



Dual blockade of macropinocytosis and asparagine bioavailability shows synergistic anti-tumor effects on KRAS-mutant colorectal cancer

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ABSTRACT

Mutations of *KRAS* gene are found in various types of cancer, including colorectal cancer (CRC). Despite intense efforts, no pharmacological approaches are expected to be effective against *KRAS*-mutant cancers. Macropinocytosis is an evolutionarily conserved actin-dependent endocytic process that internalizes extracellular fluids into large vesicles called macropinosomes. Recent studies have revealed macropinocytosis's important role in metabolic adaptation to nutrient stress in cancer cells harboring *KRAS* mutations. Here we showed that *KRAS*-mutant CRC cells enhanced macropinocytosis for tumor growth under nutrient-depleted conditions. We also demonstrated that activation of Rac1 and phosphoinositide 3-kinase were involved in macropinocytosis of *KRAS*-mutant CRC cells. Furthermore, we found that macropinocytosis was closely correlated with asparagine metabolism. In *KRAS*-mutant CRC cells engineered with knockdown of asparagine synthetase, macropinocytosis was accelerated under glutamine-depleted condition, and albumin addition could restore the glutamine depletion-induced growth suppression by recovering the intracellular asparagine level. Finally, we discovered that the combination of macropinocytosis inhibition and asparagine depletion dramatically suppressed the tumor growth of *KRAS*-mutant CRC cells *in vivo*. These results indicate that dual blockade of macropinocytosis and asparagine bioavailability could be a novel therapeutic strategy for *KRAS*-mutant cancers.

1. Introduction

Mutations of *KRAS* gene are found in various types of cancer, including pancreatic ductal cell adenocarcinoma (PDCA), non-small cell lung cancer (NSCLC) and colorectal cancer (CRC). Nearly half of CRC cases harbor *KRAS* mutations. Because several clinical trials have shown that *KRAS* mutations in CRC can predict a lack of responses to the anti-epidermal growth factor receptor (EGFR)-based therapy, the use of anti-EGFR antibodies is now limited to CRC patients with wild-type *KRAS* [1, 2]. Recently, selective inhibitors targeting *KRAS* G12C mutation have

been developed and are entering clinical evaluation [3]. However, despite intense efforts, no pharmacological approaches are expected to be effective against *KRAS* G12D, G12V and G13D mutations, which account for most *KRAS* mutations in CRC. Therefore, the development of novel therapies for *KRAS*-mutant CRC is urgently needed.

CRC is highly heterogeneous, and therefore, the identification of subtype-specific vulnerabilities is needed to design tailored treatment. The International CRC Subtyping Consortium reported that CRC could be classified into four consensus molecular subtypes (CMSs) (i.e., CMS1, CMS2, CMS3, and CMS4) and that CMS3 is characterized by aberrant metabolism and correlated with *KRAS* mutations [4]. Recent evidence

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Abbreviations

CRC	colorectal cancer
PDCA	pancreatic ductal cell adenocarcinoma
NSCLC	non-small cell lung cancer
EGFR	epidermal growth factor receptor
CMS	consensus molecular subtype
ASNS	asparagine synthetase
CCLC	Cancer Cell Line Encyclopedia
PIK3CA	phosphoinositide 3-kinase catalytic subunit alpha
PTEN	phosphatase and tensin homolog
PI3-kinase	phosphoinositide 3-kinase
TMR	tetramethylrhodamine
MI	macropinocytosis index

EIPA	5-(N-ethyl-N-isopropyl) amiloride;
PIP3	phosphatidylinositol 3,4,5-trisphosphate
FRET	Förster Resonance Energy Transfer
siRNA	small interfering RNA
FBS	fetal bovine serum
BSA	bovine serum albumin
shRNA	small hairpin RNA
L-Asp	L-asparaginase
ALL	acute lymphocytic leukemia
KO	knockout
NHE	Na ⁺ /H ⁺ exchanger
MEF	mouse embryo fibroblast
HPLC	high performance liquid chromatography
FLD	fluorescence detector

has revealed that *KRAS* mutations promote metabolic reprogramming through glycolysis, glutaminolysis, nutrient uptake, nucleotide synthesis, and fatty acid synthesis [5–8], indicating that targeting metabolic processes may be a potential strategy for *KRAS*-mutant CRC. Glutamine is the most abundant amino acid in human sera. Many cancer cells, notably those driven by *Myc* and *KRAS*, consume glutamine at exceedingly high rates to fulfill their energetic requirement, contributing to glutamine addiction [9–11]. We previously demonstrated that *KRAS*-mutant CRC cells became adaptive to glutamine depletion through asparagine biosynthesis by upregulating asparagine synthetase (ASNS), and that the combination of ASNS inhibition and asparaginase resulted in tumor shrinkage [12]. Recently, analyzing metabolite profiling of 928 cell lines in the Cancer Cell Line Encyclopedia (CCLE) database revealed that low asparagine levels caused by suppressed ASNS expression sensitize subsets of gastric cancer and hepatocellular carcinoma to asparagine therapy [13]. These results indicate that targeting asparagine bioavailability can be a promising approach for a subset of cancers.

Macropinocytosis is an evolutionarily conserved actin-dependent endocytic process that internalizes extracellular fluids into large vesicles called macropinosomes [14]. Although several studies using transformed fibroblasts have shown that macropinocytosis can be induced by oncogenic transformation (e.g., *RAS* mutation, phosphoinositide 3-kinase catalytic subunit alpha (*PIK3CA*) mutation, and activated v-*Src* expression) or loss of function in tumor suppressors (loss of phosphatase and tensin homolog (*PTEN*)) [15–19], the biological relevance of macropinocytosis in cancer has recently been elucidated. Recent studies have provided evidence of macropinocytosis's important role in metabolic adaptation to nutrient stress in cancer cells harboring *RAS* mutations, such as PDCA and NSCLC [20–24]. Macropinocytosis serves as a route of nutrient uptake by which extracellular proteins (e.g., serum albumin) are internalized and subsequently degraded within lysosomes to meet nutrient demands for tumor growth.

In the present study, we showed that *KRAS*-mutant CRC cells enhanced macropinocytosis for tumor growth under nutrient-depleted conditions. We also demonstrated that activation of *Rac1* and phosphoinositide 3-kinase (PI3-kinase) were involved in macropinocytosis of *KRAS*-mutant CRC cells. Furthermore, we found that macropinocytosis was closely correlated with asparagine metabolism in *KRAS*-mutant CRC cells. Finally, we discovered that the combination of macropinocytosis inhibition and asparagine depletion dramatically suppressed tumor growth *in vivo*. These results indicate that dual blockade of macropinocytosis and asparagine bioavailability could be a novel therapeutic strategy for *KRAS*-mutant CRC.

2. Materials and methods

2.1. Cell lines and cell culture

The CRC cell lines, HCT116 and DLD1, were obtained from the American Type Culture Collection. HKE-3, HKh-2, e3-MKRas, DKO-4 and DKs-8 cell lines were established as described previously [25,26]. Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (glucose 25 mM, glutamine 4 mM) (043–30085, Wako) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Media without glutamine were prepared by using glutamine-free DMEM (glucose 25 mM, glutamine 0 mM) (043–32245, Wako) containing 10% FBS and 1% penicillin/streptomycin.

2.2. Cell starvation assay

Cells were plated in 96-well plate at a density of 3000–5000 cells/well. After 24 h incubation in DMEM (glucose 25 mM, glutamine 4 mM) containing 10% FBS, cells were maintained in the indicated starvation medium. FBS-starvation medium was DMEM (glucose 25 mM, glutamine 4 mM) containing 1% FBS. Glutamine-starvation medium was glutamine-free DMEM (glucose 25 mM, glutamine 0 mM) containing 1% FBS. Each starvation medium was supplemented with or without 2% BSA (Fraction V, fatty acid-free, nuclease- and protease-free, #126609, Calbiochem) and 0.1 mM asparagine. Cells were incubated in the indicated medium with or without 5-(N-Ethyl-N-isopropyl) amiloride (EIPA, Merck A3085) for 72 h. Cell proliferations were analyzed with Cell Counting Kit-8 (CCK-8, Dojindo) according to the manufacturer's protocol.

2.3. Time-lapse Förster resonance energy transfer (FRET) imaging

FRET imaging data were analyzed as previously reported [27,28]. The detailed material and method are described in Supplementary Information.

2.4. *In vivo* xenograft studies

A total of 2×10^6 HCT116 cells were suspended in 75 μ L PBS and an equal volume of Matrigel and subcutaneously injected into both dorsal flanks of 7–8 weeks old female KSN/slc nude mice (Japan SLC). In cancer spheroid xenografts model, we referred to previous reports [29,30]. Cancer spheroids were cultured in 24-well plate until confluent culture and transferred to a 10 ml tube together with Matrigel. After centrifuge and re-suspend, we checked the density of the suspension. 2×10^6 spheroids were cultured in 12-well plate with 75 μ L Matrigel. Next day, each well spheroids were transferred to a 1.5 ml tube together with Matrigel and suspended in 75 μ L PBS. Spheroid suspensions were

injected into nude mice subcutaneously. At the time of EIPA treatment, mice were treated with intraperitoneal injection of EIPA (100 µg/each mouse, every day), and the other mice were vehicle-treated. Treatment was started the day after inoculation. At the time of EIPA and L-Asparaginase (L-Asp, Kyowa Hakko Kirin) treatment, mice were treated with intraperitoneal injection of L-Asp (1500 U/kg, every day) and EIPA (150 µg/each mouse, every day). Treatment was started when the tumor volume reached about 100 mm³. Tumor sizes were measured with calipers twice a week, and the tumor volumes were estimated with the formula: $0.5 \times L \times W^2$, where L = length, W = width. The animal experiment was approved by the Animal Care and Use Committee of Kyoto University.

2.5. Macropinosome visualization and quantification in vitro

Cells were seeded onto glass coverslips. After 48–72 h incubation in DMEM (glucose 25 mM, glutamine 4 mM) containing 10% FBS, cells were washed briefly and incubated with FBS-free DMEM. Macropinosomes were marked utilizing a high molecular weight TMR-dextran (D1818, Thermo Fisher) as described previously [31]. After 16 h incubation in FBS-free DMEM, cells were incubated in FBS-free DMEM with TMR-dextran, final concentration of 1 mg/ml, for 30 min at 37 °C. Z62954982 (Merck 553511) was treated at 50 µM 4 h before dextran addition. LY294002 (Cayman 70920) was treated at 2 µM 4 h before dextran addition. EIPA was treated at the indicated concentrations 30 min before dextran addition. After incubation in dextran-containing medium, cells were washed 5 times with cold PBS and immediately fixed in 3.7% formaldehyde. Cells were incubated with DAPI, and coverslips mounted onto slides using Mounting Media (DAKO). BZ-9000 (Keyence) was used to capture the images, and ImageJ (NIH) was used to analyze the captured images. The area of particles (TMR-dextran) per the area of cells was defined as the MI for each field, and the average MI of 10–15 fields collected at random was defined as the MI of each cell. We analyzed at least 400 cells, which typically consists of 10–15 fields. The intracellular uptake of polymeric fluorescent dextran was observed under a microscope as a marker of macropinosomes and quantified using the analysis software ImageJ to compare the MI.

2.6. Macropinosome visualization and quantification in vivo

To evaluate macropinocytosis *in vivo*, TMR-dextran was injected into the xenograft tumors of mice, as described previously [31]. After 1 h-interval following injection of TMR-dextran (20 mg/ml, 100 µL), tumors were harvested. Specimens were dipped in OCT compound for frozen sections. After fixation with formaldehyde, primary Ab reaction with anti-CK8 Ab and secondary Ab reaction with Alexa-488 Ab were performed. Finally, nuclear staining was performed with DAPI. Images were captured using a BZ-9000 and analyzed using ImageJ. The area of particles (TMR-dextran) per cells was defined as the MI for each field, and the average MI of 10–15 fields collected at random was defined as the MI of each cell. We analyzed at least 400 cells, which typically consists of 10–15 fields.

2.7. Statistical analysis

All values are expressed as mean values ± standard error of the mean (SEM). All *in vitro* experiments were performed three times. The statistical significance of difference was determined by Student's t-test, as appropriate. All analyses were two-sided, and a p value < 0.05 was considered to be statistically significant.

3. Results

3.1. KRAS mutation enhances macropinocytosis in CRC cells

Macropinocytosis is an endocytic process that mediates the uptake of

extracellular fluids into large vesicles, called macropinosomes. To investigate whether *KRAS* status could affect macropinocytosis in CRC, we used human CRC cell lines with mutant *KRAS* G13D (HCT116 and DLD-1) and their isogenic lines in which targeted homologous recombination disrupted the mutant *KRAS* allele [12,25]. HKh-2 and HKe-3 are two independent HCT116 clones with the mutant *KRAS* allele disrupted; DKO-4 and DKs-8 are two independent DLD-1 clones with the mutant *KRAS* allele disrupted. These paired lines differ only in 1 base pair of *KRAS* gene, which allowed us to examine normal or mutant *KRAS* proteins at physiological levels. Macropinosomes were quantified as “macropinocytosis index (MI)” by visualization of tetramethylrhodamine-labeled dextran (TMR-dextran), an established marker of macropinosome [31]. We first evaluated MI in each CRC cell line and found that HCT116 and DLD-1, parental lines with mutant *KRAS*, exhibited significantly higher MI than did their isogenic lines containing wild-type *KRAS* allele (HKe-3 and HKh-2 for HCT116; DKO-4 and DKs-8 for DLD-1) (Fig. 1A and B). In addition, the decrease in MI induced by wild-type *KRAS* allele in HKe-3 was significantly recovered in the HKe-3-derived transfectants expressing activated *KRAS* (e3-MKRas clone) [26]. We also investigated the cell proliferating status of each cell under the same condition as the macropinocytosis assay (i.e., 16 h incubation in FBS-free DMEM), and found that there was no significant difference among them (Supplementary Fig. S1A). To further validate the MI alterations caused by mutant *KRAS*, we performed RNA interference experiments. We introduced two kinds of small interfering RNA (siRNA) constructs targeting *KRAS* (siKRAS #1 and #2) into HCT116 and DLD-1 cells (Supplementary Fig. S1B), and verified that both siKRAS constructs significantly decreased MI (Fig. 1C and D). We also found that dextran uptake was inhibited in a dose-dependent manner by EIPA, a selective macropinocytosis inhibitor [32,33] (Fig. 1E and Supplementary Fig. S1C), which indicate dextran uptake was employed through macropinocytosis. To examine whether *KRAS* status could affect macropinocytosis *in vivo*, we measured MI in the transplanted xenograft tumors in mice. TMR-dextran was injected into tumors, and intracellular uptake was assessed by microscopy of tissue sections. The MI was significantly lower in tumors derived from HKe-3 and HKh-2 cells than in HCT116-derived tumors, which was not observed in e3-MKRas clone-derived tumors (Fig. 1F).

3.2. Extracellular albumin is taken up into the cell via macropinocytosis

Recent studies have reported that macropinocytosis uptake and subsequent degradation of extracellular proteins (e.g., serum albumin being the most abundant in blood and tissues) could lead to the generation of amino acids to sustain cell survival and proliferation in PDCA cells [20–22]. Therefore, we examined whether the uptake of extracellular albumin in CRC cells could be performed through macropinocytosis. To monitor the internalization of albumin, CRC cells (HCT116, DLD-1, and their isogenic lines) were incubated with FITC-labeled bovine serum albumin (FITC-BSA) and TMR-dextran. As shown in Fig. 2A, FITC-BSA was incorporated into the intracellular structures that essentially co-localized with TMR-dextran. Uptake index of BSA was significantly higher in HCT116 and DLD-1 cells than in their isogenic lines with wild-type *KRAS* allele (HKe-3 and DKO-4 cells, respectively), which was almost the same as MI (Fig. 2B). Moreover, EIPA treatment significantly suppressed albumin uptake in all cell lines (Fig. 2C and D), indicating that albumin internalization could occur through macropinocytosis in CRC.

3.3. Macropinocytosis plays a role in tumor growth under nutrient-limiting conditions

Next, we examined whether internalized albumin through macropinocytosis could contribute to tumor growth under nutrient-limiting conditions. Under the medium with Fetal Bovine Serum (FBS) starvation (1% FBS), we found that cell proliferation was significantly rescued

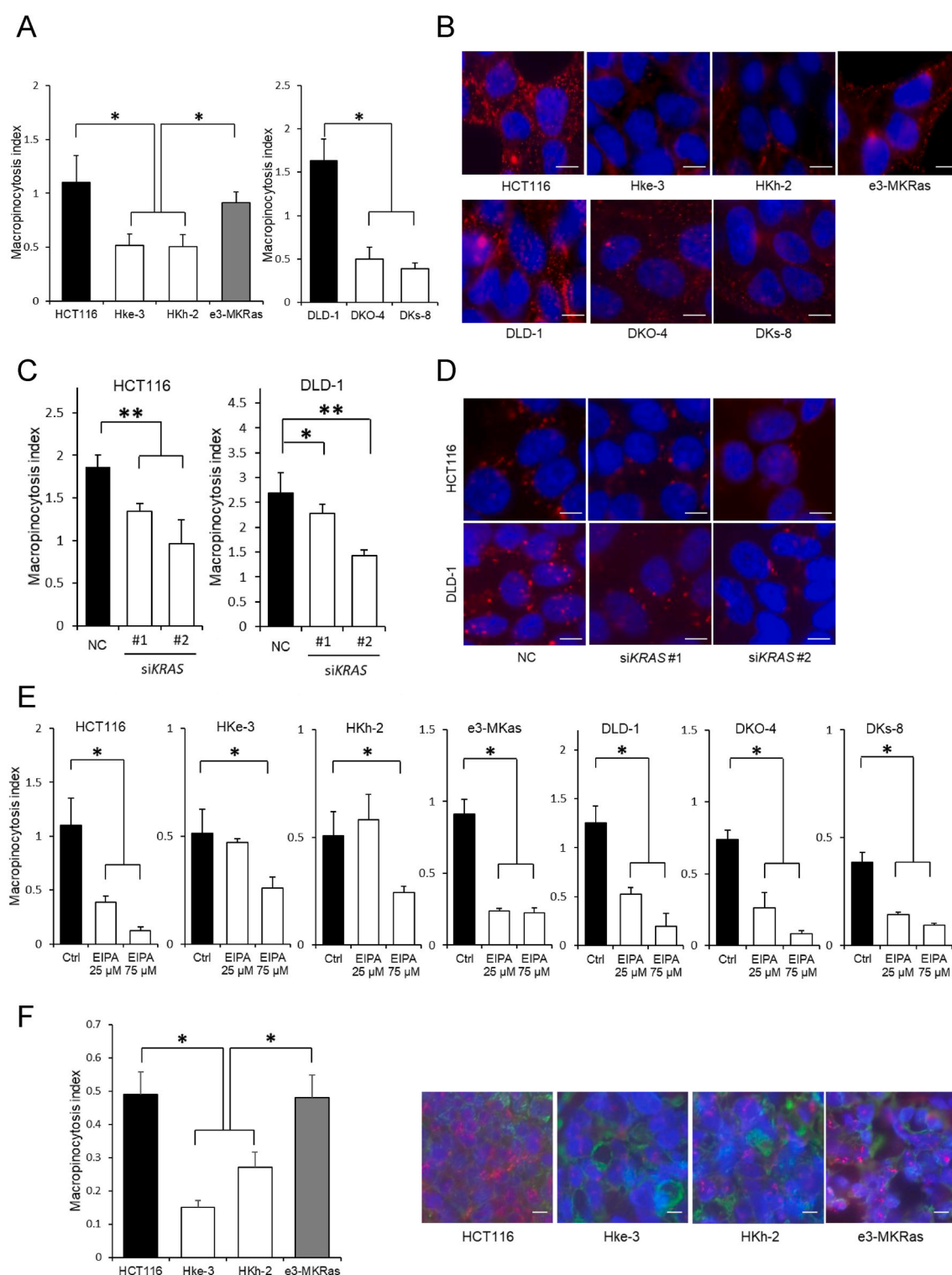


Fig. 1. KRAS mutation enhances macropinocytosis in CRC cells.

A. Quantification of TMR-dextran uptake (macropinocytosis index) in HCT116-derived lines (left) and DLD-1-derived lines (right). Results are presented as the means $\pm$ SEM of triplicate measurements. * p < 0.01 by Student's t-test. B. Macropinocytic uptake of TMR-dextran (red). The nuclei are stained by DAPI (blue). Scale bar, 5 μ m. C. Quantification of TMR-dextran uptake (macropinocytosis index). HCT116 cells (left) and DLD-1 cells (right) were treated with two independent siRNA construct (#1 and #2) targeting KRAS and NC (negative control) siRNA. Results are presented as the means $\pm$ SEM of triplicate measurements. * p < 0.05 and ** p < 0.01 by Student's t-test. D. Macropinocytic uptake of TMR-dextran (red). The nuclei are stained by DAPI (blue). Scale bar, 5 μ m. E. Quantification of TMR-dextran uptake (macropinocytosis index) in indicated cells treated with 0 (control: Ctrl), 25 or 75 μ M EIPA. Results are presented as the means $\pm$ SEM of triplicate measurements. * p < 0.01 by Student's t-test. F. Quantification of macropinocytosis *in vivo* within xenograft tumors derived from indicated cells (left). Representative fluorescence images from sections of TMR-dextran (red)-injected xenograft tumors stained with DAPI (blue) and anti-CK8 (green) are shown (right). Cytokeratin was stained with CK-8 staining. Mean; bar, $\pm$ SEM, n = 3 mice in each group with at least 400 cells scored. * p < 0.01 by Student's t-test. Scale bar, 5 μ m. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

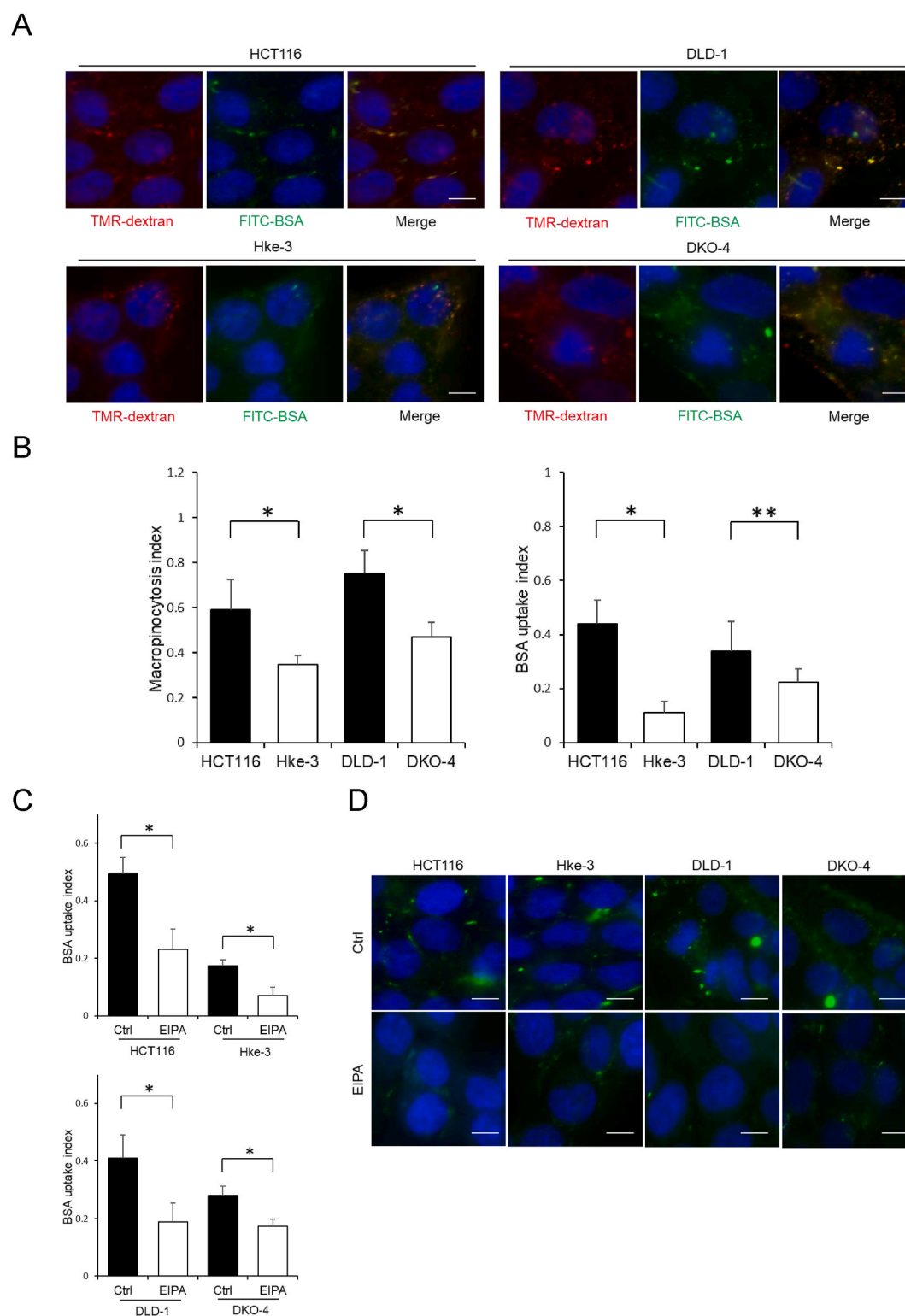


Fig. 2. Extracellular albumin is taken up into the cell via macropinocytosis.

A. Representative fluorescence images. FITC-BSA (green) is internalized into discrete puncta that co-localize with TMR-dextran (red). B. Quantification of TMR-dextran uptake (macropinocytosis index) and FITC-BSA uptake (BSA uptake index) in indicated cells. Results are presented as the means $\pm$ SEM of triplicate measurements. * $p < 0.01$ and ** $p < 0.05$ by Student's t-test. C. Quantification of FITC-BSA uptake (BSA uptake index) in indicated cells treated with Ctrl (control) or 75 μ M EIPA. Results are presented as the means $\pm$ SEM of triplicate measurements. * $p < 0.01$ by Student's t-test. D. Representative fluorescence images of FITC-BSA uptake (green). The nucleuses are stained by DAPI (blue). Scale bar, 5 μ m. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

by supplementation with 2% albumin in cells containing mutant *KRAS* allele (i.e., HCT116, e3-MKRas, and DLD-1 cells) and that this growth recovery effect was inhibited by EIPA treatment, which was not observed in their isogenic cells with wild-type *KRAS* allele (HKE-3 and HKh-2; DKO-4 and DKs-8) (Fig. 3A). These observations were more remarkable in medium with both FBS starvation (1% FBS) and glutamine depletion (Fig. 3B). We also investigated MI in each cell line under both glutamine-depleted and glutamine-sufficient conditions. Glutamine depletion increased MI to some extent in almost all cell lines, although the increase rate of MI was more remarkable in parental lines with mutant *KRAS* (Supplementary Fig. S1D).

Next, we sought to evaluate the role of macropinocytosis in *in vivo* tumor growth directly. Mice bearing HCT116 xenografts were treated with EIPA or vehicle. As shown in Fig. 3C, EIPA treatment significantly suppressed the growth rate of HCT116 xenografts. On day 28 post-inoculation, the tumor size in vehicle-treated mice was $1536 \pm 164 \text{ mm}^3$, whereas that in EIPA-treated mice was $912 \pm 101 \text{ mm}^3$ ($p < 0.01$). We found that the tumors collected from EIPA-treated mice displayed a significantly lower MI than those collected from vehicle-treated mice (Fig. 3D), and that the number of Ki-67-positive tumor cells was significantly lower in EIPA-treated mice than in vehicle-treated mice (Fig. 3E). We also examined the effect of EIPA on tumors containing wild-type *KRAS* allele, and found that EIPA treatment did not suppress the growth of HKE-3 xenografts (Supplementary Fig. S2A).

Recently, we established several CRC patients-derived spheroid lines [29,30,34–36]. To confirm the anti-tumor effect of EIPA treatment, we next extended these results to the patient-derived cancer spheroids and their xenografts. HC17T is a spheroid line derived from a CRC patient with mutant *KRAS* [29]. EIPA treatment significantly suppressed cell proliferation *in vitro* of HC17T in a dose-dependent manner (Supplementary Fig. S2B). Similar to the results of HCT116 xenografts, EIPA treatment tended to suppress the growth rate of HC17T xenografts compared with vehicle-treated mice (Supplementary Fig. S2C; $p = 0.17$).

3.4. Activation of Rac1 and PIP3 is involved in macropinocytosis of *KRAS*-mutant CRC cells

Macropinocytosis is initiated by actin-driven protrusions of the plasma membrane ruffling, followed by pinching off vesicles from the plasma membrane and macropinosome formation. Activation of RAS or PI3-kinase signaling pathways can drive macropinocytosis [15,17,18]. The small GTPase Rac1 is a key regulator of membrane ruffling and macropinocytosis [37]. To investigate the molecular mechanism of macropinocytosis in CRC, we examined whether inhibition of Rac1 or PI3-kinase could affect macropinosome formation, and found that either a Rac1 inhibitor (Z62954982) or PI3-kinase inhibitor (LY294002) significantly suppressed MI in HCT116 and DLD-1 cells (Fig. 4A and B). To further elucidate the role of Rac1 and PI3-kinase in macropinocytosis, we performed a FRET-based approach using the biosensors Rac1 and phosphatidylinositol 3,4,5-trisphosphate (PIP3). Time-lapse live-cell imaging showed that addition of 2% albumin dramatically induced FRET activation of Rac1 and PIP3 as well as morphological changes in the cell membrane (Fig. 4C and Supplementary Videos 1 and 2). Notably, the increase in FRET activity of Rac1 and PIP3 was significantly larger in HCT116 and e3-MKRas cells compared with in HKE-3 cells (Fig. 4D and E), indicating that Rac1 and PI3-kinase are involved in macropinocytosis in *KRAS*-mutant CRC cells.

Supplementary video related to this article can be found at <https://doi.org/10.1016/j.canlet.2021.09.023>.

3.5. Macropinocytosis is closely correlated with asparagine metabolism in CRC

Recent evidence demonstrates that increased dependency on glutamine metabolism is a hallmark of Ras-transformed cancer cells [5–8]

and that macropinocytosis of proteins serves to sustain tumor growth as an amino acid supply route in Ras-transformed cells [20–22]. We recently demonstrated that *KRAS*-mutant CRC cells became adaptive to glutamine depletion by up-regulating ASNS, an enzyme that synthesizes intracellular asparagine from aspartate [12]. Given that both macropinocytosis and ASNS activity play important roles in metabolic adaptation to nutrient stress, we hypothesized that macropinocytosis could interact with ASNS activity under glutamine-depleted condition in *KRAS*-mutant CRC cells. Therefore, we established HCT116 cells engineered with ASNS knockdown by lentiviral transduction of two small hairpin RNA (shRNA) constructs (referred to as shASNS#1 and shASNS#2) (Supplementary Fig. S2D). We found that, under glutamine-depleted condition, both shASNS transfectants significantly decreased cell proliferation compared with the scramble controls. Moreover, the addition of 0.1 mM asparagine (the ASNS product) was sufficient to recover the growth inhibition caused by glutamine depletion in shASNS transfectants (Supplementary Fig. S2E). We next investigated the MI of these cells (Fig. 5A and B), and found that glutamine depletion significantly increased MI in all cell lines. Of note, the MI of shASNS transfectants was significantly higher compared with that of the scramble controls under glutamine-depleted condition, whereas the MI of shASNS transfectants was almost similar to that of the scramble controls under glutamine-sufficient condition. We also found that the addition of 2% albumin as well as 0.1 mM asparagine significantly recovered the growth inhibition caused by glutamine depletion in shASNS transfectants (Fig. 5C). We also measured intracellular levels of asparagine and glutamine using high performance liquid chromatography (HPLC)-fluorescence detector (FLD) (Fig. 5D), and found that, under glutamine-depleted condition, intracellular asparagine level in shASNS#1 transfectants was significantly lower than that in scramble controls. In shASNS transfectants, intracellular asparagine level was significantly decreased by glutamine depletion. Moreover, addition of 2% albumin as well as 0.1 mM asparagine significantly recovered the intracellular asparagine level, which was inhibited by EIPA treatment.

3.6. Dual blockade of macropinocytosis and asparagine bioavailability dramatically suppresses tumor growth of *KRAS*-mutant CRC cells *in vivo*

L-asparaginase (L-Asp), an FDA-approved key drug for acute lymphocytic leukemia (ALL), catabolizes L-asparagine to L-aspartate, leading to depletion of extracellular asparagine [38,39]. We recently found that the combination of L-Asp and ASNS inhibition markedly suppressed tumor growth of *KRAS*-mutant CRC cells by inducing asparagine-depleted state [12]. To further investigate whether the combination of macropinocytosis inhibition and asparagine depletion could be effective, we next assessed the ability of CRC cells to grow as xenografts *in vivo*. We newly established HCT116 and DLD-1 cells engineered with ASNS knockout using CRISPR-Cas9 editing to investigate through *in vivo* experiments (Supplementary Fig. S2F), because the knockdown effect of ASNS in shASNS HCT116 transfectants could not continue long enough to be used in *in vivo* experiments. We subcutaneously implanted ASNS-knockout HCT116 (ASNS KO#1)-derived xenografts into nude mice. After the tumors reached an average volume of 100 mm^3 , we treated the mice in the 4 treatment groups: vehicle, EIPA alone, L-Asp alone, or the combination of EIPA plus L-Asp (Fig. 6A). We confirmed that L-Asp dramatically suppressed the serum asparagine level *in vivo* using HPLC-FLD (Supplementary Fig. S2G). Relative to control tumors, those from EIPA-treated mice or L-Asp-treated mice exhibited a significantly suppressed growth rate. Notably, the combination of EIPA plus L-Asp almost completely inhibited tumor growth. On day 24 post-inoculation, the tumor size in vehicle-treated, EIPA-treated, L-Asp-treated, and combination (EIPA plus L-Asp)-treated mice were $592 \pm 69 \text{ mm}^3$, $407 \pm 17 \text{ mm}^3$, $197 \pm 38 \text{ mm}^3$, and $94 \pm 12 \text{ mm}^3$, respectively. We also examined the *in vivo* inhibitory effects of EIPA and/or L-Asp using ASNS-knockout DLD-1 (ASNS KO#1)-derived xenografts, and found results similar to those of ASNS-knockout HCT116

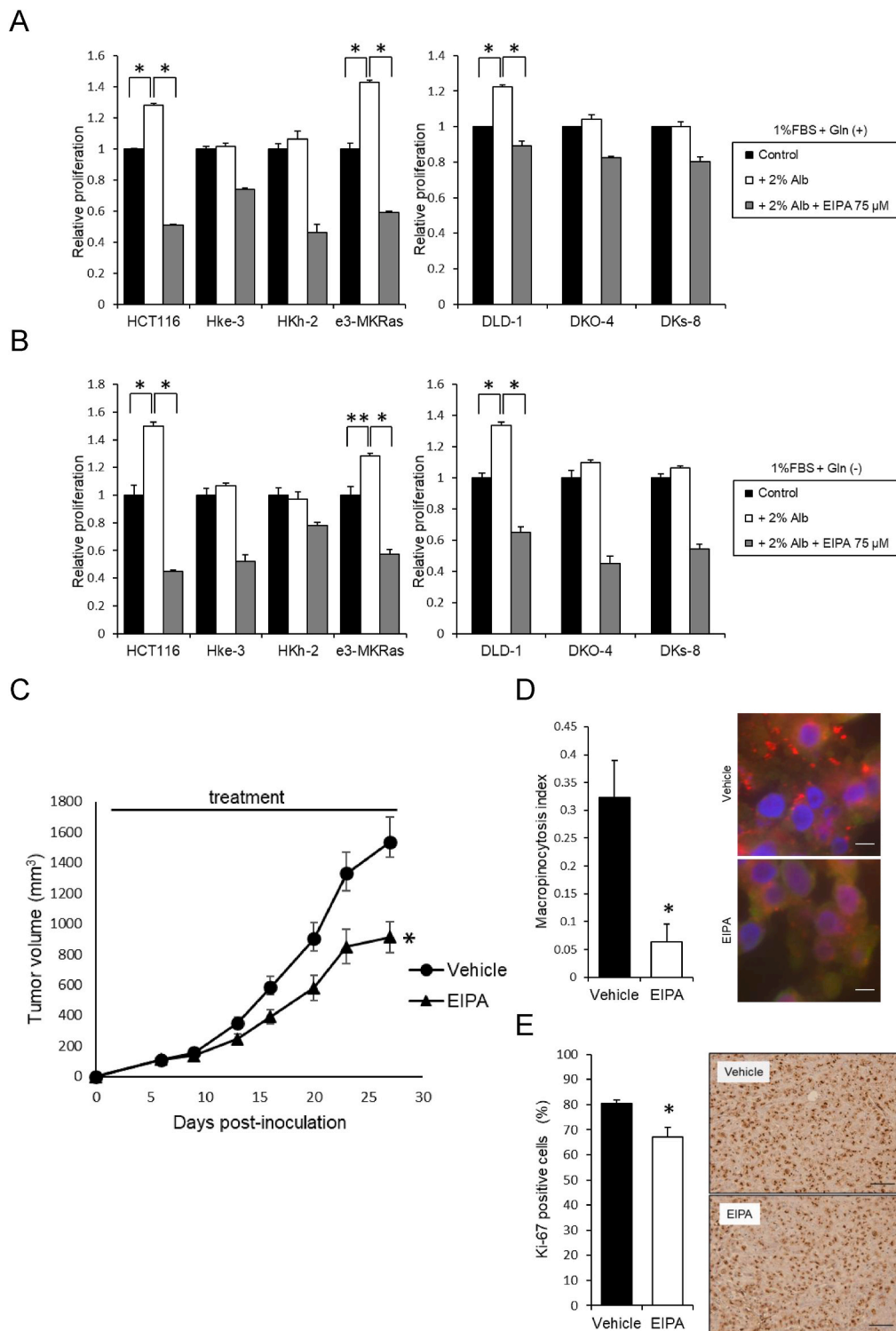


Fig. 3. Macropinocytosis plays a role in tumor growth under nutrient-limiting conditions.

A. Cell proliferation measured by CCK-8 assay. Under DMEM medium containing 1% FBS, indicated cells were treated for 72 h with vehicle, 2% albumin, or 2% albumin plus 75 μ M EIPA. Cell proliferation in each condition was normalized to that in vehicle (control). Results are presented as the means $\pm$ SEM of triplicate measurements. * p < 0.01 by Student's t-test. B. Cell proliferation measured by CCK-8 assay. Under glutamine free-DMEM medium containing 1% FBS, indicated cells were treated for 72 h with vehicle, 2% albumin, or 2% albumin plus 75 μ M EIPA. Cell proliferation in each condition was normalized to that in vehicle (control). Results are presented as the means $\pm$ SEM of triplicate measurements. * p < 0.01 and ** p < 0.05 by Student's t-test. C. Xenograft size of HCT116 cells treated with vehicle or EIPA. From the next day after tumor inoculation, EIPA (100 μ g/each mouse) was daily injected intraperitoneally. Mean; bar, $\pm$ SEM, n = 10 mice in each group. * p < 0.01 by Student's t-test. D. Quantification of macrophinocytosis *in vivo* within xenograft tumors (left). Representative fluorescence images from sections of TMR-dextran (red)-injected xenograft tumors stained with DAPI (blue) and anti-CK8 (green) are shown (right). Cytokeratin was stained with CK-8 staining. Scale bar, 5 μ m. Mean; bar, $\pm$ SEM, n = 3 mice in each group with at least 400 cells scored. * p < 0.01 by Student's t-test. E. Rate of Ki-67 positive cells (left) and immunohistochemical staining for Ki-67 (right) in xenograft tumors. Scale bar, 50 μ m. Mean; bar, $\pm$ SEM, n = 3 mice in each group with 5 fields (X40) scored. * p < 0.01 by Student's t-test. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

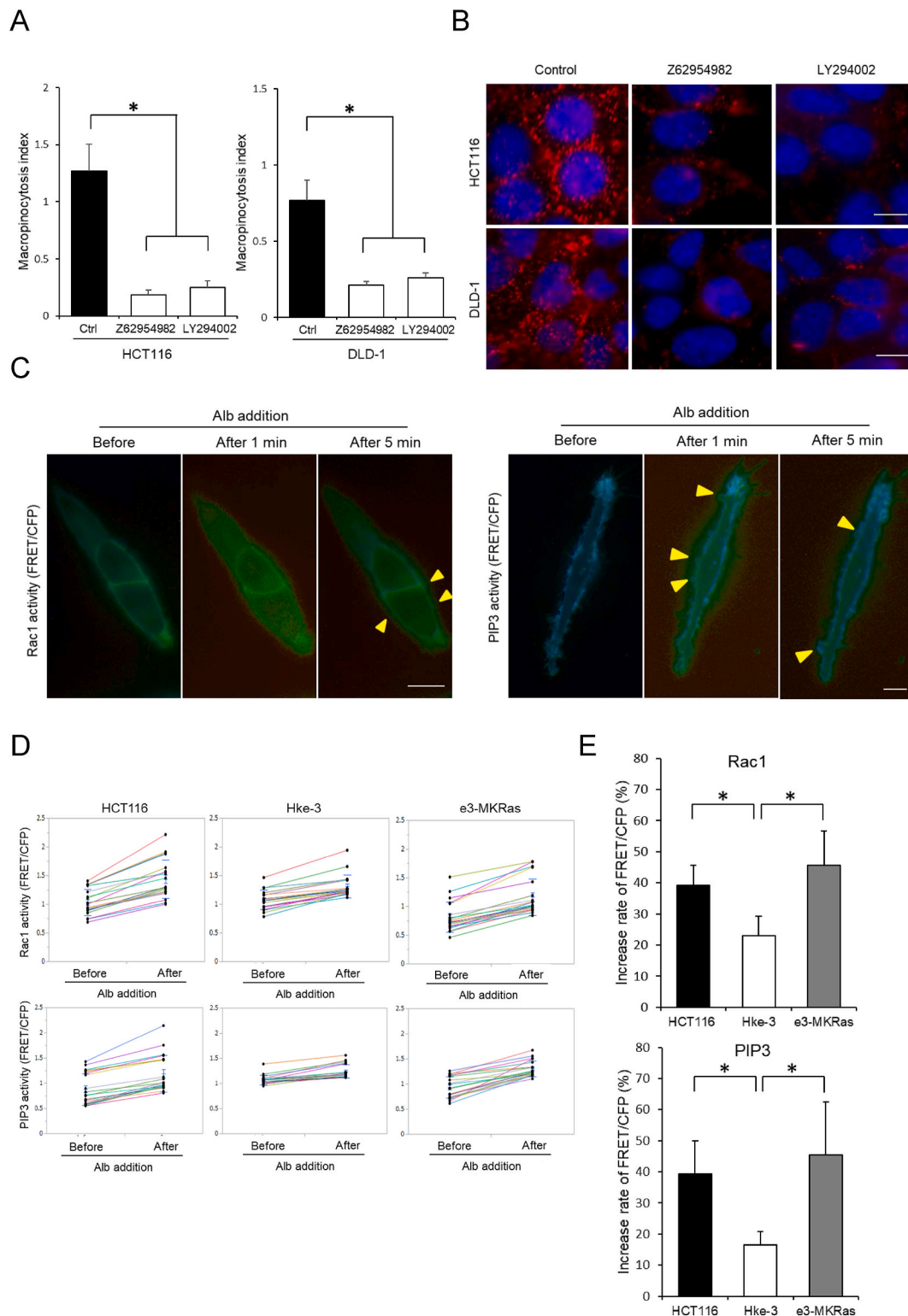


Fig. 4. Activation of Rac1 and PIP3 is involved in macropinocytosis of KRAS-mutant CRC cells.

A. Quantification of TMR-dextran uptake (macropinocytosis index) in HCT116 (left) and DLD-1 (right) treated with Ctrl (control), Z62954982 (Rac1 inhibitor) or LY294002 (PI3K inhibitor). Results are presented as the means $\pm$ SEM of triplicate measurements. * $p < 0.01$ by Student's t-test. B. Macropinocytic uptake of TMR-dextran (red). The nucleuses are stained by DAPI (blue). Scale bar, 5 μ m. C. FRET-based live cell imaging using the biosensors Rac1 and PIP3 (FRET/CFP) in HCT116 cells. HCT116 cells were serum-starved for 12 h and stimulated with 1 mg/ml albumin. FRET/CFP ratio images at the indicated time points are shown in the intensity-modulated display mode. Scale bar = 5 μ m. Yellow arrowheads indicate membrane ruffles. D. Rac1 and PIP3 activity (FRET/CFP) in HCT116, HKe-3 and e3-MKRas cells before and after albumin addition. Each dot represents each cell. Total 20 cells were analyzed. E. Increase rates in Rac1 and PIP3 activity (FRET/CFP) of HCT116, HKe-3 and e3-MKRas cells before and after albumin addition. Results are presented as the means $\pm$ SEM of triplicate measurements. * $p < 0.01$ by Student's t-test. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

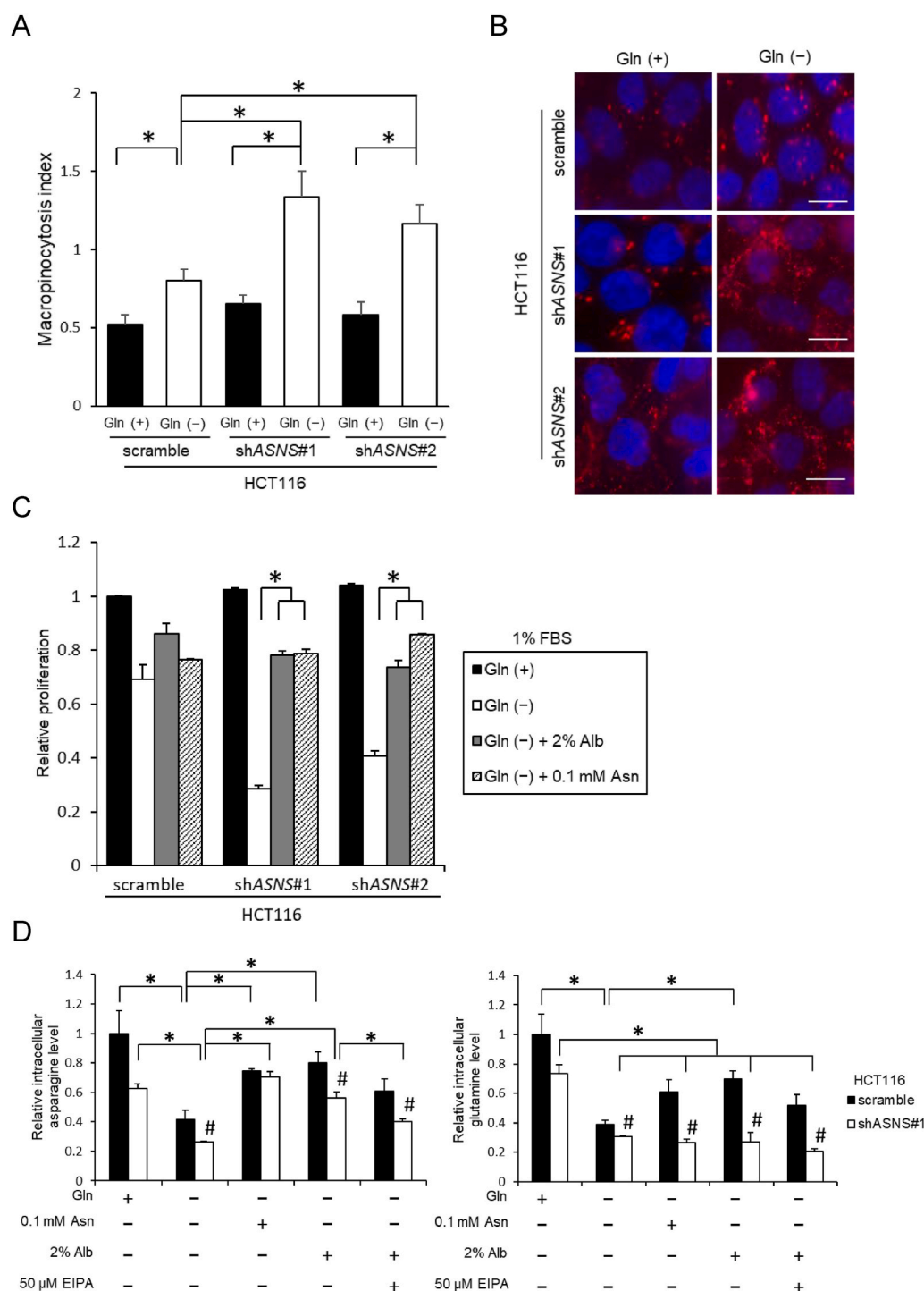


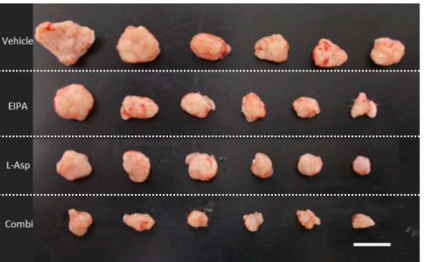
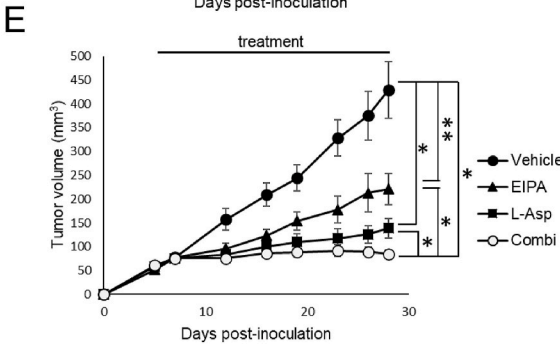
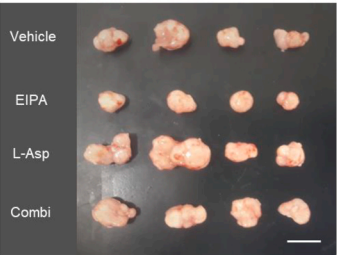
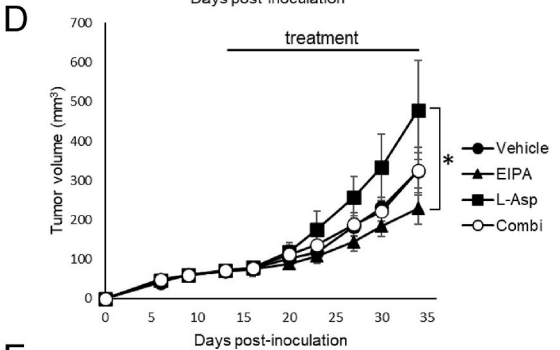
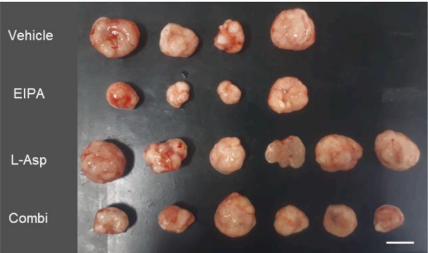
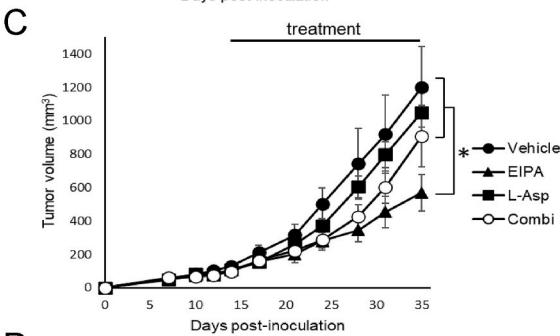
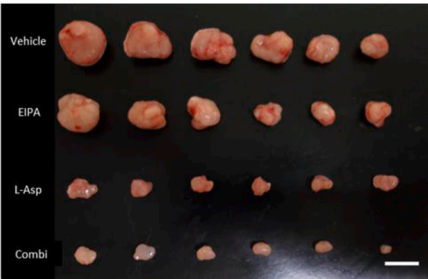
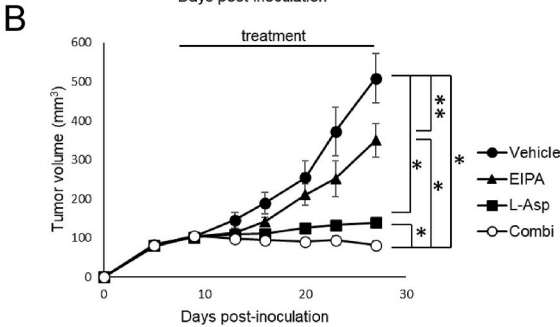
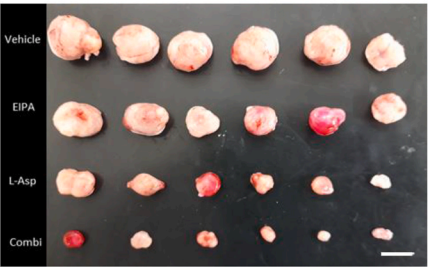
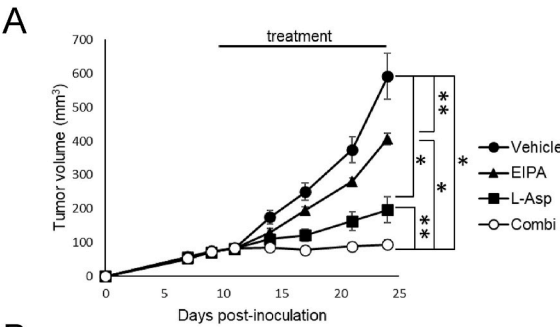
Fig. 5. Macropinocytosis is closely correlated with asparagine metabolism in CRC.

A. Quantification of TMR-dextran uptake (macropinocytosis index). HCT116 cells transfected with scramble control or two independent shASNS vectors (shASNS#1 and shASNS#2) were maintained in the presence or absence of glutamine. Results are presented as the means $\pm$ SEM of triplicate measurements. * $p < 0.01$ by Student's t-test. B. Macropinocytic uptake of TMR-dextran (red). The nuclei are stained by DAPI (blue). Scale bar, 5 μ m. C. Cell proliferation measured by CCK-8 assay. Under DMEM medium containing 1% FBS with indicated conditions, indicated cells were treated for 72 h with vehicle, 2% albumin, or 0.1 mM asparagine. Cell proliferation in each condition was normalized to that of scramble control cells in the presence of glutamine. Results are presented as the means $\pm$ SEM of triplicate measurements. * $p < 0.01$ by Student's t-test. D. Intracellular amino acid concentration. The cells were cultured in DMEM containing 1% FBS with indicated conditions for 72 h. Then, cells were harvested and counted. Each cell pellet was used for HPLC analysis. Intracellular amino acid concentration in each condition was normalized to that of scramble control cells in the presence of glutamine (4 mM). Results are presented as the means $\pm$ SEM of triplicate samples. * $p < 0.05$ by Student's t-test. # $p < 0.05$ by Student's t-test between glutamine-sufficient condition and glutamine-depleted condition. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

xenografts (Fig. 6B). The combination of EIPA plus L-Asp exhibited dramatic suppression in tumor growth, and in some cases regression. On day 27 post-inoculation, the tumor size in vehicle-treated, EIPA-treated, L-Asp-treated, and combination (EIPA plus L-Asp)-treated mice were 508 ± 62 mm³, 350 ± 43 mm³, 139 ± 6 mm³, and 81 ± 10 mm³, respectively. We also examined the *in vivo* inhibitory effect of EIPA and/or L-Asp using CRC cells with wild-type ASNS (i.e., empty vector-transfected HCT116 and DLD-1), and found that neither L-Asp alone nor the combination of EIPA plus L-Asp had anti-tumor effect *in vivo*, although EIPA alone suppressed the tumor growth to some extent (Fig. 6C and D). TMR-dextran was delivered intratumorally, and the tumors were excised for *in vivo* macropinocytosis assay. We confirmed

that tumors collected from EIPA-treated mice displayed a significantly lower MI than those from vehicle-treated mice (Supplementary Figs. S3A and B). We also found that tumors collected from L-Asp-treated mice displayed a significantly higher MI than those from vehicle-treated mice, which was not observed in combination (EIPA plus L-Asp)-treated mice. Immunohistological analysis indicated that the number of Ki-67-positive tumor cells was significantly lower in combination (EIPA plus L-Asp)-treated mice compared with in the other three groups (Supplementary Figs. S3C and D).

Furthermore, we investigated whether the above-mentioned observations could be applied to CRC patient-derived spheroid lines. We established a HC17T spheroid line engineered with ASNS knockdown by



(caption on next page)

Fig. 6. Combination of macropinocytosis inhibition and asparagine depletion dramatically suppresses tumor growth of *KRAS*-mutant CRC cells *in vivo*. A. Xenograft size of ASNS-knockout HCT116 cells treated with vehicle, EIPA alone (150 µg/each mouse), L-Asp alone (1500 U/kg), or combination of both drugs (left). Representative macroscopic views of the xenografts 24 days after inoculation (right). Mean; bar, ± SEM, *n* = 6 mice in each group. **p* < 0.01 and ***p* < 0.05 by Student's *t*-test. Scale bar, 1 cm. B. Xenograft size of ASNS-knockout DLD-1 cells treated with vehicle, EIPA alone (150 µg/each mouse), L-Asp alone (1500 U/kg), or combination of both drugs (left). Representative macroscopic views of the xenografts 27 days after inoculation (right). Mean; bar, ± SEM, *n* = 6 mice in each group. **p* < 0.01 and ***p* < 0.05 by Student's *t*-test. Scale bar, 1 cm. C. Xenograft size of empty vector-transfected HCT116 cells treated with vehicle, EIPA alone (150 µg/each mouse), L-Asp alone (1500 U/kg), or combination of both drugs (left). Representative macroscopic views of the xenografts 35 days after inoculation (right). Mean; bar, ± SEM, *n* = 4–6 mice in each group. **p* < 0.05 by Student's *t*-test. Scale bar, 1 cm. D. Xenograft size of empty vector-transfected DLD-1 cells treated with vehicle, EIPA alone (150 µg/each mouse), L-Asp alone (1500 U/kg), or combination of both drugs (left). Representative macroscopic views of the xenografts 35 days after inoculation (right). Mean; bar, ± SEM, *n* = 4 mice in each group. **p* < 0.05 by Student's *t*-test. Scale bar, 1 cm. E. Xenograft size of ASNS-knockdown HCT116 spheroid lines treated with vehicle, EIPA alone (150 µg/each mouse), L-Asp alone (1500 U/kg), or combination of both drugs (left). Representative macroscopic views of the xenografts 28 days after inoculation (right). Mean; bar, ± SEM, *n* = 6 mice in each group. **p* < 0.01 and ***p* < 0.05 by Student's *t*-test. Scale bar, 1 cm.

lentiviral transduction of shASNS construct (Supplementary Fig. S4A), and examined the *in vivo* inhibitory effect of EIPA and/or L-Asp (Fig. 6E). As anticipated, we found that either EIPA or L-Asp significantly suppressed the growth rate of xenografts compared with the control, and that the combination of EIPA plus L-Asp entirely inhibited tumor growth. On day 28 post-inoculation, the tumor size in vehicle-treated, EIPA-treated, L-Asp-treated, and combination (EIPA plus L-Asp)-treated mice were $429 \pm 60 \text{ mm}^3$, $221 \pm 33 \text{ mm}^3$, $139 \pm 21 \text{ mm}^3$, and $85 \pm 9 \text{ mm}^3$, respectively. The combination group did not show any weight loss in mice compared with the other three groups (Supplementary Fig. S4B). Taken together, these results suggest that macropinocytosis is closely associated with asparagine metabolism, and that the combination of macropinocytosis inhibition and asparagine depletion could be a novel therapeutic strategy for *KRAS*-mutant CRC.

4. Discussion

Recent studies have revealed that *KRAS*, one of the representative oncogenes, can influence several cellular processes including proliferation, motility, survival, and metabolic alterations. Metabolic reprogramming is recognized as one of the hallmarks of cancer cells. Despite a great deal of effort, targeting mutant RAS protein has been, so far, unsuccessful. Recently, macropinocytosis has been reported to function as a nutrient-scavenging pathway for the increased metabolic needs of *KRAS*-driven cancer cells, such as PDCA and NSCLC [20–24]. Pharmacological and genetic inhibition of macropinocytosis could inhibit tumor growth, presenting a potential therapeutic target. Recent work has also reported that loss of PTEN and Wnt signaling cause macropinocytosis in bladder cancer, prostate cancer, and CRC [19,40–42]. On the other hand, there are few reports to elucidate the relationship between *KRAS* mutations and macropinocytosis in CRC. Therefore, in the present study, we investigated whether *KRAS* status could affect macropinocytosis in CRC, and found that macropinocytosis was accelerated in *KRAS*-mutant CRC cells to confer growth advantage under nutrient-depleted conditions (Fig. 1–3). Using time-lapse observation of the FRET biosensor, we also found that increased activities of Rac1 and PIP3 were associated with macropinocytosis in *KRAS*-mutant CRC cells (Fig. 4). We have not examined other well-known regulators of macropinocytosis (e.g., Cdc42 and Pak1); therefore, further investigation is required. A recent study showed that glutamine depletion-induced potentiation of EGFR-Pak signaling could regulate inducible but not constitutive macropinocytosis in a subset of PDCA tumor cells [43]. In Fig. 5 A–D, we found that the MI of shASNS transfectants was significantly higher than that of the controls under glutamine-depleted but not glutamine-sufficient condition, and that albumin addition recovered the growth inhibition caused by glutamine depletion in shASNS transfectants, indicating that glutamine depletion-inducible macropinocytosis may be correlated with asparagine metabolism. Using many CRC cell lines, we also examined the relationship between *KRAS* mutational status and glutamine depletion-inducible macropinocytosis, but no clear correlation was observed (Supplementary Fig. S4C). Further research is needed to assess the mechanisms of glutamine depletion-inducible macropinocytosis in CRC. Several aspects of

macropinocytosis have functional similarities to autophagy, a vesicular pathway that mediates the breakdown of intracellular macromolecules [44,45]. Notably, recent studies showed that autophagy inhibition could synergize with pharmacological targeting of the *KRAS* downstream effector, MEK1/2 or ERK, preventing *KRAS*-driven PDCA [46,47]. Progress in the study of autophagy in CRC is expected in the future.

Nutrient limitation is widely recognized as a potential therapeutic strategy against rapidly proliferating cancers. Many cancer cells, notably those driven by *Myc* and *KRAS*, are metabolically reprogrammed to take up more glutamine and to shift from catabolic to anabolic utilization of glutamine. Thus, the mechanism by which cancer cells can adapt to glutamine depletion has been actively investigated. In some cell types, asparagine is essential for adaptation to glutamine depletion by suppressing apoptosis [48,49]. We found that addition of 0.1 mM asparagine significantly recovered the growth inhibition in HCT116 and DLD-1 cells under glutamine-depleted condition (1% FBS) (Supplementary Fig. S4D). The effect of asparagine addition was partially inhibited by EIPA treatment, but the rate of inhibition was lower than that of albumin addition. Asparagine also plays a role in the exchange of extracellular amino acids, especially serine, arginine, and histidine [50]. ASNS is an enzyme that synthesizes asparagine from aspartate using glutamine as a nitrogen source. Knott et al. recently reported that ASNS expression was positively correlated with later metastatic relapse of patients with breast cancer, and that asparagine bioavailability could govern metastatic potential in a mouse model of breast cancer [51], suggesting that ASNS can be a crucial regulator of metastatic progression. As the role of ASNS in *KRAS*-driven NSCLC, Gwinn et al. recently reported that mutant *KRAS* altered amino acid uptake and asparagine biosynthesis through ATF4 regulation under nutrient deprivation stress, and that ASNS, the target of ATF4, could contribute to apoptotic suppression, protein biosynthesis and mTORC1 activation [52]. They also found that the AKT inhibitor, MK-2206, suppressed ASNS expression, and, combined with depletion of extracellular asparagine using L-Asp dramatically decreased tumor growth, indicating that ASNS could be an important target for metabolic regulation of *KRAS*-driven tumors. Under glutamine-sufficient condition, shASNS transfectants did not have much effect on MI and cell proliferation (Fig. 5A and B), suggesting that the effect of ASNS in tumorigenesis seems to be cell-type dependent. We previously reported that ASNS was up-regulated by mutant *KRAS* via the PI3K-AKT-mTOR pathway, that *KRAS*-mutant CRC cells acquired adaptation to glutamine depletion through asparagine biosynthesis by ASNS, and that asparagine depletion (i.e., combination of ASNS inhibition and L-Asp treatment) could markedly suppress tumor growth of *KRAS*-mutant CRC xenografts *in vivo* [12]. In the present study, we found that asparagine depletion (i.e., combination of ASNS knockdown/knockout and L-Asp treatment) combined with macropinocytosis inhibition (i.e., EIPA treatment) could efficiently suppress tumor growth in *KRAS*-mutant CRC cells (Fig. 6), thus representing a novel promising approach for the treatment of *KRAS*-mutant CRC.

Extracellular depletion of asparagine using L-Asp is well-established in ALL [38,39], because most ALL cells lack constitutive ASNS expression and are dependent on plasma asparagine. On the other hand, most solid tumors harbor ASNS, and are resistant to L-Asp [53]. However,

recent analysis of the CCLE database revealed that low ASNS expression in subpopulations of gastric cancer and hepatocellular carcinoma raises the possibility of repurposing L-Asp as a therapeutic option [13]. Regarding the inhibitors of ASNS and macropinocytosis, there are still many problems. Because potent ASNS inhibitors available for clinical use have not been reported yet [54,55], development of effective ASNS inhibitors is desirable. The most commonly used preclinical inhibitors for macropinocytosis are the Na^+/H^+ exchanger inhibitors, EIPA, and amiloride. NHE promotes actin polymerization mediated by Rac1 and Cdc42 [33], which induces membrane ruffling, a critical early step of macropinocytosis. Rac1 inhibitor, EHT1864, was reported to inhibit macropinocytosis in NSCLC cell lines and mouse embryo fibroblast (MEF) [19,56]. Pak1 inhibitors, FRAX597 and IPA-3, also showed the ability to inhibit macropinocytosis in bladder cancer cells and *PTEN* knockout MEF [19,40,57]. Interestingly, systematic screening using 640 FDA-approved compounds has identified that an antidepressant, imipramine, specifically inhibits macropinocytosis and membrane ruffling without cytotoxic effects [58]. Given the lack of macropinocytosis inhibitors available for clinical use, imipramine could be a potential therapeutic drug as a macropinocytosis inhibitor. Recent studies highlight newly discovered membrane molecules, such as V-ATPase and syndecan 1, provide a link between mutant *KRAS* and RAC1 activation in macropinocytosis [59,60]. These findings might lead to the more selective pharmacological strategies to inhibit macropinocytosis in *KRAS*-mutant cancers.

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Author contributions

K.K. conceived the idea, designed the experiments, analyzed the data, and wrote the manuscript. K.H. designed the experiments, performed most experiments, analyzed the data, and wrote the manuscript. G.N. designed the experiments, performed some experiments, and analyzed the data. K.T., R.M., and Y.I. designed the experiments, and analyzed the data. H.M., Y.N., H.M., W.H., M.O., Y.K., S.H., R.O., H.M., T.S., and S.S. assisted in performing the experiments. M.M.T. and K.O. supervised the study. Y.S. designed the experiments, and supervised the study. All authors discussed the results and commented the manuscript.

Declaration of competing interest

The authors declare no competing interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.canlet.2021.09.023>.

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