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(Citation)
Biochemical and Biophysical Research Communications,543:15-22

(Issue Date)
2021-03-05

(Resource Type)
journal article

(Version)
Accepted Manuscript

(URL)
https://hdl.handle.net/20.500.14094/90007984

Biochemical and Biophysical Research Communications

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--Manuscript Draft--

Manuscript Number:	
Article Type:	Short communication/Research article
Keywords:	Ras; myosin II; NRK-52E; cancer cell invasion; basal extrusion
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- Ras-transformed NRK-52E cells are basally extruded when mixed with normal cells
- Basal extrusion is accompanied by active migration of cancer cells
- Basal protrusion and migration occur in a non-cell-autonomous manner
- PI3K and myosin II play essential roles in the invasive behavior of cancer cells

Non-cell-autonomous migration of RasV12-transformed cells towards the basal side of surrounding normal cells

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Abbreviations: NRK-52E, normal rat kidney 52E

The number of words and characters are as follows;

Characters in the title: 108 (including space)

Abstract: 120 words

The length of the manuscript (including references and figure legends): 4,186 words

Abstract

Oncogenic transformation enables cells to behave differently from their neighboring normal cells. Both cancer and normal cells recognize each other, often promoting the extrusion of the former from the epithelial cell layer. Here, we show that RasV12-transformed normal rat kidney 52E (NRK-52E) cells are extruded towards the basal side of the surrounding normal cells, which is concomitant with enhanced motility. The active migration of the basally extruded RasV12 cells is observed when surrounded by normal cells, indicating a non-cell-autonomous mechanism. Furthermore, specific inhibitor treatment and knockdown experiments elucidate the roles of PI3K and myosin IIA in the basal extrusion of Ras cells. Our findings reveal a new aspect of cancer cell invasion mediated by functional interactions with surrounding non-transformed cells.

Keywords: Ras, myosin II, NRK-52E, cancer cell invasion, basal extrusion

Introduction

The epithelium is one of the cellular compartments known for fast cell turnover in the body. Continuous rapid cell division increases the probability of mutations. Most human cancers arise from single epithelial cells that acquire mutations in oncogenes or tumor suppressor genes [1]. For transformed cells to escape elimination and progress to malignancy, they must acquire migration and invasion properties to metastasize to other tissues. Metastasis of tumor cells within the human body is one of the most dangerous pathological events that leads to mortality [2]. Therefore, extensive studies have been conducted to better understand the molecular mechanisms of cancer cell migration and invasion. Recent studies have focused on the role of interaction between transformed cells and the microenvironment comprising non-transformed cells [3–5].

One of the most known mutations that initiate cancer transformation is the mutation of the GTPase Ras. The activation of oncogenic Ras facilitates all aspects of malignancy, including cell proliferation and cell invasion [6]. Studies on the interaction between Ras-transformed epithelial cells and surrounding normal cells showed that the majority of transformed cells

were apically extruded from the epithelial monolayer in an apoptosis-independent manner [7]. However, a minority of the transformed Ras cells were basally extruded [7]. These Ras cells formed protrusions and showed a phenotype favoring cell invasion.

In this study, we show that NRK-52E cells expressing RasV12 are extruded to the basal side of normal NRK-52E cells when co-cultured. The basally extruded Ras cells exhibit motility only when surrounded by normal NRK-52E cells, indicating a non-cell-autonomous mechanism. We also demonstrate that the knockdown of myosin IIA and the inhibition of PI3K suppress basal extrusion of Ras cells. Decreased motility of myosin IIA-depleted Ras cells is correlated with the apical extrusion of cancer cells from normal cells.

Materials and methods

Reagents and antibodies

Rabbit anti-Akt (pan), rabbit anti-phospho Akt (T308), rabbit anti-SAPK/JNK, rabbit anti-phospho SAPK/JNK (T183/Y185), rabbit anti-p38, rabbit anti-phospho p38 (T180/Y182), rabbit anti-p44/42 MAPK (ERK1/2), and rabbit anti-phospho p44/42 MAPK (T202/Y204) were from Cell Signaling. Other antibodies were purchased as follows; rabbit anti-non-muscle myosin IIA (Abcam), mouse anti-Myh10 (myosin IIB, SantaCruz Biotechnology), mouse anti-E-cadherin (BD Transduction Laboratories), and rabbit anti-β-actin (MBL Life science). Acti-stain555 were from Cytoskeleton, Inc. NucBlue Fixed Cell ReadyProbes Reagent (DAPI) were from ThermoFisher Scientific. All of the inhibitors were from Sigma-Aldrich and used at the following concentrations: z-VAD (50 μM), blebbistatin (50 μM), LY294002 (25 μM), SP600125 (10 μM), PD98059 (10 μM), SB202190 (10 μM).

Cell culture

Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Nacalai Tesque) supplemented with 10% fetal bovine serum (Sigma-Aldrich). NRK-52E cell line was obtained from the Japanese Collection of Research Bioresources (JCRB). GP2-293 cell line

was purchased from ThermoFisher Scientific.

Retroviral expression and stable cell lines

To establish NRK-52E cells that express GFP or GFP-RasV12 in an inducible manner, Retro-X Tet-One Inducible Expression System was used (Takara bio). Briefly, GP2-293 cells were simultaneously transfected with pVSV-G and pRetroX-TetOne-Puro plasmid containing GFP or GFP-RasV12 for 48 h. Retroviral supernatant was added to NRK-52E cells with 4 µg/ml of polybrene. Serial dilutions were performed for the selection of stable cell lines in the presence of 1 µg/ml puromycin. Expression of GFP or GFP-RasV12 was induced by 0.4 µg/ml doxycycline.

RNA interference

SMARTpool-ON-TARGETplus Rat Myh9 and Myh10 siRNAs from Dharmacon were used to knockdown myosin IIA and IIB, respectively. Cells were transfected with 25 nM siRNAs using Lipofectamine RNAiMAX reagent (Life Technologies) for 72 h, and the expression levels of myosin IIA and IIB were assessed by western blotting.

Western blotting

Cells were washed twice with PBS, lysed in a lysis buffer, briefly sonicated, and boiled at

95°C for 5 min. Proteins were then separated by SDS-polyacrylamide gel electrophoresis and then transferred to PVDF membrane using the iBlot gel transfer device (ThermoFisher Scientific). After blocking with 5% milk in TBS-T at room temperature (RT) for 1 h, the membrane was incubated with primary antibody overnight at 4°C. The membrane was further incubated with HRP-conjugated secondary antibodies at RT for 1 h, and detected by Chemi-Lumi One L (Nacalai Tesque). Quantification was carried out using ImageQuant LAS 4000 mini (GE Healthcare).

Immunofluorescence and microscopy

Cells grown on a glass-bottom dish with or without collagen-coating were fixed with 4% paraformaldehyde (PFA)/PBS for 15 min at RT, washed three times in PBS, and permeabilized in 0.5% Triton X-100/PBS for 20 min. Cells were then washed three times in PBS and incubated in a blocking buffer (1% BSA in PBS) for 1 h. Nucleus and F-actin were stained with DAPI and Acti-stain 555, respectively. Immunofluorescence microscopy images were obtained using a laser confocal microscope (Olympus Fluoview FV1000) equipped with 405, 473, and 559 nm diode lasers through an objective lens (×60 silicone oil objective, numerical aperture = 1.30, ×100 oil immersion objective, numerical aperture = 1.40).

Collagen assays

Type-I collagen (Cultrex Rat Collagen I) was neutralized on ice according to the manufacturer's instructions. 35 mm Glass bottom culture dishes (Iwaki) were coated with 50 µl of neutralized collagen then incubated for 40 min at 37°C to solidify. Around 3×10^5 cells were plated per well on top of the collagen gel. NRK-52E RasV12 cells were mixed with normal cells at a ratio of 1:100. After incubation for 24 h at 37°C, doxycycline with or without inhibitors was added to induce RasV12 expression. Cells were incubated for 48 h and fixed with PFA. In the case of knockdown experiments, 72 h after siRNA transfection, RasV12-expressing cells were trypsinized, mixed with normal cells, and seeded on collagen.

Time-lapse microscopy

NRK-52E cells stably expressing GFP or GFP-RasV12 were mixed with normal cells at a ratio of 1:100 and plated at a density of 2×10^5 cells per well in 35 mm glass-bottom culture dishes (Iwaki). Mixed cells were incubated for 24 h at 37°C before transferred to a doxycycline-containing FluoroBrite medium (ThermoFisher Scientific). Where indicated, cells were analyzed in the presence of z-VAD or LY249002. To obtain time-lapse images, a Keyence BZ-X710 All-in-One Fluorescence Microscope was used. Images were captured

and analyzed using BZ-X viewer and BZ-X Analyzer (Keyence). To calculate the velocity, cells were tracked using the manual tracker plug-in in Fiji and analyzed using chemotaxis and migration tool by ibidi.

Statistical Analysis:

GraphPad Prism 8 was used for statistical analysis. Statistically significant differences were determined using student's t-test or ordinary one-way ANOVA for multiple comparisons. P-value < 0.05 was considered statistically significant.

Results

The motility of RasV12-expressing NRK-52E cells is enhanced when surrounded by normal cells

We established normal rat kidney 52E (NRK-52E) epithelial cells that expressed GFP (GFP cells) or GFP-RasV12 (RasV12 cells) in a doxycycline-inducible manner (see Materials and methods). Time-lapse microscopy revealed that cell–cell contact was disrupted as GFP-RasV12 was expressed (Fig. S1A). Consistently, the protein level of E-cadherin was significantly decreased in RasV12 cells as evidenced by western blotting (Fig. S1B, C).

Next, the migratory behavior of RasV12 cells was assessed in the presence of normal cells surrounding them. To this end, GFP (as a control) or RasV12 cells were mixed with normal NRK-52E cells at a ratio of 1:100 and cultured to confluency on the next day, when doxycycline was added to induce the expression of GFP-RasV12. The behavior of RasV12 cells was monitored using time-lapse microscopy. It was observed that according to the expression of GFP-RasV12 (between 4 and 8 hours after doxycycline addition), RasV12 cells exhibited movement among the neighboring normal NRK-52E cells, which was concomitant

with active membrane protrusions (Fig. 1A–C, Movie 1, and 2). As a control, GFP cells that were cultured with normal NRK-52E cells did not show any abnormal morphology or behavior (Fig. 1A–C and Movie 3). Intriguingly, when RasV12 cells were cultured alone, their motility was significantly suppressed (Fig. 1B, C, Fig. S1A and Movie 4), indicating a non-cell-autonomous migration of RasV12 cells enhanced by the surrounding normal cells.

To confirm whether a cell density close to confluency (3.3×10^4 WT cells/cm^2 vs. 3.3×10^2 RasV12 cells/cm^2) was essential for occurrence of this behavior, RasV12 cells were plated with normal NRK-52E cells at a lower density (1.1×10^3 WT cells/cm^2 vs. 1.1×10 RasV12 cells/cm^2), and time-lapse images were acquired. The results showed that when RasV12 cells resided at the edge of normal NRK-52E cell islands, they merely migrated, without any apparent morphological changes. However, once RasV12 cells were entirely surrounded by normal NRK-52E cells, the cancer cells exhibited movement with robust membrane protrusions even when plated at a lower density (Fig. S2 and Movie 2). This observation also confirms the non-cell-autonomous nature of this migration.

We observed that RasV12 cells were often fragmented when migrating among normal NRK-52E cells (Fig. S2 and Movie 2). To examine if this fragmentation occurred due to apoptosis, cells were mixed and then treated with doxycycline in the presence of the caspase inhibitor z-VAD. Neither fragmentation nor motility of RasV12 cells was blocked (Fig. S3 and Movie 5), indicating that this phenomenon occurred in an apoptosis-independent manner.

Collectively, these data demonstrate that RasV12-expressing NRK-52E cells show increased cell motility only when surrounded by normal NRK-52E cells.

RasV12-expressing NRK-52E cells are basally extruded from surrounding normal cells

RasV12-expressing Madin-Darby canine kidney (MDCK) cells are apically extruded when surrounded by normal MDCK cells [7–9]. To check whether the motility of RasV12-expressing NRK-52E cells occurred beneath or above the epithelial monolayer, RasV12 cells were mixed with normal NRK-52E cells, and plated on collagen gel. The xz images were obtained by confocal microscopy. Interestingly, most of the RasV12-expressing NRK-52E cells were found beneath the layer of normal NRK-52E cells (Fig. 2A, B). When RasV12-

expressing NRK-52E cells were plated alone, they formed a monolayer or a cluster of cells (Fig. 2A). These results indicate that RasV12-expressing NRK-52E cells are basally extruded from the epithelial monolayer to become motile.

Involvement of PI3K pathway in the basal extrusion of RasV12-expressing NRK-52E cells

Both PI3K/Akt and MAPK function downstream of the Ras signaling pathway. To understand the molecular mechanism of basal protrusion by RasV12-expressing NRK-52E cells, western blot analyses were performed using phospho-specific antibodies. As a result, we found that the phosphorylation of Akt, JNK, and ERK1/2 was significantly increased when RasV12 was expressed (Fig. 3A, B). This indicated that either of these pathways might be involved in the basal extrusion and motility of RasV12-expressing cells.

To determine the identity of the pathway that was essential for basal extrusion, RasV12 cells were mixed with normal NRK-52E cells, plated on collagen gel, and then treated with kinase inhibitors. Interestingly, treatment with the PI3K inhibitor LY294002 significantly reduced

196 basal extrusion and increased the apical extrusion of RasV12 cells (Fig. 3C–E, Movie 6, and
197 7). On the other hand, treatment with the JNK inhibitor SP600125, MEK inhibitor PD98059,
198 or p38 inhibitor SB202190 did not show any effects on the behavior of RasV12 cells (Fig.
199 3C). These data suggest that the PI3K/Akt pathway is involved in the basal extrusion of
200 RasV12-expressing NRK-52E cells.
201
202 **Myosin IIA is essential for basal extrusion of RasV12-expressing NRK-52E cells**
203 Previous studies have demonstrated that myosin II is involved in the apical extrusion of
204 RasV12-expressing MDCK cells [7,10,11]. To check whether it was involved in the basal
205 extrusion of RasV12-expressing NRK-52E cells, cells were treated with a myosin II inhibitor,
206 blebbistatin. The treatment resulted in a significant increase in the apical extrusion of
207 RasV12-expressing NRK-52E cells (Fig. 4A, B), indicating the involvement of myosin II in
208 basal extrusion.
209
210 Myosin II has three different isoforms, namely IIA, IIB, and IIC, with the two most
211 intensively studied isoforms being IIA and IIB. We aimed to identify the isoform in either

212 normal or RasV12-expressing NRK-52E cells that was essential for basal extrusion. To
213 achieve this, myosin IIA and IIB were knocked down using siRNA either in normal cells or
214 in RasV12 cells, and were then mixed (Fig. S4A, B). We found that RasV12 cells that are
215 deficient in myosin IIA, but not IIB, showed a significant decrease in basal extrusion but an
216 increase in apical extrusion (Fig. 4C, D, S4C, Movie 8, and 9), similar to the effect observed
217 with blebbistatin. However, the deficiency of myosin IIA or IIB in normal cells had no effect
218 on the behavior of RasV12 cells (Movie 10–12). These data demonstrate that myosin IIA is
219 essential in RasV12-expressing NRK-52E cells for basal extrusion.
220
221 We observed that myosin IIA-deficient RasV12 cells moved slower than control RasV12
222 cells even when surrounded by normal cells (Movie 9). To confirm this observation, we
223 tracked myosin IIA and IIB knockdown cells in mixed cultures. Indeed, myosin IIA deficient
224 cells showed significantly reduced velocity (Fig. 4E, F and S4C). Taken together, these
225 results demonstrate that myosin IIA is essential for RasV12 cells to exhibit efficient motility
226 when surrounded by normal cells.

Discussion

It has been reported that RasV12-expressing MDCK cells are often extruded to the apical side of the epithelial layer when surrounded by normal cells [7]. This process is dependent on the function of cytoskeletal, signaling, and metabolic regulators, including MAPK, myosin II, EPLIN, VASP, PKA, and filamin, in either transformed or normal cells [7,9,10,12]. In this study, we found that NRK-52E cells that expressed RasV12 were extruded predominantly towards the basal side of surrounding normal cells. As the basal extrusion of transformed cells is related to the onset of cancer metastasis, our findings provide new insights into the molecular mechanism of this malignant disease [13].

One of the mechanisms underlying cell extrusions is the role of circumferential actomyosin belt. The actomyosin belt is collectively formed by the normal cells surrounding apoptotic cells [14] or RasV12-transformed cells [15] to produce a force for squeezing out the abnormal cells from the epithelial cell layer. The position of the formed actomyosin belt determines the direction in which the abnormal cells are expelled [13,16]. We demonstrated that knockdown of myosin IIA only in RasV12-expressing NRK-52E cells effectively blocked the basal

243 extrusion of the cancer cells. Notably, treatment of the mixed culture of normal and RasV12-
244 expressing NRK-52E cells with blebbistatin also blocked the basal extrusion and further
245 directed the transformed cells to be extruded apically. These data indicate that actomyosin
246 belt formation by normal cells is not directly involved in the basal extrusion of RasV12-
247 expressing NRK-52E cells. Instead, myosin II activity seems to be essential for the migration
248 of cancer cells beneath normal cells. The mechanism by which the RasV12 cells are apically
249 excluded by blebbistatin treatment is unclear. We cannot exclude the possibility that cancer
250 cells are subjected to cell death upon mechanical stress applied by the surrounding normal
251 cells. While our data showed no effect of the caspase inhibitor zVAD, a caspase-independent
252 cell death pathway such as necroptosis [12] could be involved, and this should be examined
253 in future studies.
254
255 Our data support a non-cell-autonomous mechanism of the motility of RasV12-expressing
256 NRK-52E cells. While the Ras cells were non-motile when cultured alone, they actively
257 migrated beneath normal cells in a co-culture condition. This suggests that specific
258 interactions, either direct or indirect, may exist between RasV12- and surrounding normal

cells, which promote the motility of the cancer cells. We speculate that such a hetero interaction may be mediated by cell surface proteins and extracellular matrix, such as integrins [17,18] and laminins [18,19]. Another, but not mutually exclusive, possibility is that the confined space between the normal cells and the substrate collagen or glass surface might enhance the motility of the RasV12-transformed cells [20]. In support of this, we often found robust fragmentation of the migrating Ras cells (Fig. S2 and Movie 2), indicating that substantial shear stress was applied to the cancer cells. A similar kind of fragmentation or "cytoplast" formation has also been observed *in vivo* [21], suggesting a common feature of metastatic cancer cells.

In summary, we have demonstrated that the non-cell-autonomous motility of RasV12-expressing NRK-52E cells is induced by the surrounding normal cells. This study expands our knowledge of the mechanism of cancer invasion, depending on the local microenvironment comprising non-transformed epithelial cells.

Acknowledgments

We are grateful to Hideyuki Mukai for discussions and Natsuko Shirai for technical assistance. Funding: This work was supported by the Japan Society for the Promotion of Science (JSPS) [grant numbers JP26114008, JP19K06638, JP16H04785, JP19K06541].

Competing interests

The authors declare no competing interests.

Author contributions

I.J. and T.I. contributed to the design of the project, performed and analyzed experiments, and wrote the manuscript. K.T. and Y.F. provided materials and analyzed experiments.

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354

Figure legends

Fig. 1. Motility of RasV12-expressing NRK-52E cells surrounded by normal cells.

A. Time-lapse images of GFP-expressing NRK-52E cells (WT vs GFP) or GFP-RasV12-expressing cells (WT vs GFP-RasV12) surrounded by normal cells at a high density in the presence of doxycycline. Timestamps are hour:minute. Yellow arrows and white asterisks show tracked cells. White arrows indicate membrane protrusions formed by Ras cells. Scale bar, 20 μm. **B.** Representative movement tracks of GFP-RasV12 cells alone, GFP cells surrounded by normal cells, or GFP-RasV12 cells surrounded by normal cells. **C.** Quantitative analysis of cell migration velocity (μm/min) calculated by movement tracking. Data are mean±SD of three independent experiments (presented by gray, magenta, and cyan plots, respectively). **** *P*-value < 0.0001

Fig. 2. NRK-52E RasV12 cells are extruded to the basal side of normal cells.

A. Confocal images of xz sections of GFP-RasV12-expressing NRK-52E cells only (top), GFP-expressing NRK-52E cells surrounded by normal cells (middle), and GFP-RasV12-expressing NRK-52E cells surrounded by normal cells (bottom). Forty-eight hours after

doxycycline addition, cells were stained by Acti-stain (red) and DAPI (blue). Scale bar, 10 μm. **B.** Quantification of apical/basal extrusion of GFP- or GFP-RasV12-expressing NRK-52E cells surrounded by normal cells, n=3.

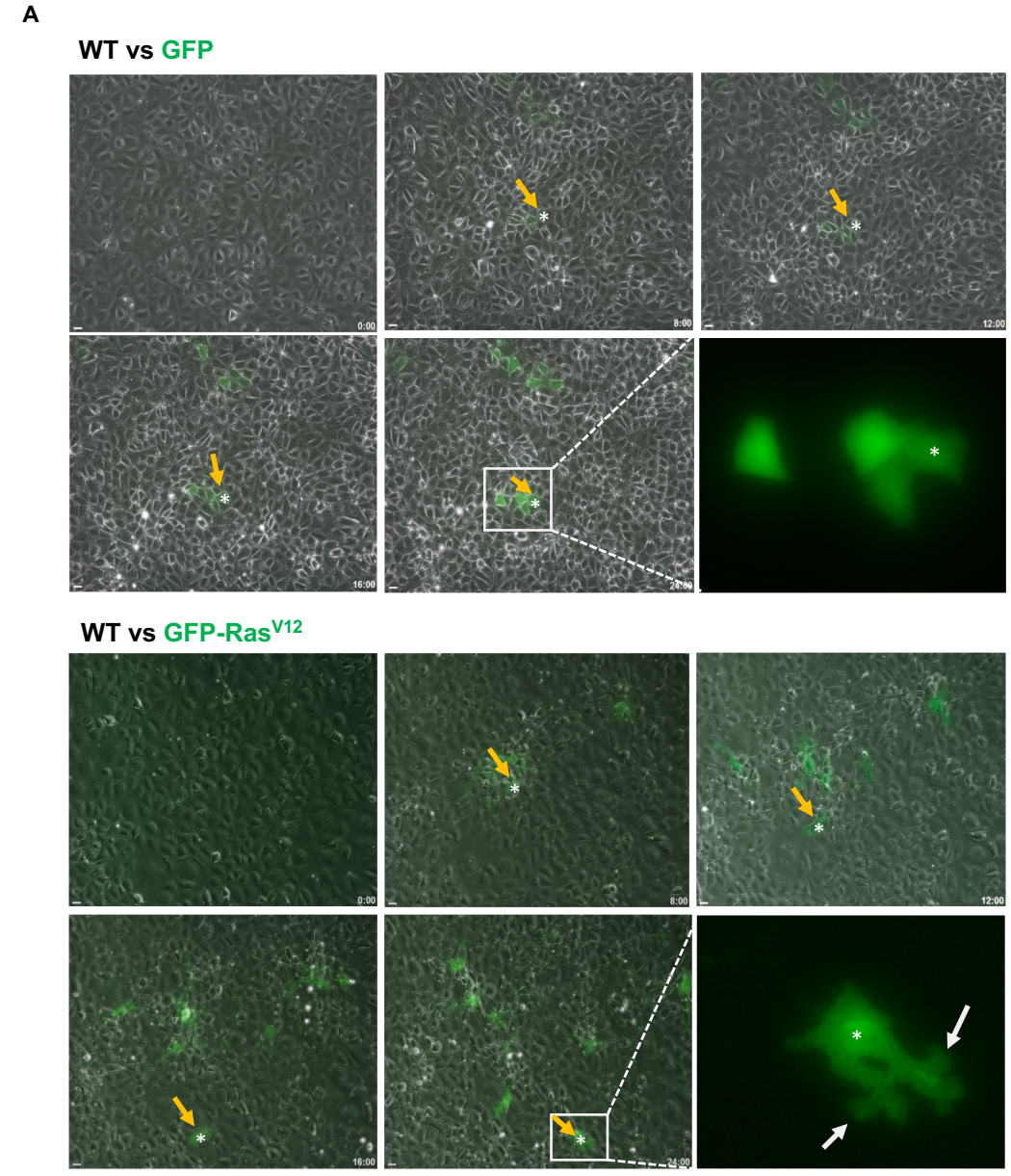
Fig. 3. Inhibition of PI3K suppresses the basal extrusion of RasV12 cells surrounded by normal cells.

A. Western blots of total and phosphorylated proteins of Akt, JNK, p38, and ERK1/2 in normal (WT) and GFP-RasV12 NRK-52E cells without (-Dox) or with (+Dox) the addition of doxycycline. **B.** Quantitative analysis of the ratio of p-Akt to total Akt, p-JNK to total JNK, p-p38 to total p38, and p-ERK to total ERK. Data are mean±SD of three independent experiments. **C.** Confocal images of xz sections of GFP-RasV12-expressing NRK-52E cells surrounded by normal cells in the presence of kinase inhibitors. Forty-eight hours after doxycycline addition, cells were stained by Acti-stain (red) and DAPI (blue). Scale bar, 10 μm. **D.** Time-lapse images of GFP-RasV12-expressing NRK-52E cells surrounded by normal cells in the presence of 25 μM LY294002. Timestamps are hour:minute. White arrows indicate the apically extruded RasV12 cells. Scale bar, 20 μm. **E.** Quantification of apical

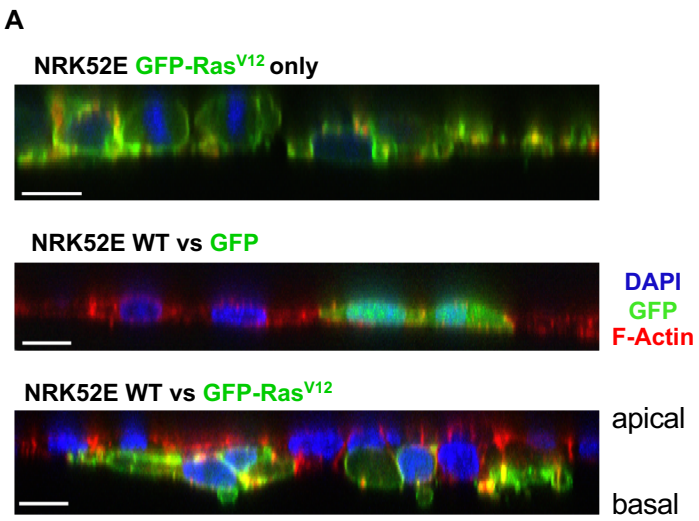
extrusion of GFP-RasV12-expressing NRK-52E cells surrounded by normal cells. Data are mean±SD of three independent experiments. ** *P*-value < 0.01

Fig. 4. Myosin IIA is essential for basal extrusion of RasV12-expressing NRK-52E cells. **A.** Confocal images of xz sections of GFP-RasV12-expressing NRK-52E cells surrounded by normal cells in the presence of DMSO or 50 μm blebbistatin. Scale bar, 10 μm. **B.** Quantification of apical extrusion of GFP-RasV12-expressing NRK-52E cells shown in **A**. Data are mean±SD of three independent experiments. **C.** Confocal images of xz sections of GFP-RasV12-expressing NRK-52E cells (siCTRL, upper), myosin IIA-depleted RasV12 cells (siMyh9, middle) or myosin IIB-depleted RasV12 cells (siMyh10, lower) surrounded by normal cells. Scale bar, 10 μm. **D.** Quantification of apical extrusion of control (siCTRL), myosin IIA- (siMyh9) or myosin IIB-depleted (siMyh10) RasV12 cells. **E.** Representative tracks of migrating control (siCTRL), myosin IIA-depleted (siMyh9) or myosin IIB-depleted (siMyh10) RasV12 cells surrounded by normal cells. **F.** Quantitative analysis of velocity (μm/min) of the tracked cells. Data are mean±SD of three independent experiments (presented by gray, magenta, and cyan plots, respectively). * *P*-value < 0.05, ** *P*-value <

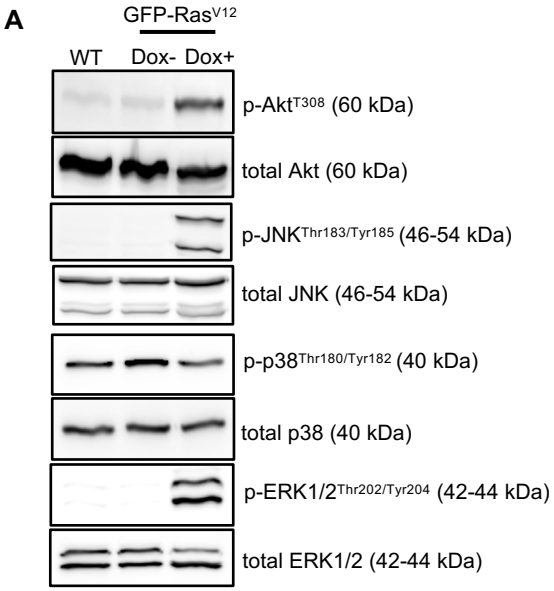
403 0.01, *** *P*-value < 0.001.

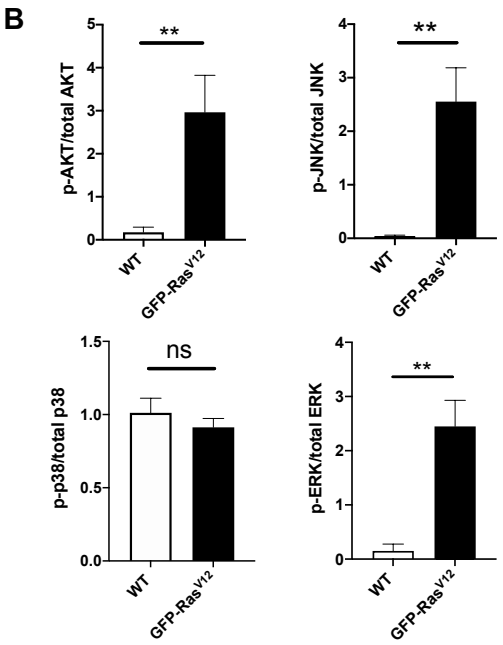


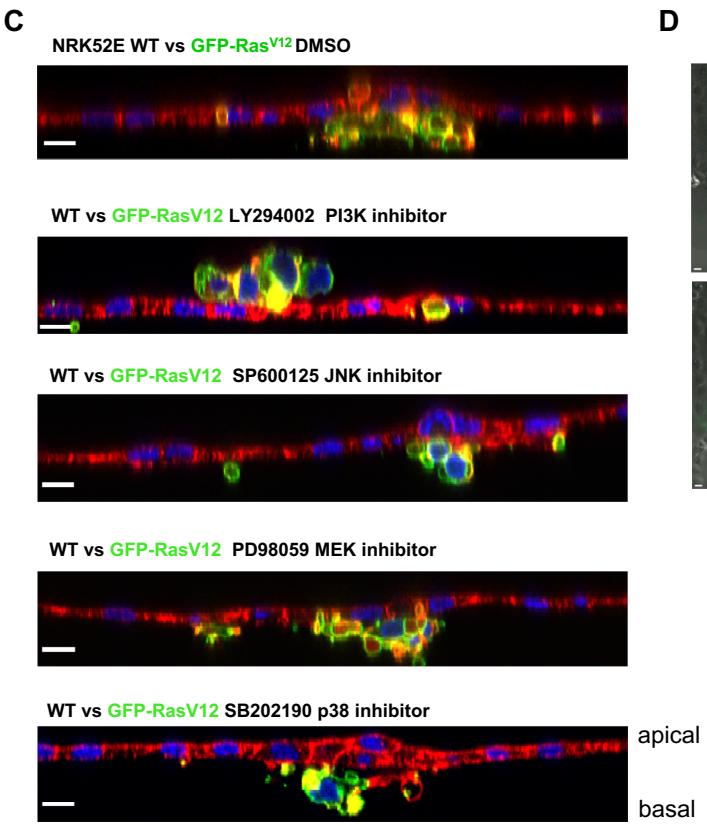
Jebri et al. Figure 1

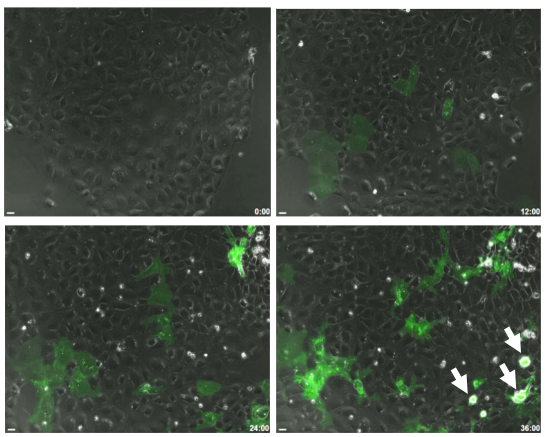


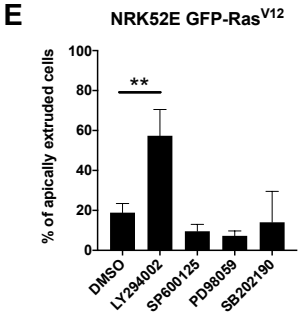
Jebri et al. Figure 2



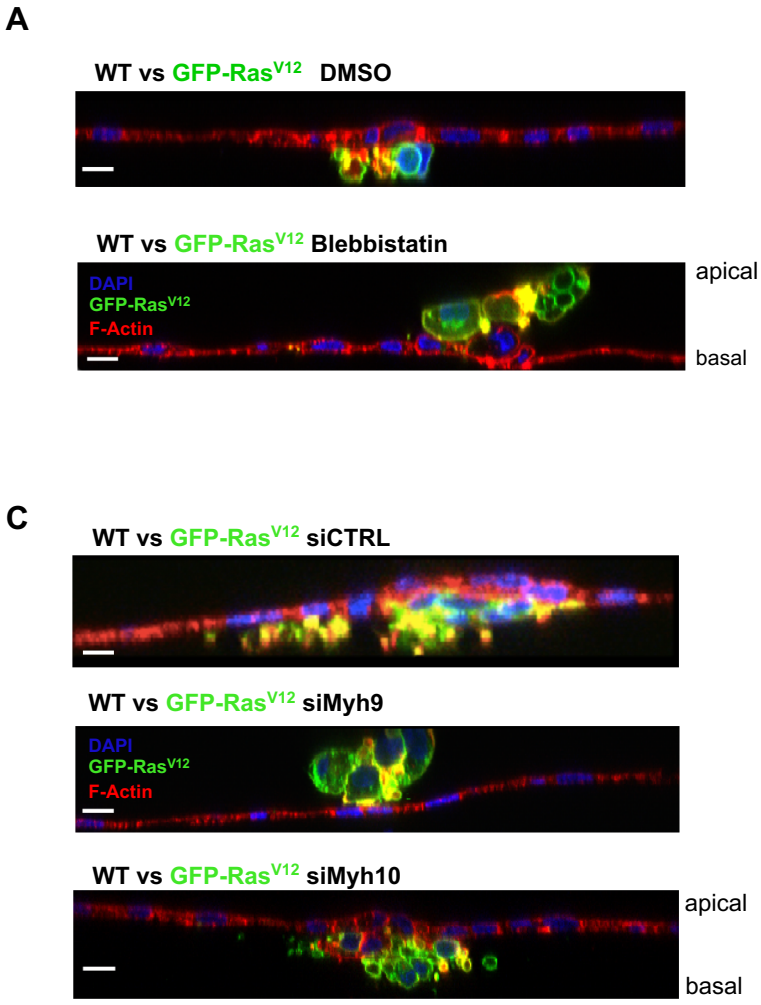








Jebri et al. Figure 3



Jebri et al. Figure 4