The Endosomal Protein CEMIP Links WNT Signaling to MEK1–ERK1/2 Activation in Selumetinib-Resistant Intestinal Organoids

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Abstract

MAPK signaling pathways are constitutively active in colon cancer and also promote acquired resistance to MEK1 inhibition. Here, we demonstrate that BRAFV160E-mutated colorectal cancers acquire resistance to MEK1 inhibition by inducing expression of the scaffold protein CEMIP through a β-catenin– and CRAF-dependent pathway. CEMIP was found in endosomes and bound MEK1 to sustain ERK1/2 activation in MEK1 inhibitor–resistant BRAFV160E-mutated colorectal cancers. The CEMIP-dependent pathway maintained c-Myc protein levels through ERK1/2 and provided metabolic advantage in resistant cells, potentially by sustaining amino acids synthesis. CEMIP silencing circumvented resistance to MEK1 inhibition, partly, through a decrease of both ERK1/2 signaling and c-Myc. Together, our data identify a cross-talk between Wnt and MAPK signaling cascades, which involves CEMIP. Activation of this pathway promotes survival by potentially regulating levels of specific amino acids via a Myc-associated cascade. Targeting this node may provide a promising avenue for treatment of colon cancers that have acquired resistance to targeted therapies.


Introduction

Colorectal cancer is the second leading cause of death from cancer in Western countries and arises from a variety of genetic alterations that result in the constitutive activation of both Wnt and ErbB-dependent oncogenic signaling pathways. Among the underlying genetic alterations are loss-of-function mutations of the adenomatous polyposis coli (APC) gene, which leads to β-catenin activation and constitutive Wnt signaling, followed by gain-of-function mutations in KRAS or BRAF proto-oncogenes (1). RAS signals though the Raf/Thr kinase family and triggers the subsequent activation of the mitogen-activated protein/extra-cellular signal–regulated kinase 1 and 2 (MEK1/2) as well as the extracellular signal–regulated kinase 1 and 2 (ERK1/2). This signaling cascade gained significant attention due to the high frequency of KRAS and BRAF mutations found in human cancers (2, 3). Indeed, activating mutations of KRAS are found in 40% of advanced colorectal cancer (4). In addition, the BRAF valine 600 (BRAFV600E) mutation, which leads to constitutive activation of BRAF, is found in approximately 11% of colorectal cancers and confers poor prognosis (5–7). As the pharmacologic inhibition of KRAS remains challenging, alternative approaches targeting downstream RAS effectors (RAF and MEK1) have been proposed but were poorly effective in monotherapy for the treatment of colorectal cancer, largely because of a feedback reactivation of MAPK signaling (8, 9). This reactivation occurs through the amplification of the driving oncogene KRAS or BRAF in colorectal cells treated with MEK1 inhibitors (10, 11). Other mechanisms involve the EGFR/HER1–dependent reactivation of MAPK in BRAFV600E-mutated colorectal cancer cells treated with a BRAF inhibitor (12, 13). Similarly, MAPK reactivation in KRAS-mutated colorectal cancer cells subjected to MEK1 inhibition also results from the induction of HER3 (14). Clinical trials in which RAF and EGFR or RAF and MEK are cotargeted to suppress the feedback reactivation of MAPK signaling were carried out but patients...
showing initial benefit nevertheless developed resistance and recurrence in disease progression (15). Here again, resistant colorectal cancer cells had KRAS or BRAF amplification as well as an activating MEK1 mutation (16). The Ras–RAF–MEK1–ERK1/2 cascade is critically reliant on scaffold proteins, which assemble pathway molecules to regulate signaling. Among them are Ras GTPase-activating-like protein (IQGAP1) as well as kinase suppressor of Ras (KSR; refs. 17–20).

Another scaffold protein is KIAA1199, now referred to as CEMIP ("Cell Migration-inducing and hyaluronan-binding protein"), whose expression is enhanced in cervical, breast, and colorectal cancer (21–25). CEMIP promotes cell survival and invasion, at least through EGFR-dependent MEK1 and ERK1/2 activation in cervical and breast cancer cells (23). It remains unclear which scaffold proteins, if any, are specifically involved in MAPK reactivation in colorectal cells showing intrinsic or acquired resistance to BRAF or MEK1 inhibitors.

It is intuitive that both Wnt- and MAPK-dependent signaling pathways are interconnected in promoting resistance to targeted therapies. Here we define CEMIP as a MEK1-binding protein induced by Wnt signaling. CEMIP promotes the acquired resistance to MEK1 inhibition in BRAFV600E-mutated colorectal cancer cells, at least through ERK1/2 signaling and Myc. This CEMIP-dependent cascade is essential for amino acid synthesis in resistant cells. Collectively, our data define CEMIP as a key driver of resistance to MEK1 inhibition in BRAFV600E-mutated colorectal cancer that acts upstream of ERK1/2 and Myc cascade.

Materials and Methods

Cell culture and reagents

Colorectal cancer cell lines (HT-29, HCT116, SW480, and COLO-205) were purchased from ATCC in 2009. These cells were characterized by ATCC, using a comprehensive database of short tandem repeat (STR) DNA profiles. Frozen aliquots of freshly cultured cells were generated and experiments were done with resuscitated cells cultured for less than 6 months. All cell lines were mycoplasma tested. HT-29 and HCT116 cells were cultured in McCoy 5A supplemented with 10% heat-inactivated FBS (HI-FBS; Gibco, Life Technologies) and 100 U/mL penicillin/streptomycin. SW480 cells were cultured in DMEM supplemented with 10% HI-FBS, 1% glutamine, and 100 U/mL penicillin/streptomycin. COLO-205 cells were cultured in RPMI1640 supplemented with 10% HI-FBS, 1% glutamine, and 100 U/mL penicillin/streptomycin. Selumetinib (AZD6244), vemurafenib (PLX4032, RG7204), PD98059, and PNU-74654 were from Selleck Chemicals.

Intestinal epithelial cell extraction and ex vivo organoid cultures

Intestines and colons were extracted from C57BL/6 (Wnt OFF) or Apc<sup>−/−Mm</sup> (Wnt ON) mice. All our studies were approved by the Institutional Animal Care and Use Committee of the University of Liege (Liege, Belgium). Bowels were washed for 10 minutes at 37°C in a PBS-DTT (1 mmol/L) buffer and then incubated for 15 minutes at 37°C in a HBSS-EDTA buffer (30 mmol/L). Cells were harvested, washed twice in PBS, and flushed frozen. For the generation of ex vivo organoid cultures, small pieces of intestine were incubated in 2 mmol/L EDTA-PBS for 30 minutes at 4°C. Crypts were extracted, washed twice in PBS, and cultured in Matrigel (BD Biosciences). DMEM/F12 supplemented with EGF (20 ng/mL), Noggin (100 ng/mL), and R-Spondin (500 ng/mL) was added every 2 days. Apc-mutated organoids were cultured in DMEM/F12 supplemented with EGF (20 ng/mL), Noggin (100 ng/mL) without R-Spondin. The enrichment of Igf5<sup>+</sup> stem cells in ex vivo organoids generated with intestinal crypts from C57BL/6 mice was carried out by treating them with a combination of valproic acid (1 mmol/L) and CHIR99021 (3 μmol/L), a GSK3 inhibitor.

Generation of selumetinib-resistant colorectal cancer cell lines

Four colorectal cancer cell lines (HT-29, COLO-205, SW480, and HCT116) were used as parental cell lines (HT-29/P, COLO-205/P, SW480/P, and HCT116/P), from which were generated the selumetinib-resistant cell lines (HT-29/SR, COLO-205/SR, SW480/SR, and HCT116/SR). These cell lines were generated by repeated subculturing cells in the presence of incrementally increasing concentrations of selumetinib (from 0.05 to 1.5 μmol/L for HT-29/P and SW480/P cells; from 0.05 to 2 μmol/L for HCT116/P cells; from 0.005 to 0.3 μmol/L for COLO-205/P) for 6 months. For the maintenance of selumetinib-resistant colorectal cancer cell lines, the maximum concentration of selumetinib, namely 1.5 μmol/L (HT-29/SR and SW480/SR cells), 2 μmol/L (HCT116/SR cells), and 0.3 μmol/L selumetinib (COLO-205/SR cells) was added into the normal medium.

For the generation of selumetinib-resistant ex vivo organoids, organoids generated from Apc<sup>−/−Mm</sup> mice were first cultured with 1 μmol/L of selumetinib for two weeks. The concentration was then increased by 0.5 μmol/L every two weeks to reach a final concentration of 5 μmol/L.

Lentiviral cell infection

Control shRNA, CEMIP, Myc, TAK1 and FRA-1 shRNA lentiviral plK01-puro plasmid constructs were purchased from Sigma. Control shRNA and CEMIP shRNA lentiviral plK01-puro-iptG-inducible plasmid constructs were also purchased from Sigma. Lentiviral infections were carried out as described previously (23).

For lentiviral infections of ex vivo organoids, they were manually disrupted, washed with PBS to eliminate debris, and subsequently trypsinized for 30 minutes at 37°C. After washing with PBS, cells were washed via strainers (70 μmol/L) with 20 mL of PBS and centrifuged (200 × g) for 5 minutes at 4°C. They were then diluted in 500 mL of full media for organoid growth and 500 mL of infectious supernatants were added, mixed, and incubated in a CO<sub>2</sub> incubator for 12 hours. Organoids were subsequently centrifuged for 5 minutes at 4°C; washed once with 1 mL of PBS, and plated as usual. 24 hours later, full media containing 2 μg/mL of puromycin was added.

Quantitative real-time PCR

Total RNAs were extracted using the E.Z.N.A Total RNA kit (Promega). cDNAs were synthesized using the Revert aid H minus reverse transcriptase kit (Thermo Scientific) and real-time PCR analyses were performed as described previously (23). mRNA levels in control organoids or cells were set to 1 and mRNA levels in other experimental conditions were relative to the control after normalization with β-actin. Data from at least two independent experiments performed in triplicates are shown.
MTS assay
Cells counted using the TC20 Automated Cell Counter (Bio-Rad) were plated in 96-well flat-bottom plates at a density of 2,000 cells per well in triplicate and then treated with various concentrations of selumetinib or vemurafenib for 72 hours. Cell viability was determined using the MTS assay reagent (CellTiter 96 AQueous One Solution Cell Proliferation Assay; Promega) according to the manufacturer's protocol. The absorbance was measured at 490 nm using a Wallac Victor™ 1420 Multilabel counter (Perkin Elmer). Absorbance of untreated cells was designated as 100% and the number of viable cells in other experimental conditions was relative to the untreated cells.

Clonogenic assay
Cells were seeded in 60-cm dishes at a density of 3,000 cells per dish in duplicate. Twenty-four hours after plating, various concentrations of selumetinib or vemurafenib were added to each dish. After treatment for 24 hours, cells were washed with PBS and further incubated for 15 days. Cells were subsequently stained with 0.5% crystal violet in 25% methanol-containing PBS. Colonies were examined under a light microscope and counted after capturing images.

Western blot analysis
Cells were lysed in a buffer containing 20 mmol/L Tris-HCl, 0.5 mol/L NaCl, Triton X-100, 1 mmol/L EDTA, 1 mmol/L EGTA, 10 mmol/L β-glycophosphate, 10 mmol/L NaF, 300 μmol/L Na3VO4, 1 mmol/L benzamidine, 2 μmol/L PMSF, and 1 mmol/L DTT. Western blots were carried out as described, using antibodies listed in Supplementary Table S1 (23).

Caspase-3/7 activity assay
Caspase-3/7 activity was quantified using the Caspase-3/7 Glo Assay (Promega). Cells were treated with selumetinib or vemurafenib for the indicated periods of time and caspase-3/7 activity was quantified from cell lysates. Luminescence was measured at 490 nm using Wallac Victor™ 1420 Multilabel counter (Perkin Elmer). Luminescence values in vehicle-treated control samples were set to 1 and values obtained in other experimental conditions was relative to the untreated cells.

Extraction of cytoplasmic and nuclear proteins
Cells were incubated on ice for 10 minutes in cytoplasmic lysis buffer (10 mmol/L HEPES pH 7.9, 10 mmol/L KCl, 0.1 mmol/L EDTA, NP-40 0.3%, and Protease inhibitor). After centrifugation at 3,000 rpm for 5 minutes at 4°C, the supernatant fraction (cytoplasmic extract) was harvested and the pellet was resuspended in nuclear lysis buffer (20 mmol/L HEPES pH 7.9, 0.4 mol/L NaCl, 1 mmol/L EDTA, Glycerol 25%, and Protease inhibitor), incubated on ice for 10 minutes, and centrifuged at 14,000 rpm for 5 minutes at 4°C and supernatant containing the nuclear fraction was retained.

Biochemical fractionation
Cells were resuspended and homogenized in a Dounce homogenizer with the lysis buffer (150 mmol/L NaCl, 5 mmol/L DTT, 5 mmol/L EDTA, 25 mmol/L Tris HCl pH 7.4, protease inhibitors) and centrifuged at 1,000 × g for 10 minutes at 4°C. The supernatant was adjusted to 1% Triton X-100 and left on ice for 30 minutes. 4 vol of OptiPrep were added to 2 vol of supernatant. OptiPrep was diluted with the lysis buffer plus 1% Triton X-100 to give 35%, 30%, 20%, and 5% (w/v) iodixanol. A total of 0.6 mL of each sample as well as the four gradient solutions were layered in tubes for the swinging-bucket rotor. Samples were centrifuged at 30,000 rpm for 16 hours. Fractions of equal volume were collected for subsequent Western blot analyses.

For the second fractionation experiment, the OptiPrep Density Gradient centrifugation kit was purchased from Sigma Aldrich. Briefly, about 300 million HT-29 cells showing some acquired resistance to selumetinib were trypsinized, washed in PBS, and centrifuged at 600 × g for 5 minutes. Cells were then lysed with the extraction buffer, homogenized using the Dounce homogenizer, and centrifuged at 1,000 × g for 10 minutes. The supernatant was collected and centrifuged at 20,000 × g for 30 minutes. The pellet (which includes ER, lysosomes, peroxisomes, mitochondria, and endosomes) was diluted to a 19% Optiprep Density gradient solution and centrifuged on an Optiprep Density Gradient at 100,000 × g for 8 hours. Fractions of equal volume were collected for subsequent Western blot analyses.

Immunoprecipitation
Anti-MEK1, -BRAF and -IgG (negative control) antibodies were first coupled noncovalently to a mixture of Protein A/G-Sepharose. The antibody-Protein A/G-Sepharose conjugates were then pelleted by centrifugation at 5,000 rpm for 2 minutes, the supernatant removed, and the beads washed with 0.1 mol/L sodium borate pH 9.3. This was repeated four times, after which the beads were resuspended in 20 mmol/L dimethyl pimelimidate dihydrochloride (DMF) freshly made in 0.1 mol/L sodium borate pH 9.3 and gently mixed on a rotating wheel for 30 minutes at room temperature. Following centrifugation at 5,000 rpm for 2 minutes, supernatant was removed and fresh 20 mmol/L DMP/0.1 mol/L sodium borate pH 9.3 solution was added to the beads, which were then gently mixed for a further 20 minutes. The beads were then spun down at 5,000 rpm for 2 minutes, the supernatant removed, and four washes with 50 mmol/L glycine pH 2.5 carried out to remove any antibody coupled noncovalently. Afterwards, the beads were washed twice with 0.2 mol/L Tris-HCl pH 8.0 (neutralization step) and then resuspended in the same solution and mixed gently on a rotating wheel at room temperature for 2 hours. The beads were then used immediately for immunoprecipitation analyses as described previously (23).

Kinase assay
Control or CEMIP-depleted HT29 cells showing acquired resistance to selumetinib were subjected to anti-MEK1 or -IgG (negative control) immunoprecipitation. Selumetinib was added as control in some experimental conditions to inhibit MEK1 activity. The kinase assay was conducted at 30°C for 30 minutes with 1 μg of GST-ERK2 substrate (Thermo Fisher Scientific), 10 μCi of [γ-32P] ATP in 20 μL of kinase buffer (25 mmol/L HEPES, pH 7.5, 10 mmol/L MgCl2, 25 mmol/L β-glycophosphate, 1 mmol/L Na3VO4, and 1 mmol/L dithiothreitol). ERK2 phosphorylation was revealed by autoradiography.

Proximal ligation and chromatin immunoprecipitation assays
Parental and resistant HT-29 cells or control and CEMIP-depleted resistant HT-29 cells were plated in 8-chamber cell culture dishes and proximal ligation assays were performed as described previously (23).
Chromatin immunoprecipitation (ChIP) was performed using anti-TCF4 or IgG antibody as a negative control. A TCF4-binding site (site #1 located 839 bp upstream of the transcriptional start site on the CEMIP promoter) was identified through in silico analysis (MatInspector, Genomatix). TCF-binding sites #2, #3, and #4 located 26417, 75344, and 79348 bp downstream of the transcriptional start site within intron 1 respectively, were described previously (26). A negative binding site was randomly chosen in exon 1 of the CEMIP sequence. Primer sequences used are available upon request.

Tumor xenograft experiments

HT-29/SR cells were infected with an IPTG-inducible CEMIP shRNA (HT-29/SR-iCEMIP shRNA) or control shRNA (HT-29/SR-iControl shRNA). HT-29/SR-CEMIP shRNA or HT-29/SR-iControl shRNA cells (1.5 × 10⁶) mixed with Matrigel at a ratio of 1:1 were injected subcutaneously into the right or left flank of 6- to 8-week-old NOD/SCID male mice, respectively. Mice were monitored and tumors tracked via caliper measurements. Tumor volume was determined using the following formula: length × width × height × 0.5236 (n = 4 mice/group). Mice were treated with a combination of 10 mmol/L IPTG in their drinking water and 25 mmol/L ITC (200 μL) via intraperitoneal injection and either untreated or treated with selumetinib (20 mg/kg) via intraperitoneal injection five days a week for two weeks. Mice from all treatment groups were euthanized and tumors were excised and tissue archived for immunofluorescence and real-time PCR analysis.

Establishment of metabolomic profiles by targeted metabolomics

For targeted metabolomics analysis of ex vivo organoids, each sample was washed three times with cold PBS, collected into an Eppendorf tube, frozen in liquid nitrogen, and stored at −80°C until extraction. The extraction solution used was 50% methanol, 30% ACN, and 20% water. The volume of extraction solution added was calculated from the cell count (2 × 10⁶ cells per mL). After addition of extraction solution, samples were vortexed for 5 minutes at 4°C, and immediately centrifuged at 16,000 g for 15 minutes at 4°C. The supernatants were collected into an Eppendorf tube, frozen in liquid nitrogen, and stored at −80°C. The two-tailed Student t-test was applied for statistical analysis when only two groups of interest were compared. Results were
plotted as mean ± SD and were significant in all experiments at P < 0.001 (**), P < 0.01 (*), and P < 0.05 (#).

**Results**

**Ex vivo organoids with some acquired resistance to MEK1 inhibition show enhanced CEMIP expression**

Cancer stem cells contribute to resistance to targeted therapies. To make the link between cancer stem cells and acquired resistance to targeted therapies, we subjected *ex vivo* organoids showing constitutive Wnt signaling (i.e., generated with extracts of intestinal crypts from *ApC*<sup>Min</sup>/Min mice) to increasing concentrations of selumetinib to generate resistant organoids (Fig. 1A). The maintenance of these *ex vivo* organoids relies on the self-renewal potential of cancer stem cells. Resistant organoids were larger in size but without changes in cell proliferation (as judged by the percentage of Ki-67<sup>+</sup> cells) and were protected from caspase-3–dependent cell death, compared with parental organoids (Figs. 1A and B). The scaffold protein CEMIP may connect protumorigenic Wnt- and MAPK signaling pathways as it is the most robust Wnt-induced gene candidate involved in MAPK reactivation. *Ex vivo* organoids treated with a combination of valproic acid and CHIR99021, a GSK3 inhibition, to induce Wnt signaling showed elevated mRNA levels of Wnt target genes such as *Lgr5* and CEMIP while the level of Dclk1, a marker of differentiated Tuft cells, was downregulated (Fig. 1C). CEMIP induction by these drugs was also detected at the protein level (Fig. 1C). Immunofluorescence confirmed that treatment with valproic acid and CHIR99021, which enriches Lgr5<sup>+</sup> cells in *ex vivo* organoids, decreased the number of Dclk1<sup>+</sup> Tuft cells (Fig. 1C). Therefore, CEMIP expression is transcriptionally induced by Wnt signaling. Importantly, resistant organoids showed increased CEMIP, SOX9, HER2, and BRAF expression as well as enhanced activation of MEK1, ERK1/2, and mTOR (as judged by 4EBP1 phosphorylation; Fig. 1D and E, respectively). CEMIP was actually increased at the mRNA level in resistant organoids (Supplementary Fig. S1). Moreover, Myc, which controls protein synthesis and organ size, was increased at the protein but not mRNA levels (Fig. 1D; Supplementary Fig. S1, respectively). Of note, we did not detect any mutation on *BRAF* or on *MEK1* in these resistant organoids. Resistant organoids were enriched in CD24<sup>−</sup>/CD133<sup>−</sup> cancer stem cells (Fig. 1F), which fits with the upregulation of CD133 in colon cancer cells showing hyperactivation of the RAS–RAF–MEK1 cascade (30). Therefore, selumetinib-resistant organoids show all molecular features classically associated with the acquired resistance to MEK1 inhibition.

**CEMIP is connected to ErbB/MEK1-, LEF1-, and Myc-dependent pathways in colon adenocarcinoma**

To explore whether CEMIP links Wnt-dependent gene transcription to MEK1 signaling, we depleted CEMIP in *BRAF*<sup>V600E</sup>-mutated HT-29 colorectal cancer cells and carried out RNA-seq experiments combined with gene set enrichment analyses (GSEA; Fig. 2A–C; Supplementary Fig. S2A). In agreement with our previous observations (23), CEMIP expression was linked to ErbB/MEK1 signaling as a signature of genes induced through ErbB2, KRAS, or MEK1 was lost upon CEMIP deficiency (Fig. 2C and D). Genes controlled by the transcription factor LEF1 were also identified to be regulated by CEMIP (Fig. 2C; Supplementary Fig. S2A). We next carried out an iRegulon analysis to identify all genes coexpressed with CEMIP in colon adenocarcinoma and found 285 candidates (Fig. 2E). Interestingly, many of them are regulated by the Myc family of transcription factors (Fig. 2E). We also carried out an Ingenuity analysis (31) on these 285 coexpressed genes and found a significant enrichment of genes controlled by p53, Myc, and β-catenin among others (Supplementary Fig. S2B). Therefore, CEMIP expression is linked to ErbB-, Myc-, and β-catenin–dependent pathways.

**CEMIP promotes Myc expression through ERK1/2 activation**

To explore how CEMIP and Myc are linked, we assessed the consequences of CEMIP deficiency in *ex vivo* organoids. The depletion of CEMIP in parental or selumetinib-resistant organoids severely impaired their maintenance (Supplementary Fig. S3; Fig. 3A, respectively). Consistently, the pool of CD24<sup>−</sup>/CD133<sup>−</sup> cancer stem cells was impaired upon CEMIP deficiency (Fig. 3B). CEMIP deficiency downregulated HER3, Cyclin D1, Cyclin D2, Myc, SOX9, and phosphorylated ERK1/2 levels (Fig. 3C). To explore whether the link between CEMIP and Myc was found in other experimental systems, we generated *BRAF*<sup>V600E</sup>-mutated COLO205 cells with some acquired resistance to MEK1 inhibition by subjecting parental cells to increasing concentrations of selumetinib (Fig. 3D). Selumetinib-resistant COLO205 cells showed elevated CEMIP mRNA and protein levels as well as decreased levels of pMEK1, pERK1/2, and pMyc (Fig. 3E and F). Importantly, CEMIP deficiency in these cells also impaired pMEK1 and ERK1/2 activation and decreased protein levels of Myc (Fig. 3G). Of note, effects on MEK1 activation were largely due to decreased total levels of MEK1 in CEMIP-deficient cells, which was not the case in *ex vivo* organoids (Fig. 3G and C, respectively). These selumetinib-resistant COLO205 cells were also resistant to PD98059, another MEK1 inhibitor, as pERK1/2 levels barely decreased at high concentrations of this inhibitor (Supplementary Fig. S4). Here also, CEMIP deficiency in these cells decreased pMyc, pERK1/2, as well as Myc protein levels (Supplementary Fig. S4). Conversely, the ectopic expression of CEMIP alone in DLD-1 cells enhanced both pERK1/2 and Myc protein levels without impacting on Myc mRNA levels and also protected from cell death triggered by selumetinib (Supplementary Figs. SSA and SSB, respectively). Therefore, CEMIP maintains Myc protein levels in multiple experimental models.

**CEMIP expression is induced through BRAF, ERK1/2, and FRA-1 upon acquired resistance in *BRAF*<sup>V600E</sup>- but not KRAS<sup>G13D</sup> or *G12A*-mutated colorectal cancer cells**

CEMIP expression is increased in *ex vivo* organoids as well as in *BRAF*<sup>V600E</sup>-mutated COLO205 cells, both with resistance to MEK1 inhibition. To explore whether this also applies to other experimental models, we cultured parental HT-29 cells (HT-29/P) with increasing concentrations of selumetinib and generated highly resistant HT-29 cells (HT-29/SR; Fig. 4A). These cells showed decreased E-cadherin levels, suggesting that they underwent epithelial–mesenchymal transition (EMT), a known feature of chemoresistance (Supplementary Fig. S6A). Importantly, CEMIP mRNA and protein levels were strongly induced in resistant HT-29 cells (Fig. 4B). We next looked at the nuclear levels of transcription factors that drive CEMIP gene transcription, namely...
Figure 1.
Selumetinib-resistant organoids show elevated levels of CEMIP and reactivation of the MEK1/ERK1/2 pathway. A, Selumetinib-resistant organoids are larger in size. Ex vivo organoids with intestinal crypts extracted from Apc<sup>þ/Min</sup> mice were treated with increasing concentrations of selumetinib. Quantification of their size is illustrated. Statistical analysis was performed as described in Materials and Methods. B, Resistant organoids do not proliferate more but show less apoptotic cells. Ki67 and activated caspase-3 staining were carried out to quantify the percentage of proliferative and apoptotic cells, respectively. Data from 20 organoids are illustrated. C, CEMIP expression is induced by Wnt signaling in ex vivo organoids generated from intestinal crypts of C57BL/6 mice. Ex vivo organoids were untreated or treated with valproic acid (1 mmol/L) and CHIR999021 (3 μmol/L) to enrich for Lgr5<sup>+</sup> stem cells. Top, mRNA levels of indicated candidates were quantified by real-time PCR analysis (see Materials and Methods for the quantification). Bottom, immunofluorescence analysis of the tuft cell marker Dclk1. Anti-CEMIP Western blot analysis using extracts from untreated or valproic acid and CHIR999021-stimulated ex vivo organoids is shown. D and E, Selumetinib-resistant organoids show elevated levels of CEMIP, HER3, BRAF, SOX9, and c-Myc and enhanced activation of MEK1, ERK1/2, and mTOR. Protein extracts from parental and resistant ex vivo organoids were subjected to Western blot analysis. F, Selumetinib-resistant organoids were enriched in CD24<sup>+</sup>/CD133<sup>+</sup> cancer stem cells. FACS analysis was conducted to quantify the percentage of CD24<sup>+</sup>/CD133<sup>+</sup> cells. Data from four experiments are illustrated.

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NF-xB and AP-1 family members (23, 32). Both p65 and BRAF-V600E but not BCL-3 and c-JUN levels were increased in nuclear extracts from resistant HT-29 cells (Fig. 4C). Of note, cytoplasmic BRAF showed elevated levels in resistant cells (Supplementary Fig. S6B), which reflects intrachromosomal amplification (10). These molecular changes persisted even in circumstances in which cells were constantly cultured with selumetinib (Supplementary Fig. S6C). Therefore, CEMIP expression is induced in resistant HT-29 cells in which mutated BRAF-V600E, nuclear p65, and BRAF-1 protein levels are increased. BRAF-1, but not p65 was actually driving CEMIP expression in these cells as BRAF-1 but not p65 deficiency impaired CEMIP expression at both mRNA and protein levels (Supplementary Figs. S7A and S7B). BRAF-1 controls the expression of several candidates such as WNT10, DKK-1, and DVL-1 acting in the canonical Wnt pathway in colon cancer cells (33). As CEMIP transcription is robustly induced upon Wnt activation (29), we hypothesized that BRAF-1 indirectly controls CEMIP expression through Wnt signaling. BRAF-1 deficiency indeed impaired nuclear b-catenin levels and both WNT10 and DKK-1, two b-catenin target genes downregulated upon BRAF-1 deficiency in HT-29/SR cells (Supplementary Figs. S7C and S7D). BRAF-1 deficiency also triggered cell death of HT-29/SR cells, as judged by clonogenic assays, at least due to caspase-3/7 activation (Supplementary Figs. S7E and S7F). As CEMIP expression is induced in BRAF-V600E-mutated cells, we reasoned that a BRAF inhibitor may decrease CEMIP expression. CEMIP mRNA and protein levels were severely decreased in HT-29/SR cells subjected to vemurafenib (Supplementary Figs. S8A and S8B). As a consequence, vemurafenib triggered some cell death and interfered with the capacity of these cells to form colonies (Supplementary Figs. S8C and S8D). Taken together, our results define BRAF-V600E, BRAF-1, and b-catenin as upstream actors that drive CEMIP transcription in BRAF-V600E-mutated resistant colorectal cancer cells. Selumetinib decreased CEMIP expression in both selumetinib-resistant organoids and HT-29 cells (Fig. 3C and 4D, respectively), we looked at BRAF-1 proteins levels upon MEK1 inhibition in HT-29/SR cells. BRAF-1 but also p65, c-JUN, b-catenin, and TCF4 were downregulated upon MEK1 inhibition while the epithelial marker E-cadherin was increased, (Fig. 4D). Moreover, TCF4 promotes CEMIP expression as CEMIP protein levels severely decreased upon TCF4 deficiency in selumetinib-resistant HT-29 cells (Supplementary Fig. S9A). Consistently, the treatment of two selumetinib-resistant colon cancer cell lines with PNU-74654, which inhibits the Wnt/b-catenin pathway by blocking the interaction between b-catenin and TCF4, decreased CEMIP mRNA and protein levels (Supplementary Fig. S9B). Both TCF4 and b-catenin were actually recruited at TCF-binding sites located on the CEMIP promoter as well as on intron 1 (Fig. 4E). Therefore, selumetinib decreases CEMIP expression, at least by negatively regulating protein levels of both BRAF-1 and TCF4.

**Figure 2.**

CEMIP controls the expression of ErbB-, RAS-, MEK1-, and LEF1-dependent signaling cascades. A, Generation of CEMIP-depleted colorectal cancer-derived HT-29 cells. Cells were transduced with the indicated shRNA lentiviral constructs. Real-time PCR analysis was performed to assess mRNA expression of CEMIP. B, CEMIP controls the expression of gene candidates linked to ErbB, RAS, and LEF1 signaling. A scatter plot of RNA-seq data obtained with RNA extracted from control versus CEMIP-deficient HT-29 cells is illustrated. C, Gene set enrichment analysis of RNA-seq expression data obtained with total RNA from control and CEMIP-depleted HT-29 cells. Candidate genes up- or downregulated are illustrated in red and blue, respectively. D, Listing of the most robust up- or downregulated candidates (red and blue rectangles, respectively) upon CEMIP deficiency in HT-29 cells. E, Identification of CEMIP coexpressed genes in colon adenocarcinoma through Regulon analysis. The transcription factors (TF) known to regulate these coexpressed genes (N = 285) are listed.
Figure 3.
CEMIP is required for the maintenance of selumetinib-resistant ex vivo organoid cultures and for Myc expression. **A**, CEMIP deficiency impairs the growth of selumetinib-resistant ex vivo organoids. Images of control and CEMIP-depleted ex vivo organoids are illustrated. The number of organoids per dish was calculated in each experimental condition. **B**, CEMIP deficiency impairs the pool of CD24+/CD133+cancer stem cells in selumetinib-resistant ex vivo organoids. FACS analysis was conducted to quantify the percentage of CD24+/CD133+ cells in control and CEMIP-depleted resistant organoids. Data from two experiments are illustrated. **C**, CEMIP deficiency impairs HER3, Myc, SOX9, cyclin D1/2 levels, and ERK1/2 activation in selumetinib-resistant ex vivo organoids. (Continued on the following page.)
To explore whether KRASG13D or G12A-mediated colorectal cancer-derived cell lines showing some acquired resistance to selumetinib also show elevated levels of CEMIP, parental HCT116, and SW480 cells were also treated with increasing concentrations of selumetinib to generate resistant HCT116 and SW480 cells, respectively (HCT116/SR and SW480/SR; Supplementary Fig. S10A). Both resistant HCT116 and SW480 cell lines did not upregulate CEMIP, in contrast to resistant HT-29 cells (Supplementary Fig. S10A). Yet, ERK1/2 reactivation was seen in all resistant cells (Fig. 4F). RSK1 activity was also specifically induced in BRAFV600E−, but not in KRASG13D or G12A-, mutated resistant cells (Fig. 4F). Both p56 and FRA-1 were not dramatically induced in both HCT116- and SW480-resistant cells (Fig. 4F; Supplementary Fig. S10B). Levels of another scaffold protein, IQGAP-1, remained unchanged in resistant HT-29 cells (Fig. 4F). BRAFV600E-mutated resistant HT-29 cells, and to a less extent KRASG13D-mutated HCT116 cells, also enhanced HER3 and MET reactivations (Fig. 4G). Finally, a somatic mutation in MEK1 exon 3 (H119R) was found in resistant HT-29 cells (Fig. 4H). The H119R mutation, among others in the same domain of MEK1, was demonstrated to underlie resistance to the MEK1 inhibitor, PD184352 (34). Taken together, our data indicate that BRAFV600E, but not KRASG13D or G12A-mutated colorectal cancer cells reactivate MAPK signaling and potently induce CEMIP gene transcription upon acquired resistance to MEK1 inhibition.

CEMIP deficiency sensitizes BRAFV600E-mutated resistant HT-29 cells to MEK1 inhibition

To explore whether CEMIP contributes to the resistance to selumetinib in BRAFV600E-mutated colorectal cancer cells, we depleted CEMIP from HT-29/SR cells and subjected them to selumetinib. MEK1 inhibition decreased CEMIP mRNA levels (Fig. 5A). CEMIP deficiency enhanced DNA damage, as evidenced by increased pH2A.X levels (S139) as well as caspase-3/7-dependent apoptotic cell death upon selumetinib treatment (Fig. 5B and C, respectively). CEMIP-depleted HT-29/SR cells generated less colonies when subjected to selumetinib (Supplementary Fig. S11A). To assess whether this was also relevant in vivo, we carried out xenograft experiments in immunodeficient mice with control and CEMIP-depleted HT-29/SR cells and subsequently treated mice with selumetinib. CEMIP mRNA levels were expectedly decreased in cells infected with the inducible shRNA construct (Supplementary Fig. S11B). Also, selumetinib failed to significantly trigger tumor regression in vivo in mice injected with HT-29/SR cells (Fig. 5D). Importantly, CEMIP deficiency, combined with selumetinib, caused significant tumor regression (Fig. 5D). This was due to DNA damage and apoptosis, as evidenced by anti-pH2A.X (S139) and cleaved caspase-3 immunofluorescence (Supplementary Fig. S11C). CEMIP-deficient HT-29/SR cells showed lower levels of HER3, even after treatment with selumetinib and failed to maintain levels of phosphorylated ERK1/2 and RSK1 compared to selumetinib-resistant cells (Fig. 5E). Moreover, E-cadherin levels increased upon CEMIP deficiency, indicating that CEMIP is required for EMT maintenance, a process linked to chemoresistance (Fig. 5E). CEMIP expression also decreased at the protein level in selumetinib-treated cells, similar to FRA-1 and also to BRAF, but not KRAS levels, suggesting again that BRAF and FRA-1 controls CEMIP expression (Fig. 5E). Therefore, CEMIP contributes to the acquired resistance to selumetinib, at least by promoting MEK1–ERK1/2 signaling.

CEMIP is an endosomal protein

We next carried out biochemical fractionation to identify cell compartments from which CEMIP contributes to MEK1 and ERK1/2 reactivation in resistant cells. CEMIP cofractionated with EEAI and APPL1, two signaling endosome markers, and to a less extent with lysosomal markers (Rab7 and LAMP2) and with Rab11, a recycling endosome marker (Fig. 6A). CEMIP also cofractionated with PDI, an endoplasmic reticulum marker, as described previously (24). Phosphorylated forms of MEK1 were also detected in CEMIP-positive fractions, suggesting that signaling endosomes are critical for MEK1 reactivation (Fig. 6A). In contrast, CEMIP did not cofractionate with Caveolin-1 and Flotillin-1, two lipid raft markers (Fig. 6A). To explore in which endosomes CEMIP is mainly located, we conducted a second fractionation experiment in which organelles of interest (ER, peroxisomes, mitochondria, and endosomes) were enriched from cell extracts and separated on a gradient by ultracentrifugation. CEMIP mainly cofractionated with EEAI+ endosomes and to a much less extent with Rab5+/7+ or APPL1+ endosomes (Fig. 6B). A SNAP-CEMIP construct expressed in HCT116 cells also partially colocalized with EEAI+ endosomes and with the ER, as assessed by immunofluorescence (Fig. 6C). CEMIP was the only endosomal protein to be upregulated in HT-29/SR cells, as both EEAI and APPL1 levels remained unchanged (Supplementary Fig. S12A). CEMIP associated with MEK1 in HT-29/SR cells, as evidenced by coimmunoprecipitation (Fig. 6D) and BRAF more weakly bound MEK1 in resistant versus parental HT-29 cells, more likely due to disengagement (Fig. 6E). Of note, pERK1/2 levels were totally abolished after 0.5 and 1 hour of treatment with selumetinib in both parental and resistant HT-29 cells but pERK1/2 levels were again detectable after 24 hours of MEK1 inhibition (Supplementary Fig. S12B). CEMIP actually contributes to MEK1 activity as an anti-CEMIP immunoprecipitate from CEMIP-depleted HT-29/SR cells was less potent at phosphorylating ERK2 (Supplementary Fig. S12C). CEMIP failed to bind mutated BRAFV600E in resistant HT-29 cells (Supplementary Fig. S13A). Although more...
BRAF<sup>V600E</sup> dimers were detected by the proximal ligation assay in resistant versus parental HT-29 cells. CEMIP was dispensable for BRAF<sup>V600E</sup> dimerization (Supplementary Fig. S13B and S13C). Therefore, CEMIP is localized in several cell compartments and contributes to MEK1 reactivation from signaling endosomes in resistant BRAF<sup>V600E</sup>-mutated colorectal cancer cells as a MEK1-binding protein.

CEMIP promotes metabolic reprogramming potentially through Myc

To explore the biology downstream of CEMIP, we established the metabolomic signature of both parental and resistant organoids. Severe metabolic reprogramming was detected in resistant organoids as they showed elevated levels of TCA intermediates (fumarate, malate, citrate, and succinate; Fig. 7A). Multiple nucleotides, whose synthesis relies on Myc (33), were increased in resistant organoids (Fig. 7A). Proline, whose degradation is inhibited by Myc (36), was detected at higher levels in resistant organoids (Fig. 7A). Moreover, levels of arachidonic acid, which is regulated by Myc in lung cancer (37), were also elevated in resistant organoids as well as levels of other unsaturated fatty acids such as oleic acid, a candidate reported to be upregulated in colon cancer (Fig. 7A; ref. 38). Finally, levels of cystathionine, which is generated by cystathionine β-synthase (CBS), an enzyme downregulated in gastrointestinal and hepatocellular malignancies (39, 40), were decreased in resistant organoids (Fig. 7A). Therefore, metabolic reprogramming is seen in selumetinib-resistant intestinal organoids.

Importantly, CEMIP expression contributes to this process as the production of lactate as well as levels of multiple amino acids was impaired upon CEMIP deficiency in these ex vivo resistant organoids (Fig. 7B). To better define CEMIP as an upstream regulator of Myc, we reasoned that Myc deficiency would mimic CEMIP deficiency in selumetinib-resistant ex vivo organoids. Indeed, the depletion of Myc impaired ERK1/2 activation and also downregulated CEMIP protein but not mRNA levels, suggesting that CEMIP and Myc mutually posttranscriptionally control their expression (Fig. 7C; Supplementary Fig. S14A). Myc was also critical for the maintenance of selumetinib-resistant ex vivo organoids, similar to CEMIP (Supplementary Fig. S14B). Moreover, Myc depletion had a profound effect on the levels of multiple metabolites as levels of lactate and numerous amino acids were significantly downregulated in Myc-depleted cells (Supplementary Fig. S14C). A comparison of the metabolic signatures of selumetinib-resistant ex vivo organoids with depleted Myc or CEMIP confirmed that both proteins control the production of multiple metabolites such as amino acids (methionine, threonine, tryptophan, valine, proline, histidine, asparagine, phenylalanine, isoleucine, leucine, glycine, and L-alanine) and lactate among other candidates (Fig. 7D). Therefore, CEMIP may promote the acquired resistance to MEK1 inhibition, in part by potentially regulating levels of specific metabolites via a Myc-associated signaling pathway.

Discussion

We describe here the characterization of CEMIP as an endosomal protein that links Wnt-dependent gene transcription to MEK1–ERK1/2 signaling to promote acquired resistance to MEK1 inhibition in BRAF<sup>V600E</sup>-mutated colorectal cancer cells. CEMIP expression is induced in resistant cells through BRAF<sup>V600E</sup>, MEK1, RSK1, and FRA-1, which provides a mechanism by which these signaling proteins promote resistance to inhibitors of RAS effectors. In addition, CEMIP regulates levels of multiple amino acids seen in resistant cells, at least through Myc. Multiple transcription factors govern CEMIP transcription, including the NF-κB proteins BCL-3 and p65 in cervical cancer cells (23). Functional NF-κB- and AP-1–binding sites were also identified on the CEMIP promoter in breast cancer cells (32). We define here FRA-1, one member of the AP-1 family of transcription factors as well as TCF4 as key drivers of CEMIP expression in resistant BRAF<sup>V600E</sup>-mutated colorectal cancer cells. The BRAF inhibitor, which indirectly turns off ERK1/2 activity, also decreases FRA-1 protein levels. This observation fits with the fact that ERK1/2 signaling stabilizes FRA-1 by preventing its proteasome-dependent degradation in colorectal cancer cells (41). Therefore, interfering with ERK1/2 signaling downregulates CEMIP transcription, at least through the destabilization of FRA-1 in resistant BRAF<sup>V600E</sup>-mutated colorectal cancer cells. This signaling cascade critically drives CEMIP transcription to establish a positive loop as CEMIP physically binds MEK1 (but not BRAF) to sustain MEK1 activity.

Our resistant BRAF<sup>V600E</sup>-mutated colorectal cancer cells have several features linked to acquired resistance. They show enhanced phosphorylation of MET and HER3, elevated levels of BRAF<sup>V600E</sup> as well as a MEK1 mutation, all events contributing to ERK1/2 reactivation. The upregulation of CEMIP has been detected in all tested BRAF<sup>V600E</sup>- but not KRAS<sup>G12D/G12A</sup>-mutated colorectal cancer cells showing some acquired resistance to MEK1.
inhibition. Yet, the induction of CEMIP upon acquired resistance was more severe in resistant BRAF<sup>V600E</sup>-mutated colorectal cancer HT-29 cells in which the MEK1 mutation within exon 3 (H119R) was found. Therefore, the combination of both BRAF<sup>V600E</sup> and MEK1 mutations may be key genetic events to efficiently drive CEMIP expression upon acquired resistance.

**Figure 5.**
CEMIP promotes acquired resistance to selumetinib in BRAF<sup>V600E</sup>-mutated colorectal cancer cells. A, Selumetinib downregulates CEMIP expression in resistant HT-29 cells. Control or CEMIP-depleted HT-29/SR cells were untreated or stimulated with selumetinib at indicated concentrations for 12 hours and real-time PCR analysis was conducted on total RNA to assess CEMIP expression. B, CEMIP deficiency enhances selumetinib-induced DNA damage in resistant HT-29 cells. Control or CEMIP-depleted HT-29/SR cells were untreated or stimulated with selumetinib at indicated concentrations for 24 hours. Protein extracts were subjected to Western blot analysis. C, CEMIP deficiency induces apoptotic cell death by selumetinib in resistant HT-29 cells. Control or CEMIP-depleted HT-29/SR cells were untreated or treated with selumetinib (3 μmol/L) for 24 hours. Caspase-3/7 activity in control and unstimulated cells was set to 1 and caspase-3/7 activity in other experimental conditions that were relative to the control. Data from three independent experiments performed in duplicate are shown. D, CEMIP deficiency significantly enhances tumor regression induced by selumetinib. A stable IPTG-inducible control or CEMIP-depleted HT-29/SR cell line (2 × 10<sup>5</sup> cells) was injected subcutaneously into NOD/SCID male mice. Mice were treated with IPTG from day 3 postinjection and treated with selumetinib (20 mg/kg) from day 6 postinjection for two weeks, as described in Materials and Methods (n = 4/group). Top, representative images of tumors excised at day 20 postinjection. Bottom, tumor weights were quantified. E, CEMIP deficiency increases E-cadherin expression and impairs selumetinib-dependent HER3 expression in resistant HT-29 cells. Control or CEMIP-depleted HT-29/SR cells were untreated or stimulated with selumetinib at indicated concentrations for 24 hours. Protein extracts were subjected to Western blot analysis.
Figure 6.
CEMIP is an endosomal protein that binds MEK1 in resistant BRAFV600E-mutated HT-29 cells. A, A pool of CEMIP is found in signaling endosomes. Protein extracts from HT-29/SR cells were biochemically fractionated on an OptiPrep gradient as described in Materials and Methods and the resulting fractions were subjected to Western blot analysis. B, CEMIP mainly co-fractionates with EEA1^+ endosomes. A fractionation experiment in which some pellets enriched with organelles of interest were further separated into fractions by ultracentrifugation (see Materials and Methods). Fractions were subjected to Western blot analysis. C, CEMIP partially colocalizes with ER and endosomal markers. HCT116 cells were transfected with the SNAP-CEMIP construct and immunofluorescence was conducted on resulting cells. Arrows, colocalization of CEMIP with EEA1^+ endosomes and with the ER using the PDI marker. D, CEMIP binds MEK1 in resistant HT-29 cells. Protein extracts from parental and resistant HT-29 cells untreated or treated with selumetinib for the indicated periods of time were subjected to immunoprecipitation (IP) with anti-IgG (negative control) or anti-MEK1 antibodies, followed by Western blot analysis. Whole-cell extracts (WCE) were also subjected to Western blot analysis. E, Enhanced MEK1 activation in resistant BRAFV600E-mutated HT-29 cells leads to disengagement from BRAF. Protein extracts from parental and resistant HT-29 cells treated or not with selumetinib for the indicated periods of time were subjected to immunoprecipitation with anti-BRAF or anti-IgG antibodies, followed by Western blot analysis carried out on the immunoprecipitates or on whole-cell extracts.
The metabolomic profile was established in parental and resistant organoids and is presented as heatmap visualization and hierarchical clustering analysis of the top 50 compounds with P ≤ 0.05, Student t test. Rows, metabolites; columns, samples; color key indicates metabolite expression value (blue, lowest; red, highest). Data with triplicates is presented. C, Myc deficiency impairs CEMIP and ERK1/2 activation in selumetinib-resistant ex vivo organoids. Extracts from control and Myc-depleted resistant ex vivo organoids were subjected to Western blot analysis. D, CEMIP and Myc control the production of specific metabolites. The metabolic signatures established in control, Myc-depleted, and CEMIP-depleted selumetinib-resistant ex vivo organoids were compared. Control (CEMIP) and Control (Myc) experimental conditions represent selumetinib-resistant ex vivo organoids with control shRNA and were used to compare CEMIP and Myc-depleted organoids, respectively. The data are presented as heatmap visualization and hierarchical clustering of the top 50 compounds with P ≤ 0.05 Student t test. Rows, metabolites; columns, samples; color key indicates metabolite expression value (blue, lowest; red, highest).
CEMIP is found in signaling endosomes and is essential for ERK1/2 reactivation in BRAFV600E-, but not KRASG12D or G12A-, mutated cells, at least through binding to MEK1. Whether or not ERK1/2 activation downstream of tyrosine kinase receptors occurs from endosomes or from the cytoplasmic membrane has been the subject of an intense debate. While some studies support the notion that MAPK scaffold complexes found in endosomes are critical for signal transduction, other reports state that signaling from a tyrosine kinase receptor occurs from the cytoplasmic membrane (42, 43). In support with this later hypothesis, EGFR endocytosis in endosomes helps to terminate Ras-dependent signaling to ERK1/2 as endogenous Ras is primarily located at the cytoplasmic membrane in low EGFR-expressing cells (44). BRAFV600E does not bind CEMIP, which fits with the hypothesis that CEMIP only binds signaling proteins such as EGFR or MEK1 found in endosomes but not candidates such as BRAF, which is activated at the cytoplasmic membrane (23, 45). The enhanced CEMIP expression that we specifically see in resistant BRAFV600E-mutated colorectal cancer cells may help to recycle HER3 and MET at the cytoplasmic membrane to sustain ERK1/2 signaling and/or to favor the assembly of a specific endosomal signaling platform for ERK1/2 reactivation.

A previous study showed that a pool of CEMIP can be found in the ER (24). These results, combined with our study revealing CEMIP in endosomes, raise some questions on mechanisms by which a scaffold protein localized in two distinct cell compartments, promotes survival and chemoresistance. The answer may come from the existence of membrane contacts between endosomes and the ER, a process that contributes to EGFR-dependent signaling (46). These physical contacts may help CEMIP to bring signaling proteins together to sustain ERK1/2 activation in resistant BRAFV600E-mutated colorectal cancer cells.

One key mechanism through which CEMIP deficiency circumvents the acquired resistance to MEK1 inhibition in BRAFV600E-mutated colorectal cancer cells may be through Myc, which is in agreement with the fact that the pharmacologic inhibition of Myc circumvents the acquired resistance to c-Met inhibition (47). Our correlative metabolic data show that Myc is a key effector downstream of CEMIP as CEMIP and Myc similarly control the production of multiple metabolites including lactate as well as amino acids such as glycine, which has been defined, among others, as a driver of cancer pathogenesis (48). We demonstrated that selumetinib-resistant ex vivo organoids show high levels of multiple amino acids, which can be metabolized as a source of carbon and nitrogen for biosynthesis of fatty acids, lipids, nucleotides, and proteins to support proliferation and survival (49). Essential amino acids such as leucine, tryptophan, and phenylalanine, whose levels are controlled by both CEMIP and Myc in selumetinib-resistant organoids, has been defined as signaling molecules for mTOR activation (50). Therefore, CEMIP and its downstream effector Myc may indirectly control mTOR signaling through the production of specific essential amino acids to support acquired resistance to MEK1 inhibition.

In conclusion, our study defined the scaffold and endosomal protein CEMIP as an upstream regulator of Myc that links Wnt- and MEK1-dependent signaling pathways. As CEMIP is linked to Myc and to specific metabolic reprogramming seen in resistant cells, this oncogenic pathway may hold therapeutic interest.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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The Endosomal Protein CEMIP Links WNT Signaling to MEK1–ERK1/2 Activation in Selumetinib-Resistant Intestinal Organoids

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