrate that sequential oncogenic mutations can also influence the cell competition phenotype. Under the wild-type background, newly emerging RasV12-transformed cells are often eliminated from epithelia (Hogan et al., 2009; Kon et al., 2017; Sasaki et al., 2018). In contrast, under the Scribble-knockdown background, RasV12-expressing cells induce apoptosis and

engulfment of the adjacent cells, representing the super-competition-like phenotype. Thus, the fate of RasV12-mutant cells can be converted from loser to winner depending on the preceding mutant background. At the mid-stage of carcinogenesis, when an additional mutation occurs within precancerous lesions, the newly mutated cells might replace the preexisting benign cells by cell competition, leading to clonal expansion and facilitating cancer development (Figure 2-27). Moreover, when Scribble knockdown occurs under the RasV12 mutant background, the RasV12/Scribble-KD double-mutant cells are eliminated from the monolayer of Ras-transformed epithelia, implying that the order of oncogenic mutations, which mutations occur first or second, is another factor that can affect the occurrence of cell competition. During the development of human malignant tumors such as colon and pancreatic cancer, the oncogenic mutations are inclined to accumulate in a certain order, which varies between tissues. It is plausible that cell competition is involved in this process of cancer evolution, but further *in vivo* studies on various combinations of oncogenic mutations would be required to clarify this issue.

In this study, I show that Scribble-KD/RasV12 double-mutant (winner) cells have increased mitochondrial membrane potential, which acts upstream of the ROS and mTOR pathways, playing a crucial role in the cell competition phenotypes (Figure 2-

28). The difference in mitochondrial activity between winner and loser cells has been also demonstrated in other studies on cell competition (Kon et al., 2017). In early mouse embryos, loser epiblast cells have lower mitochondrial membrane potential, and manipulating mitochondrial function alone is sufficient to cause cell competition. In contrast, in MDCK cells, modulation of mitochondrial membrane potential alone by knockdown of pyruvate dehydrogenase does not induce cell competition (Kon et al., 2017), implying that, at least in the cell culture model, additional factors are also involved in the induction of cell competition between single- and double-mutant cells. Another intriguing, phenotypic feature of Scribble-KD/Ras cells is profound phagocytic activity; the greedy winner cells engulf either dead or live loser cells. The comparable 'cell-in-cell' structures are also observed in another cell culture system with human cells (Bartosh et al., 2016; Sun et al., 2014) as well as in histopathological analyses of various types of human cancer (Fais and Overholtzer, 2018). The appearance of cell-in-cell structures in cancer, also termed tumor cell cannibalism, is correlated to poor prognosis, thus being a potential therapeutic target. Our cell culture system would be a suitable experimental model to elucidate the underlying molecular mechanisms of tumor cannibalism during cancer development.

As shown in Figure 2-5, I have often observed that Scribble-KD cells undergo cell death upon the contact with the tip of protrusions from Scribble-KD/RasV12 cells, which I term 'death touch' (Figure 2-28). Suppression of the protrusion formation by expression of dominant-negative Rac1 diminishes the number of cleaved caspase-3-positive Scribble-KD cells, supporting a role for protrusions in the induction of apoptosis. According to the immunofluorescence analysis with anti-Fas or anti-TNF antibody, these extrinsic apoptosis-inducers are not accumulated on the protrusions from Scribble-KD/RasV12 cells (unpublished observations). The intracellular ROS level is increased in some of the neighboring Scribble-KD cells, and treatment with Trolox suppresses cell death. Therefore, one possibility is that the death touch of protrusions from winner cells somehow induces the temporal ROS elevation in loser cells, causing apoptotic cell death. Another important player is mTOR (Figure 2-28). The mTOR activity is non-cell-autonomously elevated in Scribble-KD/RasV12 cells surrounded by Scribble-KD cells, and treatment with Rapamycin significantly suppresses apoptosis, indicating that the elevated mTOR activity in winner cells positively regulates cell death of loser cells. And I also show that protein synthesis ability of Scribble-KD/RasV12 cells is higher than that of Scribble-KD cells. Previous reports also showed that the difference in the mTOR activity can be involved in cell

competition (Bowling et al., 2018; Sanaki et al., 2020), though the underlying molecular mechanism still remains elusive.

As shown in Figure 2-25, phosphorylation of myosin light chain is upregulated in the surrounding Scribble-KD cells, but not Scribble-KD/RasV12 cells. This study shows that Scribble-KD/RasV12 double-mutant cells can engulf Scribble-KD single-mutant cells via entosis. Entosis is a process by which a live cell is ingested by a neighboring cell, presenting a characteristic “cell-in-cell” phenotype. During entotic event, the mechanical property of cells profoundly affects the fate of decision. Previous report shows that cells with higher tension tend to become losers and are engulfed by winner cells with lower tension. Moreover, another report shows that entotic cells provide the key driving force, through actin polymerization and myosin contraction, to promote the ingestion process itself. Therefore, upregulation of phosphorylation of myosin light chain in the surrounding Scribble-KD cells may promote tension, and entotic event. But, in this study, I cannot show the direct relationship between Rho activation and entotic event.

Further studies will be required to elucidate the functional mode of death touch, mTOR pathway, and the Rho pathway, which would open new avenues for therapeutic strategy that blocks the clonal expansion of more malignant populations within tumors.

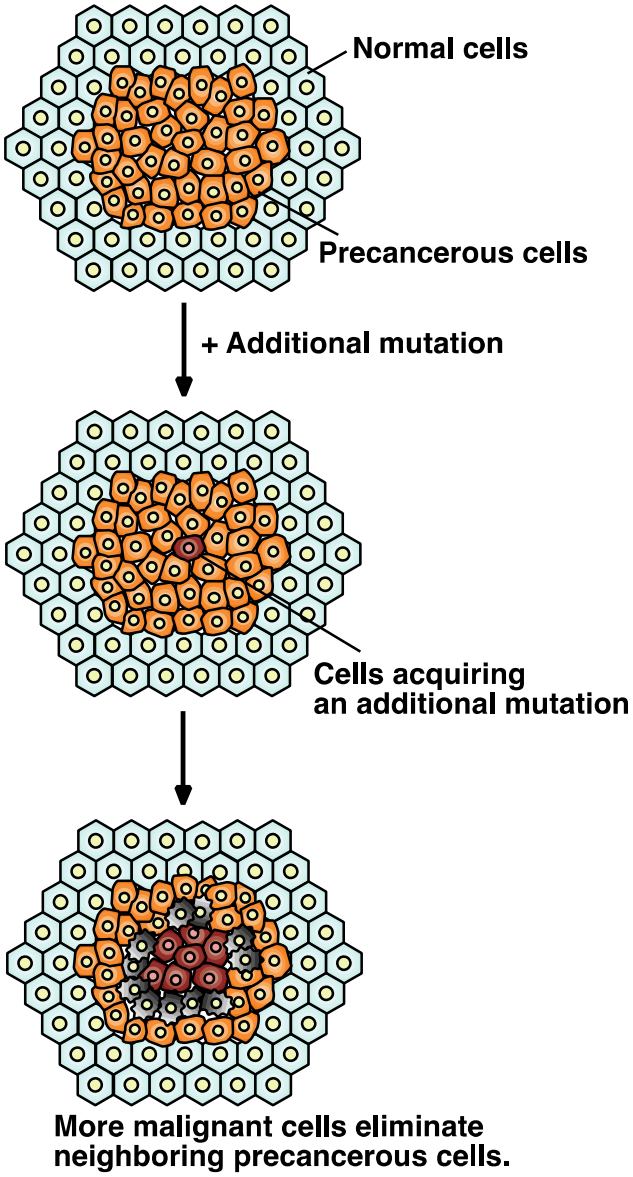

Figure 2-27. A schematic model for cell competition-mediated cancer progression driven by sequential oncogenic mutations.

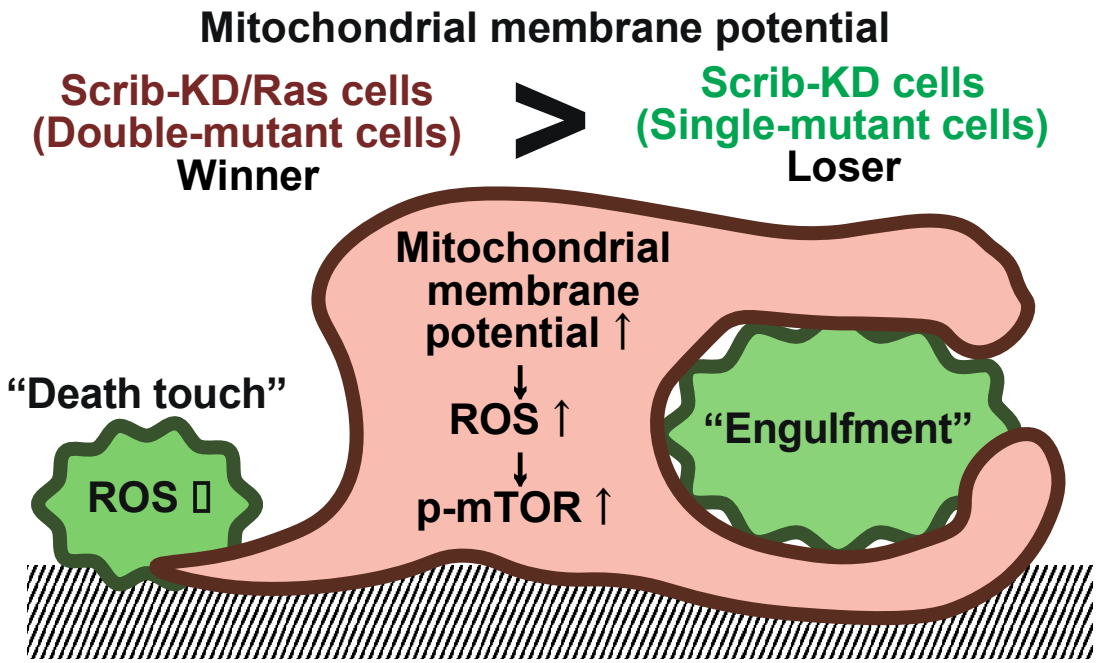

Figure 2-28. A schematic model for molecular mechanisms of sequential oncogenic mutations-induced cell competition between Scribble-knockdown/RasV12 (double-mutant) cells and Scribble-knockdown (single-mutant) cells.

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3. Conclusion

Previously, our group reported that normal tissue has the ability to sense the cancerous cells and eliminate them from epithelia by cell competition. Furthermore, normal epithelial cells actively eliminate mutant cell by generating force to squeeze them, which termed EDAC (Epithelial defense against cancer). During cancer development, multiple oncogenic mutations accumulate within epithelial tissues. However, it remains elusive whether and how cell competition is also involved in this process.

In this study, using a mammalian cell culture model system, I revealed that sequential oncogenic mutations can induce cell competition between single- and double-transformed cells and that the fate of transformed cells can be converted from loser to winner depending on the preceding mutant background.

Taken together, these results imply a notion that cell competition could be involved not only in the initial stage of carcinogenesis but also in cancer progression. At the mid-stage of carcinogenesis, when an additional mutation occurs within precancerous lesions, the newly mutated cells might replace the preexisting benign cells by cell competition, leading to clonal expansion and facilitating cancer development. To our knowledge, this is the first report demonstrating that sequential oncogenic mutations can affect cell competition.