BRAF-induced tumorigenesis is IKKα dependent but NF-κB independent.

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One Sentence Summary: The endosomal IKKα pathway is induced and required downstream of oncogenic BRAF.
Abstract

Tumor cells carrying KRAS mutations activate the NF-κB pathway by different mechanisms contributing to the acquisition of essential cancer properties. The BRAF kinase, a downstream mediator of KRAS, is also mutated in a subset of colorectal cancers (CRC), which predicts bad prognosis and therapy resistance. However, whether NF-κB participates of BRAF-mediated tumorigenesis remains unknown. We here found that, in contrast to KRAS, mutant BRAF does not trigger canonical or alternative NF-κB signaling, but induces p45-IKKα activation in CRC cells instead. Moreover, IKKα activity is required for BRAF-induced transformation and to support BRAF-dependent transcription. Activation of p45-IKKα downstream of BRAF requires the TAK1 kinase, and is associated to the endosomal compartment. Inhibition of endosomal V-ATPase abolishes p45-IKKα phosphorylation, and induces apoptosis of BRAF mutated CRC cells. Additional in vivo experiments demonstrate that pharmacologic inhibition of endosome acidification reduces the growth and the metastatic capacity of BRAF mutant tumor cells in an orthotopic xenograft model.
INTRODUCTION

Colorectal cancer (CRC) is the second leading cause of death from cancer in Western populations. Progression from adenoma to invasive CRC requires a number of genetic alterations including those of adenomatous polyposis coli (APC) gene, SMAD4 (encoding a transcription factor of the TGFβ pathway) or the guanosine triphosphatase (GTPase) KRAS. Activating mutations of KRAS are present in about 40% of advanced CRC. Clinically relevant RAS mutations are difficult to target and preclude the use of EGFR inhibitors such as cetuximab or panitumumab in advanced metastatic CRC (1-3). RAS signaling through RAF induces the subsequent activation of the mitogen activated protein/extracellular signal-regulated kinase 1 and 2 (MEK1/2), extracellular signal-regulated kinase 1 and 2 (ERK1/2) and a variety of targets including key growth factors. The importance of the RAS/RAF pathway and the identification of BRAF mutations in various types of cancer, including 5-15% of all CRC cases, pointed out the possibility of using RAF inhibitors for therapeutic purposes. BRAF inhibitors have been approved to treat metastatic melanoma patients (4, 5). Unfortunately, results in CRC patients are disappointing probably due to the fact that some RAF inhibitors that block proliferation of BRAF mutant cells are ineffective against RAS mutant cells, and even activate the MAPK pathway in the presence of wild-type RAF (6-9). The efficacy of BRAF inhibitors in CRC can also be suppressed by HGF secreted by the tumor microenvironment (10) through overexpression of CRAF (11) or EGFR (12), thus most of the tumors that initially respond to RAF inhibition tend to develop drug resistance. Better outcomes are obtained with combinations of BRAF and MEK inhibitors, especially in melanoma (13), and/or with different MEK inhibitors preferentially targeting KRAS or BRAF mutant tumors (10, 14). Yet, the identification of new druggable elements downstream of RAS is a primary line of research in the cancer field.

The NF-κB pathway is a key regulator of innate and acquired immunity but also of cancer progression. Activation of NF-κB lays downstream of the Inhibitor of κB Kinase (IKK) complex composed of two catalytic subunits, IKKα and IKKβ, and the regulatory subunit IKKγ/NF-κB essential modulator (NEMO). Phosphorylation-mediated degradation of the inhibitor of kappaB (IκB) is induced
by IKKβ downstream of the TGFβ activated kinase 1 (TAK1) leading to nuclear translocation of the NF-κB factor (mostly p65/p50) and canonical NF-κB activation. Activation and recruitment of TAK1 to the IKK complex is induced by K63-linked polyubiquitination of the TNFα receptor associated factor (TRAF) and NEMO, which acts as a bait for the TAK1 adaptors TAB2/3 [reviewed in (15)]. Alternative NF-κB pathway is IKKβ, NEMO and TAK1-independent, but requires the activation of IKKα by the NIK kinase, following NIK protein stabilization. Active IKKα (and NIK) phosphorylates p100, thus inducing its proteolytic processing into p52 that translocates to the nucleus together with RelB to activate transcription (16). Additional substrates for IKKα are histone H3 (17, 18), the kinase Aurora A (19), or the nuclear corepressors SMRT (20) and NCoR (21). In squamous cell carcinoma, IKKα induces HOX and IRX transcription through the chromatin release of SUMOylated IκBα (22), and in CRC IKKα facilitates transcription of the Notch-targets HES1 and HEY1 and the anti-apoptotic gene clAP2 associated with nuclear corepressor inactivation (21, 23). In contrast, nuclear active IKKα represses the metastasis-related gene Maspin in prostate cancer cells (24).

Oncogenic KRAS is known to induce canonical NF-κB thus inhibiting apoptosis in different cancer systems (25-29). In lung cancer, KRAS through PKC and p62 induces TRAF6 and NEMO polyubiquitination leading to TAK1-dependent phosphorylation of IKKβ (27, 30), whereas in pancreatic cancer a paracrine loop involving IL1α is responsible for activating NF-κB (25). Inhibition of TAK1 (26) or other NF-κB elements (31, 32) reverts cancer progression in the presence of KRAS mutations. However, little is known about the putative contribution of NF-κB or particular NF-κB constituents to BRAF-driven tumorigenesis. Of note that general NF-κB inhibitors are extremely toxic and they can either suppress or promote cancer in a context-dependent manner, which stands against their possible use in human therapies (33). Recently, we identified a proteolytic fragment of IKKα, which we called p45-IKKα, that is generated in the early endosomes of CRC cells by the proteolytic activity of cathepsins, and whose in vitro processing is favored at acidic pH. Phosphorylated p45-IKKα associates with NEMO and full-length IKKα to phosphorylate the nuclear corepressor SMRT.
and histone H3, thus promoting specific gene transcription. Supporting the functional relevance of P-p45-IKKα, an IKKα mutant that is not processed to the p45 form (IKKα3M) fails to drive specific transcription leading to increased apoptosis of CRC cells (34). Here, we study the mechanisms that regulate p45-IKKα activation in CRC cells with different mutations for KRAS or BRAF, and test the possibility of using this information to design specific therapies for a subset of cancer patients.

RESULTS

BRAF is associated to p45-IKKα activity in CRC cells but has no impact on NF-κB

We previously demonstrated that nuclear P-IKK is associated with advanced disease in human CRC (34). By analyzing 184 adenoma and carcinoma samples and the corresponding paired distal normal mucosa from 98 patients, we now found that 70% of samples with high abundances of nuclear P-IKK were also positive for P-ERK1/2 staining, compared with 20% of P-ERK1/2 positivity in samples with negative or low nuclear P-IKK (Fig. 1, A and B), suggestive of a link between IKK activation and the MAPK pathway. In a set of 93 samples that were examined for KRAS, BRAF and PI3K mutations, we observed that BRAF mutations significantly associate with higher nuclear P-IKK abundances (Fig. 1, C and D), further connecting signaling through the BRAF–MAPK pathway with IKK activity in CRC cells. Mutant BRAF is commonly associated with the serrated pathway in CRC. Thus, we sub-classified our set of samples to determine whether nuclear IKK activity was restricted to any specific lesion subtype. The group of samples with high amounts of nuclear P-IKK contained all serrated lesions (including all BRAF mutated polyps), but also several conventional polyps and carcinomas including those containing the BRAF^{V600E} mutation (Fig. 1E).

Next, we determined the abundance of active IKK in CRC cell lines carrying mutations in KRAS at codon 13 (HCT116 and DLD1) or codon 12 (SW480 and LS174T) or in BRAF (WiDr and HT29), or in cells carrying no mutations in these
genes (LIM1215). We found that cells with mutant BRAF contained the highest amounts of activated IKK (mostly corresponding to the previously described p45-IKKα), although activated p45-IKKα was also detectable in KRAS mutant cells (fig. S1, A and B). To directly test whether BRAF regulates the phosphorylation of p45-IKK, we treated CRC cells carrying the BRAF\textsuperscript{V600E} mutation with the RAF inhibitor AZ628. AZ268 abrogated the detection of phosphorylated p45-IKKα in these cells, compared with the partial effects of MEK1/2 or PI3K inhibition (Fig. 1F). Inhibition of BRAF also reduced p45-IKKα phosphorylation in KRAS mutated cells after 2 hours treatment (fig. S1B). Of note that neither phosphorylation of full-length IKKα/β (85-87 kD) nor the abundance of IκBα and p100/p52 were affected by AZ628, indicating that oncogenic BRAF does not modulate NF-κB activity in CRC cells. Consistent with this interpretation, we did not detect significant NF-κB DNA-binding activity in the nuclear extracts of HT29 and WiDr cells (BRAF-mutated) by EMSA, whereas NF-κB activity in cells with KRAS codon 12 mutations was found at a comparable abundance to that in TNFα-treated WT cells (fig. 1G). Moreover, BRAF\textsuperscript{V600E} failed to activate the 3XκB-luciferase reporter construct when transduced into human embryonic kidney (HEK) 293T cells (fig. S1C).

Next, we tested whether BRAF inhibition affected p45-IKK activity by analyzing the amounts of phosphorylated serine 10 of histone H3 and SMRT (both isoforms of 150 kD and 300 kD). We found reduced abundance of both phosphorylated substrates in the presence of the BRAF inhibitor, which were partially recovered after washout and linked to p45-IKK reactivation (Fig. 1H). Time course experiments using BRAF mutant cells demonstrated that p45-IKK phosphorylation started 5 minutes after washing out the BRAF inhibitor (reaching maximum recovery at 1 hour) (fig. S1D), thus suggesting that IKK activation downstream of BRAF is cell-autonomous. Consequently, HT29 or WiDr conditioned-media only induced a marginal activation of p45-IKK and ERK1/2 in LIM1215 cells (WT for KRAS and BRAF) that was abrogated when the RAF inhibitor was added (fig. S1E).
To further investigate the contribution of IKK to BRAF signaling in CRC, we performed microarray expression analysis of HT29 and WiDr cells treated for 16 hours with the RAF inhibitor. About 5000 genes were significantly repressed in both cell lines in three independent replicates, which clustered into the functional categories of cell cycle, DNA repair and MAPK signaling, among others, as determined by Ingenuity Pathway Analysis. This analysis failed to identify the canonical or alternative NF-κB pathways (Fig. 1I), in agreement with our Western blot analysis (Fig 1F). We previously demonstrated that p45-IKK was derived from full-length IKKα (34). Hence, we knocked down IKKα in the BRAF mutant cells by specific short hairpin RNA (shRNA) (fig. S1F) and examined its effect on gene transcription and in comparison with the effects of BRAF inhibition. We identified 4450 genes whose expression was significantly reduced 72 hours after shRNA transduction, including 35% of the genes inhibited by AZ628 treatment (Fig. 1J). Genes identified as common targets of BRAF and IKKα (1768 genes) fall into the functional categories of cellular apoptosis, cell migration and cell proliferation, among others; again, the NF-κB pathway was not detected in the analysis (Fig. 1K and fig. S1G). In agreement with these results, CRC cells carrying the V600E mutation in BRAF (BRAF\textsuperscript{V600E}) were resistant to treatment with BAY65-5811 (Fig. 1L), which specifically inhibits canonical NF-κB but not the alternative IKKα and p45-IKK pathways (fig. S1, H and I). In contrast, BAY65-5811 significantly reduced the proliferation of KRAS mutant CRC cells (Fig. 1L), indicating that oncogenic KRAS but not BRAF signals through canonical NF-κB. Together, these results indicate that BRAF mutation induces p45-IKK activation but not NF-κB activity.

**p45-IKKα activity is induced by BRAF and required for BRAF-mediated transformation**

We next tested whether mutant BRAF was sufficient to induce p45-IKKα phosphorylation. Ectopic expression of the constitutively active BRAF\textsuperscript{V600E} promoted p45-IKK phosphorylation in NIH-3T3, with a minor effect on the abundance of full-length phosphorylated IKKα/β, p100/p52 and IκBα. Similar to
that found in the CRC cell lines, pharmacologic inhibition of BRAF activity but not of MEK1/2 prevented phosphorylation of p45-IKKα induced by BRAF<sup>V600E</sup> (Fig. 2A). Consistent with the fact that BRAF acts downstream of KRAS, oncogenic KRAS<sup>G12V</sup> increased the abundance of phosphorylated p45-IKKα in NIH-3T3 cells (Fig. 2B). However, and in contrast to our findings with BRAF<sup>V600E</sup>, cells transduced with KRAS<sup>G12V</sup> displayed increased amounts of full-length phosphorylated IKKα/β (85-87 kD band), suggestive of signaling through NF-κB. Treatment with the BRAF inhibitor specifically prevented p45-IKKα but not full-length IKKα/β phosphorylation in these cells (Fig. 2B). These results indicate that KRAS activates both NF-κB and the p45-IKKα pathway but that only the latter is downstream of BRAF. Similar to what we found in CRC cells, NIH-3T3 cells incubated with the supernatant of BRAF<sup>V600E</sup>-transduced NIH-3T3 cells failed to activate IKK or p45-IKKα (Fig. 2C). Further supporting the IKKα identity of activated p45-IKK, the antibody recognizing phosphorylated IKKα/β failed to detect any 45 kD band in lysates from BRAF<sup>V600E</sup>-transduced IKKα knockout cells (fig. S2A).

Next, we investigated the contribution of p45-IKKα to BRAF<sup>V600E</sup>-induced cell transformation. BRAF<sup>V600E</sup> failed to induce transformation foci in IKKα-deficient mouse embryonic fibroblasts (MEFs) compared to the WT MEFs (Fig. 2D). Ectopic expression of WT IKKα restored the transformation capacity of BRAF<sup>V600E</sup> in mutant MEFs, in contrast to the uncleavable IKKα3M construct (Fig. 2E) that is defective for p45-IKKα generation (fig. S2B). An intermediate phenotype was obtained using a point mutant (S400W) with reduced p45-IKKα processing capacity (34). Importantly, knockdown of IKKα in NIH-3T3 cells reduced their transformation capacity by BRAF but not by KRAS (fig. S2C), further supporting the notion that IKKα is specifically required for BRAF-mediated transformation.

**Activation of p45-IKKα by BRAF is mediated by TAK1**

TAK1 and NIK are the major kinases that activate the IKK complex. We found that ectopic expression of BRAF<sup>V600E</sup> did not affect NIK abundance, and NIK
over-expression did not induce p45-IKKα phosphorylation in HEK 293T cells (fig. S3A). Therefore, we tested whether activation of p45-IKKα downstream of BRAF was dependent on TAK1. Our results indicated that knocking down TAK1 with one of two shRNAs reduced P-p45-IKK amounts in HT29 and WiDr cells to values comparable with that in WT LIM1215 cells (Fig. 3A). Similarly, pharmacologic inhibition of TAK1 activity with 5Z-7-oxozeanol (16 hours of treatment) abrogated p45-IKKα phosphorylation in BRAF mutated cells, associated with a slight reduction of total p45-IKKα abundance (Figure 3B). However, 5 to 30 minutes of treatment with 5Z-7-oxozeanol significantly reduced p45-IKK phosphorylation without affecting total p45-IKKα abundance (Fig. 3C). Then we tested whether TAK1 activity was required for p45-IKK phosphorylation after BRAF inhibition and reactivation in WiDr and HT29 cells. As we previously found, phosphorylation of p45-IKK was detected 5 minutes after washing out the RAF inhibitor, but it was precluded in the presence of the TAK1 inhibitor (Fig. 3D). Consistent with the requirement for TAK1 in p45-IKK activation and function, TAK1 knockdown prevented the transformation of NIH-3T3 cells induced by oncogenic BRAFV600E (Fig. 3E), and induced apoptosis in HT29 and WiDr cells (fig. S3B).

In HT29 cells, TAK1 kinase physically associated with p45-IKKα as determined by co-precipitation of endogenous proteins. Of note that this interaction was reduced following RAF or TAK1 inhibition but restored after washing out the inhibitors (Fig. 3F). As expected, we did not detect any TAK1 or IKKα in the IgG control precipitates (fig. S3C). We also performed the reciprocal experiment using a monoclonal antibody (881H3) that preferentially precipitates the p45-IKKα complex in its native form (34). We detected small amounts of P-TAK1 in the p45-IKKα precipitates in both untreated and BRAF-inhibited cells (Fig. 3G) that significantly increased after washing out the BRAF inhibitor.

Together these results indicate that TAK1 displays a dynamic interaction with p45-IKKα that is modulated by BRAF, leading to p45-IKKα activation and function.
Inhibition of endosome acidification induces apoptosis of BRAF mutant cells in a p45-IKKα dependent manner

We previously demonstrated that p45-IKKα activation was linked to the endosomal compartment of CRC cells (34). By sucrose density gradients followed by Western blot analysis from HT29 cells, we found that IKKα (both p85 and p45) and P-p45-IKKα eluted in the fractions corresponding to the early endosomes (EE) together with non-phosphorylated full-length IKKα, NEMO and active TAK1 (Fig. 4A). Using a previously described endosome protection assay (35) that allows the recovery of endosome-associated proteins we confirmed the presence of several IKKα species [the identity of which was confirmed using IKKα-deficient MEFs (fig. S4A)], NEMO, TAK1 and BRAF in the endosomal compartment of different CRC cell lines (fig. S4B). Therefore, we asked whether inhibitors of the endosomal acidification affect p45-IKKα processing or activation, as well as the subcellular distribution of p45-IKKα and its regulatory elements in BRAF or KRAS mutant cells. Inhibition of the endosomal function by chloroquine or bafilomycin A1 reduced the total amounts of P-p45-IKKα in BRAFV600E mutant CRC cells (Fig. 4B) and BRAFV600E-transduced NIH-3T3 (fig. S4C), associated with a decrease in the total and endosomal abundance of P-TAK1 (Fig. 4C and fig. S4D). However, we detected some accumulation of NEMO and TAK1 in the endosomal extracts after bafilomycin A1 treatment (Fig. 4C).

On the other hand, inhibition of endosomal acidification does not result in a consistent reduction of P-ERK1/2 concentration, further suggesting that p45-IKKα and ERK1/2 activation follow divergent pathways downstream of BRAF. In contrast with their effect on p45-IKK and TAK1 activity, both chloroquine and bafilomycin A1 increased IκBα phosphorylation (fig. S4D), which was an indication of canonical NF-κB signaling. Detection of the endosomal/lysosomal component cathepsin B, and/or the early endosomal marker Rab5, and the absence of the cytoskeletal component tubulin, the nuclear protein Sin3A and the mitochondrial marker TOM20 confirmed the purity of the endosomal fractions in the different assays.
Chloroquine or bafilomycin A1 are used for different clinical applications and proposed as anti-cancer agents based on their anti-autophagy activity. However, we speculated that these compounds might distinctively affect CRC cells carrying BRAF mutations because of their effect on p45-IKKα activation. We found that bafilomycin A1 treatment decreased growth of BRAF mutant CRC cells (HT29 and WiDr) at concentrations that were substantially lower than those affecting KRAS mutant (HCT116, LS174T, DLD1 and SW480) and BRAF or KRAS WT cells (LIM1215 and CaCo2) (IC50≈0.5 nM for BRAF mutated cells compared with IC50>2 nM for most KRAS mutants; SW480 IC50=1.5) (Fig. 4, D and E). Comparable effects on cell proliferation and decreased p45-IKKα abundance were observed after knocking down endosomal V-ATPase by specific shRNA in BRAF mutant CRC cells (fig. S4E). In contrast, we did not observe any association between the presence of mutant BRAF and the sensitivity of CRC cells toward the autophagy inhibitors 3-MA (Fig. 4F) and MY1485 (fig. S4F). In agreement with the concept that p45-IKKα mediates the inhibitory effects of bafilomycin A1 on BRAF-mediated transformation, ectopic expression of constitutively active p45-IKKαEE reduced the sensitivity of HT29 and WiDr cells to chloroquine (Fig. 4G and fig. S4G), and recovered the transformation capacity of BRAFV600E in chloroquine-treated NIH-3T3 cells (Fig. 4, H and I). In addition, all tested BRAF and IKK common target genes were significantly repressed following 36 hours of bafilomycin A1 treatment (Fig. 4J).

The proliferative inhibition imposed by endosomal inhibitors in BRAF-mutant cells was likely due to increased apoptosis as determined by PARP cleavage and active caspase 3 detection (Fig. 4K), whereas no significant changes in the cell cycle profile were detected. Accumulation of the autophagy marker LC3B-II following bafilomycin A1 treatment was comparable in the resistant LIM1215 cells and in the BRAF-mutated HT29 and WiDr, further indicating that enhanced bafilomycin A1 sensitivity was independent of autophagy inhibition (Fig. 4K). Conversely, treatment with the autophagy inhibitors 3-MA and MY1485 did not affect P-p45-IKK abundance (fig. S4H). Inhibition of cell proliferation in BRAF mutant cells by chloroquine and bafilomycin A1 was slightly increased in the combined treatment (fig. S4I), but was not improved by the addition of the
canonical NF-κB inhibitor BAY65-5811 (fig. S4J). We also tested whether extracellular acidosis, which is known to occur in the central areas of solid tumors and contributes to tumor progression, affects p45-IKKα abundance or activity in cultured CRC cell lines. By Western blotting analysis, we found that extracellular acidification (pH 5.0 for 24 hours) does not further increase p45-IKKα or P-p45-IKKα abundance in BRAF mutant CRC cells compared with the controls (fig. S4K), whereas some increase was observed in KRAS mutant SW480 cells.

These data indicate that p45-IKKα activation downstream of BRAF takes place in the endosomal compartment and can be blocked by V-ATPase inhibitors, which makes BRAF-mutant cells particularly sensitive to these inhibitors.

**Endosomal acidification inhibitors reduce growth and metastatic capacity of BRAF mutated CRC cells**

We have shown that endosomal acidification inhibitors induce apoptosis of CRC cells carrying a high amount of active p45-IKKα, being the most sensitive those carrying BRAF mutations. To test whether this selectivity was maintained in vivo, we performed tumor xenograft experiments. We first generated subcutaneous tumors from DLD1 and HCT116 (KRAS mutant) or WiDr and HT29 (BRAF mutant) cells. Comparable fragments of the tumors were orthotopically implanted in the wall of the cecum of immune-compromised animals. When tumors were detectable by palpation, animals carrying homogeneous tumors were randomly segregated in three groups of treatment: control, bafilomycin A1, or bafilomycin A1 with irinotecan). After 3 weeks, the animals were euthanized and the tumors were analyzed. We found that bafilomycin A1-treated mice bore tumors that were significantly smaller than the control animals, and that WiDr- and HT29-derived tumors were more sensitive to bafilomycin A1 than were DLD1 and HCT116-derived tumors. Moreover, this effect was significantly enhanced in the combined treatment with the second-line anticancer drug irinotecan (Fig. 5, A to C). In fact, it was also higher than irinotecan treatment alone, as determined in an independent set of experiments (fig. S5A). Bafilomycin A1 barred the capacity of
BRAF mutant-derived orthotopic xenografts to generate intraperitoneal implants in the animals, both in the single treatment and in the combination with irinotecan (Fig. 5D). Moreover, the few intraperitoneal tumors detected in the bafilomycin A1-treated animals were also reduced in size compared with the controls (Fig. 5D). Further indicating that bafilomycin A1 treatment affected p45-IKKα activity in vivo, IHC analysis at the end of the experiment showed detectable amounts of P-IKK in the control tumor samples that were significantly reduced in the remaining tumor mass from the animals treated with bafilomycin A1 or with the combined treatment (Fig. 5E). Nevertheless, by qRT-PCR we did not detect a significant reduction in the expression of the IKKα targets **BCL2L1, BIRC5, CD44** and **CHEK1** in the bafilomycin A1-treated tumors (fig. S5B). These results support previous publications that suggest the potential use of endosome inhibitors in anti-cancer cocktails (36-39), furthermore providing a mechanism-driven treatment for CRC patients carrying BRAF mutant tumors (Fig. 6).

**DISCUSSION**

Tumors carrying mutant KRAS or BRAF are associated with poor prognosis and therapeutic resistance. In addition, KRAS inhibitors are extremely toxic and even though the recently developed BRAF and MEK inhibitors improve survival in melanoma patients, their use is limited due to the acquisition of drug resistance (4, 5, 13). In this context, it is of paramount importance to identify novel druggable targets downstream of the EGFR–KRAS–BRAF pathway, paying special attention to molecules that are currently used in the clinic for specific anti-cancer treatments or other therapeutic applications. Our results indicate that inhibitors of endosomal acidification (such as chloroquine that is used for malaria prevention and treatment, and bafilomycin A1, an antibacterial, antifungal and immunosuppressive agent) prevent phosphorylation of p45-IKKα in CRC cells without affecting NF-κB signaling. Our detection of multiple proteolytic IKKα fragments after proteinase K treatment compared with the only detection of p85 and p45 forms in the sucrose gradient experiments strongly suggested that full length IKKα is likely associated to the outer membrane of the endosomes, although the exact localization of the different elements involved in endosomal p45-IKKα activation remains an open question. In any case, the fact that p45-
IKK\(\alpha\) inhibition by bafilomycin A1 specifically prevents the proliferation of BRAF mutant cancer cells both in culture and in vivo is clinically relevant. Moreover, the inhibition of endosomal acidification potentiates the effect of conventional chemotherapy in an orthotopic xenograft model of human CRC. Our results suggest that activation of p45-IKK\(\alpha\) by BRAF is likely to require the endosomal shuttling of specific elements of the p45-IKK\(\alpha\) complex elements and the association of p45-IKK\(\alpha\) with active TAK1. Consistently, inactivation of TAK1 rapidly abrogated phosphorylation of p45-IKK\(\alpha\), leading to a subsequent reduction of total p45-IKK\(\alpha\) quantity. These results indicate a functional link between IKK activation and TAK1 in BRAF mutated cancer cells, which could also be therapeutically exploited.

Importantly and unexpectedly, we found that KRAS and BRAF mutant CRC cells are essentially different — only KRAS was capable of inducing the NF-\(\kappa\)B pathway. In contrast, BRAF activates an IKK\(\alpha\)-dependent (NF-\(\kappa\)B independent) signature that includes the apoptosis regulators BIRC5 and BCL2L1, the migration related genes CD44, ECT2 or MCAM, and the cell cycle genes AURKA and CDC25A, as well as many other genes that regulate specific tumor features. Treatment of BRAF-mutant CRC cells with bafilomycin A1 reduced the expression of this IKK\(\alpha\)-dependent signature in culture. Unexpectedly, we did not detect a significant reduction of the IKK\(\alpha\)-dependent transcription in tumors treated with bafilomycin A1. This result would suggest (i) that a subset of bafilomycin A1-resistant cells that failed to down-regulate p45-IKK\(\alpha\) activity exists and that these cells contributed to the tumor mass in the treated animals, and/or (ii) that the drug was not homogeneously distributed throughout the tumor during the duration of the treatment, which permits the growth of the untreated/p45-IKK\(\alpha\)-active cell population therein.

Thus, our results provide a mechanistic explanation for the anti-cancer/anti-metastatic effect of p45-IKK\(\alpha\) inhibition and denote the possibility to target specific IKK\(\alpha\) functions independently of NF-\(\kappa\)B, which is essential from a therapeutic point of view (the fact that NF-\(\kappa\)B inhibitors are extremely toxic is explained in the introduction in page 4). Here, we show that endosomal
acidification inhibitors exhibit this effect and can be used in combination therapies to prevent the growth and metastasis of tumors carrying active p45-IKK\(\alpha\) without increasing the toxicity of standard treatments. Our current goal is to identify novel and more effective inhibitors of the endosomal function with higher specificity towards p45-IKK\(\alpha\). Nevertheless, the fact that endosomal acidification inhibitors do not exclusively block p45-IKK\(\alpha\) may also be advantageous for CRC treatment. For instance, they could help limit the activity of other pathways regulated by the endosomes such as Notch and Wnt, which are indeed activated in several types of cancer and regulate cancer-initiating cells.

In summary, we have identified a new signaling cascade downstream of BRAF that involves the endosomal compartment and leads to p45-IKK\(\alpha\) activation. Consistently, inhibition of endosomal activity provides a previously unexpected approach for treating BRAF-mutated cancers that warrants further studies in the clinics.

**MATERIAL AND METHODS**

**Plasmids**

The expression plasmid MT-IKK\(\alpha\) and MT-p45-IKK\(\alpha\)EE were constructed by inserting the PCR-amplified corresponding region, using a HA-IKK\(\alpha\) and HA-IKK\(\alpha\)EE respectively as a template, into the mammalian expression vector pcS2-MT. MT-IKK\(\alpha\) S400W and MT-IKK\(\alpha\)-3M were constructed by using the QuikChange\textsuperscript{TM} Site-Directed Mutagenesis Kit from Stratagene and according to manufacturer instructions. Specific shRNAs against IKK\(\alpha\) (x-2811c1 and x-2866s1c1) and TAK1 (x-2844s1c1 and x-601s1c1) were from Sigma. For the infection experiments, lentiviral expression vectors for WT BRAF and BRAF\textsuperscript{V600E} were a gift from Mario Encinas laboratory. The lentiviral plasmid for KRAS\textsuperscript{G12V} was constructed by inserting the PCR-amplified corresponding region, using HA-KRAS\textsuperscript{G12V} as a template, into the lentiviral vector MSCV-IRES-GFP. Viral particles were produced using the standard techniques.

**Antibodies and Inhibitors**
Antibodies against \( \text{I}\kappa\text{B}\alpha \) (sc-1643), NEMO (sc-8330), Cathepsin B (sc-6493-R), and Lamin B (sc-6216) were purchased from Santa Cruz Biotechnology. Antibodies recognizing P-IKK\( \alpha \)-Ser180/IKK\( \beta \)-Ser181 were from Santa Cruz Biotechnology (sc-23470) or Assay Biotechnology (A0440). Antibody recognizing IKK\( \alpha \) (OP-133) was from Oncogen. Antibodies detecting P-IKK\( \alpha \)-Ser176,180/IKK\( \beta \)-Ser177,181 (2697S), P-\( \text{I}\kappa\text{B}\alpha \)-Ser32,36 (92465), P-ERK1/2 (4370), BRAF (9433), TAK1 (4505), P-TAK1-Ser412 (9339), AKT (9272), P-AKT (9275) and LC3B (2775) were from Cell Signaling. Antibodies against P-Histone H3-Ser10 (06-570), Histone H3 (06-755), pan-Ras(Ab3)(OP40) and Active-\( \beta \)-catenin clone 8E7 (05-665) were from Millipore. Antibodies against \( \alpha \)-tubulin and \( \beta \)-catenin (c2206) were from Sigma. The antibody against HA (12CA5) was purchased from Covance, and the antibody detecting the myc-tag (MT antibody) was the 9E10 hybridoma. The antibody recognizing human p45-IKK\( \alpha \) (Millipore clone 881H3) has been described previously (34). The inhibitory compounds used in the different experiments were: BRAF inhibitor (AZ628 from Selleckchem); MEK inhibitor (UO126 from Cell Signaling); PI3K inhibitor (LY294002) and p38 inhibitor (SB203580) from Calbiochem. Bafilomycin A1, chloroquine (diphosphate salt) and the TAK1 inhibitor (5Z-7-oxozeanol) were from Sigma. All drugs were prepared as specified by the manufacturer and used at the indicated concentrations.

**Cell lines cultures and viral production**

HEK-293, HCT116, SW480, LS174, HT-29, LIM1215, WiDr, NIH-3T3, MCF10A and MEF were grown in DMEM plus 10% FBS. Recombinant lentiviruses were produced by transient transfection of HEK-293T cells according to Tronolab protocols (http://tronolab.epfl.ch/page58122.html). 20 \( \mu \)g of transfer vector, 15 \( \mu \)g of packaging plasmid (psPAX2), and 6\( \mu \)g of envelope plasmid (pMD2.G) were used. After 3 days, the supernatant was ultracentrifuged and the viral pellet resuspended in 100 \( \mu \)l of PBS. 20\( \mu \)l of fresh viral suspension was used per infection.

**Transformation assays**
Mouse embryonic fibroblasts (MEFs) from IKK\(\alpha\) WT or KO animals were seeded in 6-well plates at a density of 5 \(\times\) 10\(^4\) cells per well, and then transduced with the indicated plasmids. Cells were maintained in culture by changing the medium every other day. At the indicated endpoints (when foci turned apparent) plates were washed with PBS, fixed with 4% paraformaldehyde and stained with methylene blue. Two independent investigators determined the number of foci obtained in each condition.

**Dose-response assays in CRC cells**

Subconfluent CRC cell cultures were obtained by seeding 0.5 \(\times\) 10\(^4\) cells/well in 24-well plates. The day after, cells were treated with the different compounds (or vehicle) at the indicated concentrations. 72 hours after starting the treatment, cells were washed with PBS, fixed with 4% paraformaldehyde, stained with crystal violet for 20 minutes at room temperature, and then washed with water until the excess of dye was completely removed. Plates were then scanned and quantified using the ImageJ software. Results were then represented as number of cells relative to the vehicle-treated cultures from at least 3 independent experiments. The dose-response curves and the IC50 were calculated using the non-linear regression curve and the dose-response equation from the Prism software, respectively.

**In vivo assay for standard chemotherapy and endosomal acidification agents**

For CRC cell line xenograft experiments, 2\(\times\)10\(^4\) DLD1 or WiDr cells 2\(\times\)10\(^4\) were resuspended in matrigel and injected subcutaneously in nude mice. When palpable intraabdominal masses were detected, mice were randomized into the different treatment groups: (i) placebo; (ii) bafilomycin A1 (1mg/kg); (iii) irinotecan (30 mg/kg)+bafilomycin A1. In all experiments, irinotecan was diluted in saline, whereas bafilomycin A1 was diluted in DMSO and administered by intraperitoneal injection (i.p.) at a final 10% DMSO concentration. To minimize side effects in the combined treatment groups, irinotecan was administered at days 2, 7, 13 and 18 by tail vein injection (i.v.) and bafilomycin A1 was administered daily, but always 1 hour after irinotecan administration (when applicable). Animals were killed at day 21 after starting the treatment, and all
visible tumors were measured and photographed. Animals were kept under pathogen-free conditions and all experimental procedures approved by the Ethical Committee at Institute of Biomedical Research of Bellvitge.

**Western blot analysis and immunoprecipitation assays**

Cells were lysed 30 min at 4°C in 300 µl PBS plus 0.5% Triton X-100, 1 mM EDTA, 100 mM Na-orthovanadate, 0.25 mM PMSF and complete protease inhibitor cocktail (Roche). For immunoprecipitation, supernatants were pre-cleared 2 hours with 1% of BSA, 1 µg IgGs and 50 µL of sepharose protein A beads. Precleared lysates were incubated overnight with 3 µg of indicated antibodies. Antibody-Protein complexes were captured with 30 µL SPA beads for 2 hours. After washing, precipitates were analyzed by Western blotting using standard SDS/polyacrilamide gel electrophoresis techniques. In brief, protein samples were boiled in Laemli buffer, run in 9% polyacrilamide gels and transferred to PVDF membranes. Membranes were incubated overnight at 4°C with the appropriate primary antibodies. After washing, membranes were incubated with specific secondary horseradish peroxidase (HRP)-linked antibodies from DAKO and visualized using the enhanced chemiluminescence (ECL) reagent from Amersham.

**Cell fractionation**

Nuclei were isolated in 0.1% NP40 in PBS for 5 min on ice, followed by centrifugation at 1,900 rpm and then lysed in 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 5 mM EGTA, 5 mM EDTA, 20 mM NaF and complete protease inhibitor cocktail (Roche). Supernatants were recovered as the cytoplasmic fraction. For cytoplasm/nuclear/chromatin separations, cells were lysed in 10 mM HEPES, 1.5 mM MgCl2, 10 mM KCl, 0.05% NP40 at pH 7.9, 10 min on ice and centrifuged at 13,000 rpm. Supernatants were recovered as the cytoplasmic fraction and the pellets lysed in 5 mM HEPES, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM DTT and 26% glycerol, and sonicated 5 min three times to recover the soluble nuclear fractions. Remaining pellet included the chromatin fraction. Lysates were run in SDS/PAGE and transferred to immobilon-P transfer membranes (Millipore) for WB analysis.
Statistical analysis
Categorical data were compared by use of Fisher's exact test. A nonparametric analysis of variance was used for the analysis of the ordinal expression of the phosphorylated IKK data by applying a rank transformation on the dependent variable. The analysis was performed using SAS version 9.1.3 software (SAS Institute Inc.), and the level of significance was established at 0.05 (two sided).

TMA preparation and Immunohistochemistry
Formalin-fixed, paraffin-embedded tissue blocks of colorectal tumors were retrieved from the archives of the Bank of Tumors of the Hospital del Mar (MarBiobanc). Multiple areas of invasive carcinoma, adenomatous lesions from the same surgical sample, and normal adjacent mucosa were identified on corresponding hematoxylin-eosin-stained slides. The tissue blocks were transferred to a recipient “master” block using a Tissue Microarrayer. Each core was 0.6 mm wide spaced 0.7–0.8 mm apart. Paraffin sections of 4 µm were hydrated, permeabilized and antigen retrieval was achieved by incubating with 10 mM citrate buffer overnight at 80ºC. Primary antibodies were incubated overnight and then developed with the DAB System from DAKO.

Flow cytometry analysis and Cell Sorting
Apoptosis was determined using the Annexin V binding kit (from Pharmingen) following the manufacturer instructions. For cell cycle profile determination, cells were fixed in ethanol 2 hours on ice and then stained with Propidium Iodide overnight at 4ºC. Cells were analyzed by flow cytometry on a FACScalibur or LSRII (Becton Dickinson). All data were analyzed with the FlowJo software (Tree Star, Inc).

Endosome protection assays
To identify proteins that reside in the endosomal compartment, cells were pelleted and resuspended in a buffer containing 100 mM potassium phosphate at pH 6.7; 5 mM MgCl₂; 250 mM sucrose, and 6.5 µg/ml digitonin and incubated for 5 min at room temperature followed by 30 min on ice. After treatment, the digitonin solution was removed by centrifugation for 5 min at 13,000 rpm and the
pellets resuspended in the same buffer without digitonin. Then, samples were divided and processed in 3 different ways: maintained untreated (input control), incubated with 1 µg/ml proteinase K (Invitrogen) to eliminate all proteins that were excluded from the endosomal compartment, or with proteinase K plus 0.1% Triton X-100 to solubilize the endosomes and hydrolyze all proteins (negative control). After incubation for 10 min at room temperature, samples were boiled in loading buffer to stop the reaction, and directly used for WB analysis.

Sucrose gradients

Purification of early endosomes by sucrose gradients was performed as previously described (40). In brief, cells were washed twice with PBS and collected in homogenization buffer (250 mM sucrose, 3 mM Imidazole, pH 7.4), centrifuged and resuspended in homogenization buffer. Cells were then disrupted mechanically by using a 22-gauge needle. Nuclei were discarded by centrifugation for 15 min at 4°C at 1500 g., and post-nuclear supernatants (PNS) containing the endosomal fraction were adjusted to 40.2% sucrose (w/v) and loaded at the bottom of a SW40 ultra-centrifugation tube (Beckman Ultraclear). Consecutively, 35% sucrose, 25% sucrose and homogenization buffer were added on top of the PNS to create a gradient. After centrifugation at 35,000 g for 90 minutes at 4°C in a swing out Beckman SW40 rotor, 800 µl fractions were collected from top to bottom of the tubes followed by WB analysis.

Microarray assay

We compared the transcriptomes of WiDr and HT29 cells left untreated, treated for 3 hours with the BRAF inhibitor or transduced with sh-RNA against IKKα (all experiments were done in triplicates) using a whole human genome oligonucleotide microarray from Agilent (G4112A). Microarray data was processed with R Bioconductor statistical framework, URL http://www.R-project.org) and the Limma package was used to determine the most differentially expressed genes across conditions. Values represented for each probe are the standard deviations after mean-centering the values in each row.

SUPPLEMENTARY MATERIAL
Figure S1. The phosphorylation of p45-IKKα is induced by BRAF and is independent of NF-κB.

Figure S2. BRAF-activated p45-IKK corresponds to IKKα.

Figure S3. The phosphorylation of p45-IKKα is regulated by TAK1.

Figure S4. Active p45-IKKα is localized to the endosomal compartment.

Figure S5. Effects of bafilomycin A1 and irinotecan on xenografts.

REFERENCES


Acknowledgements: We thank Jessica González, Alba Dalmases, Ester Moragón, Ana García-Melero and Carlos Enrich for experimental and technical support, and Erika López-Aribillaga for critical reading of the manuscript and scientific support. Funding: PM is a recipient of a FPU fellowship (AP2009-2892) and LE is an investigator at the Carlos III program. This work was further supported by Instituto de Salud Carlos III Grant PI041890, AGAUR (SGR23) and RTICCS/FEDER (RD12/0036/0054 and RD09/0076/00036), and "Xarxa de Bancs de tumors" sponsored by the Pla Director d'Oncologia de Catalunya (XBTC). Author contributions: PM, CC, AV, MI, BB and MM-I performed the experiments and analyzed data. CM, RS and MP analyzed the data and designed the experiments. AB and LE conceived the study, analyzed data and wrote the manuscript. Competing interest: The authors declare no competing interests. Data and materials availability: Microarray data have been deposited to Gene Expression Omnibus ( GEO), accession number GSE57979.
FIGURE LEGENDS

Figure 1. BRAF controls p45-IKKα but not NF-κB activation in CRC cells
(A) Immunohistochemistry for phosphorylated (P-)IKK or P-ERK1/2 in serial sections of representative samples from a tissue microarray containing 184 human CRC tumors. (B) Samples were classified based on nuclear intensity of P-IKKα/β and P-ERK1/2: negative (-), positive (+), or strongly positive (++). Fisher’s exact test determined the significance of the correlation. Scale bar, 50µM. (C) Table showing the correlation between nuclear P-IKKα/β and BRAF status. (D) Representative of 13 serrated tumors stained for P-IKK. Scale bar, 50µM. (E) Classification of samples in (D) according to histology and P-IKK. (F) Cells were treated as indicated and analyzed by Western blot (WB). The ratio of P-p45-IKK/p45-IKKα was quantified from 3 independent experiments. (G) NF-κB activity measured by electrophoretic mobility shift assay (EMSA). TNFα-treated LIM1215 cells are the positive control. (H) WB analysis of subcellular fractions from HT29 cells treated with vehicle or RAF inhibitor (inh). h, hours. Where indicated, inhibitor was washed out. (I) Biological process enrichment analysis of genes repressed after RAF inhibition. (J) Venn diagram showing the overlap between genes repressed after RAF inhibition and IKKα knockdown. (K) Heatmap of selected genes repressed by either treatment. (L) Cellular density of the indicated CRC cells treated with increasing BAY65-5811 concentration. Dose-response data are means ±S.D. of 3 experiments. All blots are representative of at least 3 experiments.

Figure 2. Oncogenic BRAF promotes p45-IKKα activation that is required for transformation
(A) WB analysis of NIH-3T3 cells infected with the control or the BRAF^{V600E} construct, and then treated with RAF or MEK inhibitors for 30 min at the indicated concentrations. (B) NIH-3T3 cells were infected with the control or the KRAS^{G12V} vector, and 2 days after infection, treated with RAF or PI3K inhibitors at the indicated concentrations for 30 min. Protein extracts were analyzed by WB using the indicated antibodies. (C) NIH-3T3 cells were infected with control or BRAF^{V600E} vector and 2 days after the infection, supernatants were collected and
added to non-infected NIH-3T3 cell cultures as indicated. This procedure was repeated for 3 consecutive days, and then cells were analyzed by WB using the indicated antibodies.  

(D and E) Foci formation assay in IKKα WT or KO MEFs transduced with the BRAFV600E vector alone (D) or together with the indicated IKKα constructs (E). Representative images and the average number of foci obtained from 5 independent experiments are shown. Data are means ± S.D.; *p<0.05, ***p<0.001, by unpaired two-tailed t-test. Blots are representative of at least 3 experiments.

Figure 3. Activation of p45-IKKα by BRAF requires TAK1

(A and B) WB analysis of the indicated cells that were (A) transduced with shRNA against TAK1 or the scrambled control, or (B) treated for 2 hours (hr) with vehicle or the TAK1 inhibitor (10 µM). The ratio of P-p45-IKKα/p45-IKKα was quantified.  

(C) Cells untreated or treated several minutes (min) with vehicle or TAK1 inhibitor were analyzed by WB with the indicated antibodies.  

(D) Cells were treated for 2 hours with BRAF inhibitor (2 µM), which was then washed out (Wo) and replaced by fresh medium without or with TAK1 inhibitor. Extracts were obtained at different time points and analyzed by WB.  

(E) Foci formation assays from NIH-3T3 cells 2 weeks after transduction with the indicated constructs.  

(F) HT29 cells were either untreated (0) or treated with the indicated inhibitor (Inh) for 2 hours (2 h) or 2 hours then washed out (Wo). Samples were precipitated with an antibody to TAK1 and analyzed by WB.  

(G) Extracts from cells treated as indicated were precipitated with the antibody to p45-IKKα and analyzed by WB. Blots are representative of X experiments. Data in (A, B, and E) are means ± S.D.; ***p<0.001, by a unpaired two-tailed t-test.

Figure 4. Endosomal acidification inhibitors prevent cancer cell growth and BRAF-mediated transformation in a p45-IKKα-dependent manner

(A) WB analysis of HT29 fractions isolated from sucrose density gradients. LE, late endosomes; EE, early endosomes; PM, plasma membrane.  

(B and C) WB of HT29 cells treated with vehicle (Vehic.), cloroquine (CHL) or bafilomycin A1 (BAF) (B), or after isolation of the endosomal fraction (C, lane 2). In (C), lanes 1 and 3 are positive and negative controls, respectively. In (A) and (C), endosomal
fraction quality was confirmed by presence of cathepsin B or Rab5 and absence of tubulin, TOM20 and Sin3A. (D and E) Dose-response analysis of relative proliferation (D) and IC50 (E) in cells treated as indicated. (F and G) Dose-response curves as in (D) from cells treated with the autophagy inhibitor 3-MA (F) and either control or p45-IKKalphaE-transduced WiDr cells treated as indicated (G). (H and I) The number of foci present at day 9 in NIH-3T3 cell cultures transduced with BRAFV600E (H) or BRAFV600E plus p45-IKKalphaE (I) and treated as indicated. (J) qRT-PCR analysis of the indicated genes. Data are means ± S.D. relative to the expression of GAPDH and 28S RNA. (K) WB analysis of cells treated as indicated. Blots are representative of 3 experiments. All data are means ± S.D. from 3 independent experiments. *p<0.05, **p<0.01, ***p<0.001, by a unpaired two-tailed t-test.

Figure 5. Endosomal acidification inhibitors reduce growth of BRAF mutated cells and abrogate metastatic capacity of CRC in vivo
(A) Orthotopic xenografts were generated from DLD1, HCT116 (both KRAS mutant), HT29, or WiDr (both BRAF mutant) cells and tested for their sensitivity to the endosomal acidification inhibitor bafilomycin A1 (BAF), as a single agent or in combination with irinotecan (IRI). Two representative tumors from each condition are shown in the picture. (B and C) Tumor size (B) and weight (C) was determined at 3 weeks after starting the treatment. (D) Average weight of the intra-peritoneal (IP) implants detected in animals carrying HT29-derived tumor xenografts. (E) IHC analysis of control and treated tumors using the specific antibody to active P-IKK. Data are means ± S.D. of tumors obtained from 5 animals included in each experimental group. *p<0.05; **p<0.01; ***p<0.001; n.s., no significance; by a unpaired two-tailed t-test.

Figure 6. Model for p45-IKKalpha activation and inhibition downstream of BRAF
Our data suggest a mechanism by which mutant BRAF specifically induces activation of p45-IKKalpha in the endosomal compartment of tumor cells, most likely through TAK1. Inhibition of endosomal V-ATPase activity (and endosomal acidification) by bafilomycin A1 or chloroquine prevents p45-IKKalpha activation.
leading to increased cell death. In contrast, KRAS mutant cells are resistant to endosomal acidification inhibition, likely due to their capacity to induce NF-κB.
Figure 1

A. Sample #1 and Sample #2 with P-IKK and P-ERK1/2 staining.

B. Graph showing P-ERK1/2 positive and negative with n=184.

C. Table showing nuclear P-IKK with *** and ≥++.

D. Immunohistochemistry of BRAF WT and BRAF V600E.

E. Bar graph showing % tumor subtype with conventional, serrated, and conventional tumors.

F. Western Blot (WB) analysis of P-IKK, IKKα, p100/p52, IκBα, P-ERK, ERK1/2, P-AKT, AKT, and Tubulin in HT29 and WiDr.

G. Table showing log(p-value) for conventional polyps, serrated tumors, and serrated polyps.

H. Soluble extracts with P-IKK, P-ERK1/2, P-S10H3, P-SMRT, and nuclear extracts.

I. Log [BAY65], -log(p-value) with cell cycle, DNA repair, cellular response to stress, and regulation of MAPK activity.

J. Venn diagram showing BRAF inh, sh-IKKα, CaCo2, LIM1215, DLD1, LS174T, HCT116, SW480, LIM1215, and WiDr.

K. Heatmap showing sh-IKKα, sh-control, BRAF inh, and Vehicle for apoptosis, migration, metastasis, and proliferation.

L. Scatter plot showing cells relative to control with Log [BAY65], µM.
Figure 3

A

<table>
<thead>
<tr>
<th></th>
<th>LIM1215</th>
<th>WiDr</th>
<th>HT29</th>
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<tr>
<td>Control sh</td>
<td>+</td>
<td>-</td>
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<tr>
<td>shTAK1 #1</td>
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<td>shTAK1 #2</td>
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WB:
- P-IKK
- p85
- IkKα
- p45
- TAK1
- P-ERK
- ERK1/2
- Act. β-catenin
- β-catenin
- NEMO
- β-tubulin

B

E

2 hr BRAF Inh

Control | +TAK1 Inh

WB:
- P-IKK
- p85
- IkKα
- p45
- ERK1/2
- β-tubulin

H

IP:
- p45-IKKa
- IgH
- BRAF
- TAK1
- NEMO
- IkKα

F

G

BRAF

Number of foci

0 15 30 45 60

0 15 30 45 60

0 15 30 45 60

0 15 30 45 60

0 15 30 45 60

0 15 30 45 60
Figure 5

A

DLD1 (KRAS$^{G13D}$)  WiDr (BRAF$^{V600E}$)

Control

BAF

BAF+IRI

B

Tumor size (mm$^3$)

DLD1

WiDr

Tumor size (mm$^3$)

Control  BAF  BAF+IRI

C

Tumor weight (g)

HCT116 (KRAS)

HT29 (BRAF)

n.s.  ***

D

IP implants weight (g)

HT29 (BRAF)

Control  BAF  BAF+IRI

p=0.08

E

P-IKK

Control

BAF

BAF+IRI