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Liver Endothelium Microenvironment Promotes HER3-mediated Cell Survival in *KRAS* Wild-type and Mutant Colorectal Cancer

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Running title: Liver Endothelial cells activate HER3 in *KRAS* wild-type and mutant CRC Cells

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ABSTRACT

We previously showed that primary liver endothelial cells (ECs) secreted soluble factors in a paracrine fashion (angiocrine) and activated human epidermal growth factor receptor 3 (HER3, also known as ERBB3) mediated colorectal cancer (CRC) growth and chemoresistance. However, Ras proteins play a critical role in receptor tyrosine kinase signaling pathways, and *KRAS* mutations mediate CRC resistance to therapies targeting EGFR, another HER protein. Therefore, the role of *KRAS* mutation status in EC-induced HER3 activation and CRC survival was investigated as it has therapeutic implications. We used CRC cell lines and patient-derived xenografts harboring *KRAS* wild-type or mutant genes and demonstrated that liver EC-secreted factors promoted HER3-mediated CRC cell growth independent of *KRAS* mutation status. Also, blocking HER3 in CRC by siRNAs or a HER3 antibody seribantumab attenuated EC-induced CRC cell survival. Our findings highlight the potential of utilizing HER3-targeted therapies for treating patients with mCRC independent of *RAS* mutational status

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INTRODUCTION

In the United States, colorectal cancer (CRC) is the second-leading cause of cancer-related deaths, with estimated deaths of >50,000 patients per year [1]. Close to 25% of CRC cases are metastatic (mCRC) at the time of diagnosis and over 20% of primary or localized CRC cases will develop into mCRC. Patients with mCRC have a 5-year survival rate at 14% and they do not respond to standard therapies well, with only 50% of response rate to systemic therapies and the duration of response is only ~10 months [2, 3]. Therefore, a better understanding of the regulations of CRC cell survival is urgently needed for the development of novel therapeutic strategies for patients with mCRC.

As over 80% of mCRC occur in the liver [4], our laboratory aims to elucidate potential roles of the liver microenvironment on CRC survival pathways, with a focus on the liver endothelial cells (ECs) as they represent more than 50% of all stromal cells in the liver [5]. Preclinical studies from other groups in gastric, liver and other types of cancers used established human umbilical vein ECs showed that ECs secreted soluble factors, and activated cancer-promoting signaling pathways (such as AKT, NFκB) in adjacent cancer cells[6-9], known as angiocrine. In contrast, our laboratory isolated primary ECs from the liver to recapitulate the liver EC microenvironment and demonstrated that liver ECs secreted soluble factors and activated cancer stem cell-associated Notch and Nanog pathways in CRC cells [10-12]. More recently, we determined that liver EC-secreted factors specifically activated human epidermal growth factor receptor 3 (HER3, also known as ERBB3) and its downstream target AKT, resulting increased cell proliferation and resistance to 5-fluorouracil (5-FU) chemotherapy in CRC. Moreover, we used a pan-

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HER inhibitor AZD8931 to block HER3 activation and attenuated the EC-induced CRC cell proliferation and chemoresistance in a subcutaneous (subQ) xenograft tumor model[13].

Meanwhile, Ras proteins, encoded by *HRAS*, *KRAS*, and *NRAS* genes, are key components in mediating receptor tyrosine kinase (RTK) signaling pathways including HER3 [14], and mutations in *RAS* genes are major oncogenic alterations in CRC and other types of cancer [15]. In CRC, close to 50% of patients with mCRC have *RAS* mutations, with over 90% mutations found in *KRAS* [16]. Patients with mutant *KRAS* have markedly worse prognosis compared to those with wild-type *KRAS* [17-19]. As *RAS* mutations lead to constitutive activation in downstream targets including AKT, blocking RTKs has limited anti-cancer effects in patients with *RAS* mutations [20]. Indeed, *KRAS* mutations are approved by the FDA as resistance markers for CRC response to EGFR targeted therapies as EGFR antibodies including cexucimab and panitumumab failed to improve the outcomes of patients with *KRAS* mutant mCRC [21,22]. Since both EGFR and HER3 are RTKs in the HER protein family and recruit Ras proteins for activating downstream targets [23], the role of *KRAS* mutation status in EC-induced HER3 activation in CRC cells needs to be elucidated. Moreover, the effects of blocking HER3 on cell survival in CRC cells with different *KRAS* mutation status needs to be determined for utilizing HER3-targeted therapies for treating patients with mCRC with different *RAS* mutational status.

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In this present study, we hypothesized that liver ECs promote CRC cell survival by activating the HER3-AKT pathway independent of *KRAS* mutation status. We determined the effects of liver ECs on CRC cell functions in CRC tumors and multiple cell lines with either wild-type or mutant *KRAS* genes. Using a modified patient derived xenograft (PDX) tumor model, we validated that conditioned medium (CM) from liver ECs increased CRC tumor growth in both *KRAS* wild-type and mutant PDXs. We then used multiple CRC cell lines to demonstrate that liver ECs activated the HER3-AKT signaling pathway and increased cell proliferation and resistance to 5-FU regardless of the *KRAS* mutation status. For HER3-specific blockade, we used HER3-specific siRNAs knockdown and a fully humanized HER3 antibody seribantumab, which is currently being assessed in a clinical trial for treating cancer patients with NRG-1 gene fusion (NCT04383210). We demonstrated that HER3 inhibition, either with siRNAs or the HER3 antibody seribantumab, completely abolished EC-induced AKT activation and CRC proliferation and chemoresistance *in vitro*. We also used an orthotropic liver injection xenograft tumor model and determined that the HER3 antibody seribantumab sensitized CRC to 5-FU chemotherapy *in vivo*. These findings demonstrated that liver EC-induced HER3 activation and CRC survival are independent of *KRAS* mutation status and highlighted the potential for using a combination of chemotherapy and anti-HER3 target therapy to treat patients with *KRAS* mutant mCRC.

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RESULTS

CM from liver ECs promoted *KRAS* wild-type and mutant CRC PDX tumor growth *in vivo*

We firstly used a proof-of-principle subcutaneous (subQ) xenograft tumor model to determine the effects of liver ECs on CRC growth. CRC PDX tissues harboring either wild-type or mutant *KRAS* genes were subQ implanted in an inoculation mixture of Matrigel and concentrated control CM (CM from HCP-1 CRC cells) or CM from human primary liver ECs (EC-1). To maintain the effects of EC-secreted factors on PDXs during the study, concentrated CM were subQ injected at the inoculation sites once a week throughout the experiment. As a result, both *KRAS* wild-type and mutant PDXs inoculated and treated with liver EC CM had significant greater tumor growth compared to the control groups with CRC CM (Fig 1).

CM from liver ECs activated the HER3-AKT pathway and increased cell survival in CRC cells with different gene mutation profiles

To elucidate specific effects of EC CM on CRC cell growth and signaling pathways, we used two primary liver EC lines (EC-1 and EC-6) and multiple CRC cell lines that carried either a wild-type *KRAS* gene (SW48 and Caco2) or *KRAS* mutations (HCP-1, HCT116, and DLD-1). Cell line mutation status were determined in previous studies or from the ATCC database (Supplementary Table 1) [24-27]. CM containing EC-secreted factors were harvested and added to CRC cells, with CM from CRC cells themselves as control CM. Compared with control CM, CM from liver ECs (EC-1 and EC-6) dramatically increased

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phosphorylation of HER3 and its downstream target AKT, demonstrating activation of the HER3-AKT survival pathway in all CRC cells studied (Fig. 2a). This suggested that constitutive activation of the KRAS pathway, as a result of *KRAS* mutations, in CRC cells did not affect HER3 or AKT activation by CM from ECs. We noticed that under the control conditions, HCP-1 cells (*KRAS* mutant) had higher basal levels of phosphorylation of HER3 and AKT than all other cells. However, the other two *KRAS* mutant cell lines (HCT116 and DLD-1) had basal levels of phosphorylation of HER3 and AKT similar to those of *KRAS* wild-type cells (SW48 and Caco2). Therefore, the high basal levels of HER3 and AKT phosphorylation in HCP-1 is likely cell line-specific. The low but detectable basal levels of HER3 and AKT phosphorylation in multiple CRC cell lines suggests that HER3 and AKT are active even without extracellular stimulation, such as CM from ECs.

In addition to *KRAS* mutations, CRC cell lines used in this study harbored other genetic alterations including mutations of the *PIK3CA* and *TP53* genes, and different microsatellite instability statuses. To determine the specific roles of *KRAS* mutations in liver ECs activating HER3-AKT and promoting cell survival in CRC cells, we also used modified CRC cells with the mutant alleles knocked out (*KRAS*Δ KO) developed and characterized in a previous study [28]. Incubating these cells with control or EC CM demonstrated that these cells also responded to EC CM incubation and had increased phosphorylation of HER3 and AKT without the mutant *KRAS* alleles.

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We then sought to determine whether EC-induced HER3-AKT activation leads to alterations in cancer cell proliferation and response to chemotherapy. CRC cells were incubated with control CM or CM from liver ECs and then treated without or with the cytotoxic chemotherapy agent 5-FU. MTT assay was used to determine the relative number of viable CRC cells, presented as percent of viable cells relative to that in control groups with CRC CM only (Fig. 2b—d). In the absence of 5-FU treatment, CM from liver ECs significantly increased cell viability in CRC cells compared to control CM. In particular, CRC cells with *KRAS* mutations had greater increases in cell viability (~2.5 to 3.5-fold increases in HCP-1, HCT116 and DLD-1 cells, Fig. 2c) compared to CRC cells with wild-type *KRAS* (<2-fold change in SW48 and Caco2 cells, Fig. 2b). Meanwhile, cells in different CM were treated with 5-FU with a clinically relevant dose (2 μg/ml) [29], which resulted in sufficient induction of apoptosis in CRC cells in our previous studies [10,11,13]. The MTT assay showed that in CRC cells with wild-type *KRAS* (Fig. 2b), mutant *KRAS* (Fig. 2c), and mutant alleles knocked out (Fig. 2d), 5-FU treatment was effective and significantly led to a 50% decrease in cell viability relative to cells incubated in control CM. In contrast, CRC cells in liver EC CM had much greater viability (~100%-150%) than control groups, suggesting that the CRC cells in CM from liver ECs were more resistant to chemotherapy. Taken together, these findings suggested that CM from liver ECs increased the proliferation and chemotherapy resistance in CRC cells with different *KRAS* mutations.

HER3 mediated EC CM-induced AKT activation and cell survival in CRC cells with different gene mutation profiles

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To validate that EC CM-induced AKT activation and CRC cell survival were mediated by HER3, we used siRNAs to knock down CRC-associated HER3 and showed that in the absence of HER3 expression, EC CM could no longer induce AKT phosphorylation in CRC cells with different mutation profiles (Fig. 3). This observation was further validated by blocking HER3 activation with a HER3-specific antibody seribantumab, which has been assessed in clinical trials in solid tumors [30,31]. Similar to siRNA knockdown, seribantumab blocked HER3 and AKT phosphorylation induced by CM from two liver EC lines (Fig. 4). Taken together, these results showed that EC-induced AKT activation was inhibited by HER3 blockade, either with siRNA knockdown or seribantumab, and confirmed that HER3 mediated EC CM-induced AKT activation in CRC cells independent of the *KRAS* mutation status. We noticed that the anti-HER3 antibody did not affect the basal levels of HER3 phosphorylation in CRC cells incubated with control CM, and that neither HER3 siRNAs nor seribantumab decreased the basal levels of AKT phosphorylation. These findings supported the notion that HER3 blockade only inhibited EC-induced HER3-AKT activation in CRC cells and that cancer cells had basal levels of AKT and HER3 activation, which were possibly mediated by CRC intrinsic mechanisms or cell culture conditions.

Moreover, we used MTT assay to determine the effects of the HER3-antibody on EC CM-induced CRC cell proliferation and response to chemotherapy. First, we confirmed that seribantumab significantly blocked EC-induced proliferation in all CRC cells with different mutation profiles used (Suppl. Fig. 1). Subsequently, we incubated CRC cells with 5-FU either alone or in combination with seribantumab (Fig. 5). EC CM significantly

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increased cell viability, as expected, in all CRC cells used in this study. Single-agent treatment of 5-FU decreased cell viability in all conditions, but to a lesser extent in cells incubated with liver EC (EC-1) CM. These findings confirmed that CRC cells were more resistant to 5-FU when incubated with EC CM. In contrast, when we treated CRC with seribantumab and 5-FU together, the EC CM-induced cell viability was completely abolished, resulting in lower cell viability than that in other groups. Similar results were found in CRC cells without *KRAS* mutant alleles (Fig. 5c), confirming that the effects of HER3 inhibition on EC-induced cell proliferation and chemotherapy resistance were independent of *KRAS* mutations. Taken together, these findings demonstrated that inhibition of HER3 activity blocked EC-induced CRC cell proliferation and chemoresistance and sensitized cells to 5-FU treatment in both *KRAS* wild-type and mutant CRC cells.

HER3 inhibition sensitized CRC tumors in the liver to 5-FU *in vivo*

Because *KRAS* wild-type CRC has been proven to be susceptible to EGFR-targeted therapies such as cetuximab [32] and panitumumab [33] in the clinic, we focused on evaluating roles of the HER3-AKT pathway in *KRAS* mutant CRC tumors, especially in the context of liver metastases. We assessed the effects of blocking HER3 activity on CRC tumors in the liver using an orthotropic tumor model by injecting human CRC cells (HCP-1, *KRAS* mutant) into the livers of athymic nude mice to recapitulate CRC liver metastases in patients with mCRC. To determine if murine liver ECs activate human CRC-associated HER3, we isolated primary liver ECs from athymic nude mice and generated murine EC CM to confirm that murine liver ECs activated HER3 and AKT in

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human CRC and promoted cancer cell growth (Suppl. Fig. 2). We then injected luciferase-labeled HCP-1 cells in the livers of athymic nude mice and treated tumor-bearing animals with the anti-HER3 antibody alone or in combination with 5-FU (Fig. 6). Compared with the control group, a low dose of 5-FU (20mg/kg) resulted a noticeable decrease in tumor burden and tumor growth rate as determined by tumor-bearing liver weights and bioluminescence over time. Moreover, the combination of seribantumab and 5-FU significantly decreased tumor growth, leading to lower tumor burden and liver weights than in other groups. These findings validated that inhibition of HER3 activity by the HER3 antibody seribantumab decreased EC-induced CRC chemoresistance, and therefore, sensitized tumors to 5-FU treatment. However, we noticed that single agent treatment with seribantumab did not affect tumor development in the liver, suggesting that HER3 inhibition alone is not sufficient to block tumor growth.

DISCUSSION

Effects of the stromal microenvironment on cancer cell functions have been discussed in depth in many different types of cancer [34,35]. Also, studies have evaluated specific strategies for targeting *KRAS* mutant-bearing tumors in different types of cancer, but these strategies have remained unsuccessful for CRC [36,37]. In the present study, we sought to elucidate the role of liver ECs in mediating CRC cell survival and directly compare that among CRC cells with different *KRAS* mutation statuses. We determined that liver ECs activated cancer cell-associated HER3-AKT in both *KRAS* wild-type and mutant CRC cells. Moreover, we showed that inhibiting HER3 activation with a HER3

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antibody attenuated liver EC-induced CRC cell survival *in vitro* and sensitized *KRAS* mutant CRC tumors to 5-FU chemotherapy *in vivo*.

The canonical activation of HER3-AKT in cancer cells involves the PI3K and/or MAPK pathways [38,39]. When gain-of-function mutations of key factors occur, such as mutations in *KRAS* and *PIK3CA* genes, these pathways are highly active and are not further activated by extracellular signals. To our surprise, liver EC-secreted factors activated HER3-AKT in CRC cells independent of mutations in *KRAS* and other genes. It is possible that the HER3-AKT activation we observed was mediated by other signaling pathways. To date, neuregulin family proteins have been the only identified ligands for HER3. Because HER3 has an intracellular domain with weak kinase activity and is considered a “kinase-dead” receptor [40], neuregulin-binding leads to HER3 dimerization with HER2 and, to a lesser extent, other HER family receptors to activate downstream pathways such as AKT [23,41]. Indeed, our previous studies demonstrated that EC-induced HER3-AKT activation in CRC cells was independent of neuregulin-triggered HER2-HER3 binding [13]. In that study, we also determined that the liver EC-secreted factor that activated HER3 was larger than 150kDa, much larger than soluble neuregulins (<45kDa) [23]. Also, we did not detect HER2 phosphorylation, which is the key indicator of neuregulin-induced HER2-HER3 activation. These findings strongly suggest that EC-secreted factors activate HER3 via a mechanism that has not reported before and is potentially independent of KRAS pathway. Additional investigations are needed to further elucidate the specific mechanism of liver EC-induced HER3 activation but is beyond the scope of this study.

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In addition to *KRAS* mutations, CRC cells used in this study harbored mutations in other genes, including *PIK3CA*, *TP53* and *APC.* Preclinical studies suggested there are correlations between response to therapies and mutations in these genes [21,42-44], and the consensus molecular subtypes [45]. However, the FDA has not approved these mutations as predictive markers for response to targeted therapies in CRC. Therefore, we did not assess the effects of these genetic alterations on liver EC-induced cancer cell functions in the present study. However, our results showed that liver ECs induced HER3-AKT activation and cell survival in all cell lines we used. It suggests that the pro-survival effects of liver ECs are independent of these gene alterations and, more importantly, that HER3-targeted therapy can potentially be used for treating patients with mCRC with different mutation profiles.

Previous studies have characterized HER3 primarily in breast, ovarian, and a several other cancer types for mediating cancer cell survival[46]. In CRC, more than 75% of primary and metastatic tumors express HER3 [47,48]. cBioPortal analysis of the TCGA database showed that majority of the tumors (>94%) do not carry mutation, duplication or other alterations in HER3. Meanwhile, HER3 overexpression occurs frequently and is associated with poor prognosis in patients with CRC [49,50]. As a result, HER3 inhibition has been proposed as a promising targeted therapy strategy in different types of cancers. However, most clinical studies conducted using HER3 antibodies/inhibitors showed minor anti-tumor effect, possibly because most clinical studies so far used either HER3 antibodies/inhibitors as monotherapy or in combination with EGFR or

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HER2-targeted therapies such as cexucimab, erlotinib and trastuzumab. The HER3 antibody we used in this study, seribantumab, has been assessed in previous studies for treating breast[51], lung[52], and ovarian[53] cancer either alone or in combination with paclitaxel or EGFR/HER2 targeted therapies. Those studies showed that seribantumab was particularly effective in patients with NRG-1 gene fusion, which led to the ongoing CRESTONE trial (NCT04383210) for treating patient with any solid tumors that harbor NRG-1 fusion, which represent less than 0.2% of all cancer cases. On the other hand, there is little information in HER3-targeted therapy combining with other cytotoxic chemotherapy agents. A phase I study with 20 CRC patients used seribantumab and irinotecan but the combination did not improve patient outcomes (NCT01451632)[54]. Considering the limited number of participants in this study, the effect of combination of seribantumab and cytotoxic chemotherapy needs to be further determined. Results from our studies confirmed that even though seribantumab demonstrated potent HER3 inhibition and anti-cancer effects *in vitro*, the antibody alone only had modest effects on CRC growth in the orthotropic xenograft model. On the other hand, we showed that the treatment arm with seribantumab and 5-FU together significantly decreased CRC tumor growth compared to control or monotherapy arms. Our findings suggest that combination of HER3 inhibition and cytotoxic chemotherapy, especially with 5-FU based regiments, is likely to improve the outcomes of patients with mCRC liver metastases.

In summary, our findings demonstrated a role of liver EC microenvironment in activating HER3 and promoting CRC cell survival independent of the *KRAS* mutation status, and potentially other key oncogenic drivers. We also showed that blocking HER3 activation

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with a HER3 antibody sensitized CRC to chemotherapy *in vivo*. This work demonstrated a potential therapeutic strategy of using HER3 antibodies/inhibitors in combination with established chemotherapy for treating patients with mCRC in the clinic.

METHODS

Cell culture

The established CRC cell lines SW48 and Caco2 were purchased from ATCC (Manassas, VA, USA). Parental control and *KRAS* mutation-modified HCT116 and DLD-1 cells were described previously [28].The human CRC primary cell line (HCP-1) and human liver parenchymal primary ECs (EC-1 and EC-6) lines were isolated and established in our laboratory using MACS microbead-conjugated antibodies and separation columns (Miltenyi Biotec, Bergisch Gladbach, Germany) [10-13]. Anti-EpCAM was used for CRC cells and ant-CD31 was used for ECs. All CRC cells were cultured in MEM (Sigma-aldrich, St. Louis, MO, USA) supplemented with 5% FBS (Atlanta Biologicals, Atlanta, GA), vitamins (1x), nonessential amino acids (1x), penicillin-streptomycin antibiotics (1 x), sodium pyruvate (1x), and L-glutamine (1x), all from Thermo Fisher Scientific/Gibco (Grand Island, NY, USA). Human liver primary ECs were cultured in Endothelial Cell Growth Medium MV2 (PromoCell, Heidelberg, Germany) supplemented with 10% human serum (Atlanta Biologicals) and antibiotics-antimycotic (1x, Thermo Fisher Scientific/Gibco).

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Murine liver primary ECs were isolated from athymic nude mice also using MACS microbead-conjugated antibodies for murine CD31 (Miltenyi Biotec) and cultured in MV2 EC culture medium as described above. HCP-1 cells, human and murine liver ECs were used within 10 passages, with approximately 1 week per passage. Authentication for all cell lines were done in every 6 months by short tandem repeat (STR) tests. For primary cell lines (HCP-1 and ECs) established in our laboratory, genomic DNA from the original tissues was used for authentication. For cell lines from the ATCC, STR profiles of cell lines cultured in our laboratory were compared with the public CCSG Core Shared Resources database at University of Texas M.D. Anderson Cancer Center. All cell lines were tested for mycoplasma contamination for every 6 months.

Reagents

The fully humanized IgG2 anti-HER3 antibody seribantumab (previously known as MM-121) was provided by Merrimack Pharmaceuticals (Cambridge, MA, USA), and is now owned by Elevation Oncology Inc. (New York, NY, USA). The control human IgG antibody for *in vitro* and *in vivo* studies was from Invitrogen (Carlsbad, CA, USA). Pharmaceutical grade 5-fluorouracil (5-FU) was obtained from the pharmacy at The University of Texas MD Anderson Cancer Center. For all *in vitro* studies, 200 μg/ml seribantumab and 2 μg/ml 5-FU were used. Human *ERBB3* (HER3) specific siRNAs (si-3: 5'-GCUGAGAACCAAUACCAGA, si-4: 5'-CCAAGGGCCCAAUCUACAA) and a validated control siRNA were obtained from Sigma-Aldrich.

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Conditioned medium (CM)

0.3×10^6 of CRC cells or ECs were seeded in T25 culture flasks overnight. The next day, cells were washed two times with 1X PBS and then cultured in 3ml growth medium with 1% FBS (0.1×10^6 cells/ml) for 48 hours. CM was harvested and centrifuged at 4,000 *g* for 10 minutes to remove cell debris. CM from each CRC cell line was used as controls.

Western blotting

Cell lysates were processed and run by SDS-PAGE gel electrophoresis as described previously [11,55]. A HRP conjugated β-actin antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other antibodies were from Cell Signaling Technology (Beverly, MA, USA). For each experiment, protein lysates were loaded into two gels and processed at the same time for separate probing for antibodies specific to phosphorylated proteins and total proteins. All membranes were probed with β-actin as a loading control and a representative image was shown for each experiment. Each Western blotting figure shows representative results of at least three independent experiments.

siRNA transfection

For each transfection, 1×10^6 CRC cells were transiently transfected with 400 pmol siRNAs via electroporation using the Neon Transfection System (Invitrogen, Carlsbad, CA, USA) with 3 pulses of 10 msec at 1,600 V according to the manufacturer's instructions. Cells were recovered in 5% FBS for 24-48 hours, cultured in 1% FBS

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overnight, and then incubated in CM for 30 minutes for Western blotting, or up to 72 hours for the MTT assay.

MTT assay

CRC cells were seeded at 3,000 cells/well in 96-well plates, cultured in 1% FBS overnight and then incubated in CM for 72 hours. When seribantumab (200 μg/ml) or 5-FU (2 μg/ml) was used, cells were pretreated with seribantumab in 1% FBS medium for 6 hours, and then cultured with or without 5-FU and seribantumab in CM for 72 hours. Cell viability was assessed by adding MTT substrate (0.25% in PBS, Sigma-Aldrich) in growth medium (1:5 dilution) for 1 hour at 37 °C. Cells were washed with 1x PBS and then added with 50 μl DMSO. The optical density of converted substrate was measured at 570 nm, and relative MTT was presented as percent of control groups with cells treated with CRC CM only.

Xenograft tumor models

CRC PDXs harboring wild-type or *KRAS* G12D mutant genes were established at Case Comprehensive Cancer Center by Drs. Sanford D Markowitz, Zhenghe Wang, and Joseph Willis. Frozen PDX tumors were expanded in athymic nude mice, sliced into ~5mm^3 pieces, and implanted subcutaneously (subQ) into the right flanks of athymic nude mice in an inoculation matrix (100 μl of 1:1 mix of growth-factor-reduced Matrigel and concentrated HCP-1 or EC-1 CM). After implantation, mice were treated with

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concentrated CM by subQ injection adjacent to implanted tumors once a week. Tumor volumes were measured with a caliper.

For liver injection orthotropic xenografts, CMV-driven luciferase reporter-labeled HCP-1 CRC cells were suspended in an inoculation matrix (1:1 mix of growth factor-reduced Matrigel and serum-free MEM medium) and injected into the left lobe of the livers in athymic nude mice ($1x10^6$ cells in 50 μl/injection). After injection, tumor burden was assessed by bioluminescence with the *In Vivo* Imaging System (IVIS) and D-Luciferin substrate (Xenogen, Alameda, CA, USA) according to the manufacturer's instructions. When tumor burden was confirmed (Day 10), mice were randomized into four groups with equal tumor burden (n=10/group) and were treated with control IgG (20 mg/kg), 5-FU (20 mg/kg), or seribantumab (20 mg/kg) in 100 μl saline by intraperitoneal (I.P.) injection in every three days on Days 11, 14, 17, and 20. All mice were euthanized when three mice in any group became moribund or their tumor sizes reached $1,000mm^3$. Tumor-bearing livers were harvested for imaging and weighing.

Statistical analysis

For *in vitro* assays, all quantitative data were reproduced in at least three independent experiments, with multiple measures in each replicate. Groups were compared by two-tailed Student's *t*-test and data was expressed as means -/+ standard error of the mean (SEM) with significance of $P<0.05$. For *in vivo* assays, Wilcoxon rank-sum test was used for tumor volume and burden change over time, and one-way ANOVA was used for

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comparing liver weights between groups. Data was expressed as means -/+ standard deviation (SD) with significance of $P<0.05$.

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Michel'le Wright	collection and assembly of data
Zhenghe Wang	Resource sharing, data interpretation
Sanford D. Markowitz	Resource sharing, data interpretation
Joseph Willis	Resource sharing
Lee M. Ellis	Financial support, conception and design, data analysis and interpretation, final approval of manuscript
Rui Wang	Conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing, financial support, final approval of manuscript

COMPETING INTERESTS

The authors declare no competing interests.

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FIGURE LEGENGDS

Figure 1. Liver ECs promoted *KRAS* mutant and wild-type CRC PDX tumor growth *in vivo*.

CRC PDXs with wild-type (*KRAS* WT) and mutant (*KRAS*Δ) *KRAS* were subQ implanted with CM from HCP-1 cells (CRC CM) or primary liver EC (EC-1 CM) (EC-1 CM). **(a, e)** Tumor burden measurement over time showed that EC-1 CM promoted PDX tumor growth. Mean -/+ SD, *P<0.01, **P<0.001, ***P<0.0001 Wilcoxon rank-sum test. **(b, f)** Pictures of tumors harvested from each group. Scale bars, 1 cm. **(c, d, g, h)** Scatter plots of tumor sizes and weights after tissue harvest. Mean +/- SD, P value by one-way ANOVA.

Figure 2. CM from liver ECs activated HER3-AKT and increased cell viability and resistance to chemotherapy in CRC cells with different *KRAS* mutation profiles.

CRC cells with wild-type *KRAS* (SW48 and Caco2), mutant *KRAS* (HCP-1, HCT116, and DLD-1), and sub-clones with the mutant *KRAS* allele knocked out (*KRAS*Δ KO) were incubated with control CRC CM or CM from different primary liver ECs (EC-1 and EC-6). **(a)** Western blotting showed that treating CRC cells with EC CM for 30 minutes increased levels of HER3 and AKT phosphorylation in all cell lines. The total levels of HER3, AKT, and β-actin were used as loading controls. Data represents results of at least 3 independent experiments. **(b—d)** CRC cells were incubated with CM and treated without (Solvent) or with 5-FU in CM for 72 hours. The MTT assay showed that CM from liver ECs increased cell viability in CRC cells. Relative cell viability was presented as %

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of control groups treated with CRC CM only. Mean +/- SEM of at least three experiments, *p<0.01 *t*-test, #p<0.01 *t*-test compared to control groups treated with CRC CM only.

Figure 3. HER3 knockdown by siRNAs inhibited liver EC-induced HER3-AKT activation in CRC cells.

CRC cells were transfected with control (Si-Ctrl) or HER3-specific (Si-3 and Si-4) siRNAs and then incubated with control CRC CM or CM from different primary liver ECs (EC-1 and EC-6). The Western blotting showed that HER3 siRNAs decreased HER3 protein levels, and blocked liver EC CM-induced AKT phosphorylation in CRC cells with **(a)** wild-type *KRAS* (SW48 and Caco2), **(b)** mutant *KRAS* (HCP-1, HCT116, and DLD-1), and **(c)** sub-clones with the mutant *KRAS* allele knocked out (*KRAS*Δ KO). The total levels of HER3, ATK and β-actin were used as loading controls. Data represents results of at least 3 independent experiments.

Figure 4. HER3 antibody seribantumab blocked liver EC-induced HER3-AKT activation in CRC cells.

CRC cells were incubated in control CRC CM or CM from different primary liver ECs (EC-1 and EC-6) either in presence or absence of seribantumab. The Western blotting showed that seribantumab blocked liver EC CM-induced HER3 and AKT phosphorylation in CRC cells with **(a)** wild-type *KRAS* (SW48 and Caco2), **(b)** mutant *KRAS* (HCP-1, HCT116, and DLD-1), and **(c)** sub-clones with the mutant *KRAS* allele

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knocked out (*KRAS*Δ KO). The total levels of HER3, ATK and β-actin were used as loading controls. Data represents results of at least 3 independent experiments.

Figure 5. HER3 antibody seribantumab blocked liver EC-induced CRC cell viability and chemoresistance.

CRC cells were treated with control CM (CRC) or CM from primary liver ECs (EC-1) and with seribantumab (Seri.) or 5-FU for 72 hours. The MTT assay showed that incubation with seribantumab blocked EC CM-induced CRC cell viability and sensitized the cells to 5-FU chemotherapy in CRC cells with **(a)** wild-type *KRAS* (SW48 and Caco2), **(b)** mutant *KRAS* (HCP-1, HCT116, and DLD-1), and **(c)** sub-clones with the mutant *KRAS* allele knocked out (*KRAS*Δ KO). Mean +/- SEM of 3 experiments. Relative cell viability was presented as % of control groups treated with CRC CM only. *p<0.01 *t*-test, $^{\#}$p<0.01 *t*-test compared to control groups treated with CRC CM only.

Figure 6. HER3 antibody seribantumab sensitized *KRAS* mutant CRC tumors to chemotherapy *in vivo*.

Luciferase reporter-labeled HCP-1 CRC cells with *KRAS* mutant (*KRAS*Δ) were injected into the livers of athymic nude mice. Once tumor burden confirmed on Day 10, mice were then treated with control vehicle (Ctrl), 5-FU alone, seribantumab alone (Seri.), or 5FU and seribantumab (5FU+Seri.) in every three days (black arrow). **(a)** Tumor burdens were measured by the luminescence IVIS system over time. Mean -/+ SD, *P<0.02, **P<0.001, ***P<0.0001 Wilcoxon rank-sum test between groups on Day 21. **(b)** Picture of tumor-bearing livers harvested from each group. Scale bar, 1 cm. **(c)**

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Scatter plots of liver weighs. Mean +/- SD, P<0.03 (one-way ANOVA) for 5FU+Seri.treated group compared with other groups.

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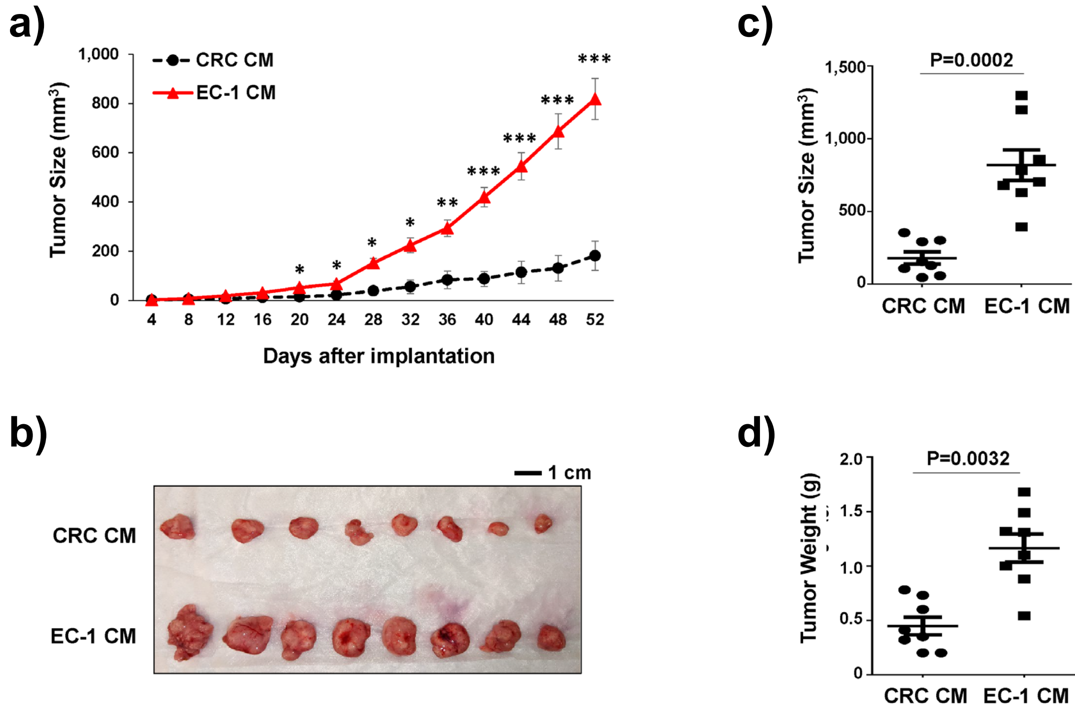
Supplementary Information

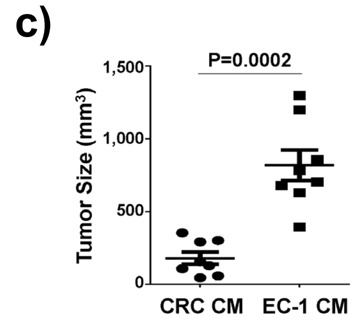
Supplementary Figure 1. HER3 antibody seribantumab blocked liver EC-induced viability of CRC cells with different mutation profiles.

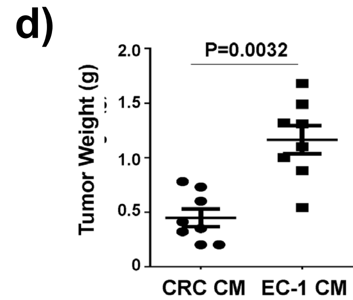
CRC cells with wild-type *KRAS* (SW48 and Caco2), mutant *KRAS* (HCP-1, HCT116, and DLD-1), and sub-clones with the mutant *KRAS* allele knocked out (*KRAS*∆ KO) where treated without or with seribantumab (Seri.) in control CM (CRC CM) or primary liver ECs CM (EC-1 CM) for 72 hours. The MTT assay showed that seribantumab significantly blocked EC CM-induced cell viability in CRC cells. Mean +/- SEM of at least three experiments, relative cell viability was presented as % of control group with CRC CM only. *$p<0.01$ *t*-test.

Supplementary Figure 2. CM from murine primary liver ECs activated HER3-AKT and induced cell growth in human CRC cells. Murine primary liver ECs were isolated from athymic nude mice and cultured for making CM. HCP-1 human CRC cells were incubated in its own CM or in murine liver EC CM. **(a)** Western blotting showed HER3 and AKT phosphorylation in human CRC cells were induced by murine EC CM after 30-minute treatment. The total levels of HER3, ATK and β-actin were used as loading controls. Data represents results of at least three independent experiments. **(b)** The MTT assay showed that CM from murine liver ECs increased human CRC cell proliferation after 72-hour treatment. *$p<0.05$ *t*-test.

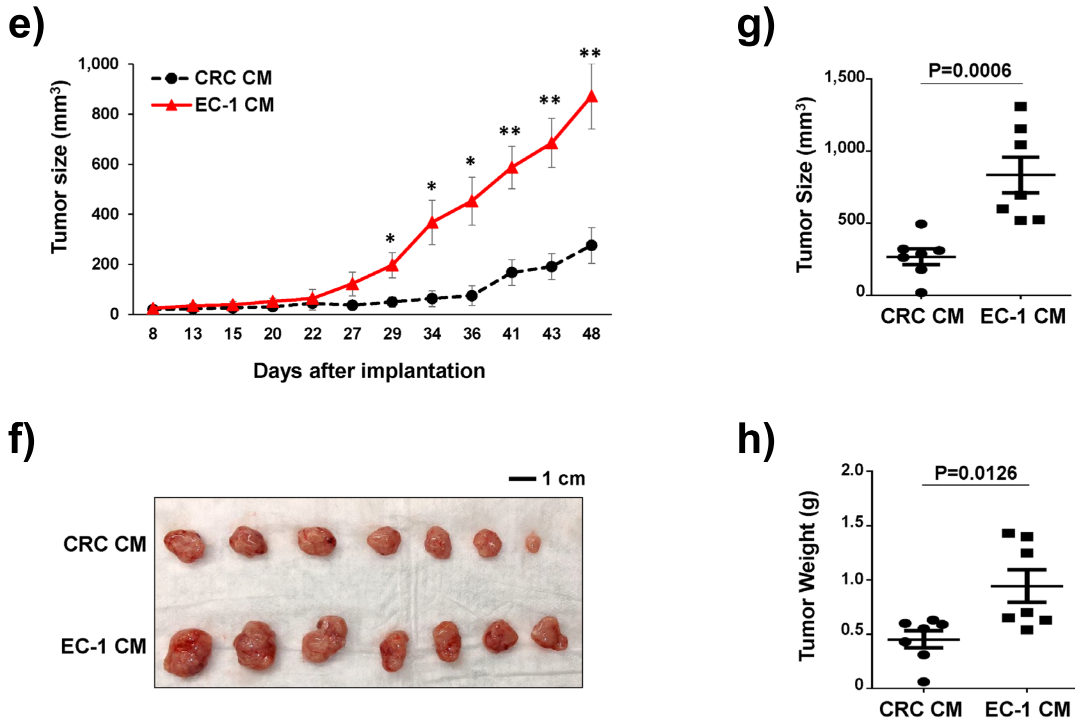
PDX (*KRAS* WT)

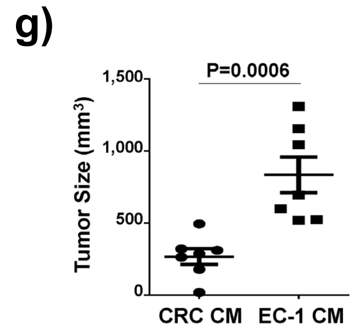






PDX (*KRAS*Δ)





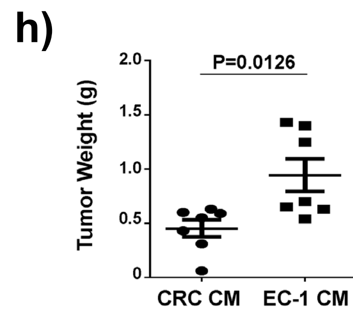
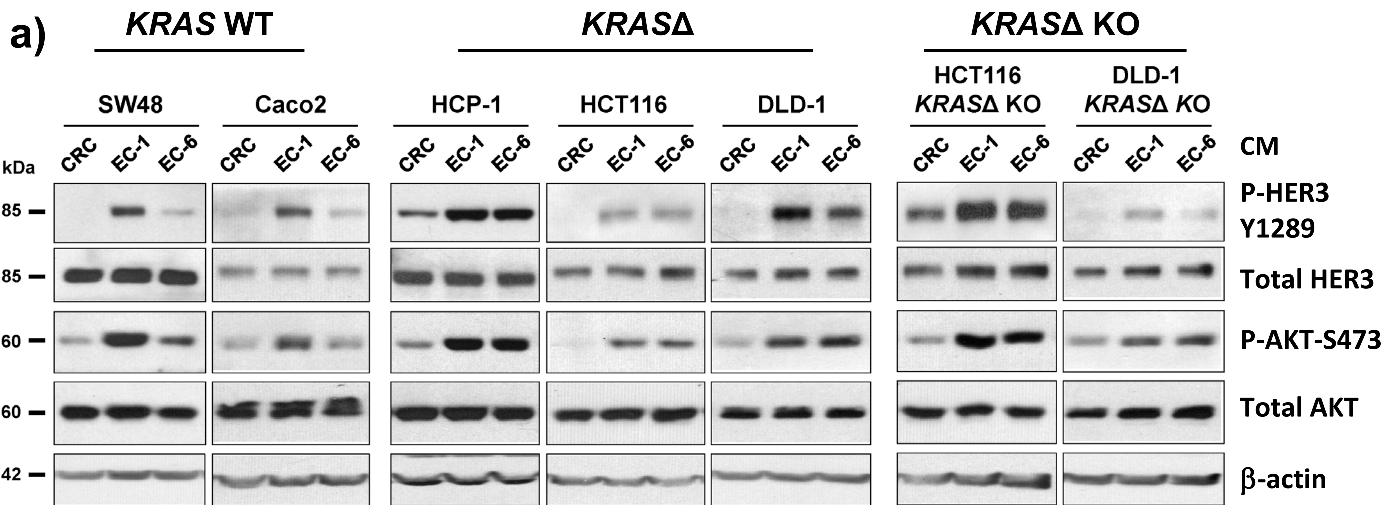
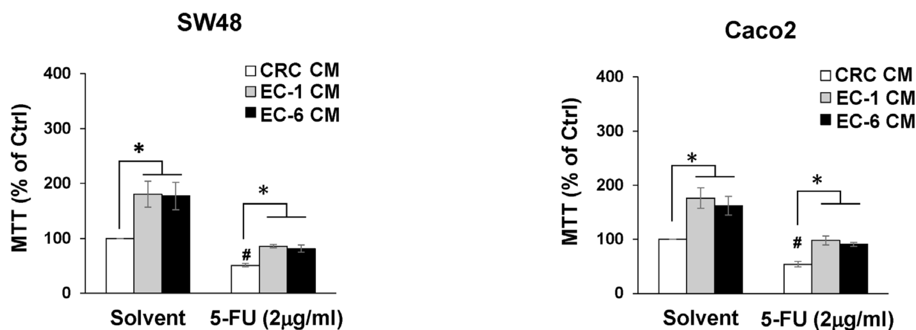


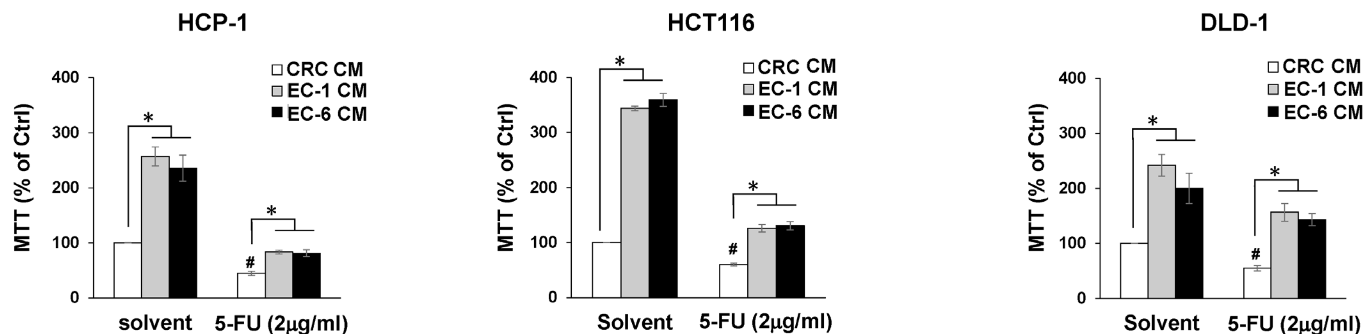
Figure 1



b) ***KRAS* WT**



c) ***KRASΔ***



d) ***KRASΔ* KO**

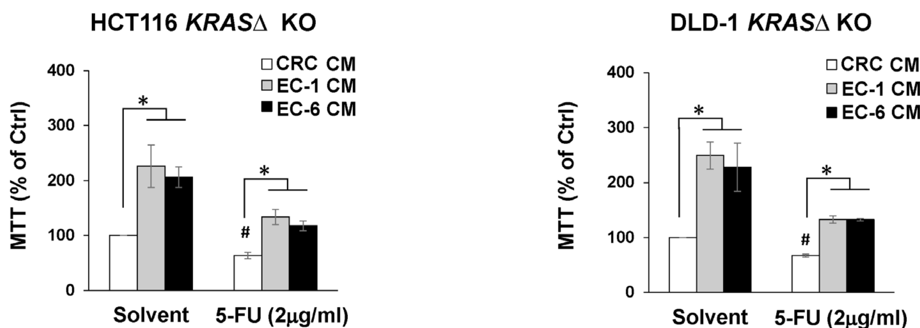
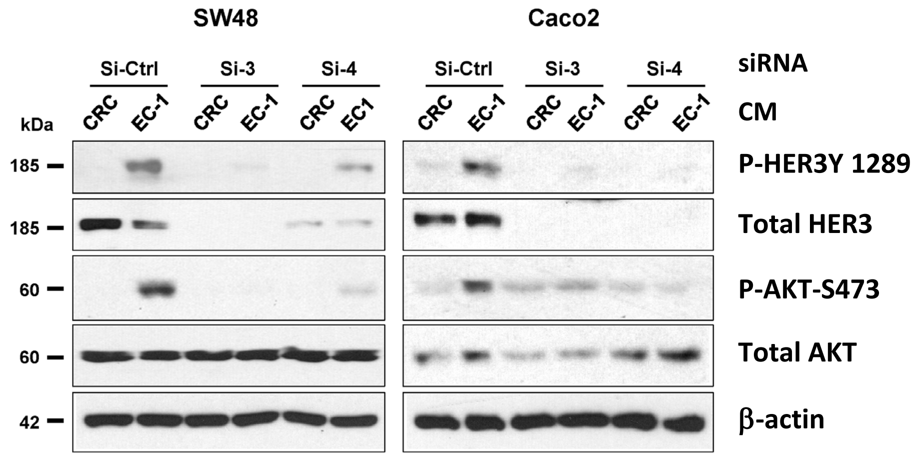
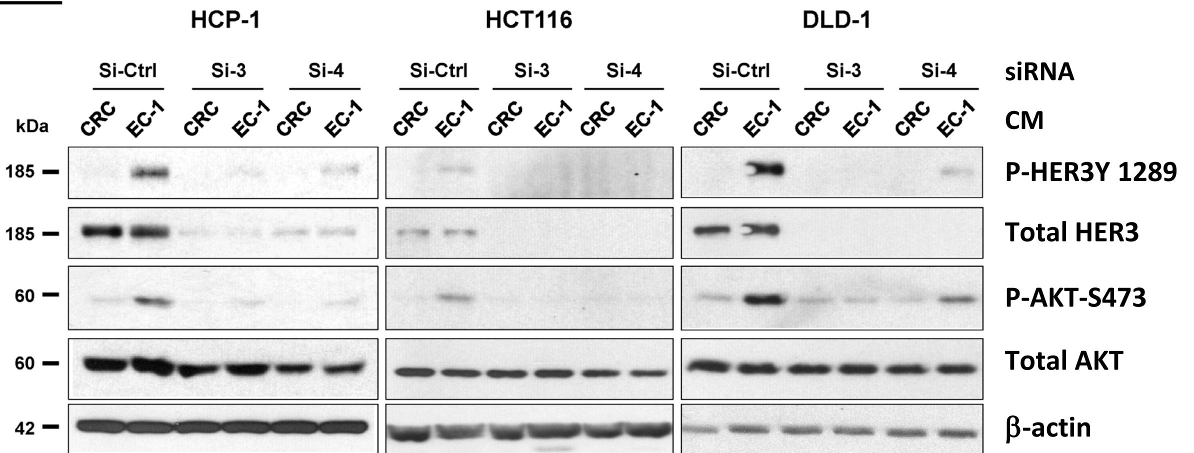


Figure 2

a) *KRAS* WT



b) *KRAS*Δ



c) *KRAS*Δ KO

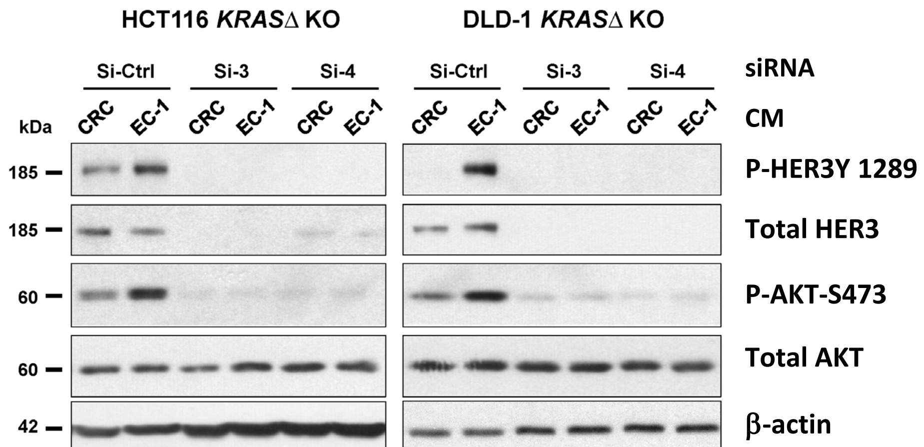
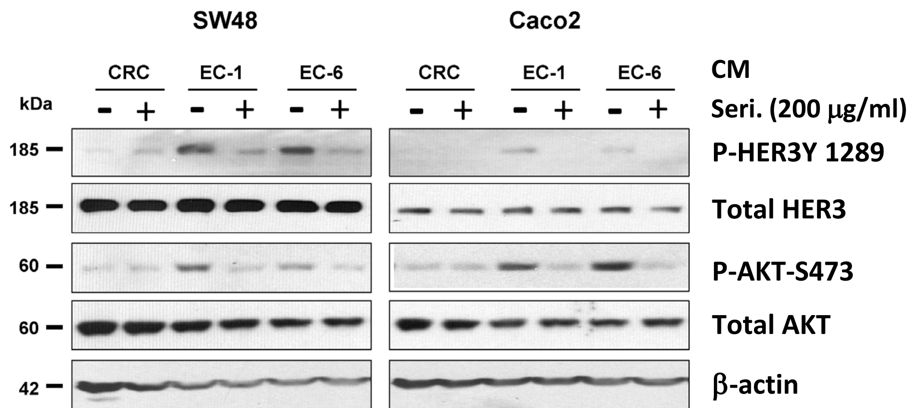
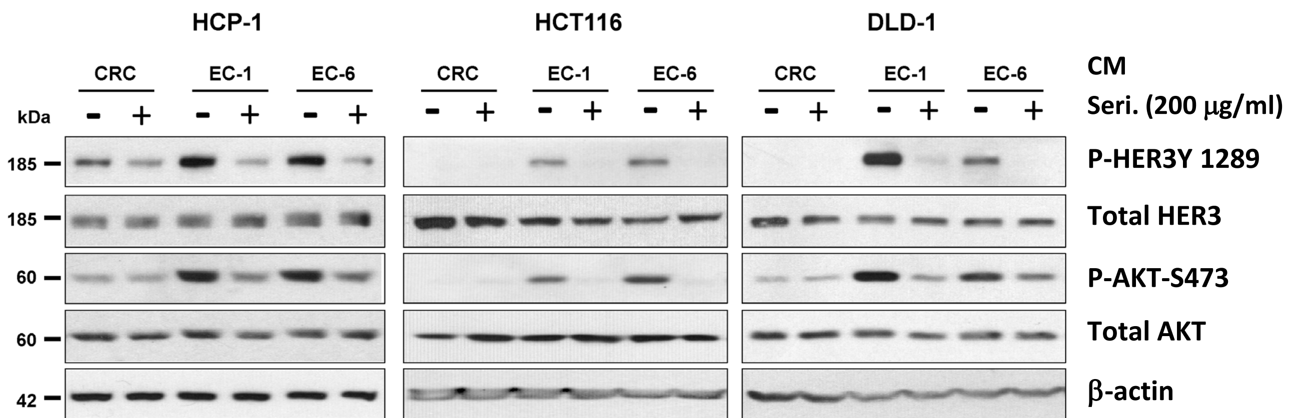


Figure 3

a) *KRAS* WT



b) *KRASΔ*



c) *KRASΔ* KO

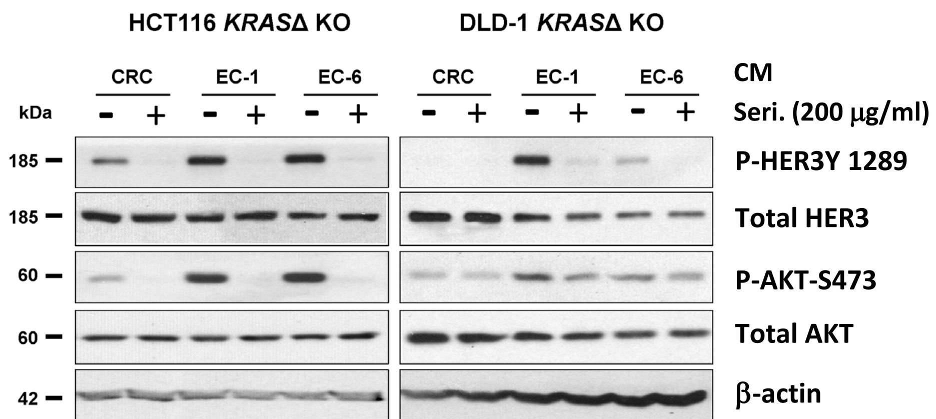
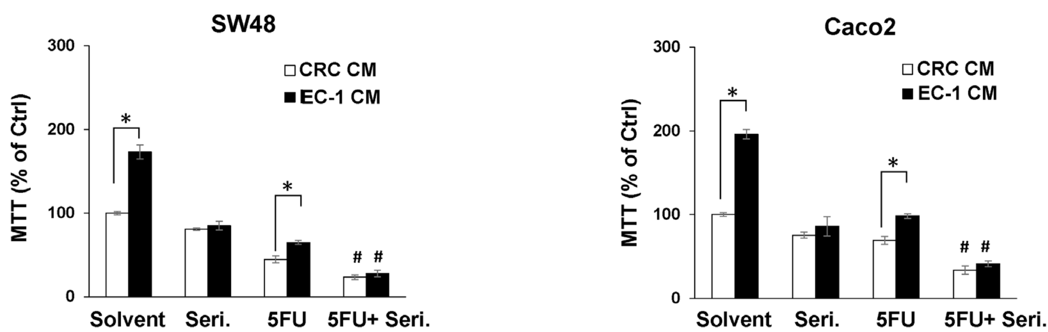
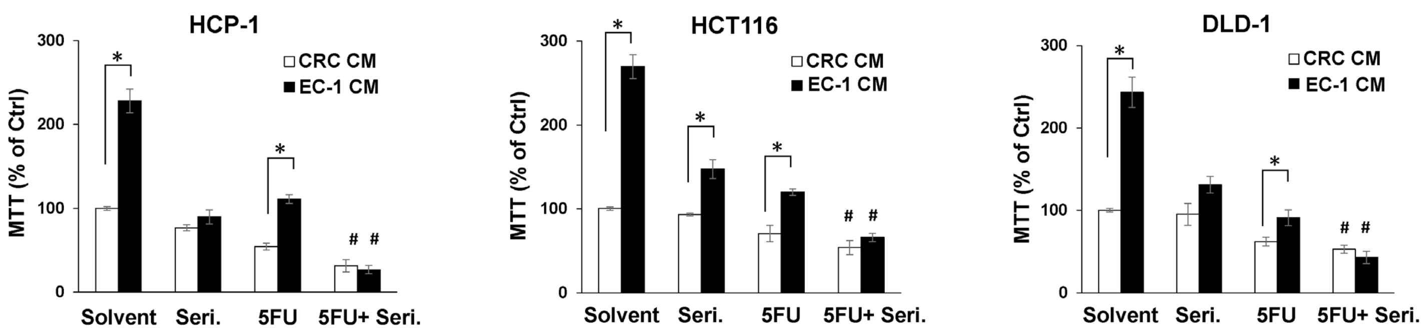


Figure 4

a) *KRAS* WT



b) *KRAS*Δ



c) *KRAS*Δ KO

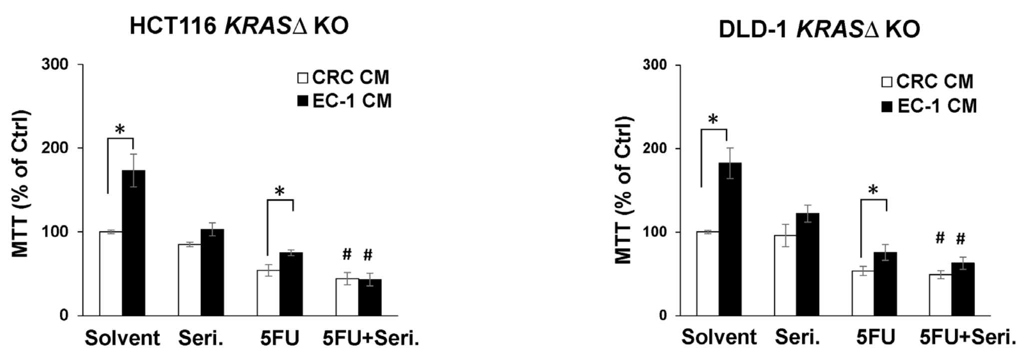
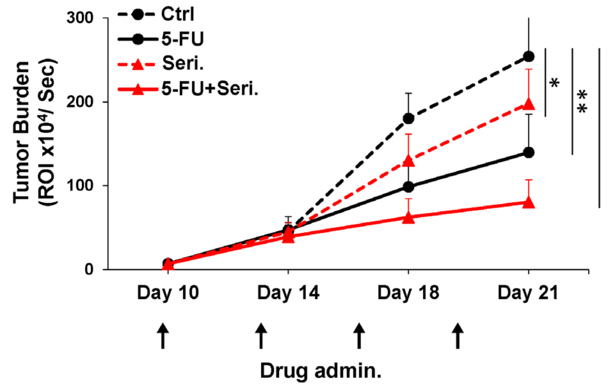


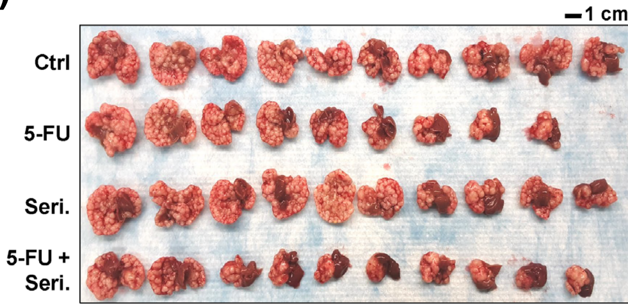
Figure 5

HCP-1 (*KRASΔ*)

a)



b)



c)

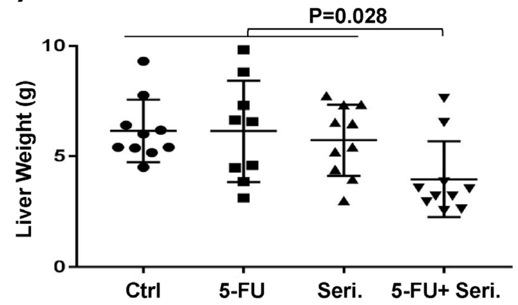


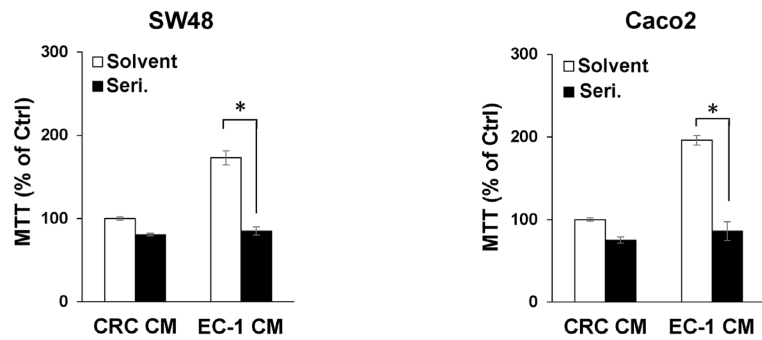
Figure 6

Table 1. Gene mutation profiles of CRC cell lines

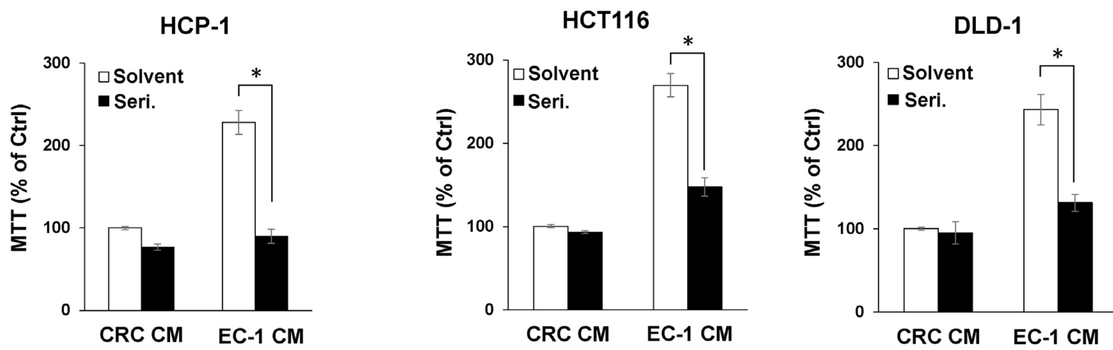
Cell Line	*KRAS* mutant	*BRAF* mutant	*PIK3CA* mutant	*TP53*	*APC*	MSI status
HCP-1	G12D	-	H1047R	NA	NA	Unstable
SW480	G12V	-	-	Δ	NA	Stable
HT29	-	V600E (-/Δ)	P449T (-/Δ)	R273H	E853# T1556 fs	Stable
SW48	-	-	-	Δ	NA	Stable
Caco2	-	-	-	Δ	NA	Stable
HCT116	G13D (-/Δ)	-	H1047R (-/Δ)	-	NA	Unstable
HCT116 *KRAS*Δ KO	(-/x)	-	H1047R (-/Δ)	-	NA	Unstable
DLD-1	G13D (-/Δ)	-	E545K (-/Δ)	S241F	NA	Unstable
DLD *KRAS*Δ KO	(-/x)	-	E545K (-/Δ)	S241F	NA	Unstable

Wildtype "-", Mutation "Δ", Knockout "x", Stop "#", Frame shift "fs", NA "not available

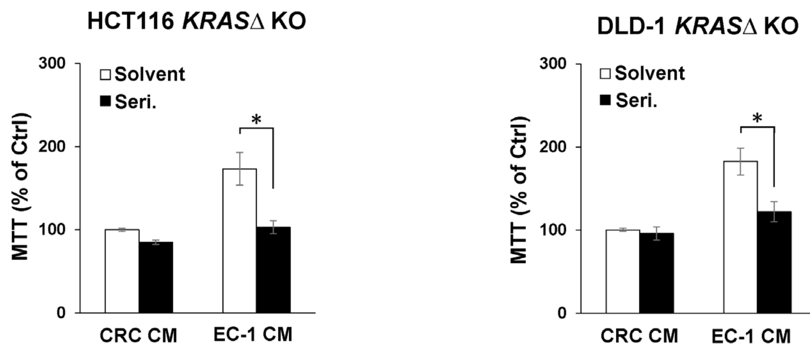
a) *KRAS* WT



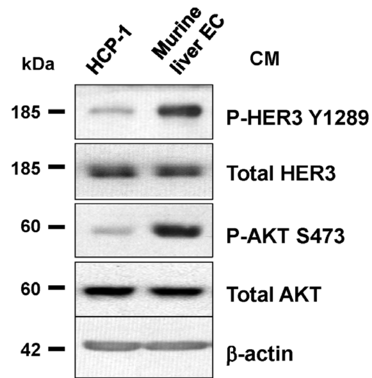
b) *KRAS*Δ

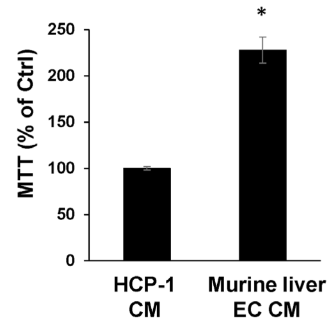


c) *KRAS*Δ KO



Supplementary Figure 1





Supplementary Figure 2