

An Unconventional KITENIN/ErbB4-Mediated Downstream Signal of EGF Upregulates c-Jun and the Invasiveness of Colorectal Cancer Cells

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Abstract

Purpose: EGF-stimulated signaling via EGF receptor (EGFR) is important in colorectal tumorigenesis and drug targeting. However, anti-EGFR therapy is not effective in a subset of patients with colorectal cancer, suggesting that unidentified EGF-stimulated pathways might play roles in colorectal cancer. Previously, we identified KAI1 C-terminal interacting tetraspanin (KITENIN) as a metastasis-enhancing gene and found it to be highly expressed in sporadic colorectal cancer tissues. We recently found that EGF further increases KITENIN-induced elevated AP-1 activity. Here we attempted to clarify this novel EGF-stimulated molecular pathway and its roles in colorectal cancer.

Experimental Design: We analyzed how EGF modulates the downstream signaling pathway of oncogenic KITENIN in colorectal cancer cells. Biological alterations following EGF treatment were identified in KITENIN-overexpressed colorectal cancer cells with or without alteration of EGFR activity.

Results: We identified the KITENIN/ErbB4-Dvl2-c-Jun axis as a novel downstream signal of EGF that is switched on under elevated KITENIN conditions in an EGFR-independent manner. This unconventional EGF signal upregulates c-Jun and enhances invasion and anchorage-independent growth of colorectal cancer cells. In addition, tumor tissues from metastatic patients with colorectal cancer who showed initial poor responses to cetuximab/chemotherapy expressed higher levels of KITENIN than did responders to therapy.

Conclusions: Our results highlight the role of an EGFR-independent EGF signal in mediating the invasiveness and tumorigenesis of colorectal cancer cells. This unconventional pathway might be related to the limited clinical efficacy of anti-EGFR agents in a subset of patients with colorectal cancer. *Clin Cancer Res*; 20(15); 4115–28. ©2014 AACR.

Introduction

EGF is released through paracrine signaling from the tumor-associated microenvironment and is critical in the facilitation of metastasis. The EGF receptor (EGFR, ErbB1) is the cell-surface receptor for members of the EGF family, and the ErbB family includes 4 different tyrosine kinases (ErbB1, ErbB2, ErbB3, ErbB4) that are activated following binding

to ErbB ligands. The ErbB tyrosine kinases mediate complex interactions between tumor cells and their microenvironment that may ultimately result in enhanced tumor progression, which thus makes ErbB an interesting target for therapeutic intervention in several human cancers (1, 2). Although EGFR is overexpressed in colorectal cancer tissues, EGFR kinase inhibitors with proven efficacy in other clinical settings often fail to block metastatic recurrence of colorectal cancer (3, 4). One reason for this lack of response is the heterogeneity of colorectal cancers. In previous studies, colorectal cancers lacking oncogenic alterations in KRAS, BRAF, PIK3CA, and PTEN genes (so-called "quadruple negative" tumors) were shown to have the highest probability of a response to anti-EGFR therapy (5, 6). However, approximately 20% of patients with metastatic colorectal cancer who show no response to anti-EGFR targeted therapies do not harbor mutations in KRAS, BRAF, and PIK3CA, nor loss of PTEN expression (7).

Such poor responses in these patients with colorectal cancer have led investigators to seek a broader understanding of kinase-independent EGFR functions that might support tumor growth. In one such study, EGFR was reported to

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Translational Relevance

EGFR-targeted therapy is an important approach in colorectal cancer therapy; however, up to half of all patients with colorectal cancer do not respond to this therapy. Our present results indicate the presence of an unconventional EGFR-independent signal of EGF, the KAI1 C-terminal interacting tetraspanin (KITENIN)/ErbB4–Dvl2–c-Jun axis, which mediates increased colorectal cancer cell invasiveness and thereby enhances tumor progression. Our results suggest that the KITENIN/ErbB4–c-Jun axis could be a molecular basis for conferring resistance to anti-EGFR agents in colorectal cancer tissues in which KITENIN is highly expressed. These findings support a rationale for combined targeting of the KITENIN complex to improve responses to cetuximab-based therapy in EGFR/KITENIN-overexpressing metastatic patients with colorectal cancer. We propose that, together with "quadruple negative" genotype (tumors lacking alterations in KRAS, BRAF, PIK3CA, and PTEN genes), lower KITENIN levels in resected tumor tissues from metastatic patients with colorectal cancer could be useful for identifying patients who would benefit from EGFR-targeted therapies.

maintain the basal intracellular glucose level independent of its kinase activity and thereby to prevent cells from undergoing autophagic death, leading to increased tumor cell survival, even in the presence of EGFR kinase inhibitors (8). Accordingly, delineation of novel downstream signals of EGF that do not involve EGFR is of tremendous importance, because such signals endow colorectal cancer cells with prosurvival responses to anti-EGFR therapies, nevertheless, these signals have not yet been identified.

Previously, we identified KAI1 C-terminal interacting tetraspanin (KITENIN) as a metastasis-enhancing gene (9, 10) that participates in the dissemination of colorectal (11, 12), squamous (13–15), gastric (16), and hepatocellular cancer cells (17). KITENIN protein expression is significantly higher in human colon (11, 12), laryngeal (14), oral cavity squamous (15), gastric (16), and hepatocellular (17) cancer tissues than in corresponding normal mucosa or early-stage cancer tissues. Interaction of KITENIN with Dishevelled (Dvl)/PKC δ is important in regulating colorectal cancer cell invasion via ERK/AP-1 activation (11) and expression of KITENIN is significantly associated with lymph node metastasis and poor survival of colorectal cancer (12). However, it is unknown how elevated KITENIN drives colorectal cancer progression and whether extracellular signals, such as EGF or HGF, from the tumor-associated microenvironment modulate the downstream signaling pathway of oncogenic KITENIN to contribute to colorectal tumorigenesis.

In this study, we investigated how extracellular signals affect elevated KITENIN-induced increases in colorectal cancer cell invasiveness and whether the action of extracel-

lular signals on KITENIN, such as EGF, is involved in the failure of anti-EGFR therapies in patients with metastatic colorectal cancer. We identified a novel EGFR-independent oncogenic signal of EGF that works under coexpressed KITENIN and ErbB4. Our present results indicate that an alternative signaling pathway of EGF stimulates the formation of a functional complex among membrane-associated scaffolding proteins, KITENIN/ErbB4, which are not cognate receptors of EGF. This unconventional EGF signal may cause a decrease in the sensitivity to treatment with anti-EGFR agents in patients with cancer.

Materials and Methods

Plasmids and cell lines

Expression constructs of HA- or V5-tagged KITENIN, GST-tagged KITENIN deletion mutants, HA-tagged ErbB4 deletion mutants, EGFR, HA-tagged Dvl2, DN-c-jun, and Nrpd1 were generated by PCR-based methods. All constructs were confirmed by sequencing. ErbB4 cDNA was kindly provided by K. Elenius (University of Turku, Finland). Human colorectal cancer cell lines (Caco2, DLD-1, HCT116, HT29, SW-480, and SW-620), Chinese hamster ovary (CHO)-K1 cells, and human embryonic kidney (293 T) cells were obtained from American Type Culture Collection and were grown as described (11). EGFR wild-type and knockout mouse embryonic fibroblasts (MEF) were kindly provided by Z. Dong (University of Minnesota, Minneapolis, MN). Denoted constructs and si-RNAs were transfected into the cells by using FuGENE6 and RNAiMax, respectively, as described (11).

We characterized how EGF modulates the downstream signaling pathway of oncogenic KITENIN in colorectal cancer cells through cell cultures, immunoblotting and immunoprecipitation, siRNA interference and RT-PCR analyses, reporter and invasion assays, immunofluorescence, and immunohistochemistry.

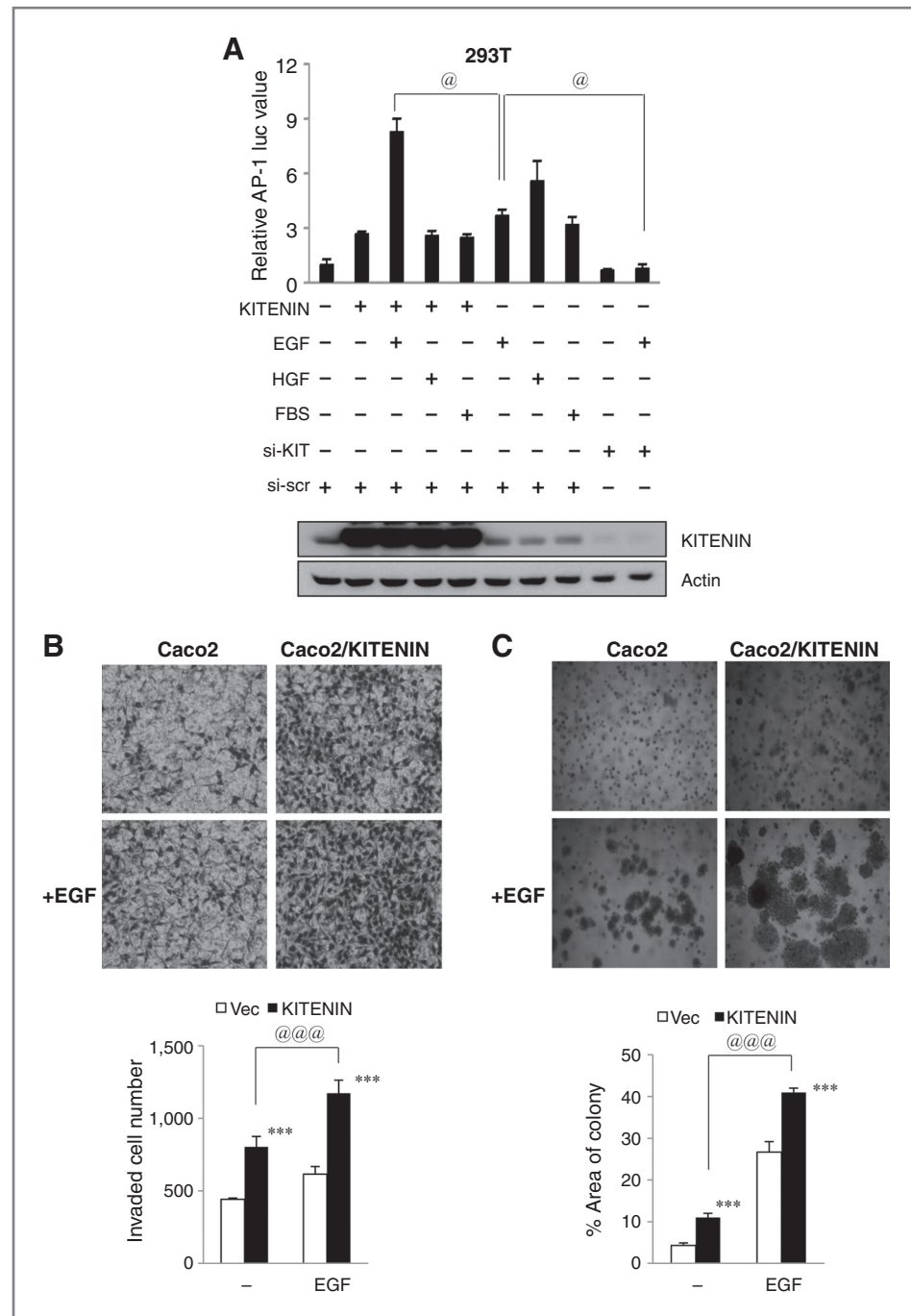
Full methods are available at Clinical Cancer Research Online.

Results

EGF induces synergic AP-1 activation and enhanced cell motility in KITENIN-transfected cells, which does not require EGFR or activated JNK

Previously, we found that transfection of KITENIN in 293 T cells causes a 3-fold increase in AP-1 activity, which is mediated by interaction with Dvl/PKC δ (11). However, it is not fully understood how Dvl transduces downstream signals for AP-1 activation, although translocation of Dvl to the plasma membrane is needed for JNK/AP-1 activation (18). To investigate whether extracellular signals also affect AP-1 activation in KITENIN-transfected cells, we chose growth factors that positively regulate colorectal cancer progression, such as EGF and HGF, as candidate substances. In 293 T cells, treatment with EGF, HGF, and FBS increased AP-1 activity (Fig. 1A). Interestingly, significant increases in AP-1 activity were noted when EGF, but not HGF or FBS, was given to KITENIN-transfected

Figure 1. EGF upregulates AP-1 and enhances cell motility in KITENIN-transfected cells. **A**, effect of growth factors on AP-1 activity under KITENIN transfection. 293 T cells were cotransfected with AP-1 reporter with or without KITENIN or following KITENIN siRNA and treated with EGF (100 ng/mL), HGF (40 ng/mL), or 10% FBS for 12 hours. Each bar represents mean \pm SEM for triplicate samples. si-scr, nonspecific scrambled siRNA (negative control). @, significant difference between groups (@, $P < 0.05$). **B**, invasion assay in Caco2 cells after EGF treatment. Caco2/vector or Caco2 cells stably transfected with KITENIN (Caco2/KITENIN) were treated with EGF (100 ng/mL) for 12 hours and subjected to invasion assay. The pictures shown represent three independent experiments. The histogram represents invading cells, which were counted at the 5 chosen areas and represented as bar graphs (mean \pm SEM, $n = 3$). The asterisk (* or @) indicates a significant difference between groups (***, $P < 0.001$; @, $P < 0.01$; @@@, $P < 0.001$). **C**, cell growth assay in Caco2 cells after EGF treatment. Caco2/vector or Caco2/KITENIN cells were subjected to anchorage-independent cell growth assay. EGF (100 ng/mL) was treated for 12 hours. The pictures shown represent three independent experiments. The areas of the stained cells were measured and represented as bar graphs (mean \pm SEM, $n = 3$).



cells compared with 293 T/parent cells, which suggests that EGF specifically sensitized KITENIN-overexpressing cells to exhibit synergistic promotion of AP-1 activity. However, increases in AP-1 activity by EGF alone were attenuated by KITENIN knockdown. In Caco2 colorectal cancer cells, cell invasion (Fig. 1B) and anchorage-independent cell growth (Fig. 1C) significantly increased after EGF treatment in KITENIN-transfected Caco2 cells compared with Caco2/parent cells.

The question thus arose as to whether EGFR is involved in the enhanced AP-1 activity and colorectal cancer cell motility observed after EGF treatment under KITENIN transfection. EGFR knockdown did not affect the elevation in AP-1 activity by EGF in either KITENIN-transfected 293 T cells (Supplementary Fig. S1A) or KITENIN-transfected Caco2 cells (Fig. 2A, left), nor the elevated Caco2 cell invasion by EGF treatment (Fig. 2A, right). Interestingly, roughly the same level of phospho-ERK was observed

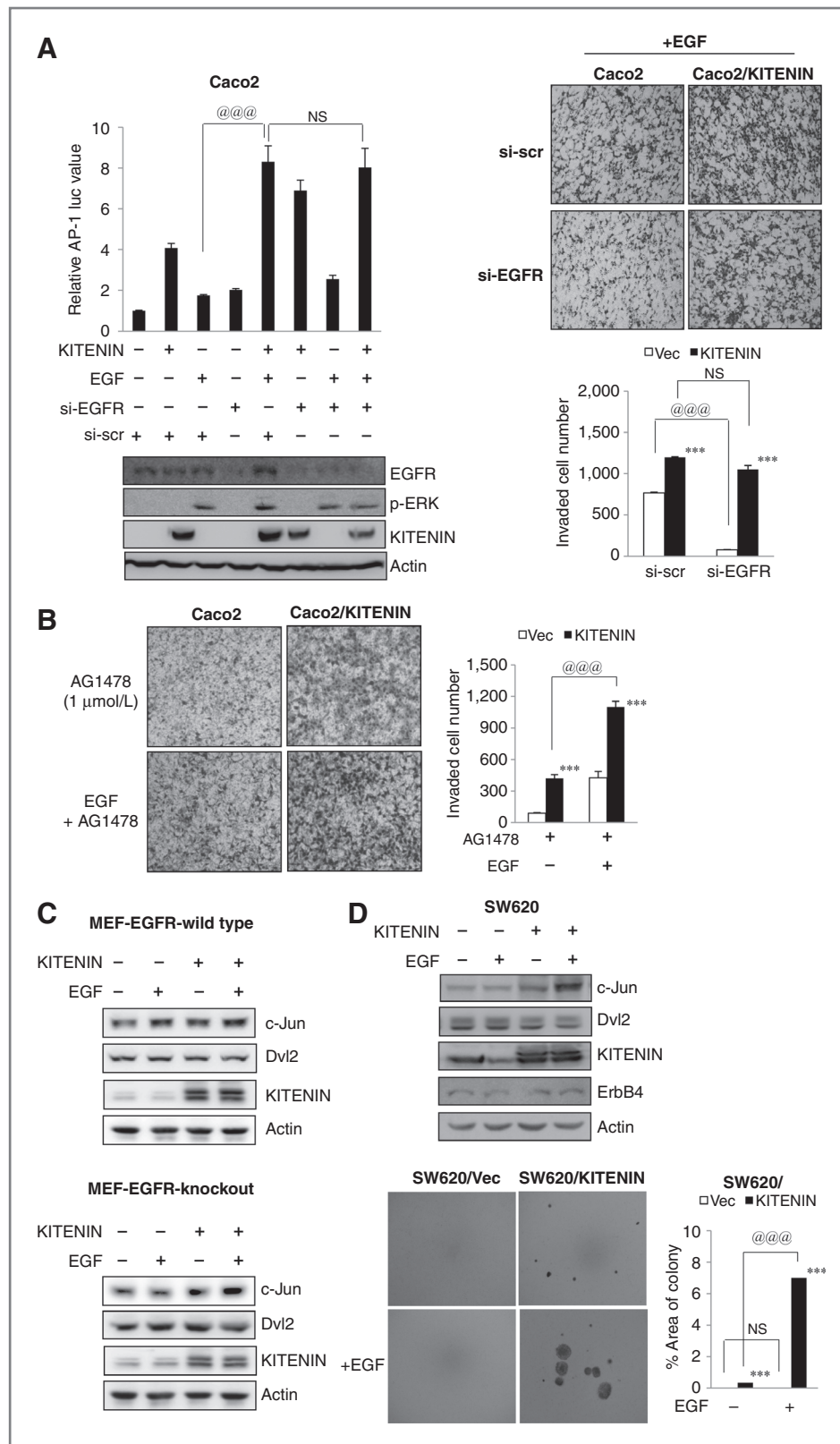


Figure 2. EGF exhibits synergic AP-1 activation and enhanced colorectal cancer cell motility under elevated KITENIN conditions in an EGFR-independent manner. A, effects of EGFR knockdown on AP-1 synergy and on enhanced cell invasion by EGF. Caco2/vector or Caco2/KITENIN cells treated with EGF for 12 hours were subjected to AP-1 reporter (left) and invasion (right) assays. NS, no significant difference between groups. B, effects of chemical blockade of EGFR on enhanced cell invasion by EGF. Caco2/vector or Caco2/KITENIN cells were treated with EGF and/or AG1478 (1 μmol/L) for 12 hours. C–D, EGF action under elevated KITENIN in MEF and SW-620 cells that do not express EGFR. C, EGFR-wild-type (top) and EGFR-knockout (bottom) MEF cells were transfected with KITENIN and treated with EGF for 1 hour. D, SW-620/vector or SW-620/KITENIN were transfected with KITENIN (SW-620/KITENIN) were treated with EGF for 12 hours and subjected to anchorage-independent cell growth assay.

between parent and KITENIN-transfected cells after EGF treatment. Although increases in phospho-ERK by EGF treatment were not affected by EGFR knockdown (Supplementary Fig. S1A and Fig. 2A), which can be explained by the report that maximal EGF signaling can be achieved with only 5% receptor occupancy (19), phospho-ERK does not seem to contribute to elevation of AP-1 activity by EGF under KITENIN transfection. Moreover, chemical blockade of EGFR via treatment of AG1478, an inhibitor of ErbB kinase activity (20), or functional blockade of EGFR via treatment of cetuximab, an anti-EGFR monoclonal antibody (4), did not affect elevations in AP-1 activity (Supplementary Fig. S1B) or in cell invasion (Fig. 2B and Supplementary Fig. S2) by EGF treatment in KITENIN-transfected cells. Thus, phospho-EGFR did not seem to contribute to elevated AP-1 activity by EGF under KITENIN transfection.

To elucidate whether EGF induces AP-1 activation under KITENIN transfection in an EGFR-independent manner, we used EGFR-knockout MEFs and EGFR-null colorectal cancer cells. However, AP-1 activity was barely detected in EGFR-knockout MEF cells and EGFR-null SW-620 cells, probably because of differences in AP-1 reporter expression; thus, c-Jun was examined following EGF treatment instead of AP-1. As a positive control, c-Jun was increased following EGF treatment in EGFR-wild-type (124% by densitometry; Fig. 2C, top, 2nd column) but not in EGFR-knockout MEF cells (100%; Fig. 2C, bottom, 2nd column). Also, greater elevation of c-Jun was observed in KITENIN-transfected EGFR-wild-type MEF cells after EGF treatment than in empty vector-transfected cells (138% vs. 124%; Fig. 2C, top). However, in EGFR-knockout MEF cells, c-Jun was increased only in KITENIN-transfected cells following EGF treatment (138% vs. 100%; Fig. 2C, bottom).

We further examined this EGF action by using SW-620 cells, an EGFR-null colorectal cancer cell line. Again, greater elevation of c-Jun was observed after EGF treatment in KITENIN-transfected SW-620 cells than in empty vector-transfected cells (Fig. 2D, top). Compared with Caco2 and HCT116 cells that expressed EGFR, SW-620 cells showed a very low chemotactic response to fibronectin; however, anchorage-independent cell growth increased after EGF treatment in KITENIN-transfected SW-620 cells (Fig. 2D, bottom). We interpreted these results to mean that EGF induced elevated c-Jun/AP-1 activity and enhanced cell motility in KITENIN-overexpressing colorectal cancer cells in an EGFR-independent manner.

Next, we investigated whether enhanced AP-1 activity by EGF under KITENIN transfection was derived from JNK activity. In HCT116 cells, which express the highest level of endogenous KITENIN among colorectal cancer cells (11), there were no actual changes in phospho-JNK despite increases in phospho-c-Jun following EGF treatment (Supplementary Fig. S3A). Also, JNK blockade via pretreatment with SP600125 did not influence the increased c-Jun level by EGF in KITENIN-transfected 293 T cells (Supplementary Fig. S3B). Therefore, EGF induces enhanced AP-1 activity/cell motility under KITENIN expression, which does not require EGFR kinase or activated JNK.

EGF induces AP-1 synergy via downregulation of KITENIN-bound phospho-Dvl2 and subsequent upregulation of c-Jun

Next, we investigated the underlying mechanism of enhanced AP-1 activation (AP-1 synergy) by EGF under KITENIN transfection. Because EGF induced AP-1 synergy in an EGFR kinase- and EGFR-independent manner, the downstream signaling pathways of EGFR, such as the PI3K-mTOR and KRAS-RAF-MAPK pathways (5, 6), might be ruled out as possible candidates. Our previous speculations as to whether PMA acts as an upstream signal to the functional KITENIN complex (in which KITENIN induces ERK/AP-1 activation via interaction with Dvl2/PKC δ ; ref. 11) prompted us to assess whether PMA affects Dvl2 for AP-1 activation in KITENIN-overexpressing cells. PMA induced 5-fold increases in c-Jun, but concurrently reduced total Dvl2 by 70% in KITENIN-transfected 293 T cells compared with parent cells by densitometry (Fig. 3A), which suggested that lowered endogenous Dvl2 can result in increased c-Jun/AP-1 activity. To explore the possibility of Dvl2 levels contributing to increased c-Jun/AP-1 activity, we modulated Dvl2 levels in 293 T cells to examine AP-1/TOPflash reporter activity and c-Jun levels. Dvl2 knockdown increased AP-1 activity and c-Jun protein, while increasing Dvl2 resulted in upregulation of TOPflash activity but not in AP-1 activity (Supplementary Fig. S4A).

We then attempted to assess whether EGF also increases c-Jun-like PMA in colorectal cancer cells. HCT116 cells were chosen and changes in Dvl2 were analyzed under phosphatase treatment because phospho-Dvl2-specific antibody was not available. At 2 hours after EGF treatment, c-Jun markedly increased to 3 times that before treatment by densitometry, whereas phospho-Dvl2 and unphospho-Dvl2 levels were reduced by half (Fig. 3B, top). EGF also led to a reproducible 3-fold increase in c-Jun and a decrease in total Dvl2 by 70% in KITENIN-transfected 293 T cells compared with parent cells (Supplementary Fig. S4B). Also, in KITENIN-transfected Caco2 cells, c-Jun increased by 120% and total Dvl2 decreased by 80% after EGF treatment (Supplementary Fig. S4C). In addition, more increases in c-Jun and lower levels of Dvl2 were observed after EGF treatment in KITENIN-transfected EGFR-null MEF and SW-620 cells, compared with those before treatment (Fig. 2C and D). To confirm whether lowered Dvl2 and increased c-Jun were responsible for AP-1 synergy by EGF under KITENIN transfection or by PMA, cotransfection of Dvl2 or a dominant negative c-Jun (DN-c-Jun) was undertaken. Transfected Dvl2 or DN-c-Jun reversed the upregulated AP-1 activity by EGF or PMA (Fig. 3C), indicating that downregulation of Dvl2 and upregulation of c-Jun is involved in EGF-induced AP-1 synergy in KITENIN-overexpressed cells.

Next, we examined the characteristics of the interactions between KITENIN and Dvl2 after EGF treatment. Only phospho-Dvl2 interacted with KITENIN. Interactions between KITENIN and Dvl2 disappeared after phosphatase treatment (Fig. 3B, bottom). At 30 minutes after EGF treatment in HCT116 cells, the level of KITENIN-bound phospho-Dvl2 initially increased but decreased after 2 hours.

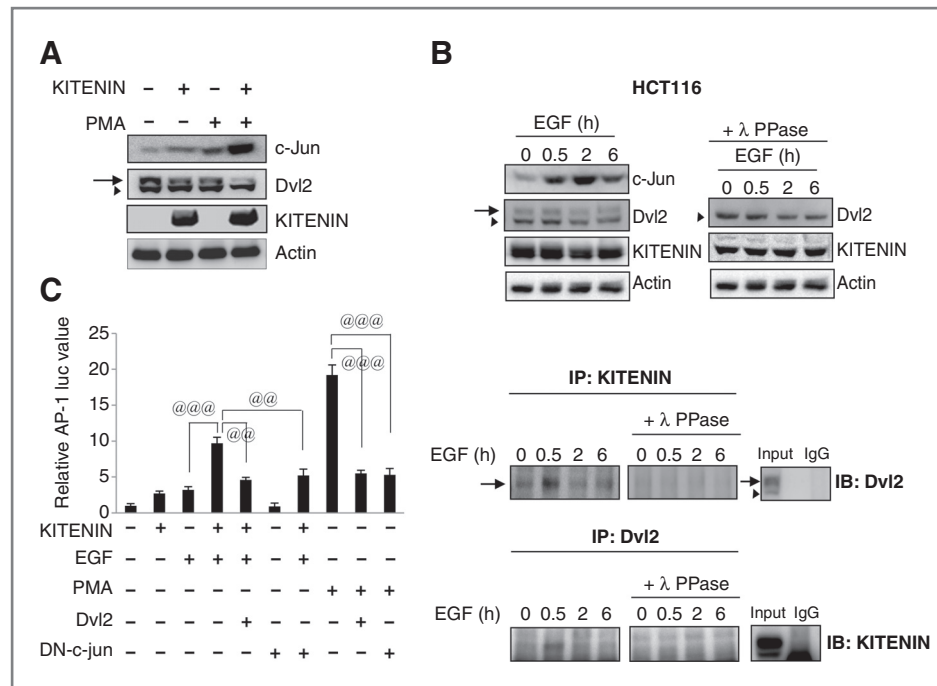


Figure 3. EGF induces increased interactions of KITENIN with Dvl2 in cytoplasmic vesicles, as well as downregulation of KITENIN-bound phospho-Dvl2 and upregulation of c-Jun. **A**, effect of PMA on c-Jun and Dvl2 in KITENIN-transfected cells. Immunoreactive Dvl2/c-Jun was examined after PMA (100 nmol/L) treatment for 4 hours in 293 T cells transfected with or without KITENIN and measured by densitometry in triplicate experiments. Phospho-Dvl2 was detected by phosphorylation-dependent mobility shift with Dvl2-specific antibody. The arrow and arrowhead indicate phospho- and unphospho-Dvl2, respectively. **B**, effects of EGF on c-Jun and Dvl2 (top) and on KITENIN-bound phospho-Dvl2 (bottom) in colorectal cancer cells. Serum-starved HCT116 cells were treated with EGF at the time points indicated. Total cell lysates (500 μ g) were treated with or without lambda phosphatase (λ PPase) and were analyzed by immunoblot (top) or immunoprecipitation (bottom). The arrow and arrowhead indicate phospho- and unphospho-Dvl2, respectively; the upper band disappeared after phosphatase treatment. **C**, effects of transfection with Dvl2 or DN-c-Jun on EGF-induced AP-1 synergy under elevated KITENIN conditions. 293 T cells were cotransfected with AP-1 reporter with or without KITENIN and with Dvl2 or DN-c-Jun, and treated with EGF or PMA (20 nmol/L) for 12 hours. (Continued on the following page.)

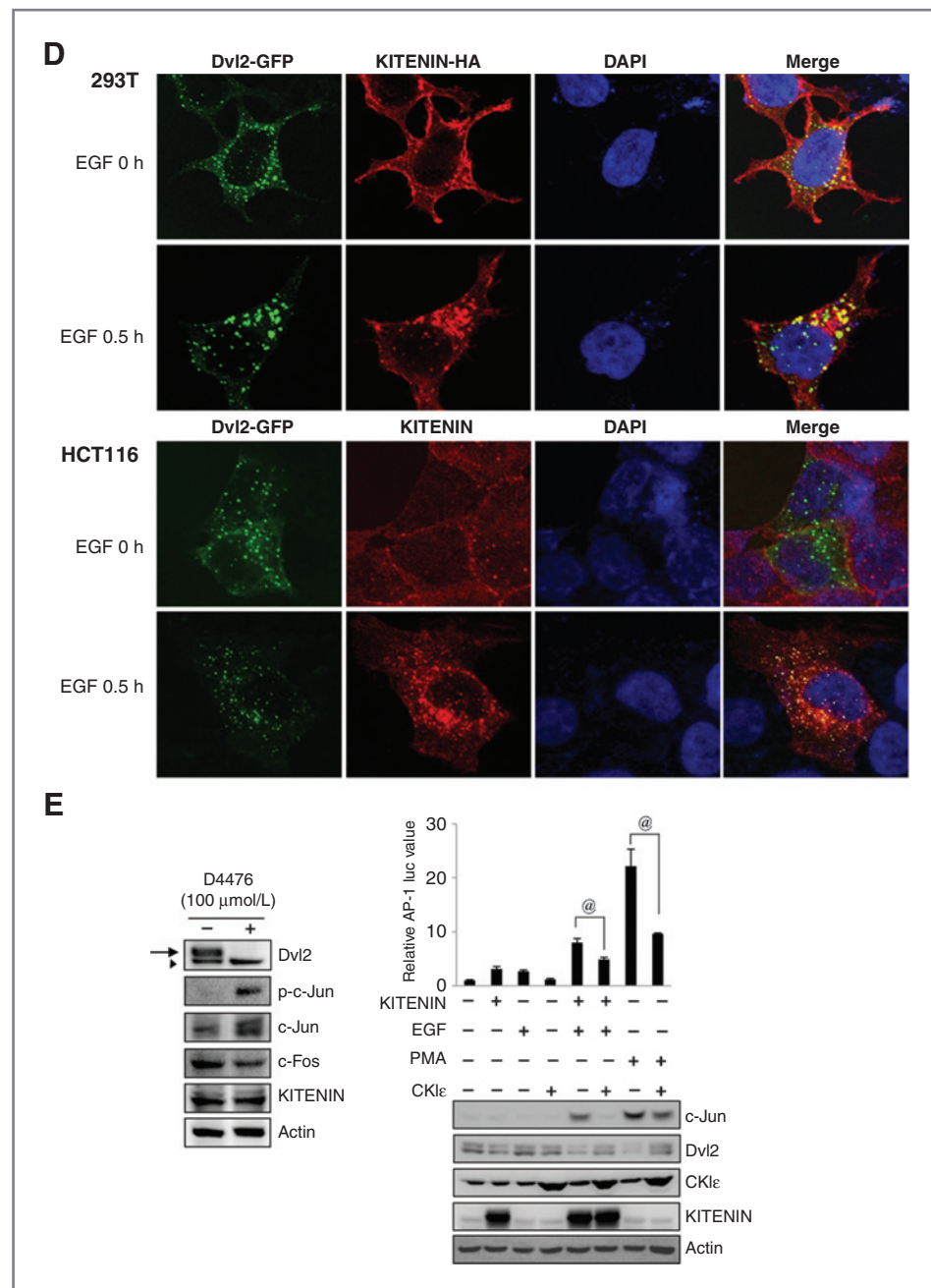
Consistently, at 30 minutes after EGF treatment in 293 T and HCT116 cells, KITENIN translocation was observed from the plasma membrane to cytoplasmic vesicles in which phospho-Dvl2 was also localized (Fig. 3D, KITENIN panels). Importantly, the number of colocalized vesicles (cytoplasmic puncta) increased upon EGF treatment (Fig. 3D, merge panels), which indicated that membrane-associated KITENIN is internalized and thereafter interacts with phospho-Dvl2. Thus, we attempted to discern whether decreases in phospho-Dvl2, rather than total Dvl2, are responsible for increased c-Jun. When phosphorylation of Dvl was blocked by treatment with D4476, a casein kinase I inhibitor, phospho-c-Jun/total c-Jun was upregulated, but not c-Fos (Fig. 3E, left). In contrast, cotransfection of casein kinase I not only attenuated AP-1 synergy by EGF under KITENIN transfection or by PMA, but also restored the increased c-Jun and lowered total Dvl2 observed after EGF treatment under KITENIN transfection or after PMA treatment (Fig. 3E, right). We interpreted these results as follows: EGF initially induces endocytosis of KITENIN and activates the formation of a KITENIN/phospho-Dvl2 complex in cytoplasmic vesicles. Thereafter, EGF downregulates KITENIN-bound phospho-Dvl2, which subsequently contributes to increased c-Jun/AP-1 activity.

Interestingly, Dvl2 knockdown did not increase transcription of c-Jun and Dvl2 did not directly interact with c-Jun (Supplementary Fig. S5A and S5B). JNK blocking also did not affect EGF action in KITENIN-transfected cells (Supplementary Fig. S3B). These data suggest that downregulation of phospho-Dvl2 after EGF treatment leads to AP-1 synergy through stabilization of c-Jun protein. Although, it is presently unknown how decreases in KITENIN-bound phospho-Dvl2 stabilize c-Jun, this mechanism is distinct from the known regulators of phospho-c-Jun, such as N-terminal phosphorylation of c-Jun by JNK or the MEK/RACO-1 pathway (21, 22).

ErbB4 is required for EGF-induced AP-1 synergy

Because we found that EGFR is not necessary for EGF-induced AP-1 synergy and that downregulation of KITENIN-bound phospho-Dvl2 is responsible for this EGF action, we attempted to address whether KITENIN may recruit other receptor tyrosine kinases (RTK) as coregulators to assist in the processing of KITENIN-bound Dvl2. To examine whether expression levels of KITENIN may affect the activation of RTK, we compared the phosphorylation levels of various RTKs between parent and KITENIN-overexpressing colorectal cancer cells and between parent

Figure 3. (Continued.) D, effect of EGF on colocalization of KITENIN with Dvl2 in the cytoplasmic vesicles. 293 T cells (top) were transiently transfected with Dvl2-GFP and KITENIN-HA constructs and HCT116 cells (bottom) were transiently transfected with Dvl2-GFP for 36 hours. Cells were visualized by fluorescent confocal microscope after EGF treatment for 30 minutes. Representative examples are shown; the yellow color in the merged panel indicates colocalization of Dvl2 with KITENIN. **E**, effects of modulating Dvl2 phosphorylation on c-Jun (left) and on AP-1 synergy by EGF (right). 293 T cells were treated with the casein kinase I inhibitor D4476 (100 $\mu\text{mol/L}$) for 4 hours to block Dvl-phosphorylation, after which Dvl2, c-Jun, and c-Fos levels were examined (left). Next, cells were cotransfected with AP-1 reporter with or without KITENIN and with casein kinase I ϵ , and subsequently treated with EGF or PMA (20 nmol/L) for 12 hours (right).



and KITENIN-knockdown colorectal cancer cells. In KITENIN-overexpressing Caco2 cells, phospho-RTKs, including ErbB4 and RON, were increased, whereas phospho-Ephrin1 was decreased. Meanwhile, phospho-ErbB4, phospho-RON, and phospho-c-Ret were decreased in KITENIN-knockdown HCT116 cells (Supplementary Fig. S6A). Phospho-c-Met was detected constantly in HCT116 cells. Thus, among the phospho-RTKs studied, RON and ErbB4 were consistently identified as being affected by KITENIN. As expected, phospho-ErbB4 and phospho-RON, but not their total forms, were increased in Caco2/KITENIN cells and decreased in HCT116/si-KITENIN cells (Supplementary

Fig. S6B). Interestingly, enhanced invasion of Caco2/KITENIN cells, compared with parent cells, indicative of a gain of function, was restored by ErbB4 knockdown, but not by RON knockdown (Fig. 4A). These results showed ErbB4 to be an essential cofactor in KITENIN signaling, enhancing colorectal cancer cell invasion.

Subsequent to the above, we next attempted to uncover whether ErbB4 is required as a binding partner to assist in KITENIN signaling. We found that KITENIN interacted with the ErbB4 via its C-terminal cytoplasmic domain (Supplementary Fig. S6C) and that interaction between KITENIN and ErbB4 increased upon EGF treatment (Fig. 4B).

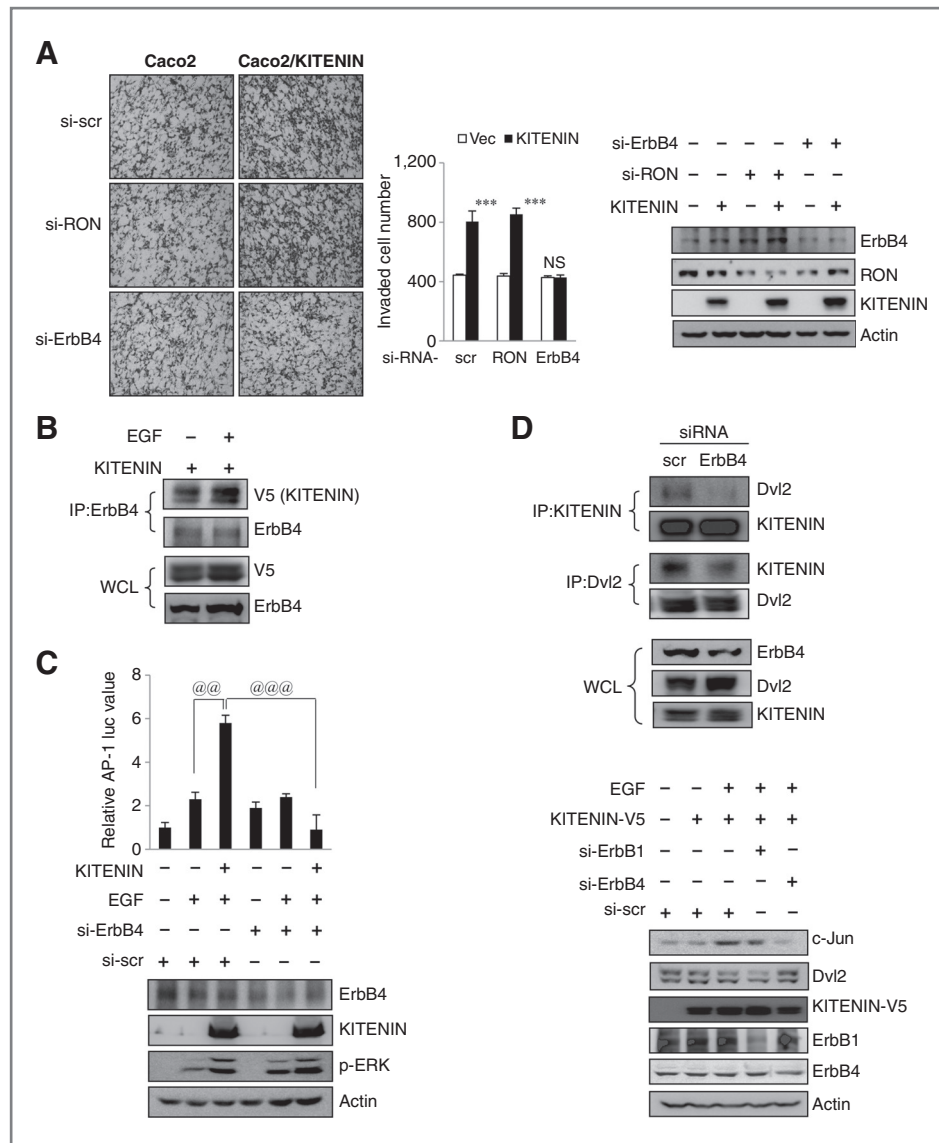


Figure 4. ErbB4 is necessary for AP-1 synergy by EGF under elevated KITENIN conditions. A, effects of RON- or ErbB4-knockdown on increased cell invasion by KITENIN. Caco2/ vector or Caco2/KITENIN cells were transfected with si-scr, si-RON, or si-ErbB4 for 48 hours and subjected to invasion assay. B, EGF increased interactions of KITENIN with ErbB4. Caco2/KITENIN cells were treated with EGF for 12 hours, and immunoreactive KITENIN and ErbB4 were examined after immunoprecipitation with anti-ErbB4 antibody. C, ErbB4 is necessary for EGF-KITENIN-AP-1 signaling. 293 T cells were cotransfected with KITENIN and AP-1 reporter with or without ErbB4-siRNA treatment and subsequently treated with EGF for 12 hours. D, ErbB4-knockdown decreased the interactions of KITENIN with Dvl2 (top) and restored the EGF action under elevated KITENIN conditions (bottom). Caco2/KITENIN cells were transfected with Dvl2 and treated with ErbB4-siRNA for 36 hours. Immunoreactive Dvl2 or KITENIN was examined after immunoprecipitation with anti-KITENIN or anti-Dvl2 antibody, respectively (top). Caco2/KITENIN cells were treated with ErbB1-siRNA or ErbB4-siRNA for 36 hours. Immunoreactive Dvl2 and c-Jun were examined after EGF treatment (bottom).

Importantly, ErbB4 knockdown attenuated EGF-induced AP-1 synergy in KITENIN-transfected cells (Fig. 4C), indicating that ErbB4 is required for EGF-induced AP-1 synergy. Moreover, EGF treatment induced significant increases in AP-1 activity in KITENIN/ErbB4-cotransfected cells, compared with cells transfected with EGFR alone, ErbB4 alone, or KITENIN/EGFR (Supplementary Fig. S7). Consistently, ErbB4 knockdown decreased interactions between KITENIN and Dvl2 (Fig. 4D, top), and reversed EGF-induced increases in c-Jun and decreases in Dvl2, which were not affected by EGFR knockdown (Fig. 4D, bottom). Thus, we discerned ErbB4 to be necessary in elevating KITENIN-Dvl2 interactions after EGF treatment and assisting in the processing of KITENIN-bound Dvl2, and thereby to be responsible for increased c-Jun after EGF treatment under KITENIN transfection. Also, EGF stimulated the formation of KITENIN/ErbB4 complexes.

The EGF-KITENIN/ErbB4-c-Jun axis upregulates colorectal cancer cell invasion

We finally examined whether the delineated EGF-KITENIN/ErbB4-c-Jun axis works in an EGFR-independent/ErbB4-dependent fashion by using CHO cells, a known all ErbB-null cell line. In KITENIN/ErbB4-cotransfected CHO cells, more increases in c-Jun and decreases in Dvl2 were observed following EGF treatment than in parent or KITENIN alone-transfected cells (Fig. 5A). This further confirmed that the KITENIN/ErbB4-mediated downstream signal of EGF increases c-Jun in an EGFR-independent manner. Moreover, our observations that c-Jun increases following EGF treatment in both KITENIN-transfected EGFR-knockout cells (Fig. 2C) and EGFR-null SW-620 cells (Fig. 2D) and that roughly the same AP-1 activity was observed following EGF treatment between KITENIN/EGFR-cotransfected cells and EGFR alone-transfected cells

(Supplementary Fig. S7) indicated that the axis of EGF-KITENIN/ErbB4–c-Jun might not be regulated by asymmetric EGFR-ErbB4 heterodimer formation.

We next investigated whether coexpression of ErbB4/KITENIN led to further increased colorectal cancer cell invasion as a result of strengthening the EGF-KITENIN/ErbB4–c-Jun axis. To do so, we chose HCT116 cells, which have the highest endogenous KITENIN levels and also harbor a KRAS mutation. Cell invasion was markedly increased in ErbB4-transfected HCT116 cells following EGF treatment compared with HCT116/parent cells (Fig. 5B). However, this elevation pattern was abolished after KITENIN knockdown, although HCT116 cells still expressed elevated ErbB4. Thus, KITENIN may be a more important component than ErbB4 in regulating colorectal cancer cell invasiveness via the EGF-KITENIN/ErbB4–c-Jun axis. Also, treatment of AG1478 in KITENIN/ErbB4-cotransfected Caco2 cells did not attenuate the action of EGF on the increased cell invasion (Fig. 5C), as in KITENIN-transfected Caco2 cells (Fig. 2B). This confirmed that the upregulation of cell invasiveness by EGF under coexpressed KITENIN/ErbB4 does not require modulation of ErbB kinase activity.

Higher levels of KITENIN/ErbB4 are coexpressed in tumor tissues from patients with metastatic colorectal cancer who show poor responses to cetuximab/chemotherapy

The use of an anti-EGFR monoclonal antibody has been implemented as the only effective tool in clinical practice for treating patients with metastatic colorectal cancer since 2004, but objective response rates even for those of KRAS wild-type status are below 20% (5–7). Accordingly, we attempted to elucidate the functional significance of the KITENIN/ErbB4 complex outlined in this study in cetuximab-resistant patients with metastatic colorectal cancer. To do so, we examined the expression levels of KITENIN and ErbB4 in resected tumor tissues obtained from patients with metastatic colorectal cancer before treatment. These patients exhibited wild-type KRAS and were primarily treated with cetuximab/chemotherapy (Supplementary Table S1). The immunohistochemical coexpression of KITENIN/ErbB4 was great in tumor tissues from patients with metastatic colorectal cancer who showed progression of disease (PD) at initial stages (2 months) after treatment with cetuximab, compared with those who exhibited a partial response (PR). However, the expression level of ErbB4 in patients with colorectal cancer who showed a PR was not matched with that of KITENIN (Fig. 6A).

To assess quantitatively the immunohistochemical expression of KITENIN and ErbB4 in colon tissues, a score was calculated by using the intensity of the staining and the size of the positively stained area. The KITENIN expression score was significantly greater in tumor tissues from patients who showed PD than in those who exhibited a PR (Fig. 6B; 8.05 ± 0.30 , $n = 22$ vs. 4.42 ± 0.33 , $n = 33$; $P = 5.9 \times 10^{-10}$). ErbB4 was highly expressed in tumor tissues from

both groups, with no significant difference in ErbB4 scores (Fig. 6B; 6.05 ± 0.51 , $n = 22$ vs. 6.18 ± 0.41 , $n = 33$; $P = 0.84$). Patients showing good early response had a significantly longer progression-free survival (PFS) than patients with poor response (Supplementary Table S1, $P = 0.014$). Thus, our data suggest that early chemoresistance to cetuximab in patients highly expressing KITENIN may originate from the existence of an unconventional EGF signal, which is switched on under the elevated KITENIN condition.

KITENIN/ErbB4–c-Jun axis confers resistance to cetuximab in colorectal cancer cells in which KITENIN is highly expressed

The novel EGF signal described here may endow colorectal cancer cells expressing higher KITENIN with enhanced survival in response to EGFR-targeted therapies. To test this possibility, we first examined the susceptibility of various colorectal cancer cells to increasing doses of cetuximab. We found that HCT116 and Caco2 cells expressing higher levels of endogenous KITENIN were more resistant to cetuximab than were DLD1 and SW620 cells expressing lower KITENIN (Supplementary Fig. S8A). To examine whether the expression levels of KITENIN affected the survival of colorectal cancer cells in response to cetuximab, we first chose Caco2 colorectal cancer cells, a KRAS/BRAF wild-type cell line (23), as these would be suitable models for the clinical setting where EGFR-targeted therapy is used (5, 6). Although there was no difference in survival to cetuximab between empty vector- and KITENIN-transfected Caco2 cells, highly KITENIN-expressing Caco2 cells were resistant to cetuximab, whereas KITENIN-knockdown Caco2 cells showed increased sensitivity (Supplementary Fig. S8B). We also compared the proliferation rate to cetuximab in colorectal cancer cells with mutant KRAS/BRAF, such as DLD1 and HCT116 cells. We found that highly KITENIN-expressing DLD1 cells were resistant to cetuximab, whereas KITENIN-knockdown HCT116 cells showed sensitivity to cetuximab (Supplementary Fig. S8C). Thus, these results indicated that KITENIN/ErbB4–c-Jun axis confers resistance to cetuximab in Caco2 cells with KRAS/BRAF wild type in which KITENIN is highly expressed. Also, our results suggest that this axis can contribute to the early acquisition of resistance to cetuximab in colorectal cancer cells with mutant KRAS/BRAF.

Discussion

Among the ligands known to interact with EGFR, TGF α exhibits an EGFR-independent action, in which it protects Naked2 from proteasomal degradation (24). However, EGFR-independent actions for EGF have not yet been explored. In this study, we found a novel EGFR-independent action for EGF that works under elevated expression of KITENIN/ErbB4: EGF stimulates the formation of KITENIN/ErbB4 complexes that target phospho-Dvl2 for degradation, which then leads to the upregulation of c-Jun and

colorectal cancer cell motility (Fig. 5D). Although the detailed mechanisms of what exactly EGF binds to, how ErbB4 assists in processing KITENIN-bound phospho-Dvl2, and how lowered phospho-Dvl2 within the KITENIN/ErbB4 complex stabilizes c-Jun are unknown, the downstream signal of EGF, the KITENIN/ErbB4-Dvl2-c-Jun pathway, that we identified in this study differs from the previously reported downstream pathways of EGF that require binding to EGFR (3–6). Thus, we have identified a new EGFR-independent promotility signal of EGF in colorectal cancer cells.

The lack of response to anti-EGFR agents in "quadruple-negative" patients may be because of multiple reasons, including the oncogenic deregulation of the same 4 genes by mechanisms other than mutations, providing an

alternate pathway of survival and proliferation (5, 6, 25). As for the new molecular determinants of resistance to anti-EGFR agents, proto-oncogene securin promotes resistance to gefitinib-induced apoptosis via an EGFR-independent pathway in human cancer cells (26) and TGF β provides an intrinsic EGFR-independent survival signal that protects squamous cancer cells from cetuximab-dependent cellular cytotoxicity (27). In this study, we observed that KITENIN was significantly highly expressed in tumor tissues from metastatic colorectal cancer patients with wild-type KRAS who showed poor responses to cetuximab/conventional chemotherapy after the initial stages of treatment. Furthermore, this novel downstream signal of EGF endowed colorectal cancer cells expressing higher KITENIN with enhanced survival

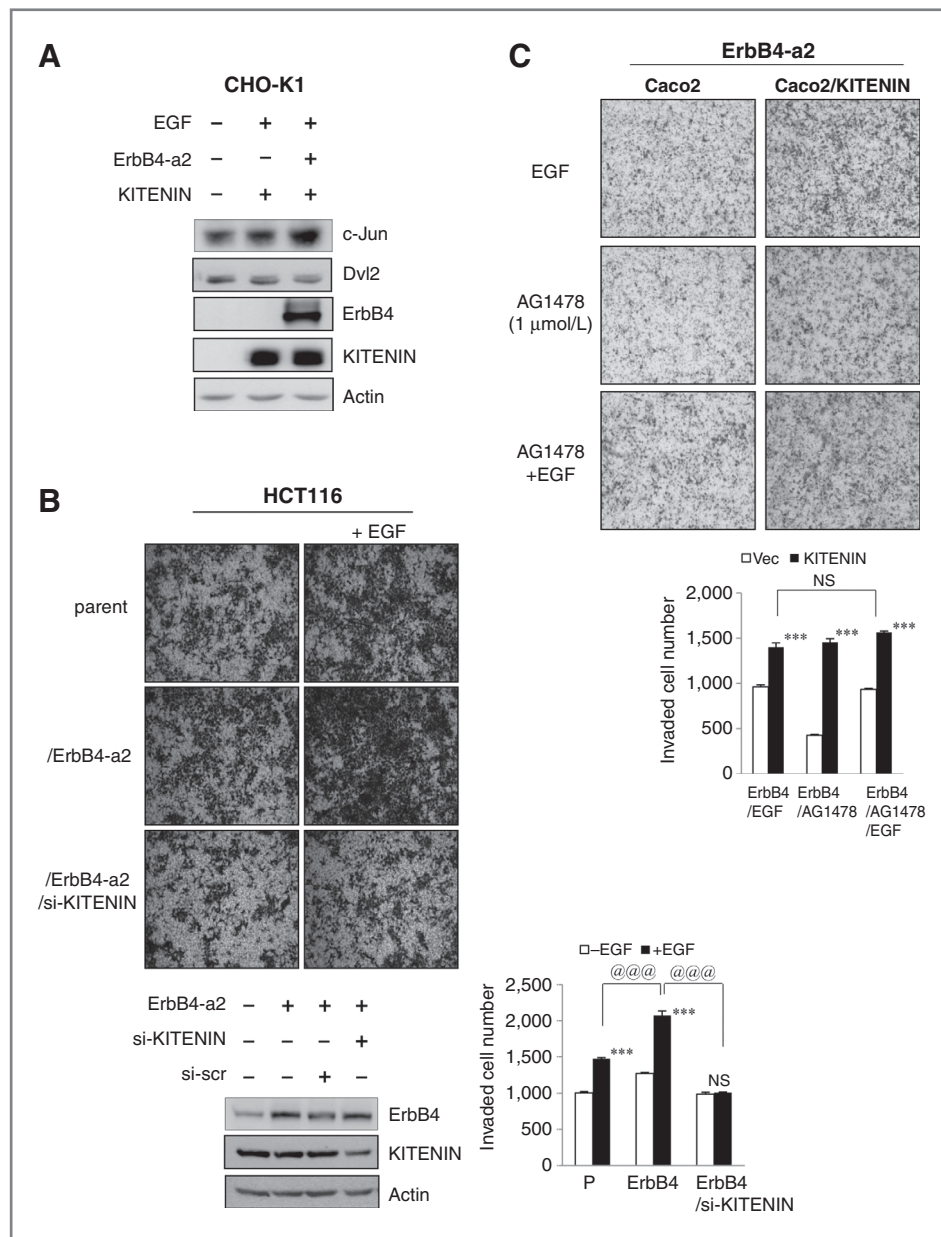


Figure 5. EGF-KITENIN/ErbB4-c-Jun axis upregulates colorectal cancer cell invasion. A, EGF-KITENIN/ErbB4-c-Jun axis works in CHO cells. CHO-K1 cells were cotransfected with KITENIN with or without ErbB4 JM-a/CYT-2 (a2) for 48 hours, and treated with EGF for 12 hours. B, invasion assays in ErbB4-transfected HCT116 cells after EGF treatment with or without KITENIN knockdown. HCT116 cells were transfected with ErbB4 isoform (a2) or cotransfected with si-KITENIN/ErbB4 isoform (a2). Parent or transfected HCT116 cells were treated with EGF for 12 hours and subjected to invasion assay. C, effects of ErbB kinase blocker on elevated cell invasion by EGF under KITENIN/ErbB4 conditions. Caco2/ErbB4(a2) or Caco2/KITENIN/ErbB4(a2) cells were treated with EGF and/or AG1478 (1 μmol/L) for 12 hours and subjected to invasion assays. (Continued on the following page.)

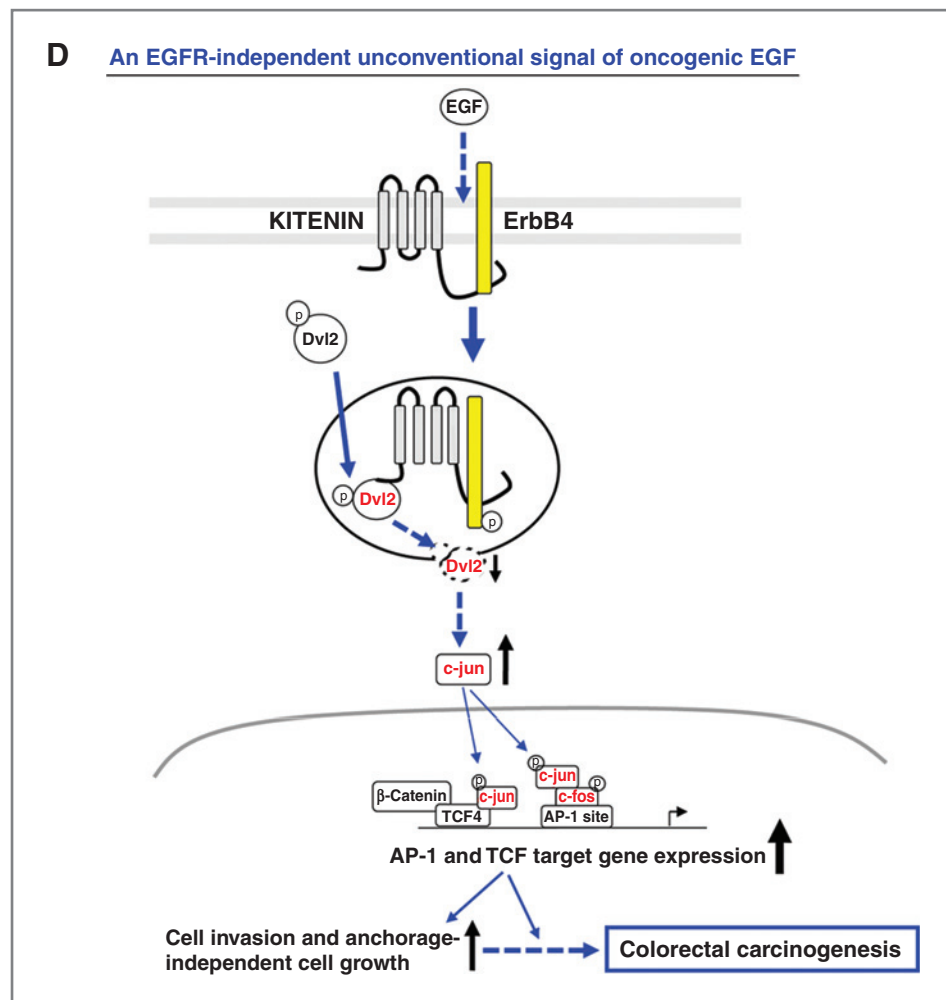


Figure 5. (Continued.) D, schematic showing the novel downstream signal of oncogenic EGF: stimulation of the KITENIN/ErbB4-Dvl2-c-Jun axis by EGF results in the upregulation of c-Jun and colorectal cancer cell invasion. Unidentified pathways are indicated by dotted lines.

to cetuximab. Therefore, we suggest that the KITENIN/ErbB4-c-Jun axis could be a molecular basis for conferring resistance to anti-EGFR agents in colorectal cancer tissues in which KITENIN is highly expressed.

c-Jun belongs to the dimeric AP-1 family of transcription factors, which are critical regulators of the gene expression that defines the invasive phenotype of cancer (28). Phospho-c-Jun interacts with TCF4 to form a c-Jun-TCF4-β-catenin complex that enhances intestinal tumorigenesis, whereas deletion of *c-Jun* in intestinal tissue reduces *APC^{min/+}*-mediated tumor formation (29). Thus, elevated c-Jun resulting from the EGF-driven KITENIN/ErbB4-Dvl2-c-Jun axis may enhance colorectal tumor progression by increasing colorectal cancer cell invasiveness. Currently, the upstream mechanism that regulates the phosphorylation of c-Jun is largely unknown (30, 31), yet we present evidence of a possible mechanism for the stabilizing of c-Jun through the degradation of phospho-Dvl2 within the functional KITENIN/ErbB4 complex.

Gene amplification or overexpression is a common mechanism of RTK deregulation in many cancers that not only yields increased surface abundance and signaling, but that is also likely to alter the distribution, clustering, and

dimeric partnering of RTKs (32). Structural and other studies have indicated that many RTKs form dimers and higher-order oligomers in the absence of ligand, which suggests more sophisticated mechanisms of ErbB partnering, activation, and ligand-independent clustering (33, 34). Our present results suggest that overexpressed KITENIN alters the complex equilibrium of ErbB4-containing clustering to form a KITENIN/ErbB4 complex, which consequently increases c-Jun signaling output in an EGFR-independent manner. Although the underlying mechanism is unknown, this is thought to occur via physical collaboration between KITENIN and ErbB4.

Recently, the emergence of KRAS mutations and amplification was shown to be associated with primary and acquired resistance to cetuximab or panitumumab (5, 6, 25, 35, 36). Previously, we observed that the functional KITENIN complex is indispensable for invasion of DLD1 and HCT116 cells harboring KRAS mutation (11), indicating that the KITENIN complex is also required as a downstream effector for increased invasiveness by KRAS. We here found that highly KITENIN-expressing DLD1 and HCT116 cells were also more resistant to acute exposure of cetuximab. It thus suggests that blockers of

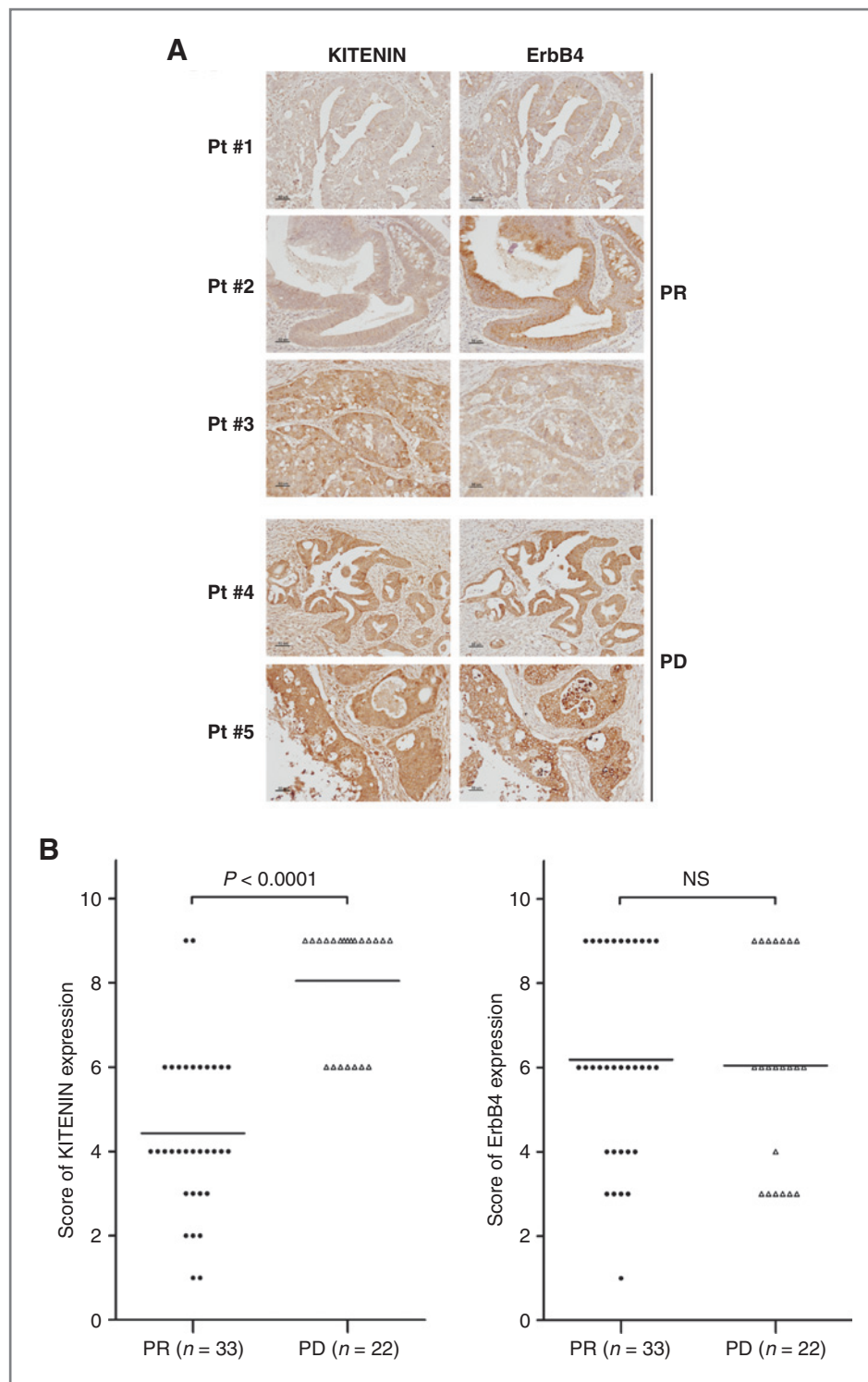


Figure 6. Higher levels of KITENIN/ ErbB4 are coexpressed in tumor tissues from metastatic patients with colorectal cancer with poor responses to treatment with cetuximab. A, KITENIN and ErbB4 expression by immunohistochemistry (dark brown color) in serially sectioned colon tissues from patients with metastatic colorectal cancer with wild-type KRAS who were primarily treated with cetuximab. Representative examples are shown: #1, #2, and #3 are derived from patients with stage IV colorectal cancer who exhibited PR at 2 months after treatment with cetuximab; #4 and #5 are from patients who showed PD over the same period. Scale bar: 50 μ m. B, positive correlation between the expression score of KITENIN and poor responses to cetuximab at initial stages after treatment. To assess the immunohistochemical level of KITENIN and ErbB4, a score was calculated and the significance thereof between the PR and PD groups was tested as described in the Materials and Methods. NS, not significant.

the KITENIN complex may be effective in patients with colorectal cancer with KRAS mutation who exhibit poor responses to anti-EGFR agents. Therefore, other than the EGFR pathway, our results support the rationale for combined targeting of the KITENIN complex to improve

responses to cetuximab-based therapy in EGFR/KITENIN-overexpressing patients with metastatic colorectal cancer. Such a strategy is expected to lower resistance to ErbB inhibitors in patients with metastatic colorectal cancer, regardless of KRAS status.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Study supervision: J.A. Bae, S.-Y. Park, K.K. Kim

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