Supplementary Information

Supplementary Figure legends

Figure 1. EGF induces synergic AP-1 activation under elevated KITENIN in an EGFR- and EGFR-kinase-independent manner. A, effects of EGFR-knockdown on AP-1 synergy by EGF. 293T cells were co-transfected with KITENIN and AP-1 reporter with or without EGFR siRNA (si-EGFR) and treated with EGF for 12 h. NS, no significant difference between groups. B, effects of chemical blockade of EGFR on AP-1 synergy by EGF. 293T cells were co-transfected with AP-1 reporter with or without KITENIN and treated with EGF (100 ng/ml) and increasing doses of AG1478 (0.1 to 10 μg/ml) for 12 h. The asterisk (@) indicates a significant difference between groups (@@@P<0.001). NS, not significant.

Figure 2. Effects of functional blockade of EGFR via treatment of an anti-EGFR monoclonal antibody on enhanced cell invasion by EGF. A, Caco2 cells were treated with EGF (100 ng/ml) and increasing doses of cetuximab (1, 3, 10 μg/ml) for 12 h. Maximal effect of cetuximab on suppression of increased p-EGFR by EGF treatment was observed at a higher concentration (10 μg/ml). B, Caco2/vector or Caco2/KITENIN cells were treated with EGF and/or cetuximab (10 μg/ml) for 12 h and subjected to invasion assay. The asterisk (* or @) indicates a significant difference between groups (***P<0.001, @@@P<0.001).

Figure 3. EGF-induced AP-1 synergy under elevated KITENIN conditions does not require activated JNK. A, effect of EGF on phospho/total-JNK and on phospho/total-

c-Jun. Serum-starved HCT116 cells were treated with EGF at the time points indicated. B, effect of JNK blocker on EGF action under elevated KITENIN conditions. Parent or KITENIN-transfected 293T cells were pretreated with SP600125 (50 ng/ml), a JNK blocker, and the effects of EGF (100 ng/ml for 12 h) on c-Jun and Dvl2 were examined.

Figure 4. Effects of Dvl2-knockdown on c-Jun and of EGF on c-Jun/Dvl2 level in KITENIN-transfected cells. A, 293T cells were transfected with increasing amounts of Dvl2 or Dvl2 siRNA for 48 h, and AP-1 or TOPflash reporter activity and c-Jun level were examined. Each bar represents the mean ± SEM for triplicate samples. B-C, immunoreactive Dvl2 and c-Jun were examined after EGF treatment for 8 h in 293T (B) and Caco2 (C) cells under transfection with or without KITENIN and measured by densitometry in triplicate experiments.

Figure 5. Dvl2-knockdown does not increase transcription of c-Jun and Dvl2 does not interact with c-Jun. A, effect of Dvl2-knockdown on c-Jun promoter activity. Dvl2 siRNA was co-transfected into 293T cells for 48 hr with c-Jun, AP-1, or TCF reporter, and c-Jun (left), AP-1 (middle), and TOPflash (right) activity was measured. PMA (20 nM, 8-h treatment) or LiCl (40 mM) was used as a stimulator of c-Jun/AP-1 and TOPflash activity, respectively. Values represent mean ± SEM (*n*=3). B, Dvl2 does not directly interact with c-Jun. Lysates (500 μg) from parent or KITENIN-transfected 293T cells were immunoprecipitated with anti-c-Jun or anti-Dvl2 antibody to detect interaction between c-Jun and Dvl2.

Figure 6. Modulation of multiple RTKs in KITENIN-overexpressed Caco2 or KITENIN-knockdown HCT116 CRC cells. A, ErbB4 and RON are positively regulated by KITENIN. Total cell lysates (500 μg) from Caco2/KITENIN (left) or HCT116/si-KITENIN (right) cells cultured under serum-starved conditions for 24 h were subjected to phospho-RTK array. The two dots in each corner indicate positive controls, and the eight dots at the lower right indicate negative controls (indicated by circles and the number 5). B, phospho-ErbB4 and phospho-RON are regulated by KITENIN. Expression levels of KITENIN and total RTK in each cell line were confirmed by immunoblot analysis. To detect phospho-RTK, cell lysates (500 μg) from Caco2/KITENIN or HCT116/si-KITENIN cells were immunoprecipitated with each antibody for RTK and subsequently immunoblotted with 4G10 antibody, which detects phosphotyrosine. C, KITENIN interacts with ErbB4. GST pull-down assay was performed in lysates from 293T cells with various KITENIN-GST fusion proteins (1-240, N-terminal cytoplasmic plus transmembrane region; 111-240, transmembrane region; 111-524, transmembrane plus C-terminal cytoplasmic region), which were immunoblotted with anti-ErbB4 antibody (left). 293T cells were co-transfected with KITENIN-myc and various deletion mutants of ErbB4-HA (WT, wild-type; ECD, extracellular region plus transmembrane region; CTF, cytoplasmic C-terminal region). Each lysate was immunoprecipitated with anti-myc antibody and immunoblotted with anti-HA antibody (right).

Figure 7. Co-transfection of both KITENIN and ErbB4 induced greater elevation in AP-1 activity after EGF treatment. 293T cells were co-transfected with AP-1 reporter and KITENIN, EGFR, or ErbB4 isoform (a2), and treated with EGF for 12 h. In another set, 293T cells were co-transfected with AP-1 reporter and either

KITENIN/EGFR or KITENIN/ErbB4(a2), and treated with EGF for 12 h. Up-regulation of AP-1 activity was observed in 293T cells transfected with EGFR alone or ErbB4 alone after EGF treatment. The asterisk (@) indicates a significant difference between groups (@@@P<0.001).

Figure 8. Higher expression levels of KITENIN affect the survival of CRC cells to the anti-proliferative effects of cetuximab. A, susceptibility of various CRC cells to cetuximab. CRC cells were treated with increasing doses of cetuximab (10, 30, 100 μg/ml) for 96 h and subjected to cell viability assay. HCT116 cells expressed the highest levels of endogenous KITENIN and were most resistant to cetuximab. DLD1 and SW620 cells expressing lower KITENIN were most sensitive to cetuximab. B, expression levels of KITENIN and the survival of Caco2 CRC cells, which is KRAS/BRAF wild-type cell line, to cetuximab. Proliferation rates were compared between empty vector- and KITENIN-transfected Caco2 cells, and between control siRNA- and KITENIN siRNA-transfected Caco2 cells after treatment with increasing doses of cetuximab (10, 30, 100 μg/ml) for 96 h. C, expression levels of KITENIN and the survival of DLD1 and HCT116 CRC cells, which are KRAS/BRAF mutant cell lines, to cetuximab. Proliferation rates were compared between empty vector- and KITENIN-transfected DLD1 cells (left side), and between control siRNA- and KITENIN siRNA-transfected HCT116 cells (right side) after treatment with increasing doses of cetuximab (10, 30, 100 μg/ml) for 96 h. The asterisk (*) indicates a significant difference between groups (*P<0.05, **P<0.01).

Supplementary Table 1. Characteristics of 55 patients treated with cetuximab and conventional chemotherapy.

	No. of patients	%
Age		
<58	27	49.1
≥58	28	50.9
Gender		
Male	32	58.2
Female	23	41.8
Location		
Colon	34	61.8
Rectum	21	38.2
Differentiation		
Well	12	21.8
Moderate	38	69.1
Poor	5	9.1
Metastatic site		
Liver	32	58.2
Lymph node	37	67.3
Lung	21	38.2
Peritoneum	18	32.7
Early chemotherapy response		
Good response (CR+PR)	33	60
Poor response (SD+PD)	22	40
Chemotherapy regimen		
FOLFOX-Cetuximab	24	43.6
FOLFIRI-Cetuximab	31	56.4

Median age: 58

All patients Median PFS 8.00 months (95% CI 6.256-9.744)

All patients Median OS 22.53 months (95% CI 0.000-45.240)

	Good response (CR+PR)	Poor response (SD+PD)	*p-value*
Median OS (months)	42.00 (95% CI 20.10-63.90)	18.00 (95% CI 12.13-23.86)	0.076
Median PFS (months)	8.30 (95% CI 6.657-9.943)	3.00 (95% CI 1.357-4.643)	0.014

Supplementary Table 2. Primer (Forward/Reverse) and siRNA specific sequences.

RT-PCR
KITENIN
5’-CTACAATGTAGATGGCCCC-3’ / 5’-AGCCTCATCACTGACAAGCC-3’
EGFR
5’-TGGAAAACCTGCAGATCATC-3’ / 5’-TTGCTGAGAAAGTCACTGCT-3’
mErbB4-JM
5’-CAGTGTGAGAAAATGGAAGATGGACTCCTC-3’ /
5’-GCACGTTTCTTTTTGATGCTCTTTCTTCTG-3’
mErbB4-CYT
5’-AAGAATGGCTAGAGACCCTCA-3’ /
5’-GGACCGCTGGAAGCACTGTGATA-3’
Genomic-PCR for KITENIN
primer 1, 5'-CGACGGCCACTGCTCTCACAT-3'
primer 2, 5'-ATGACAACTGGGGAGAGACCA-3'
primer 3, 5'-CAGCTCCTCCCTCCACAGGA-3'
siRNA
Human *KITENIN*
5'-GCUUGGACUUCAGCCUCGUAGUCAA-3'
Human *Dvl2*
5'-AACUUUGAGAACAUGAGCAAC-3'
Human *ErbB1*
5'-UGAUCUGUCACCACAUAAUUACGGG-3'
Human *ErbB4*
5'-UAUAGAUGUUUCCUGCGCUGAUUUC-3'
Human *RON*
5'-UGAAAGAGAAGCCUCUCAGCACGGA-3'

Supplementary Materials and Methods

Growth factors

Human recombinant EGF (R&D systems) and HGF (Invitrogen) were used at concentrations of 40~100 ng/ml. For all experiments, cells were first serum-starved overnight and then stimulated with the appropriate concentration of growth factors in 1% serum medium.

Reporter assay

For the reporter assay, 293T cells were plated at a density of 5×10^4 cells/well at 24 h before transfection, and were subsequently transiently transfected with 50 ng of reporter (TOPFLASH, AP-1-luc, and c-Jun-luc promotor), 1 ng of phRL-CMV, and effector constructs (50 to 200 ng) using FuGENE6. Empty vector DNA was added to equalize the total amount of DNA. After incubation for 24 or 48 h, luciferase activity was measured and normalized to Renilla activity for transfection efficiency as previously described (11). All data represent the mean ± SEM for triplicate samples.

Antibodies and immunoprecipitation

Antibodies to ErbB1, ErbB4, c-Met, RON, and Nrdp1 (Santa Cruz Biotech); c-Jun, c-fos, p-JNK, total-JNK, p-ERK, p-ErbB4, Dvl2, and beta-catenin (Cell Signaling Technol); anti-V5 and anti-myc (MBL); anti-HA and anti-actin (Sigma); and rabbit KITENIN (9) or Vangl1 (Atlas) were used with appropriate secondary antibodies (Amersham biosciences). For transient transfection analyses, 293T cells were transfected with various plasmids and harvested for immunoblot analysis 36 h after transfection. For most assays using stable cell lines, we used mixed polyclonal cells to exclude clonal variation. Cellular proteins were separated, transferred, and

immunoblotted as previously described (9). The blot was reprobed with anti-actin antibody to control for loading. Cell lysates from 293T, Caco2, and HCT116 cells were used for immunoprecipitation experiments as previously described (11).

Cell invasion assay

Cell invasion was measured by using the Transwell migration apparatus as previously described (10). Briefly, cultured cells pretreated with siRNA (for 48 h) or stably overexpressed cells were loaded into the top of a 24-well invasion chamber assay plate (Costar). Conditioned DMEM medium containing 20 μg/ml of fibronectin (Calbiochem) was added to the bottom chamber as a chemoattractant. After 24 h of incubation, the cells were stained. Cells at the top surface of the filters were wiped off with a cotton ball, and migrated cells on the bottom surface were counted in six random squares of 0.5 mm × 0.5 mm for each filter. The results are represented as the mean ± SEM of the number of cells per field for at least three independent experiments.

Soft agar colony forming assay

Caco2 (1×10^5) and SW-620 (1×10^5) cells were suspended in 1.5 ml of soft agar (0.35% agarose in DMEM complete medium), plated onto 1.5 ml of solidified agar (0.6% agarose in DMEM complete medium) in six-well plates and cultured for 3 and 2 weeks, respectively. Cells were fed two times per week with cell culture media containing EGF or not. Pixel intensity of colony area was measured by IMT iSolution software (IMT i-Solution Inc) from random microscope views for each plate. To measure % area of colony, the numbers of pixels of the colony area were normalized by the given pixel*pixel square. Data represent the average of three experiments.

Cell viability assay

The method for growth and survival of CRC cells is based on the colorimetric quantification of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. Briefly, CRC cells were plated and cultured in 96-well plates (5×10^3 cells/well). The cells were treated with different concentrations of cetuximab (0, 10, 30, 100 μg/ml) for 96 h. After culture, the assay was performed as described (9).

Immunofluorescence

Cells were seeded on fibronectin-covered cell culture slides (Becton Dickinson). 293T cells were transfected with expression vectors encoding Dvl-GFP and HA-tagged KITENIN, and HCT116 cells with Dvl-GFP. After 24 h, cells were treated with EGF (100 ng/ml) for 30 minutes in a serum-starved condition. After incubation, monolayered cells were washed, fixed, permeabilized, and stained with either a monoclonal anti-HA coupled to a red fluorescent-labeled Alexa-fluor 568 secondary antibody and blue fluorescent nuclear staining with DAPI. Cells were then examined with a Laser Scanning Confocal Microscope (Leica) as previously described (11).

RT-PCR and RNA interference

RNA preparation and reverse transcription were performed as previously described (11). The RT-PCR exponential phase was set for 30 cycles to allow for quantitative comparison of the various cDNAs developed from identical reactions on a GeneAmp PCR system (Eppendorf). For the siRNA interference experiments, we designed siRNA specific to human KITENIN, Dvl2, ErbB1, ErbB4, and RON. For the nonspecific scrambled siRNA (si-scr), we used the All Stars Negative Control siRNA

(Qiagen). The siRNA duplexes were prepared and transfected according to a protocol provided by Lipofectamine^{TM} RNAiMAX. Cells were treated for 48 to 72 h to allow for maximum knockdown, followed by RT-PCR and Western blot analysis or use in invasion assays. The PCR primers and siRNA sequences are listed in Supplementary Table S2.

Patients and immunohistochemistry

We reviewed the cases of 55 patients with unresectable recurrent or metastatic colorectal adenocarcinoma who had been primarily treated with cetuximab plus irinotecan, fluorouracil and leucovorin (FOLFIRI), or cetuximab plus oxaliplatin, fluorouracil, and leucovorin (FOLFOX-4) chemotherapy as a first-line agent from January 2007 to December 2011 at Chonnam National University Hwasun Hospital (Kwangju, Korea) and Yonsei University Hospital (Seoul, Korea). The specimens of Yonsei University Hospital were obtained from the archives of the Department of Pathology, Yonsei University and from the Liver Cancer Specimen Bank of the National Research Resource Bank Program of the Korea Science and Engineering Foundation of the Ministry of Science and Technology, and authorization for the use of the tissues for research was obtained from the Institutional Review Board of Yonsei University of College of Medicine.

The paraffin wax-embedded tumor tissue at diagnosis was available for all included cases. Patients were staged by using a combination of colonoscopy, computed tomographic scans of the chest and abdomen, and positron emission tomography or bone scans, when clinically indicated. Data regarding patient demographics, chemotherapeutic regimen, chemotherapy response, progression-free survival (PFS), and overall survival (OS) were obtained by medical record review.

The study protocol was reviewed and approved by the Institutional Review Board of Chonnam National University Medical School Research Institution (2013-19). The recommendations of the Declaration of Helsinki for biomedical research involving human rights were followed. Tissue sections were deparaffinized, rehydrated, rinsed, stained with KITENIN and ErbB4, and examined as previously described (9).

Assessment of KITENIN and ErbB4 staining in CRC specimens

For semiquantification of immunohistochemical KITENIN and ErbB4 expression, a score was attained by multiplying the intensity of the staining by the size of the area that stained positively as previously described (11). The intensity of cell staining appearing as dark brown color was graded according to the following scale: 0, no staining; 1+, mild staining; 2+, moderate staining; 3+, marked staining. The area of staining was evaluated on the following scale: 0, <5% of cells stained positive; 1+, 5% to 25% stained positive; 2+, 25% to 50% stained positive; 3+, >50% stained positive. The maximum combined score was 9, and the minimum was 0. Generally, specimens with scores >4 were considered as positive for a candidate protein expression, but this criterion was not used in this study.

Chemotherapy

In total, 31 patients received cetuximab-FOLFIRI chemotherapy, while 24 patients received cetuximab-FOLFOX-4 chemotherapy. The patients in the FOLFIRI group received a 30- to 90-minute infusion of irinotecan at a dose of 180 mg/m^2 on day 1, an infusion for 120 minutes of leucovorin at a dose of 200 mg/m^2, followed by fluorouracil in a bolus of 400 mg/m^2, as well as continuous infusion for 46 hours of 2400 mg/m^2 during days 1 and 2. The patients in the FOLFOX-4 group received a

120-minute infusion of oxaliplatin at a dose of 85 mg/m^2 on day 1, an infusion for 120 minutes of leucovorin at a dose of 200 mg/m^2, followed by fluorouracil in a bolus of 400 mg/m^2, as well as 600 mg/m^2 infusion for 22 hours on days 1 and 2. Cetuximab was administered via an initial 120-minute infusion on day 1 of 400 mg/m^2, followed by 60-minute infusions of cetuximab at a dose of 250 mg/m^2, once weekly. FOLFIRI or FOLFOX was given after the cetuximab infusion on day 1 of each period. Each regimen was repeated every 2 weeks.

The schedule was repeated until either an observation of disease progression, lack of clinical benefit, unacceptable toxicity, or patient refusal. Hematological and non-hematological adverse events were evaluated. Management of adverse events and subsequent dose reductions of chemotherapeutic agents was carried out in a conventional manner.

Response evaluation

The clinical tumor response was assessed radiologically by computed tomography every 8 weeks, according to the Response Evaluation Criteria in Solid Tumors (ver. 1.0) (37). PFS was defined as the period from the start of chemotherapy to documentation of disease progression or death from any cause, whichever occurred first. If neither event had occurred at the time of the last record, the patient was censored at that time. OS was calculated from the start of chemotherapy to death from any cause.

Statistical analysis

Experimental differences were tested for statistical significance by using ANOVA followed by Tukey HSD post hoc test or Student's *t* test. All statistical tests were two-

sided, and *P*-values of less than 0.05 were considered statistically significant. Statistics was performed with PASW Statistics 20 (SPSS, an IBM Company, Chicago, IL) software.

Reference

37. Therasse P, Arbuck SG, Eisenhauer EA, Wanders J, Kaplan RS, Rubinstein L, et al. New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. J Natl Cancer Inst 2000;92:205-16.