IDENTIFICATION AND CHARACTERIZATION OF AN ONCOGENIC FORM OF IKKα IN COLORECTAL CANCER

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Per vosaltres, que sempre heu confiat en mi

"Que tot està per fer i tot és possible"

Míquel Martí i Pol

"I a vegades ens en sortim,
i a vegades ens en sortim.
I a vegades una tonteria de sobte ens indica que ens en sortim.
I a vegades se'ns baixa la verge i de sobte ens revela que ens en sortim.
I a vegades contra tot pronòstic una gran bestiesa
capgira la traca i amb lògic tot fent evident que per un moment
ens en sortim"

Manel - Captatio benevolentiae

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ABSTRACT

Nuclear IKKα regulate gene transcription and it has been linked to cancer progression and metastasis, although the mechanistic connection remains poorly understood. We have found that nucleus of tumor cells contains an active IKKα isoform of 45kD (p45-IKKα). Active nuclear p45-IKKα forms a complex with non-active IKKα and NEMO that mediates phosphorylation of SMRT and Histone H3. Generation of p45-IKKα is required to prevent apoptosis of CRC cells and to sustain tumor growth. We have observed that KRAS through BRAF induces TAK1-mediated activation of p45-IKKα, with minor impact on NF-κB. Mechanistically, BRAF promotes NEMO ubiquitination and association of active TAK1 to the p45-IKKα complex. We also demonstrate that p45-IKKα is required for KRAS/BRAF-mediated cell transformation and inhibitors of the endosomal function abolished TAK1 and p45-IKKα activities. Using a human orthotopic xenograft model we demonstrate the efficacy of these drugs in preventing the metastatic capacity of primary CRC cells from a patient with acquired resistance to standard chemotherapy.

RESUM

L'IKKα nuclear regula la transcripció gènica i ha estat relacionada amb la progressió del càncer i la metàstasis, tot i que la connexió mecanística no s'entén massa bé. Hem observat que les cèl·lules tumorals tenen una forma d'IKKα activada (p45-IKKα) al nucli. Aquesta p45-IKKα activada i nuclear forma un complex amb IKKα no activada i NEMO que media la fosforilació d'SMRT i d'histona H3. La formació de p45-IKKα és necessària per tal de prevenir l'apoptosis de les cèl·lules de càncer colorectal i per tal de mantenir el creixement tumoral. També hem observat que KRAS a través de BRAF indueix l'activació de p45-IKKα mediada per TAK1, amb un mínim efecte sobre NF-κB. Mecanísticament, BRAF promou la ubiquitinació de NEMO i l'associació de la forma activada de TAK1 al complex de p45-IKKα. També hem desmostrat que p45-IKKα és necessària per la transformació cel·lular mediada per KRAS/BRAF i que inhibidors de la funció endosomal suprimeixen l'activitat de TAK1 i de p45-IKKα. Treient partit d'un model ortotòpic xenògraft humà hem demostrat l'eficàcia d'aquestes drogues en prevenir la capacitat metastàtica de cèl·lules primàries de càncer colorectal d'un pacient amb resistència adquirida al tractament quimioteràpic estàndard.

PROLOGUE

The work presented in this thesis was done from July 2008 to date under the supervision of Dr. Lluís Espinosa and Dr. Anna Bigas, at Institut Hospital del Mar d'Investigacions Mèdiques IMIM.

Colorectal cancer (CRC) is the fourth most common cause of death by cancer. Thus, understanding the molecular mechanism leading to CRC generation and progression is of crucial importance to develop new and more specific drugs for treating patients. NF-κB activity has long been related to several types of cancer including CRC, but work from different groups has demonstrated that NF-κB inhibitors are very toxic. During the last 5 years I have had the opportunity to work in the characterization of an oncogenic form of IKKα in colorectal cancer, which was first identified by a former member of the lab during her thesis. My work has been focused on the characterization of this form; the mechanism involved in its generation, its biochemical properties, and its role in colorectal cancer. In addition, we have found that specific inhibition of oncogenic IKKα shows low toxicity against normal cells but synergizes with conventional anti-cancer cocktails in preventing tumor cell growth and metastasis.

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INTRODUCTION

INTRODUCTION

1. Colorectal Cancer (CRC)

Normal intestinal epithelium is capable of continuously self-renew, while maintaining a fine balance between proliferation, differentiation, cell migration and cell death. When one of these processes is altered intestinal tumorigenesis can be initiated. Colorectal cancer is characterized by uncontrolled cell growth in the large intestine, mainly in the colon or in the rectum. Symptoms of this type of cancer can include the bleeding of the rectum and anemia which can be sometimes associated with changes in bowel habits and weight loss.

Incidence and mortality

Colorectal cancer is the third most common cancer in men accounting for 663.000 cases that represent a 10% of the total worldwide. In women it is the second most common type of cancer accounting for 571.000 cases that represent 9.4% of the total worldwide (Colorectal Cancer Incidence, Mortality and Prevalence Worldwide in 2008. GLOBOCAN 2008). The incidence across the world varies depending on the geographical location. This is due to differences in diet, particularly the consumption of red and processed meat, fiber and alcohol, as well as bodyweight and physical activity[1–6]. The highest mortality rates are estimated in Central and Eastern Europe (20.1 per 100,000 for male, 12.2 per 100,000 for female), and the lowest in Middle Africa (3.5 and 2.7 respectively).

Colorectal cancer development and progression

CRC is initiated in the colon (or in the rectum) as an epithelial hyperplasia that becomes increasingly dysplastic resulting in aberrant crypt foci[7]. It is

now firmly established that most, if not all, colon carcinomas develop from a preceding noninvasive adenoma[8], which is defined by an expansion of the proliferating compartment and a lack of differentiation of epithelial cells that migrate toward the luminal surface of the crypts. Thus, the strict correlation between cellular phenotype and position along the vertical axis is disrupted in the adenoma as indicated by the aberrant expression of a variety of markers of gastric epithelial differentiation[9–13].

The earliest recognizable adenomatous lesion is the single-crypt adenoma, or dysplastic aberrant crypt focus[14–17]. A single-crypt adenoma contain all the different epithelial cell lineages that can be found in the normal epithelium[15]. Single-crypt adenomas initially expand through crypt fission, a process of crypt multiplication that occurs through budding and subsequent elongation to form two separate crypts[18,19]. Crypt fission most likely results from a symmetrical stem-cell division in which both daughter cells retain their stem-cell characteristics and each stem cell forms its own separated crypt. After the initial growth by crypt fission, the developing tumor later expands and occupies the space of the adjacent normal crypts.

Aberrant crypt foci expand over time to form macroscopically visible adenomatous polyps. During tumor progression, mutant cells lose their differentiation capacity, and acquire the ability to invade the basement membrane into the lamina propria (carcinoma in situ). Overtly invasive carcinomas often represent the first clinical presentation of colorectal tumors. Finally these carcinomas are able to colonize other organs both locally or at distance through the blood circulation (metastatic carcinoma)(Figure 1).

Crypts in the adult normal mucosa rarely show fission[20] but its frequency increases during intestinal growth and in situations of mucosal damage and repair[21,22].
Studies of the clonal composition of human CRCs have demonstrated that tumors tend to have a monoclonal composition[23], in contrast with the normal colonic crypts that are polyclonal.

Two different models propose the origin and growth of dysplastic aberrant crypt foci. Vogelstein and colleagues suggested a top-down morphogenesis model in which mutant cells at the surface epithelium of the colon spread laterally and downward to form new crypts[24].
This view was challenged with an alternative model proposing that adenomas grow initially in a bottom-up pattern[15].

Genetics of sporadic colon cancer

From a molecular-genetic point of view, colorectal cancer is one of the best-understood solid malignancies, which has been facilitated by the fact that different stages of the disease can coexist in the same patient. This has led to define the sequence of mutational events that characterize the transition from normal colon epithelium to premalignant adenoma and then invasive adenocarcinoma[25]. Several studies support the conclusion that tumor progression in the intestine (and most likely in other tissues) is propelled by the selection of specific genetic and epigenetic alterations that accumulate in strict sequence. Indeed, while mutation events are stochastic, the order in which they accumulate is non-random, supporting the argument that only certain mutations confer a selective advantage at a given stage of a tumor's natural history (Figure 1).

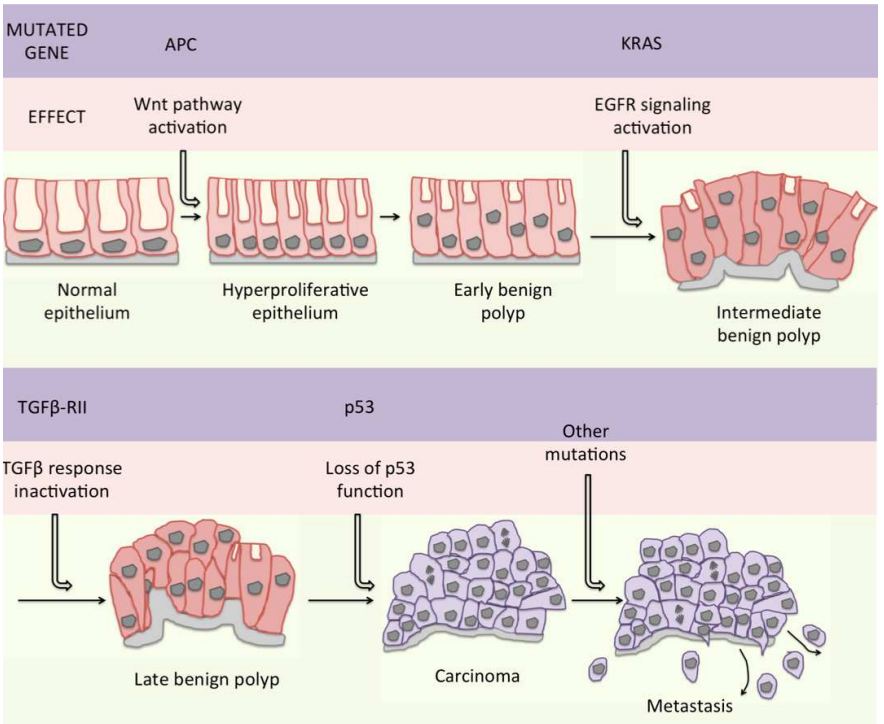

Figure 1. Genetic changes associated with colorectal tumorigenesis. Adapted from Fearon and Vogelstein, 1990.

The sequence of genetic mutations

The model proposed by Fearon and Vogelstein[25] proposes that colorectal tumors are originated from mutational activation of different oncogenes combined with the inactivation of tumor-suppressor genes (Figure 1).

Activation of the **Wnt** signaling pathway is an early event required for colon adenoma formation. Loss of function of the adenomatous polyposis coli (APC) gene, a protein that functions as a suppressor of Wnt signaling by catalyzing the degradation of β-catenin[26,27] is found in up to 85% of all cases of CRC. Alternatively, some colon cancers activate Wnt signaling through mutations in the β-catenin gene. Genes induced by Wnt activation include *c-myc*, *cyclin D1*, and *PPAR-δ*[26]. On the other

hand, most of the cellular β-catenin protein is found associated with the cell-adhesion transmembrane glycoprotein E-cadherin in the adherens junctions. E-cadherin is downregulated in a significant number of patients leading to altered β-catenin regulation[28,29].

RAS, mainly the **KRAS** isoform, a GTPase that controls cell proliferation, is mutated in about 40-50% of cases of CRC[30] leading to aberrant activation of the MAPK pathway.

Mutations inactivating the **TGF-β** receptor II (TGFβ-RII) gene arise in 30% of colon cancers and are temporarily coincident with the further progression of colon adenomas to colon carcinomas[31,32]. SMAD4 or SMAD2 transcription factors, that are phosphorylated by the TGF-β receptor complex, can also be mutated[31]. Unexpectedly, it was recently demonstrated that high levels of TGF-β in the blood of CRC patients is a bad prognosis factor. The reason is that the activity of TGF-β on stromal cells increases the efficiency of organ colonization by CRC cells[33].

TP53 mutations tend to be late events and increase the resistance of cancer cells to apoptosis. Mutational inactivation of p53 is also coincident with progression of colon adenomas to carcinomas. Greater than 50% of colon cancers bear p53 mutations, and these mutations, first discovered in human colon cancers, are now recognized as the most common genetic event in human cancer.

Loss of **MUC2** expression, a member of the mucin family, is also a frequent event in human colon adenomas and carcinomas[34]. In this same sense, it has been recently demonstrated that the mucus barrier organized around MUC2 constrains the immunogenicity of gut antigens by delivering tolerogenic signals[35].

Finally, overexpression of the **PRL-3** tyrosine phosphatase has been observed in 25% of all metastatic colon cancer cases, likely as a result of

gene amplification suggesting that PRL-3 is a direct genetic target contributing to metastatic progression[36].

A different group of sporadic tumors include those with genetic instability or MicroSatellite Instability (MSI)[37], (13% of sporadic colon cancers)[27,31] which is commonly associated with mismatch repair gene inactivation. The most common cause of these sporadic MSI tumors is epigenetic methylation affecting both *hMLH1* alleles[31]. Such epigenetic modification can serve as a tumor marker and has been detected preoperatively in blood from MSI colon cancer patients[38]. Not only promoter methylation but also mutations in different mismatch repair genes have been detected in CRCs[37,39,40].

Hereditary colon cancer

In agreement with that found in sporadic CRC, germline mutations in the *Apc* gene cause the inherited Familial Adenomatous Polyposis (**FAP**) syndrome, in which hundreds to thousands of colonic adenomas develop during the third to fourth decade of life, and the lifetime risk of colon cancer approaches 100%[26,27]. *Apc* is a classic tumor suppressor gene, with FAP tumors showing inactivation of the wild-type germline allele, most often by deletion[26,27]. The majority of FAP associated *Apc* mutations are nonsense mutations that truncate the APC protein amino terminal to the β-catenin interacting domain, allowing β-catenin accumulation.

Germline mutations in components of the DNA Mismatch Repair (MMR) complex are the genetic basis of hereditary nonpolyposis colon cancer (**HNPCC**)[27,31,39–41]. Carriers of these autosomal dominant mutations have an 80% lifetime risk of colon cancer, most often localized to the

ascending colon, and an increased risk of gastric and endometrial cancers. HNPCC tumors show somatic inactivation of the second germline MMR allele. MMR inactivation induces a nearly 1000-fold increased spontaneous gene mutation rates[42]. This "mutator" phenotype reduces the time for colon cancer development to less than 36 months, creating mutational hotspots in the coding regions of some tumor suppressor genes. The prototypical example is the biallelic mutational inactivation of the type II TGF-β receptor[31,32].

2. NF-κB pathway

More than 25 years ago, NF-κB was discovered in the laboratory of David Baltimore[43]. The name account for Nuclear Factor kappa-light-chain-enhancer of activated B cells. It was discovered biochemically as a DNA-binding activity in activated B cells with affinity for the transcriptional enhancer of the immunoglobulin κ light-chain gene, and initially thought to be an important regulator of antibody production.

Subsequent studies revealed that B cells derived from a variety of NF-κB knockout mice are able to produce antibodies containing κ light chain. In any case the name NF-κB remains, however it is somehow misleading since NF-κB is neither a critical regulator of the κ light-chain gene, nor B-cell specific. Moreover, it is not a truly nuclear factor[44] since it only localizes in the nucleus after specific stimulation, which leads to NF-κB-mediated gene transcription[45].

Studies from different laboratories have contributed to understand the complexity of the NF-κB pathway that can regulate hundreds of genes in a context dependent manner. Control of NF-κB involves specific post-

translational modifications of several elements of the pathway, and it has critical roles in many physiological and pathological processes such as cancer[46].

Regulators of the NF-κB pathway

NF-κB transcription factors

Five member of the transcription factor NF-κB can be found in mammals: RelA (**p65**), **RelB** and **c-Rel**, and the precursor proteins NF-κB1 (**p105**) and NF-κB2 (**p100**), which are processed into **p50** and **p52**, respectively. These proteins are characterized by a highly conserved Rel homology domain (RHD), which mediates dimerization, DNA binding, and interaction with IκB (for Inhibitor of κB) proteins[47,48].

RelA, c-Rel, and RelB are synthesized in their mature form and are sequestered in the cytoplasm by IκB. All IκB proteins contain six or seven ankyrin repeats that mediate binding to the RHD of NF-κB factors thus masking their nuclear localization sequences (NLSs).

On the other hand, NF-κB1 (p50) and NF-κB2 (p52) are synthesized as large precursors, called p105 and p100 respectively, containing an N-terminal RHD and a C-terminal IκB-like region with multiple ankyrin repeats. Proteolysis of the C-terminus is required to obtain the mature p50 and p52 subunits, consisting of the N-terminal RHD[49–51] but lacking the inhibitory ankyrin domains.

p105 is constitutively processed to generate p50, in a process that is likely ubiquitin-independent[52]. Instead, p100 processing is tightly regulated through the activation of specific TNF receptor (TNFR) family members, such as CD40-ligand, which will be later discussed (page 45).

While RelA, c-Rel, and RelB contain transactivation domains, known to be required for gene transcription, p50 and p52 do not contain transactivation domains and serve primarily as dimerization and DNA-binding partners[48].

Functional characterization of NF-κB transcription factors

Targeted disruption of different NF-κB family genes in mice[53,54] has revealed a certain degree of redundancy, as expected from their ability to form homo- and heterodimers to recognize a common DNA sequence motif, the κB consensus[55].

Mice deficient for **p105** and **p50** develop normally without obvious histopathological defects[56]. However, they exhibit multiple functional defects in the immune system, such as poor splenic B cell proliferation after activation with lipopolysaccharide (LPS) and soluble CD40.

Mice deficient for **p100** and **p52** also develop normally, with a defect in the splenic and lymph node architecture[57,58].

Mice lacking **c-Rel** have a normal development, although it is essential for a variety of functions in hematopoietic cells, such as activation of mature lymphocytes and macrophages[59,60].

RelB deficiency led to multiple pathological lesions and defects in acquired and innate immunity[61,62].

However, **p65** deficiency led to embryonic death due to massive TNFα-induced apoptosis in the liver, indicating an essential, non-redundant anti-apoptotic role for p65 in response to TNFα stimulation[63].

Inhibitor of κB proteins (IκBs)

Activation of all NF-κB dimers except p52:RelB is controlled by IκB proteins, which prevent nuclear entry and DNA binding by masking the

NLS near the C terminus of the RHD. IκB family comprises four main members: **IκBα**, **IκBβ**, **IκBε**, and **IκBNS**[48,64,65].

A critical regulatory event in NF-κB signaling is the site-specific phosphorylation of IκB (α, β, and ε) by the IKK complex, which leads to their polyubiquitination and proteasomal degradation. IκBs are in turn regulated through NF-κB-dependent de novo synthesis, leading to a tight feedback regulation that is crucial for a proper termination of the NF-κB response[66–68].

Stimulus-induced degradation of IκB proteins starts through the phosphorylation by the IκB kinase (IKK) complex. Phosphorylation of IκB occurs in two conserved serine (S) residues located in its N terminal region, S32 and S36, leading to recognition by the β-TrCP F-box-containing component of a Skp1-Cullin-F-box (SCF)-type E3 ubiquitin-protein ligase complex, called **$SCF^{\beta TrCP}$**, resulting in polyubiquitination and degradation of phosphorylated IκBα by the 26S proteasome[69]. This liberates bound NF-κB dimers that now are able to translocate to the nucleus and activate transcription of target genes.

IκBβ and IκBε are also phosphorylated in its N-terminal part, but the kinetics of the phosphorylation and degradation are much slower in comparison with IκBα, which may reflect different specificities of the IKK complex[70].

IκB kinase (IKK)

The IKK complex consists of two catalytically active kinases, **IKKα** and **IKKβ**, and the regulatory subunit IKKγ, also called **NEMO** (NF-κB essential modulator)[71–75]. Both IKKα and IKKβ were identified to form a 700- to 900-kD protein complex that shows TNFα-induced IκBα specific kinase

activity[71,76]. This basic trimolecular complex may also contain an additional substrate-targeting subunit named **ELKS**[77].

IKKα and IKKβ share 50% sequence identity, 70% protein similarity and both contain N-terminal protein kinase domains. IKKβ has C-terminally located ubiquitin-like domain (ULD) and a α-helical scaffold/dimerization domain (SDD), both of them mediating a critical interaction with IκBα that restricts substrate specificity, and catalytic activity (ULD)[78]. IKKα is predicted to have a similar structure. Both proteins show kinase activity toward IκBα.

Despite their homologies and the fact that are part of the same complex, IKKα and IKKβ have non-overlapping functions, mainly due to substrate specificities. IKKβ is the major IKK catalytic subunit for the phosphorylation of IκBα by pro-inflammatory stimuli, such as TNFα, IL-1, and Toll-like receptor (TLR) agonists, such as LPS. IKKα phosphorylation is not critical for activation of the classical IKK complex or for NF-κB activation by most pro-inflammatory stimuli[79,80]. Nonetheless, there are situations in which catalytically active IKKα forming part of the classic IKK complex is more critical than is IKKβ for inducible IκB phosphorylation and degradation. For instance, NF-κB activation in mammary epithelial cells in response to RANK-L requires the kinase activity of IKKα but not that of IKKβ[81]. This activity triggers a classical NF-κB activation pathway that drives cyclin D1 expression. The factors that dictate the subunit specificity for each stimulus are currently unknown[82].

On the other hand, IKKα kinase activity is primarily required for activation of p52:RelB (more detail in page 45) Whereas the IKKβ-dependent pathway is essential for activation of innate immunity, the IKKα-dependent pathway is more important for regulation of adaptative

immunity and lymphoid organogenesis[83]. Whereas IKKβ-dependent degradation of IκBα occurs within minutes, IKKα-dependent processing of p100 requires several hours.

Two other protein kinases have been identified with sequence similarity to IKKα and IKKβ, the so-called IKK-related kinases, **IKKε** (or IKK-i)[84,85] and **TBK1** (also called NAK for NF-κB-activated kinase or T2K for TRAF2-associated kinase)[86–88]. The IKK-related kinases activate NF-κB but also two other transcription factors, IRF3 and IRF7[89–92], which are critical for the expression of type I interferon (IFN) genes. Thus, the IKK-related kinases are important mediators of antiviral responses and, together with the original IKKs, coordinate the host defense response.

NEMO, the regulator of the IKK complex

The third member of the IKK complex is NEMO, a 48-kD regulatory unit[74]. It was identified biochemically as a component of the IKKα/β-containing complex and by genetic complementation of a cell line nonresponsive to NF-κB-activating stimuli[74,93]. NEMO is essential for activation of the classical NF-κB pathway and is required for the formation of the large IKK complex and activation in the classical NF-κB pathway, but it is not necessary for the activation of the alternative NF-κB pathway[94]. Binding of IKKα and IKKβ to NEMO is conferred by a C-terminal decapeptide motif that recognizes residues located in the N-terminal region of NEMO[95]. This motif was named NEMO-binding domain (NBD) and was shown to be required for IKK assembly and activation[95]. It has been recently shown that NEMO directs IKKβ activity towards IκBα[96].

Activating the IKK complex

Both IKKα and IKKβ need to be phosphorylated in order to become active[71]. The kinase domains of both IKKα and IKKβ contain an activation loop that is subject to phosphorylation at two serines, resulting in a conformational change that leads to kinase activation[72,97,98]. Replacement of S177/S181 in IKKβ with alanines prevents kinase activation, whereas its substitution with phosphomimetic glutamates results in a constitutively active kinase[97]. In fact, both serines are phosphorylated in vivo in response to pro-inflammatory stimuli, being serines S176/S180 in IKKα the ones that become phosphorylated upon cell stimulation[97].

It is likely that activation of IKK involves trans-autophosphorylation by its catalytic subunits IKKα and IKKβ. Nonetheless, other mechanisms have also been proposed to regulate this initiating event, although the molecular details of IKK activation are unclear. Mainly, three different mechanisms can be suggested: direct phosphorylation of one IKK catalytic subunit at the activation loop; IKK multimerization resulting in trans-autophosphorylation; and a conformational change induced by a posttranslational modification other than phosphorylation or through protein-protein[82].

Another region involved in regulating IKK activity is the helix-loop-helix (HLH) motif, now renamed as SDD[78]. Mutations within this motif decrease IKK activity with no effect on complex assembly[73,99]. The HLH motif physically interacts with the kinase domain and seems to serve as an endogenous activator of IKK. N-Terminal to NBD and C-terminal to the HLH, both IKKα and IKKβ contain a stretch of serines that are strongly phosphorylated during IKK activation, depending on IKK activity and with

a negative autoregulatory function[97]. Given the transient nature of IKK activation, this autophosphorylation event may contribute to termination of IKK activity.

Activation of the NF-κB pathway

Activation of NF-κB through members of the TNFR family

NF-κB activation upon signaling by members of the TNFR family can be divided: the classical or canonical NF-κB pathway and the alternative or non-canonical NF-κB pathway.

Classical NF-κB pathway

The classical NF-κB pathway (Figure 2) can be activated upon **Tumor Necrosis Factor** (TNF) interaction with the TNF Receptor (TNFR). The members of the TNFR family lack enzymatic activity and thus, they rely on recruitment of intracellular adaptors and signaling molecules in order to initiate signal transduction. All the different family members interact either directly or indirectly with TNFR-associated factors (**TRAFs**), which are critical mediators of NF-κB activation[100]. Six members of the TRAF family have been identified, containing a C-terminal TRAF-C domain that confers binding capacity to upstream molecules[101]. There are some cases in which this TRAF-C domain is able to directly interact with the intracellular signaling domain of the respective TNFR (for instance, CD40), whereas in other cases, receptor-induced TRAF recruitment depends on additional adaptors, such as TNFR1-associated Death Domain (TRADD) in the case of TNFR1[101]. TRAF2-deficient cells show reduced activation of IKK[102], which might be due to a compensatory effect of

TRAF5, because cells lacking both TRAF2 and TRAF5 show no TNFα-dependent IKK activity[103].

TRADD, apart from recruiting TRAFs, is able to recruit a molecule named **RIP1** (for Receptor Interacting Protein 1)[104]. RIP1 contains different structural domains, such as a death domain (DD) that mediates interaction with TRADD, or a protein kinase domain. This protein is required for TNFR-dependent NF-κB activation[105] and RIP1-knockout cells fail to activate IKK in response to TNFα[106]. The kinase activity of RIP1 is not required for activation of NF-κB, since reconstitution of RIP1-deficient cells with a catalytically inactive RIP1 mutant confers full responsiveness[105].

RIP1 is ubiquitinated during TNFR activation and TRAF2 seems to be required for this process[107]. It is possible that TRAF2-dependent ubiquitination of RIP1 is involved in the formation of a stable supramolecular complex with TRAF2, RIP1, and IKK. Nonetheless, TRAF2 does not have E3 ubiquitin ligase activity and rather serves as a scaffold protein that recruits an E2 ubiquitin-conjugating enzyme to its target[108]. On the other hand, it has been recently shown that sphingosine-1-phosphate (**S1P**) binds to TRAF2 and renders TRAF2 competent catalyzing K63 polyubiquitination of RIP1[109]. **cIAP1** and **cIAP2** also catalyze polyubiquitination of RIP1, although these chains are not restricted to the K63 linkage[110–112].

TNFR1-mediated IKK activation might be explained by the induced proximity of IKK subunits with TRADD/TRAF2/5/RIP1 signaling complex, playing the TGFβ–activated Kinase 1 (**TAK1**) a pivotal role. TAK1 was initially identified as a kinase involved in TGF-β signaling[113], but later it was shown to be activated in response to other stimuli, such as TNFα or IL-1[114,115] and identified as a component of the IKK activating complex[116].

Overexpression of TAK1 can induce NF-κB activation, although it requires the coexpression of **TAB1**, **TAB2** and **TAB3** (for TAK1 Binding Protein)[116–118]. Experiments with TAB1- or TAB2-knockout mice failed to demonstrate a role for these proteins in TNFα activation of IKK[119], however downregulation of both TAB2 and TAB3 impairs NF-κB activation[118]. Both TAB2 and TAB3 are recruited to TAK1 during TNFα and IL-1 signaling, and both also interact with TRAF2 and TRAF6, which are involved in TNFα and IL-1 signal transduction, respectively[118]. TAB2 and TAB3 contain a ubiquitin-binding domain[120], by which can bind to RIP1-lysine-63 (K63)-linked polyubiquitin chains produced by TRAF2[121,122]

NEMO contains two ubiquitin-binding domain[123,124], one with high affinity for linear di-ubiquitin[125,126] and another at C-terminus with increased affinity for K63 polyubiquitin chains[127]. The finding that TAB2 and NEMO, being regulatory elements of the TAK1 and IKK complexes, respectively, are ubiquitin receptors further confirm the role of ubiquitination in the activation of these kinases.

Recently, a novel ubiquitin E3 ligase complex, **LUBAC**, which is composed of HOIP, HOIL-1 and Sharpin, was found to be recruited to TNFR1[128]. LUBAC and Ubc5 catalyze linear polyubiquitination of NEMO in vitro[129]. Depletion of LUBAC impairs NF-κB activation, and in fact, several groups have shown that Sharpin-deficient cells failed to activate IKK[130–132].

In addition to K63 polyubiquitin chains, other ubiquitin linkages, including K11 and linear chains, have been detected on RIP1[123,130,133,134]. It is likely that multiple E3 ligases, including TRAFs, cIAPs and LUBAC, cooperate

with or compensate each other to regulate NF-κB and control cell death in response to TNFα.

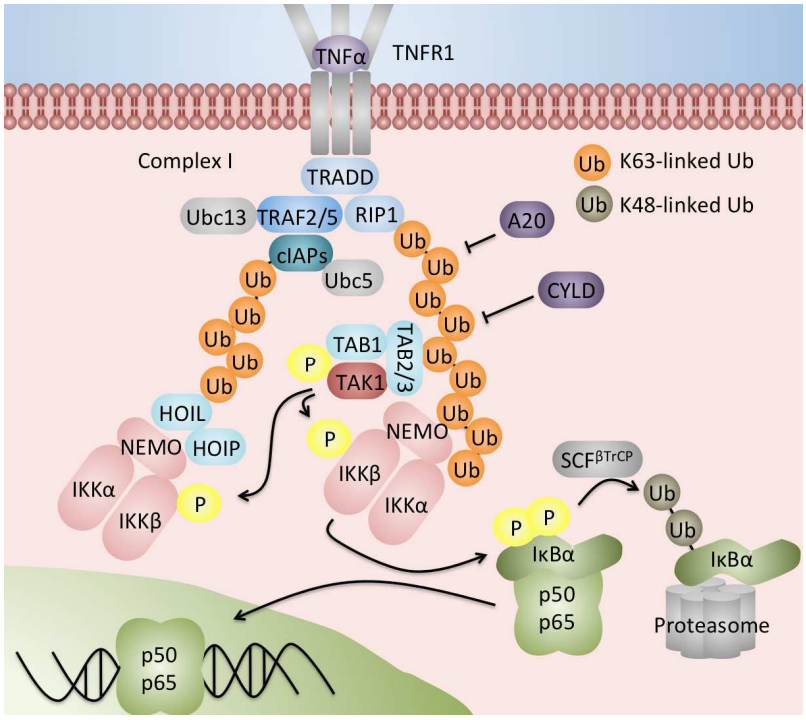

Figure 2. Putative model of TNFα activation. After TNF binding, the adaptor protein TRADD interacts with TNFR1. This implies the binding of TRAF2, cIAP1, cIAP2 and RIP1. After its interaction with TNFR1, RIP1 is rapidly modified with K63 non-degradative polyubiquitin chains, being TRAF2, cIAP1 and cIAP2 involved in the process. Newly formed polyubiquitin chains may then serve as docking sites for TAB2 and TAB3, which recruit TAK1. Binding of TAK1 through TAB2 and TAB3 may trigger the catalytic activity of TAK1. The IKK complex is then recruited through the interaction of NEMO with ubiquitinated RIP1, and this would bring TAK1 and IKK into close proximity, leading to phosphorylation of IKKα/β and its activation. TRADD, TRAF2, cIAP1 and cIAP2 are also required for the recruitment of LUBAC to Complex I. LUBAC binds linear ubiquitin chains to RIP1 and NEMO, thus activating the pathway. Adapted from Häcker and Karin, 2006.

In general, most of the E3 ubiquitin ligases that participate of the TNFR1 signaling are involved in NF-κB activation and result in cell death

inhibition. This function is counteracted by the activity of **CYLD** and **A20**, which function as deubiquitinating enzymes on RIP1 to inhibit IKK activation[135–140]. K63-linked polyubiquitination of RIP1 leads to NF-κB activation and promotes cell survival, whereas deubiquitination of RIP1 by CYLD promotes the assembly of the cytosolic complex termed Ripoptosome, which leads to cell death[141,142].

Subsequent to the rapid activation of IKK and NF-κB, the TNF receptor complex dissociates, and some of the proteins are released from the receptor to form a cytosolic complex consisting of **FADD**, **RIP1** and **procaspase-8**[143] (Figure 3). Within this complex, procaspase-8 cleaves itself to form mature caspase-8, which then cleaves caspase-3 to initiate apoptosis. Caspase-8 also cleaves RIP1, thereby preventing RIP1 from activating RIP3, which would otherwise trigger necrosis[144–146]. When caspase-8 is absent or inhibited, RIP1 phosphorylates RIP3, leading to necrosis, which is highly inflammatory. In most cells, TNFα does not induce apoptosis or necrosis because NF-κB is activated before caspase-8 can execute the cell death program. NF-κB induces the expression of cell survival factors, including apoptosis inhibitors such as cIAPs and **c-FLIP**. c-FLIP binds to caspase-8 and blocks the initiation of apoptosis[147].

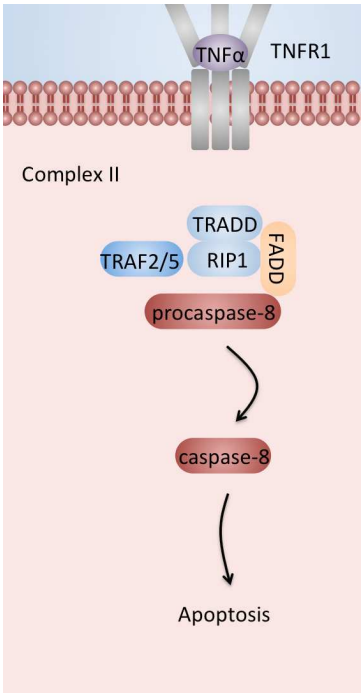

Figure 3. Formation of the death-inducing complex (Complex II)

Alternative NF-κB pathway

A limited number of TNFR family members, such as B-cell activating factor Receptor (BAFF-R activated by **BAFF**) and CD40 (activated by **CD40 ligand**) (on B cells), Receptor Activator of Nuclear Factor κB (RANK activated by **RANK-L**) (on osteoclasts), and Lymphotoxin β Receptor (LTβR activated by **Lymphotoxin α/β**) (on stromal cells), are able to activate the alternative NF-κB signaling pathway[94,148–151] (Figure 4).

Signaling through this pathway requires the processing of p100 subsequent to its phosphorylation by IKKα, which leads to $SCF^{\beta TrCP}$ recruitment, polyubiquitination and p100 targeting for proteasome-dependent proteolysis[152]. This generates mature p52 through the proteolytic degradation of the C-terminal IκB-like portion of p100, which mainly enters the nucleus as a dimer with RelB[51,153].

There are two critical kinases for the activation of the alternative NF-κB pathway: **NIK** and **IKKα**[154,155], being dispensable both IKKβ and NEMO[94]. It has been demonstrated that overexpression of NIK or the constitutive active IKKα (EE) variant induces processing of p100 through phosphorylation of specific serine residues located in its C-terminal domain, thus creating and $SCF^{\beta TrCP}$ binding site[154,155]. The response to NIK depends on the presence of catalytically active IKKα, and it has been demonstrated that in vitro, NIK is a potent IKKα-activating kinase[156]. Furthermore, IKKα and NIK are able to interact[157], and NIK is able to enhance the interaction between IKKα and p100[158,159].

Upstream regulation of NIK involves TRAF proteins, with particular focus on **TRAF3**[160]. NIK and TRAF3 are constitutively bound, resulting in NIK ubiquitination and rapid turnover[160]. Recently it has been demonstrated that CD40 forms a complex containing **TRAF2** and TRAF3, ubiquitin-conjugating enzyme Ubc13, **cIAP1/2**, NEMO, and MEKK1 upon ligation[161].

The kinase complex is only active when translocated from CD40 to the cytosol upon cIAP1/2 degradation of TRAF3. Consistently, TRAF2, TRAF3, and cIAP1/2 inhibit alternative NF-κB signaling in resting cells by targeting NIK for ubiquitin-dependent degradation[162]. Upon CD40 or BAFF-R activation, TRAF2 ubiquitinates and activates cIAP1-cIAP2 to induce degradative K48-linked polyubiquitination of TRAF3. Degradation of TRAF3 prevents targeting of newly synthesized NIK by the TRAF2-cIAP1-cIAP2 ubiquitin ligase complex, allowing NIK accumulation and activation through autophosphorylation, IKKα activation and p100 processing. The requirement for NIK protein synthesis explains the delay in activation of the alternative NF-κB signaling compared with the canonical pathway.

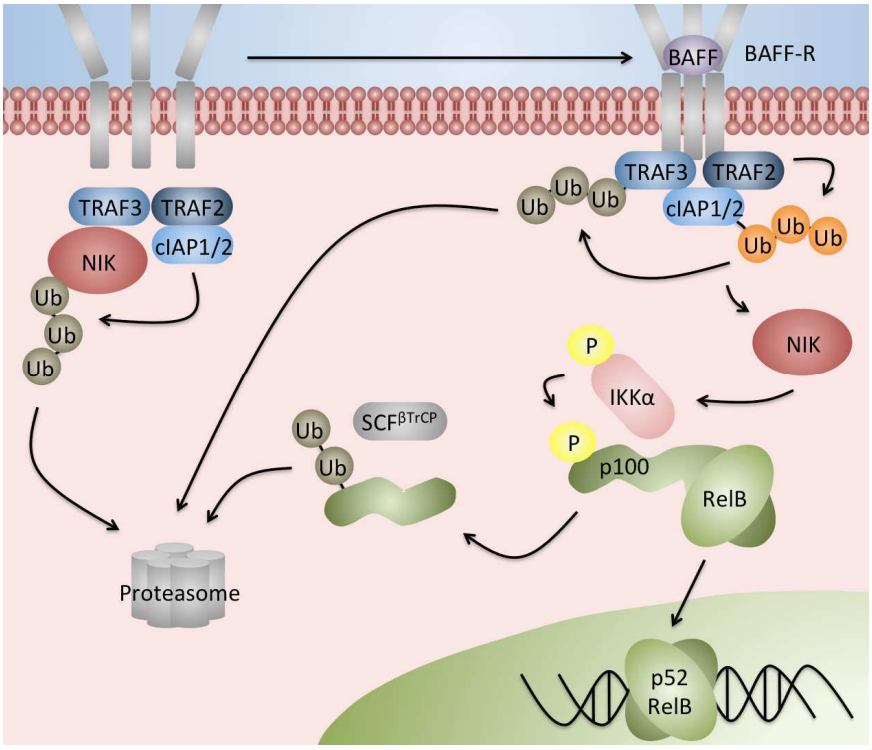

Figure 4. Activation of the alternative NF-κB pathway. Adapted from Häcker and Karin, 2006.

It has been demonstrated that p100 can also inhibit the DNA-binding activity of p65-p50 and RelB-p50 dimers, thus activation of the alternative pathway can also affect the activation of classical NF-κB pathway downstream of NIK and IKKα[163].

Activation of NF-κB through members of the IL-1R and the Toll-like Receptor (TLR) family

Members of the IL-1R family (whose activation is mediated by **IL-1**) and members of the Toll-like Receptor family (such as TLR4, whose activation is mediated by Lipopolysaccharide **(LPS)**) are potent activators of the classical NF-κB pathway. Both types of receptors share a common structural motif called TLR and IL-1R (**TIR**) homology domain[164].

TIR-containing receptors do not have catalytic activity and use intracellular adaptors and signal-transducing molecules to activate downstream effectors[165]. **MyD88** (Myeloid differentiation primary response gene 88) is essential for the induction of inflammatory cytokines triggered by all TLRs, whereas **TRIF** (TIR domain-containing adaptor inducing IFN-β) is involved in the TLR3- and TLR4-mediated MyD88-independent pathway[166]. MyD88-dependent activation of NF-κB depends on TRAF6, which is also involved in CD40 signaling[167,168].

TRAF6 is able to enhance the synthesis of K63 polyubiquitin chains by Ubc13/Uev1A[121], and this is required for IKK activation. TRAF6 interacts with TAB1, TAB2, and TAB3[116,118], to allow **TAK1**-mediated activation of the IKK complex in response to IL-1[169,170]. TAK1 kinase complex is activated by TRAF6-catalyzed K63 polyubiquitination, and once it is activated it is able to phosphorylate IKKβ at the activation loop.

TIR-mediated signaling also involves additional molecules that belong to the **IRAK** (IL-1R associated kinase) family such as IRAK1 and IRAK4 [171–173]. It is independent of the kinase activity (in case of IRAK1)[174], but essential for the recruitment of TRAF6 to MyD88[175].

On the other hand, TRIF-dependent signaling is critical for interferon (**IFN**) and interferon-related gene induction[176,177]. Thus, differential ubiquitination of TRAF3, which interacts with both MyD88 and TRIF, may determine the activation of proinflammatory cytokines versus the production of interferons[178].

Activation of NF-κB by antigen receptors

T cell receptor (**TCR**) and B cell receptor (**BCR**) are directly associated with cytoplasmic protein kinases and initiate signal transduction through tyrosine-phosphorylation events, which ultimately lead to recruitment and activation of protein kinase C isozymes (PKCθ for TCR and PKCβ for BCR). TCR-mediated activation of PKCθ depends on PDK1 (3-phosphoinositide-dependent kinase-1), which may directly phosphorylate PKCθ in its activation loop[179].

Several additional molecules are required for PKC-mediated IKK activation. **CARMA1**, localized at the plasma membrane, is required for membrane recruitment of **BCL10** and **MALT1** in response to antigen receptor activation[180,181]. PDK1 also interacts with CARMA1 and is required for IKK recruitment[179]. PKCθ and PKCβ phosphorylate CARMA1 at a central linker region, thereby inducing a conformational change that allows CARMA1 to interact with BCL10[182,183]. MALT1 activity cleaving BCL10 is required for T-cell activation[184], and this activity is constitutively active in diffuse large B-cell lymphoma (DLBCL)[185].

One mechanism proposed to be involved in MALT1-dependent activation of IKK is through the K63-linked ubiquitination of NEMO[186]. Alternatively, TRAF6-autoubiquitination was suggested to lead to activation of TAK1, which then may phosphorylate IKKβ.

Another element involved in TCR- and BCR-induced NF-κB activation is caspase-8, whose role is not required for TNFα[187,188]. Stimulation of TCR can induced recruitment of IKKα and IKKβ to BCL10 and MALT in a caspase-8 dependent manner[188].

Activation of NF-κB pathway through DNA damage

DNA double-strand breaks (DSBs) also activate the classical NF-κB pathway in a **NEMO**-dependent manner[189,190]. Free NEMO (not in a complex with IKKα and IKKβ) that represents a small fraction of the total pool of NEMO is capable to be modified by the addition of SUMO (small ubiquitin-related modifier), leading to its nuclear translocation[191]. Sumoylation of NEMO is increased in cells overexpressing **PIDD** (p53-induced protein with a death domain)[192,193], enhancing DSB-induced IκBα phosphorylation[193]. Further studies have identified protein inhibitor of activated STATγ (**PIASγ**) as the protein responsible for NEMO sumoylation[194] and calcium mobilization as an essential signal for NEMO nuclear export[195].

After being sumoylated, NEMO is phosphorylated by **ATM** to promote its ubiquitin-dependent nuclear export. Moreover, it has been shown that poly(ADP-ribose)-polymerase-1 (**PARP-1**), responsible for sensing DNA strand breaks, is the DNA proximal regulator of NEMO, PIASγ, and ATM assembly, which depends on poly(ADP-ribose)(PAR) synthesis[196]. At the same time, ATM is also exported in a NEMO-dependent manner to the

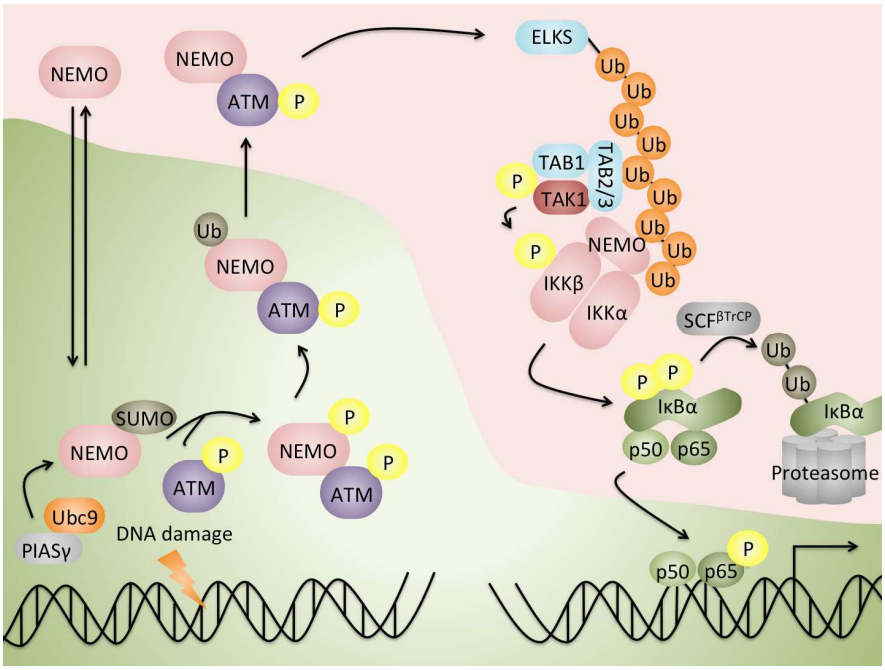

Figure 5. NF-κB activation through DNA damage. Adapted from Wu et al., 2010.

cytoplasm, where it associates to **ELKS**, which function as an IKK complex activator (Figure 5).

This results in the activation of NF-κB and the transcriptional activation of anti-apoptotic genes[197]. Different groups have now shown that ELKS ubiquitination is dependent on ATM and NEMO, and promotes the assembly of TAK1/TAB2/3 and NEMO/IKK complexes thus activating NF-κB signaling[198].

In addition, ATM activates **TRAF6** leading to Ubc13 polyubiquitination and generation of an ATM/TRAF6/cIAP1-complex that promotes TAK1 activation and NEMO monoubiquitination[199].

Alternative nuclear functions for specific NF-κB family members

Nuclear functions for IKKα.

IKKα is mainly located in the cytoplasm as part of the IKK complex. However, after several stimuli, IKKα has been shown to accumulate in the nucleus, phosphorylate different substrates and regulate gene transcription (Figure 6).

Some examples of genes regulated by IKKα through the phosphorylation of:

- **Histone H3**:
    - o NF-κB dependent (in fibroblasts):
        - *IκBα* or *IL-6* downstream of TNFα [200,201].
        - *IκBα* and *rantes* downstream of LPS stimulation and through translocation of NIK.[202]
    - o NF-κB-independent:
        - *c-fos* downstream of EGF[203]
        - *cyclin D1* and *c-myc*: in response to estrogen in breast cancer cells[204]
        - *Il17α:* during Th17 lymphocytic differentiation [205]

- **Silencing Mediator for Retinoic Acid and Thyroid hormone receptor (SMRT)** at serine 2410, thus inducing its dissociation from the chromatin, and being a prerequisite for the expression of NF-κB-dependent genes, such as *ciap-2* and *IL-8* in response to laminin attachment[206]. In basal conditions SMRT and HDAC3 are sited at the chromatin bound to p50 homodimers and promoting basal gene

repression. Upon stimulation, IKKα-mediated phosphorylation initiates transcriptional derepression by releasing SMRT and HDAC3 from the chromatin. After this, SMRT is degraded by the proteasome allowing the binding of transcriptionally active p65-p50 dimer to specific gene promoters.

In CRC cells, IKKα is aberrantly activated, localized in the nucleus of tumor cells and bound to the promoter of different Notch-dependent genes, including *hes1* and *herp2*. This chromatin-bound IKKα is able to phosphorylate SMRT leading to its release and resulting in Notch-dependent gene expression[207]. General IKK inhibition restores SMRT chromatin binding, thus inhibiting Notch-dependent gene expression, and preventing tumor growth in a xenograft model of nude mice. In a similar fashion, IKKα phosphorylates N-CoR, a very homologous nuclear corepressor, in CRC cells creating a functional 14-3-3 binding domain, and facilitating its nuclear export[208].

- **Phosphorylation of p65 (Ser536)** by chromatin-associated IKKα favors p300 recruitment that acetylates p65 at Lys310, which is required for full NF-κB-gene expression[209]. However, it can also trigger its proteasomal degradation and facilitate termination of NF-κB-dependent transcription of pro-inflammatory gene promoters[210]. In fact, it seems that in response to inflammatory stimuli such as TNF-α or LPS, IKKα phosphorylates PIAS, and this allows PIAS to associate with chromatin and repress promoter-binding and transcriptional activity of NF-κB, thus terminating NF-κB response[211].
- Phosphorylation of the **steroid receptor coactivator 3 (SRC-3)** upon TNFα stimulation results in the activation of NF-κB signaling[212], β-catenin[213–215] or FOXO3a[216].

Nuclear IKKα also plays a role in regulating proliferation:

- Phosphorylation of **CREB-binding protein (CBP)** after TNFα stimulation, favors its binding to p65 at expenses of p53[217]. Hence, NF-κB-dependent transcription is enhanced and p53-mediated gene expression is suppressed, leading to increased cell proliferation and tumor growth in lung cancer[217].
- IKKα is required for estrogen-induced cell cycle progression through regulation of PCAF-mediated acetylation of **E2F1**, thus increasing its DNA-binding activity and protein stability[218].
- IKKα plays also a role during M phase of cell cycle by inducing phosphorylation and activation of **Aurora A**, a mitotic kinase that regulate cell cycle progression[219].

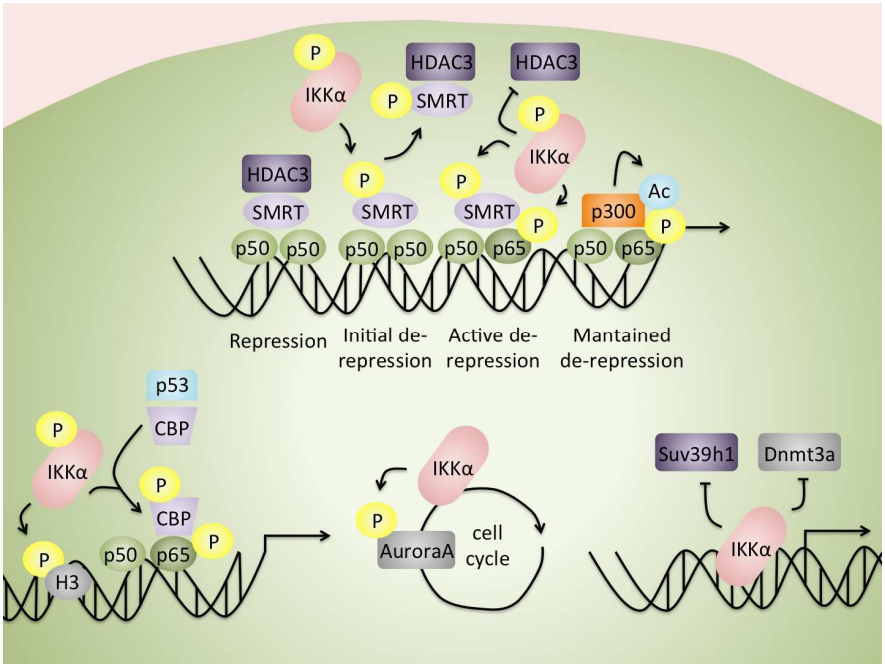

Figure 6. IKKα nuclear functions. Adapted from Espinosa et al., 2011.

IKKα and epigenetics:

- In prostate cancer IKKα negatively regulates in the nucleus the expression of *maspin*, a metastasis suppressor, presumably by facilitating the recruitment of DNA methyltransferase activity to its promoter[220].

Nuclear functions for IKKβ

Nuclear functions of IKKβ are not well documented. There is data suggesting that IKKβ is recruited to the promoter of specific NF-κB target genes in a TNFα-dependent manner, although this data is controversial[200,201]. In NIH-3T3 cells, IKKβ and IKKα, bind the promoter of the Notch target genes *hes1* and *herp2* in response to TNFα associated with their transcriptional activation[221]. Recently, it has been proven that nuclear IKKβ acts as an adaptor protein and together with β-TrCP and heterogeneous ribonucleoprotein U (hnRNP-U) promotes degradation of nuclear IκBα upon UV irradiation. NF-κB activated by the nuclear IKKβ adaptor protein suppresses anti-apoptotic gene expression and promotes UV-induced cell death[222].

In some breast cancer cells IKKβ phosphorylates FOXO3a triggering its cytoplasmic export and proteasomal degradation resulting in increased survival[216].

Finally, it was recently demonstrated that during DNA damage response to alkylating agents, a fraction of activated IKKβ translocates to the nucleus and directly phosphorylate ATM[223]. It was proposed that ATM phosphorylation by IKKβ promote DNA repair. In parallel, active IKKβ induced classical NF-κB activation, thus generating an anti-apoptotic response[223].

Nuclear functions of NF-κB elements in the skin homeostasis

Nuclear NF-κB elements most likely play roles in different tissues, however individual NF-κB members seem to play specific functions in the maintenance of skin homeostasis[224–226] as indicated by the different *in vivo* studies[79,227–229].

This includes the kinase-independent functions of IKKα such as regulation of 14-3-3σ by direct binding to the chromatin[227] and the activation of a putative soluble factor that is sufficient to rescue the skin phenotype of IKKα KO mice[229]. These IKK functions might have a tumor suppressor role, since IKKα mutations or its aberrant cytoplasmic localization are associated with poorly differentiated Squamous Cell Carcinoma (SCC) and skin papillomas[230,231].

Mechanistically, chromatin-bound IKKα at the 14-3-3σ promoter in keratinocytes prevents Suv39h1 recruitment, a protein that is responsible for Lys9 trimethylation of histone H3 leading to gene transcription. Since 14-3-3σ negatively regulates the cell cycle phosphatase CDC25, in the absence of functional IKKα, cells aberrantly proliferate, which results in the loss of skin homeostasis and increased cell transformation[227]. This matches with the IKKα requirement in skin development[79,227,228] and with the fact that the skin defects detected in the IKKα knockout mice can be rescued by reintroducing kinase-dead IKKα under the control of Keratin 14 promoter[232].

In addition, epidermal IKKα interacts with SMAD2/3 after TGF-β stimulation to facilitate transcriptional activation of cell-cycle regulators promoting cell growth arrest and keratinocyte differentiation[233].

Similarly, **IKKβ** and **NEMO** are also found in the nucleus of normal skin cells[234], but mice with skin-specific deletion of these genes do not display any alteration in keratinocyte differentiation or proliferation although they show a markedly thickened epidermis[235]. However, deletion of TNFR1 rescued the skin defects associated to IKKβ deletion, which suggests that inflammation is responsible for this phenotype[235,236]. All this data indicate that the role of IKKβ and NEMO in the skin is associated with the classical NF-κB pathway.

Another member that is required for skin homeostasis is **IκBα**. Mice deficient for IκBα, display skin disorders associated with increased keratinocyte proliferation and T-cell infiltration[237–239], although selective deletion of IκBα in the keratinocytes resulted in lower epidermal inflammation. Recently, a study has brought light into the role of IκBα in the skin[240]. Sumoylated and phosphorylated IκBα accumulates in the nucleus of keratinocytes and interacts with histones H2A and H4 at the regulatory regions of *hox* and *irx* genes. This form of IκBα regulates Polycomb recruitment and imparts their competence to be activated by TNFα. Moreover, it has been demonstrated that oncogenic transformation of keratinocytes results in cytoplasmic IκBα translocation, which associates with massive activation of *hox*, what is also observed in SCC cells associated with IKK activation and HOX upregulation.

Linking inflammation and cancer: NF-κB

Innate immunity and inflammation are protective mechanisms that have been originated and conserved in all multicellular organisms[241]. In fact it has been proposed that regulation of innate immunity by NF-κB has

evolved in parallel with organism evolution from the early days of multicellularity[242].

Shortly after its discovery, NF-κB was already postulated as a main conductor of the inflammatory responses[243,244]. However, it is now well characterized that NF-κB is not only required for triggering, amplifying and maintaining inflammation but also for its resolution, thus preserving tissue functions and homeostasis once the stimulus that originated inflammation is no longer present[245]. However, repeated tissue injury may result in persistent inflammatory response leading to excessive cell proliferation, which in different systems is linked with tumor[246].

NF-κB is involved in many aspects of tumorigenesis, exerting important activities in both the tumor cells and their microenvironment, and having both tumor-promoting and tumor-protective functions (Figure 7).

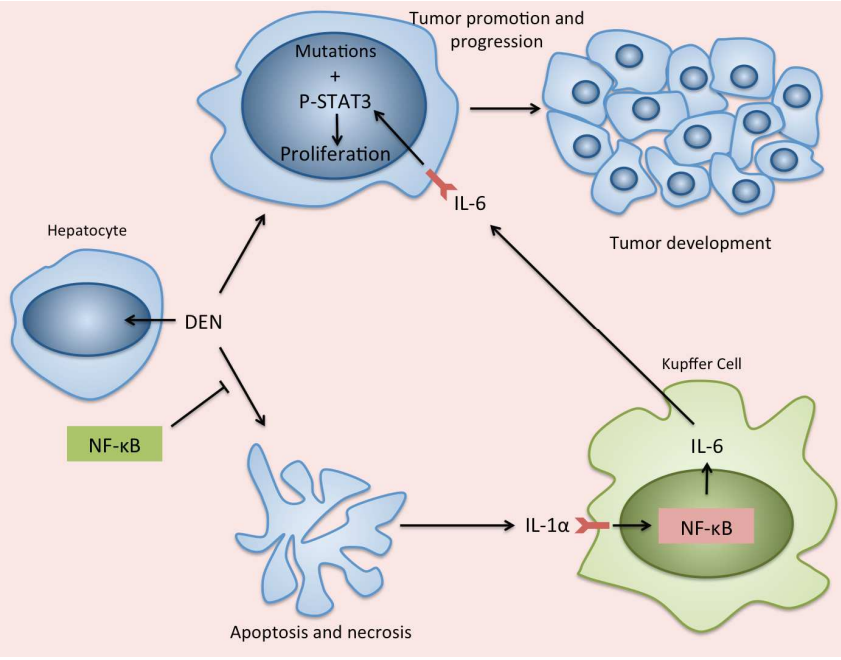

Figure 7. Example of NF-κB having both tumor-protective functions (in green) and tumor-promoting functions (in red). Adapted from Karin and Greten, 2005.

Tumor-promoting functions of NF-κB

In agreement with the possibility that inflammation has a tumor promoting effect, animals maintained under germ-free conditions or deficient for MyD88 (cannot activate NF-κB) are protected from intestinal tumorigenesis even in the presence of constitutively active β-catenin[247,248]. In this scenario, it is essential that the inflammatory response is self-limiting and self-resolving, with NF-κB regulating both the initiation of inflammation and its resolution[249–251], which is mainly achieved through specific feedback loops, such as NF-κB-dependent activation of IκBα and A20 transcription[252].

However, the first example of NF-κB as a tumor driver was the discovery of the retroviral oncogene *v-Rel* as the homolog of the gene encoding c-Rel[253], which was further supported by the discovery of mutations in the genes encoding for other **NF-κB** subunits or the **IκB** proteins in a variety of hematological malignancies[253–256]. IκBα is also frequently deleted in gliobastoma tumors[257], which is mutually exclusive with the common amplification of the epidermal growth factor receptor (EGFR).

However, the number of tumors with activated nuclear NF-κB is much larger than the subfraction of malignancies with mutations in NF-κB- or IκB-encoding genes[242]. This suggests the existence of mutations in components that regulate NF-κB activity or can be explained by the presence of inflammatory cytokines in the tumor microenvironment[258]. Examples of this include the identification of MALT lymphomas with chromosomal translocations that increase **Bcl-10**[259] and **MALT1**[260] expression, leading to constitutive NF-κB activation, the presence of activating mutations in **CARMA1** or in **MyD88** in B cell lymphoma[261–263],

and mutations leading to the activation of NIK in multiple myeloma[264–266]. Activated NF-κB has also been observed in different chronic inflammatory diseases, such as inflammatory bowel disease[267,268], rheumatoid arthritis[269], and psoriasis[270]. These diseases respond to anti-TNFα therapy[271] and the corresponding mouse models respond to inhibitors of NF-κB, which are postulated as therapeutic targets in chronic inflammation and autoimmunity diseases.

Despite these examples, the vast majority of NF-κB-positive tumors are solid malignancies derived from epithelial cells, in which NF-κB mutations are very rare. Among them, mutations and gene fusions of *IKKA* and *IKBKB* have been detected in breast and prostate cancer, respectively[272,273], playing murine IKKα a direct role in the self-renewal of breast cancer progenitors[274]. **IKKα** is also responsible for the tumor-promoting effect of progesterone in breast cancer downstream of RANKL induction[275,276] and for the metastatic spread of breast cancer cells, which depends on RANKL produced by tumor-infiltrating regulatory T cells[277].
Other oncogenic functions for IKKα in breast or prostate cancers[220,278] are not mediated through either classical or alternative NF-κB signaling but rather depend on the nuclear functions of IKKα[220,279] (see page 51).

Contribution of NF-κB to tumor progression and inflammation can have both protective and tumor promoter effects. Thus it is difficult to predict the convenience of inhibiting NF-κB in cancer therapies.
For example, colitis-associated colon cancer (CAC) is an inflammation-driven disease that accounts for about 5% of all sporadic CRCs. In this model, **NF-κB** activity in the IECs provides a survival advantage to damaged cells through the induction of antiapoptotic genes such as Bcl-

x_L[280]. However, the lamina propria macrophages and dendritic cells, as well as tumor-associated macrophages, secrete different inflammatory cytokines, such as TNF, IL-6 or IL-23 (that are also NF-κB dependent), which are responsible for tumor growth[281–283]. Finally, tumor proliferation mediated through IL-6 and related cytokines lead to the activation of STAT3 in IECs[280,281]. A similar pro-tumorigenic effect for NF-κB was observed in hepatitis-associated liver cancer[284].

In contrast, in tumors induced by chronic liver inflammation that depends on NF-κB activation in the hepatocytes, ablation of hepatocyte specific **IKKβ** prevents Hepatocellular Carcinoma (HCC) development[285]. Moreover, ablation of different elements (IKKβ, **IL-1** or **MyD88**) in Kupffer cells also inhibits HCC development[286,287] (Figure 7). This result has been linked to the inhibition in IL-6 production, upstream of STAT3[288,289].

Another example of a cell type-specific role for NF-κB in tumor progression is castration-resistant prostate cancer[278]. In this system, IKKβ activation in B cells is necessary for the production of the two subunits of lymphotoxin encoded by NF-κB target genes; lymphotoxin α/β then activate IKKα in prostate carcinoma cells that induces transcription of the prometastatic gene *Maspin*. Thus, whereas IKKβ ablation in prostate carcinoma cells has no effect on the development or recurrence of tumors, ablation or inactivation of **IKKα** delays or even inhibits the development of castration-resistant cancer[278]. Furthermore, IKKα but not IKKβ is required for the metastatic spread of prostate cancer in mice[220]. It should be noticed, that this pro-metastatic function of IKKα is NF-κB-independent and requires IKKα nuclear translocation (see page 54).

Recently it has been discovered that NF-κB signaling is also required for MLL progression by a mechanism involving coocupancy of MLL and **p65** to gene promoters that are essential for leukemia progression and the

leukemia stem cell program[290]. Importantly, NF-κB is also induced and essential in T-ALL leukemia carrying activating Notch mutations[291].

On the other hand, NF-κB can also affect microenvironment, for instance by promoting the expression of a proinflammatory gene signature in cancer-associated fibroblasts, which is important for macrophage recruitment, neovascularization and tumor growth[292]. Although some of those activities are mediated through inflammatory cytokines, others are mediated via chemokine expression.

Tumor-protective functions of NF-κB

In some cases, NF-κB inhibition induces inflammation as observed by the greater susceptibility to chemical-induced colitis of mice lacking **IKKβ** in the intestinal epithelial cells (IECs)[280] or mice lacking Intestinal Epithelial **NEMO**[293]. In these models, the epithelial barrier, which prevents the exposure of underlying tissue macrophages and dendritic cells to commensal bacteria is lost in the absence of the cell-survival functions of NF-κB[294]. Moreover, studies in mice with inducible deletion of IKKβ in myeloid cells showed hypersusceptibility to septic shock induced by either LPS or bacterial infection, similar to mice treated with specific IKKβ inhibitors[295].

In a genetic model of liver cancer, hepatocyte-specific **IKKβ** ablation led to the elimination of some premalignant cells during tumor initiation leading to a huge compensatory cell proliferation that in turn facilitates tumor progression[286]. Similarly, hepatocyte-specific ablation of NEMO or TAK1[296,297] results in spontaneous liver damage, hepatocyte death, liver fibrosis and spontaneous development of HCC. This effect is associated with ROS accumulation, and can be prevented by administrating a potent

antioxidant[286]. In general, the tumor-suppressive function of hepatocyte-specific NF-κB occurs when the main driver of liver inflammation is hepatocyte death (that is prevented by NF-κB), leading to release of IL-1α that activates Kupffer cells, which are in turn pro-tumorigenic[287] (Figure 7).

Finally over-expression of **IL-1β** (negatively regulated by NF-κB) in the stomach[298,299] results in gastric inflammation and cancer[300].

NF-κB blockade as a strategy for cancer prevention and therapy

As it was shown in the previous sections, inhibiting or activating NF-κB results in different and sometimes opposite effects. Thus, using NF-κB or elements of the pathway as therapeutic targets for cancer should be considered for each situation. This will define the therapeutic strategy for treating individual patients. That said NF-κB and its elements have emerged as potential targets for cancer therapy. Many IKK inhibitors have been developed with antitumoral effect in a variety of experimental cancer models, ranging from lymphoma and CRC to melanoma[207,301–304], but at present no such drug has been clinically approved.

More realistic is the possibility of combining IKK inhibition with the more traditional chemotherapy protocols. For instance, IKK inhibitors are effective in sensitizing ovarian, colorectal and pancreatic cancer cells to standard chemotherapy-induced death[305–308], through suppression of genes encoding antiapoptotic[309] and antioxidant molecules[310].

An alternative strategy is the use of multitarget drugs[311], which should improve therapeutic efficacy by targeting diverse regulatory pathways

essential for the proliferation and survival of cancer cells. A promising combination is blocking heat-shock protein HSP90 and inhibitors of histone deacetylases and the ubiquitin-proteasome system[312–314]. In some type of tumors in which NF-κB activation protects against genotoxic damage[315] or tunes down an exaggerated innate immune response[295], drug combination should be considered to rebalance the adverse effects of NF-κB inhibition.

Moreover tumors often benefit from NF-κB activation in the microenvironment. Hence, blocking of NF-κB activation in the tumor microenvironment (instead of in tumor cells) would be a good therapeutic strategy. This would be facilitated by the superior genomic stability of the non-transformed cells and their lower tendency to develop drug resistance[242].

A different issue is prevention and prophylactic therapy, and whether long-term suppression of low inflammation could reduce cancer risk. At this point, information is limited to Non Steroidal Anti-Inflammatory Drugs (NSAIDs), mostly aspirin. Daily aspirin use at doses of 75 mg per day for 5 years or longer diminishes death due to several common cancers, such as colorectal, pancreatic and lung carcinomas[316]. The protumorigenic effects of this low but persistant inflammation could have been underestimated, since it is believed that about 20% of all cancers are linked to inflammation[317].

3. RAS/RAF/MAPK signaling pathway

First Ras experiments can be tracked back to 1964 when it was first observed that a preparation of a murine leukemia virus obtained from a leukemic rat was able to induce sarcomas in new-born rodents[318]. In 1970s the sequences responsible for the oncogenic properties of these transforming viruses were identified[319]. From then on, many studies have tried to understand Ras molecular pathways and its role in cancer.

Some RAS history

The protein products of the *ras* oncogenes were named p21 due to its molecular weight. The studies of the RAS functional properties identified its high affinity for guanine-containing nucleotides[320] and their ability to associate with the inner side of the plasma membrane[321]. Molecular cloning allowed the analysis of its biochemical characteristics and the discovery that RAS proteins were GTPases[322]. These discoveries led to propose that RAS proteins are mediators of signal transduction across the plasma membrane, specifically in the transduction of mitogenic signals. Consistently, Epidermal Growth Factor (EGF) was able to stimulate the ability of RAS proteins to bind GTP [323]. Many Receptor Tyrosine Kinases (RTKs) have been shown to activate RAS signaling, among them EGF-R, ErbB2, Insulin-like growth factor 1 Receptor (IGF1-R) or Vascular Endothelial Growth Factor Receptor (VEGF-R)[324].

Upstream of RAS activation, a cytosolic factor (RAS-GEFs for Guanine Exchange Factor) was able to induce the release of GDP from RAS p21 proteins, thus allowing GTP to bind[325]. The main GEF for RAS is Sos,

among many others identified. Other screenings identified Growth factor receptor-bound protein 2 (GRB2) in mammals as the adaptor protein responsible for the connection between tyrosine-kinase cell-surface receptors and RAS-GEFs [326].

In 1993, RAF, a serine/threonine kinase previously identified as a retroviral oncogene[327], was found to interact with GTP-bound-active RAS proteins[328–331].

In humans, three RAS proto-oncogenes that encode four RAS isoforms (HRAS, KRAS4A, KRAS4B and NRAS) have been identified. Active RAS acts as an adaptor protein, recruiting effectors to membranes where they can interact with proteins and lipids to transduce intracellular signals. Knockout mice for NRAS, HRAS or double (N and H) deleted animals developed normally with no impact on long-term survival[332–334]. In contrast, KRAS4B knockout mice died during embryogenesis[98,332].

GTPase-Activating Proteins (GAPs) are responsible for accelerating RAS GTPase activity, thus terminating RAS activation[335]. Among them we can find Neurofibromin 1 (NF1), which functions as a negative regulator of RAS activation[336].

Downstream of RAF

One of the main downstream effectors of RAS is RAF (Figure 8). In humans three isoforms of RAF have been identified: ARAF, BRAF and CRAF. They share a common structure with a conserved kinase domain. RAS is able to directly interact with the N-terminal regulatory domain of the RAF proteins in its GTP-bound active form[337]. Since RAS is mainly bound to the inner part of the plasma membrane, RAF is also found

there. Membrane localization is not enough to initiate RAF kinase activity and RAF needs to be activated in a RAS-dependent manner.

Once RAF is activated by RAS it activates MEK1 and MEK2 (for Mitogen-Activated Protein Kinase (MAPK) and Extracellular signal-Regulated Kinase (ERK) kinase) and subsequently the MAPK/ERK proteins ERK1 and ERK2.

The three RAF isoforms share a complex regulation by phosphorylation. ARAF and CRAF share five phosphorylation sites that are required to get activated[338]. BRAF contains four conserved phosphorylation sites and T598 and S601 are required for BRAF activation[339].

BRAF has a strong basal kinase activity in comparison with CRAF[340]. This difference could account for the fact that BRAF is frequently mutated in human cancer, whereas ARAF and CRAF mutations are very difficult to find in tumors. In fact, single aminoacid changes are enough to stimulate BRAF kinase activity.

Dimerization of RAF proteins is important for their activation, being the dimers functionally asymmetric. Thus, one of the kinases acts as an activator that stimulates the activity of the partner kinase[341]. The activating kinase does not require kinase activity but depend on its N-terminal phosphorylation that acts allosterically to induce cis-autophosphorylation to the partner kinase. Since N-terminal phosphorylation of BRAF is constitutive, BRAF initially functions to activate CRAF. On the other hand, N-terminal phosphorylation of CRAF is dependent on MEK, suggesting a feedback mechanism. Hence, CRAF can only act as an activator of the other partner in the dimer once MEK has been activated.

MEK-ERK Cascade

The best-characterized pathway downstream of RAF proteins is that involving MEK-ERK kinase cascade (Figure 8). MEK proteins are activated by phosphorylation of two serine residues (S217 and S221 in MEK1) that are located in their activation segments. Once activated, MEK proteins phosphorylate ERK1 and ERK2 on threonine and tyrosine residues within their activation segments (T202 and Y204 in ERK1).

It is thought that the three RAF isoform are able to activate both MEK1 and MEK2 in vitro[338]. However BRAF is more efficient in its binding and phosphorylating capacity over MEK1 and MEK2 in comparison with ARAF and CRAF[338,342].

Once ERK1/2 is phosphorylated and activated, it can activate its substrates, which include both cytosolic and nuclear protein. ERK is able to phosphorylate ELK1, which forms part of the serum response factor and regulate the expression of FOS; moreover ERK phosphorylates c-JUN. This results in the activation of the AP1 transcription factor, which is composed of FOS and JUN heterodimers[343]. Once AP1 is activated it can regulate cell-cycle regulatory elements, inducing cell cycle progression[344]. This pathway through the regulation of transcription, metabolism and cytoskeletal rearrangements also affects cell growth, cell survival and differentiation.

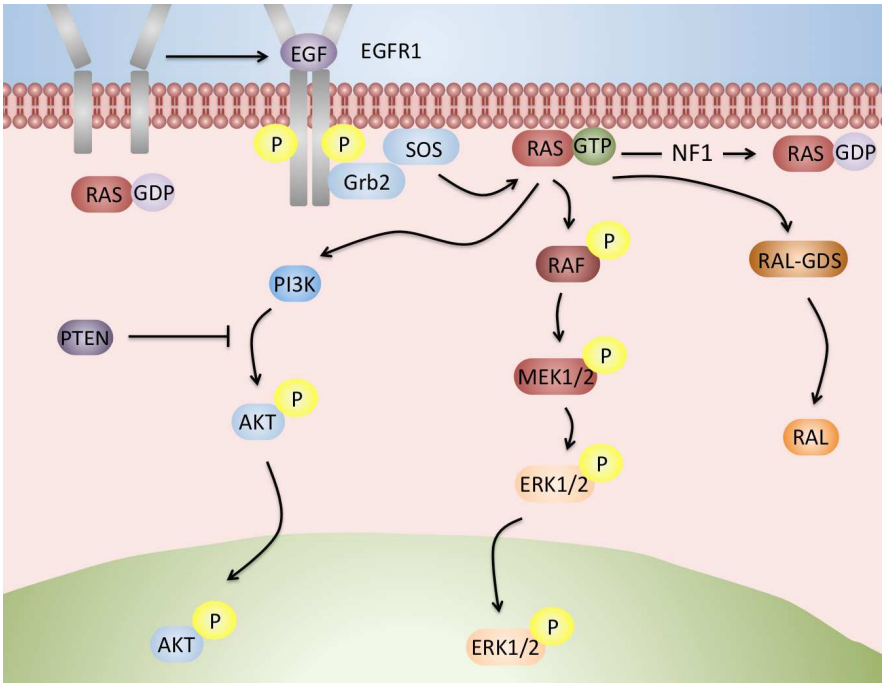

Figure 8. Elements activated downstream of RAS

Other elements downstream of RAS

RAS is also able to interact and induce the activation of type I phosphatidylinositol 3-kinases (PI3Ks)(Figure 8), by recruiting it to the membrane and inducing a conformational change[345,346]. Once activated, PI3K can control the activity of downstream effectors such as PDK1 and AKT to prevent apoptosis.

Another well-studied RAS effector family comprises three exchange factors for the RAS-related RAL proteins[347] (Figure 8). Through this family of proteins RAS is able to inhibit Forkhead transcription factors of the FoxO family, thus inducing the cyclin-dependent kinase inhibitor KIP1 (or p27) that induce cell-cycle arrest, and apoptosis through the induction of BIM and Fas ligands.

Identification of new RAS effectors has also linked RAS activity with senescence[348,349] and differentiation[350].

Aberrant RAS signaling in tumors

When RAS is mutated, it can activate the above-described pathways, promoting malignant transformation. For instance, through the induction of Cyclin D1, this in turn leads to the inactivation of retinoblastoma, as well as downregulation of cell-cycle inhibitors such as KIP1, that induce increased proliferation. In addition, mutant RAS cells become unresponsive to apoptosis through the activation of PI3K/AKT pathway, upregulate angiogenic factors by inducing angiogenesis, or increase invasiveness through the induction of metalloproteases.

Different mutational changes can induce aberrant RAS signaling. RAS is one of the oncogenes most commonly mutated in different types of cancer, being mutated in 30% of all human cancers[351]. KRAS is mutated in almost 85% of total, NRAS in 15% and HRAS in less than 1%. All the different mutations occurring in RAS genes compromise the GTPase activity of RAS, preventing GTPase Activating Proteins (GAPs) from promoting hydrolysis of GTP on RAS and therefore causing RAS to accumulate in the GTP-bound, active state.

In CRC, 35%-45% of tumors have mutations in KRAS[30,352–355]. Codon 12 and 13 account for about 95% of all mutation types in KRAS, more specifically 80% occurring in codon 12 and 15% in codon 13[356].

Although mutations in RAS are the most frequent way to activate RAS signaling, cancer cells found other ways to activate it. For instance, loss of GAPs can also lead to activation of RAS. The best known is the loss of

neurofibromin, which is encoded by the *NF1* gene[357]. Recently another study has shown that RasGAP gene, *Rasal2*, function as a tumor and metastasis suppressor and is mutated or suppressed in human breast cancer[358].

EGFR or ErbB2 (also known as Her2) are also Ras activators, thus activating mutations happens in approximately 50% of the carcinomas[359].

In the recent years BRAF has emerged as another mutated gene in human tumors; in particular it is mutated in 12% of colon carcinomas[360].

Mutations in BRAF are located in the kinase domain resulting in the aberrant activation. The mutation that substitutes valine for glutamic acid at codon 600 (V600E) of the kinase domain mimics a phosphorylated site[361] and induces a conformational change resulting in hyperactivity of the MAPK pathway[362]. BRAFV600E mutation is the most prevalent in melanomas and papillary thyroid carcinomas[363].

The frequency of mutational changes of ARAF and CRAF in human cancers is very low, except for a truncated form of CRAF[364].

Deletion of the tumor suppressor gene *Pten* induces the activation of PI3K in cancer. PTEN is deleted in about 30%-40% of human tumors[365], being the second most significant tumor-suppressor gene after TP53.

Targeting RAS signaling in tumors

RAS signaling is aberrantly overexpressed in many cancers making it one of the most interesting druggable targets. Many inhibitors have been approved or are being tested for clinical use (summarized in Table 1[366–369]), however acquisition of resistance is one of the main complications for this strategy.

	AGENT	MOLECULAR TARGET	TRIAL PHASE	TUMOR TYPE	CLINICAL ACTIVITY	REFERENCES
RAF Inhibitors	Sorafenib (BAY-43-9006)	RAF VEGFR-2 VEGFR-3 PDGR-β	III	Advanced Renal Cell Carcinoma,	3 month PFA advantage; FDA and EMEA Approved	Wilhelm et al., 2004; Mendel et a., 2003
			III	Advanced Hepatocellular Carcinoma	3 month survival advantage FDA and EMEA Approved	
	RAF265	RAF VEGFR-2	I/II	Advanced Melanoma	Ongoing	Stuart et al., 2008; Stuart et al., 2006
	PLX4032	Mutated BRAF	I/II	Advanced Melanoma	Ongoing	Flaherty et al., 2010; NCT00405587
	XL281	ARAF, BRAF, CRAF, mutant BRAF	I	Advanced Solid Tumors	Ongoing	NCT00451880
MEK Inhibitors	CI-1040	MEK	II	Breast, CRC, pancreatic, NSCLC	Stable Disease 12%	Rinehart et al., 2004
	PD0325901	MEK	I	Breast, CRC, pancreatic, NSCLC	PR 5%; SD 36%	LoRusso et al., 2007; Haura et al., 2007
			II	Advanced NSCLC (heavily pretreated)	No responses; SD23%	
	AZD6244	MEK	I	Advanced Solid tumors	SD 33% (16% >5 months	Adjei et al., 2008; MEK.
			II	Advanced Melanoma; Advanced Colorectal; Advanced NSCLC	No survival advantage	
	XL518	MEK	I	Advanced Solid Tumors	Ongoing	
	Trametinib	MEK	II/III	Metastatic Melanomas	Increased PFS	Flaherty et al., 2012
Small-molecular tyrosine kinase inhibitors (TKIs)	Gefitinib	EGFR	II/III	NSCLC, Head and Neck Cancer, Prostate Carcinoma	No improvement in overall survival; FDA approved	Herbst et al., 2002
	Erlotinib	EGFR	II/III	NSCLC	FDA approved	Hidalgo et al., 2001
Humanized antibodies	Cetuximab	EGFR	III	Metastatic CRC, HNSCC	FDA approved	Van Cutsem et al., 2009; Vermorken et al., 2008
	Panitumumab	EGFR	III	Metastatic CRC	FDA approved	Hecht et al., 2009
	Trastuzumab	ErbB2	III	Breast Carcinomas	5 months survival advantage	Slamon et al., 2001

Table 1. Inhibitors of RAS signaling. Adapted from Montagut et al., 2009

Resistance to RAS signaling inhibition

Resistance to RAF inhibitors: the melanoma example

Raf inhibitors often cause tumor regression in patients with metastatic melanoma. However, patients relapse and tumors regrow and progress in almost all patients, with a median time to progression of approximately seven months[370,371].

Several mechanisms have been proposed to understand the acquired resistance[363]. One of the most surprising resistance strategy relies on RAF inhibitor inhibition of ERK signaling in a mutation-specific manner[372–376]. While BRAF inhibitors potently inhibit ERK activation in BRAFV600E mutant cells (Figure 9), levels of activated ERK were maintained or increased in cells with wild-type BRAF (Figure 10). Activation of ERK by BRAF inhibitors in the non-mutated cells was more pronounced in cells with active RAS, either due to activation of RAS by upstream signaling components, such as RTKs, or due to RAS mutation. In this sense, RAF induction by inhibitors requires levels of RAS activity adequate to support formation of enough RAF dimers capable of transactivate.

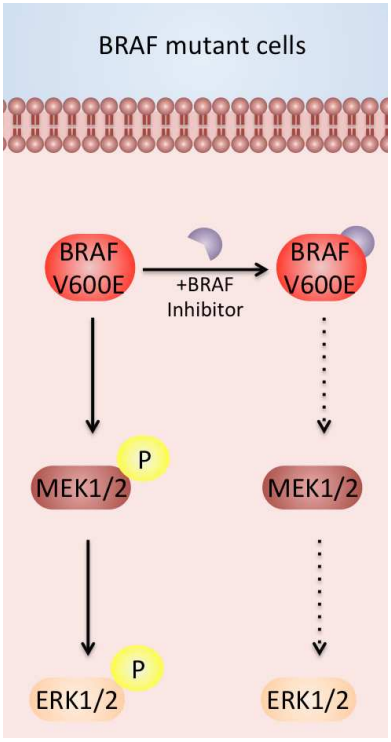

Figure 9. BRAF inhibition in BRAF mutant cells

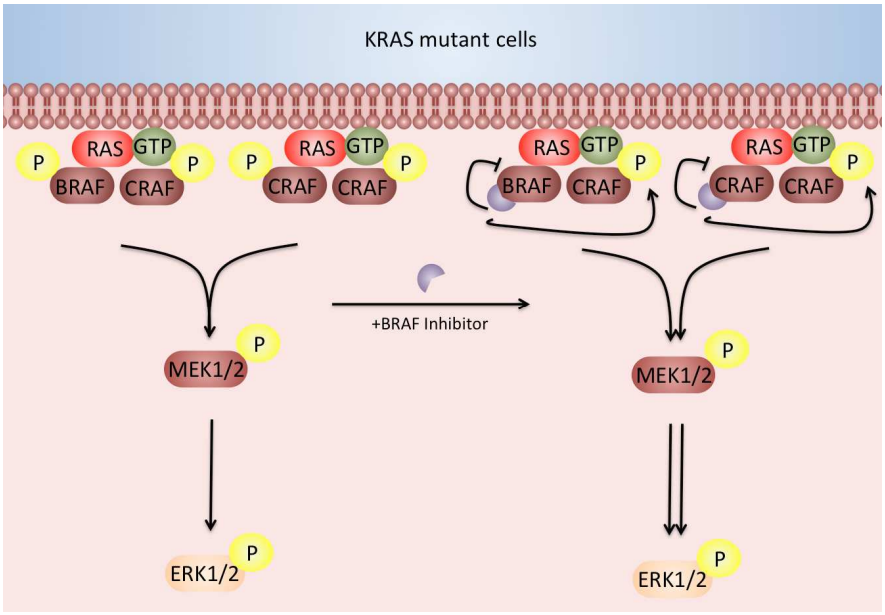

Figure 10. BRAF inhibition in KRAS mutant cells. Adapted from Molina-Arcas and Downward, 2012.

Mutant BRAF signals as a monomer. However wild type BRAF forms homodimers or heterodimers with other RAF proteins, such as CRAF, to become active. When BRAF inhibitor binds to one member of a RAF dimer, it blocks the catalytic activity of that member, but it induces transactivation of the other member of the RAF dimer, leading to an increase in catalytic activity and enhanced activation of MEK[372–376].

However, the inhibitor binds both RAFs in the dimer at high concentrations, resulting in pathway inhibition (Figure 11).

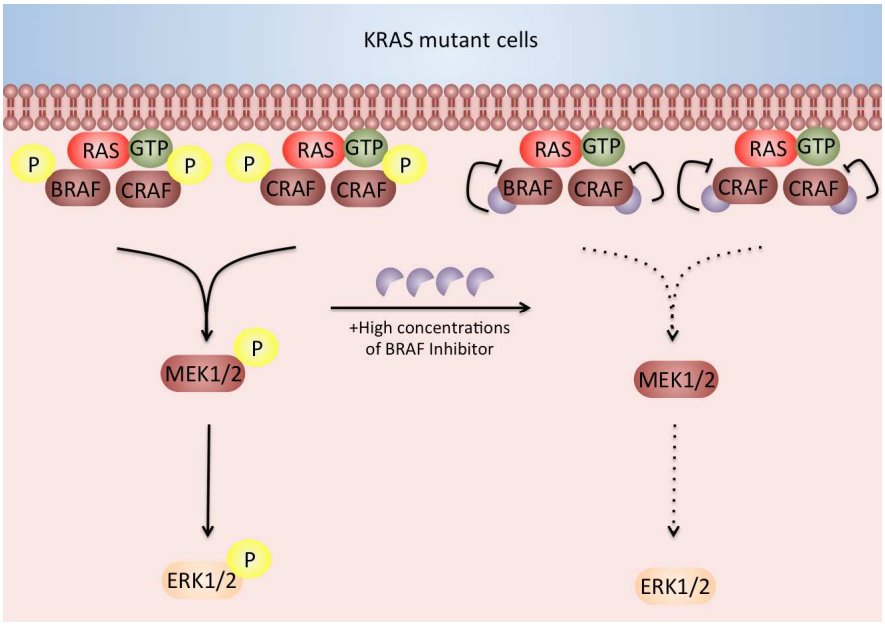

Figure 11. BRAF inhibition in KRAS mutant cells at high concentrations of the inhibidor. Adapted from Molina-Arcas and Downward, 2012.

On the other hand, BRAFV600E melanomas are dependent on high levels of ERK-dependent negative feedback loops[377] that turn cells insensitive to extracellular ligands. When these cells are treated with BRAF inhibitor and ERK is inhibited, the ERK-dependent negative feedback is lost, and the ability of ligands to activate signaling is enhanced.

Work from different groups has demonstrated that RAF inhibitor resistance is achieved by RAS mutation[376] or CRAF overexpression[378]. The clinical relevance of this mechanism has been shown in at least one patient[379]. Moreover, acquired resistance to vemurafenib (a RAF inhibitor) in BRAFV600E melanomas can be achieved by the expression of a smaller BRAFV600E transcript that results in a constitutively activated BRAFV600E dimer rather than the monomer found in the parental cells[380].

Recently it was demonstrated that activation of other pathways can induce resistance to Raf inhibition in BRAF-mutant melanoma such as activation of MAPK and PI3K pathways by stromal secretion of the hepatocyte growth factor (HGF)[381] or activation of IGF1 receptor (IGFR1)[382].

Resistance to MEK inhibitors

A patient with BRAFV600E mutant melanoma who had initially responded to the MEK inhibitor AZD6244 acquired resistance due to a MEK1 point mutation[383]. These point mutation severely attenuated MEK inhibitor ability to block ERK phosphorylation. It has been also identified selective amplification of the mutant BRAF allele as the mechanism underlying acquired resistance in two independent BRAF mutant CRC cell lines selected for resistance to the MEK inhibitor AZD6244[384,385].

Resistance to Tyrosine Kinase Inhibition

Despite the clinical response to EGFR Tyrosine Kinase Inhibitors (TKIs) in Non Small-Cell Lung Cancer (NSCLC) patients with mutations in the catalytic domain of EGFR, acquired resistance 6 to 12 months after the beginning of the therapy[386]. These patients with resistance to gefitinib or erlotinib had a secondary mutation in EGFR kinase domain[387–389], thus altering the specificity of the inhibitor for the ATP-binding pocket in EGFR. In fact, several studies have demonstrated that the mutation is present before the patient starts the therapy[390], suggesting that it may confer survival advantage to the tumor and is selected while the patient is being treated with EGFR TKIs[390–394].

It has also been demonstrated that EGFR variant III (EGFRvIII), a variant that lacks the ligand-binding domain, may contribute to gefitinib resistance[395]. Another mechanism explaining the intrinsic or acquired resistance is what is termed as 'oncogenic shift', that occurs when other membrane-bound RTK signaling pathways are contributing to the resistance[396].

On the other hand, amplification of *MET*, which prolonged the activation of HER3/PI3K/AKT axis, contribute to gefitinib and erlotinib resistance[397].

Humanized antibodies are another useful strategy to block the activation of some transmembrane receptors such as TNFR or EGFR. In CRC it has been described an acquired mutation in EGFR that prevents cetuximab binding and confers resistance to cetuximab treatment[398]. This mutant still retain binding to a different anti-EGFR antibody, panitumumab.

4. Crosstalk between NF-κB pathway and KRAS pathway

RAS induces NF-κB activation that is essential for different types of KRAS mutated cancers. Original studies showed that activated forms of RAS or RAF resulted in the activation of transcription specifically through κB sites[399]. Some years later, the same group demonstrated that oncogenic forms of HRAS activate NF-κB, not through induced nuclear translocation, but rather through the activation of the transcriptional activity of p65[400] and inhibition of NF-κB by IκBα expression blocks focus formation in NIH-3T3 cells induced by oncogenic RAS, indicating that RAS transformation required NF-κB. One explanation was that oncogenic RAS initiate a p53-independent apoptotic response that was suppressed through the activation of NF-κB[401]. RAS-transformed cells are susceptible to apoptosis

even when p53 tumor-suppressor is absent, and require NF-κB activation to avoid apoptosis.

In RAS- or RAF-transformed rat liver epithelial cells, NF-κB is activated through the canonical phosphorylation and degradation of IκBs [402]. This NF-κB activation by RAS appeared to be mediated by both IKKα and IKKβ, while IKKβ mediated RAF-induced NF-κB activation.

However, the role of NF-κB in transformation is still controversial. Both p65 and c-Rel enhance the frequency of RAS-induced cellular transformation in murine fibroblasts, however these subunits were not essential for this process [403]. Latter it was demonstrated that NF-κB was required for the development of tumors in a mouse model of lung adenocarcinoma[404]. Loss of p53 and expression of oncogenic KRAS resulted in NF-κB activation, whereas p53 restoration led to NF-κB inhibition. Inhibition of the NF-κB pathway resulted in significantly reduced tumor development. In a similar way, and also in lung cancer, it was demonstrated that deletion of p65 reduces the number of KRAS-induced tumors[405]. In a similar way, IKKβ depletion in a lung-cancer mouse model significantly attenuated tumor proliferation and prolonged mouse survival[406].

Although elevated levels of RAS activity are critical for RAS-induced tumorigenesis, the pathological threshold of RAS activity is unclear, since RAS mutations are also present in healthy individuals. In this sense, it was recently shown that in the presence of oncogenic RAS, inflammation initiates a positive feedback loop involving NF-κB that further amplifies RAS activity to pathological levels[407]. The effects of these inflammatory stimuli were disrupted by deletion of IKKβ or Cox-2.

KRAS-dependent activation of NF-κB

How KRAS activates NF-κB is still not clear. An upstream activator of NF-κB, TRAF6, was identified in an analysis of lung cancer-associated chromosomal amplifications in NSCLC and small-cell lung cancer (SCLC)[408]. Inhibition of TRAF6 in human lung cancer cell lines suppressed NF-κB activation, anchorage-independent growth, and tumor formation.

In a mouse model for pancreatic cancer, IKKβ deletion inhibited NF-κB activation and pancreatic ductal adenocarcinoma development. From the mechanistic point of view, KRAS-activated AP-1 induced *IL-1α,* which in turn activated NF-κB and its target genes *IL-1α* and *p62,* to initiate IL-1α/p62 feedforward loop for inducing and sustaining NF-κB activity[409] (Figure 12).

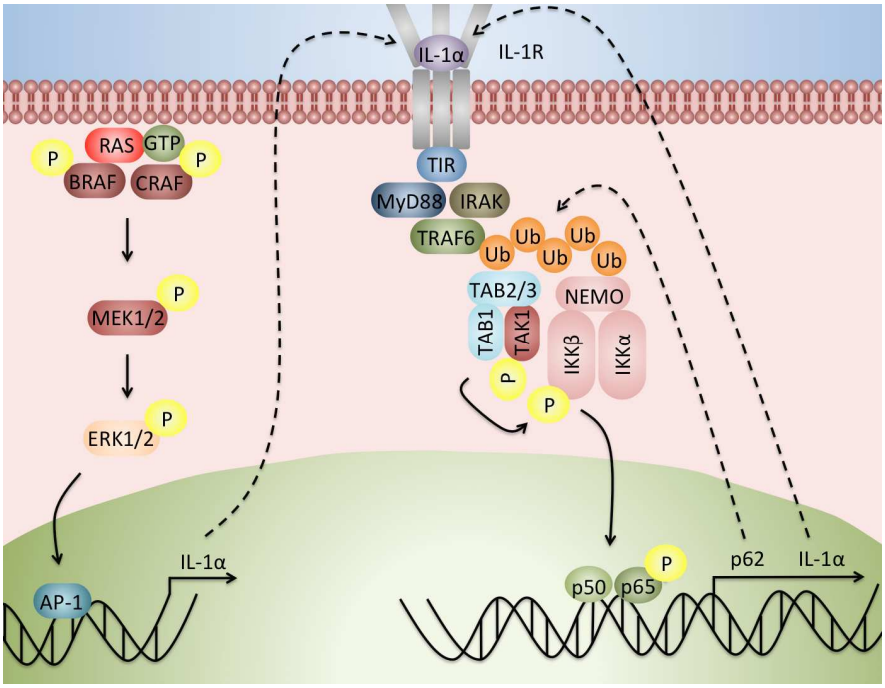

Figure 12. Crosstalk between RAS and NF-κB pathway

TAK1 was also required in APC-mutant, KRAS-dependent colon cancer cell lines for cell viability by secreting BMP-7 (downstream target of KRAS) thus inducing BMP/TGF-β signaling, leading to TAK1 activation and enhancement of Wnt-dependent transcription, and possibly NF-κB[410]. Moreover, mutant KRAS upregulates Glycogen Synthase Kinase 3α (GSK-3α), which then interacts with TAK1 leading to the stabilization of the TAK1-TAB complexes that promote IKK activity[411]. In addition, GSK-3α is required for promoting critical non-canonical NF-κB signaling in pancreatic cancer cells, and pharmacological inhibition of GSK3 suppresses growth of human pancreatic tumor explants.

Notch signaling pathway has also been involved in KRAS-dependent NF-κB activation and synergizes with IKKβ to upregulate transcription of Notch target genes, such as *hes1* or *hey1*, in a KRAS-mutant mouse model of pancreatic cancer[412]. Mechanistically, NF-κB activation resulted in phosphorylation of histone H3 at the *hes1* promoter, and Hes1 suppresses expression of Pparg, an anti-inflammatory nuclear receptor.

Most of the mechanisms reported in the literature involve the secretion of cytokines that induce canonical NF-κB activation, but they cannot explain many examples of pathological NF-κB activity downstream of K-ras. Understanding this association is of crucial importance for potential therapeutic applications.

OBJECTIVES

OBJECTIVES

Based on previous data from others and our group, our general objective was to study of the role nuclear active IKKα in CRC cells and use this information to identify new therapeutic targets for cancer therapy. This main objective has been divided in specific subjects:

1. To characterize the nuclear form of active IKKα protein
2. To determine how nuclear IKK is generated
3. To study the composition of a putative nuclear IKKα complex
4. To identify the mechanisms upstream of nuclear IKKα activation
5. To study the possibility to specifically inhibit nuclear IKKα

RESULTS

RESULTS: PART 1

A Truncated Form of IKKα Is Responsible for Specific Nuclear IKK Activity in Colorectal Cancer

Margalef, Pol; Fernández-Majada, Vanessa; Villanueva, Alberto; Garcia-Carbonell, Ricard; Iglesias, Mar; López, Laura; Martínez-Iniesta, María; Villà-Freixa, Jordi; Mulero, Mari Carmen; Andreu, Montserrat; Torres, Ferran; Mayo, Marty W.; Bigas, Anna; Espinosa, Lluís

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A novel truncated form of IKKα is responsible for specific nuclear IKK activity in Colorectal Cancer

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Running title: Novel nuclear function of truncated IKKα

Summary

Nuclear IKKα regulates gene transcription by phosphorylating specific substrates and it has been linked to cancer progression and metastasis. However, the mechanistic connection between tumorigenesis and IKKα activity remains poorly understood. We have now analyzed 288 human colorectal cancer samples and found a significant association between the presence of nuclear IKK and malignancy. Importantly, nucleus of tumor cells contains an active IKKα isoform with a predicted molecular weight of 45kD (p45-IKKα) that includes the kinase domain but lacks several regulatory regions. Active nuclear p45-IKKα forms a complex with non-active IKKα and NEMO that mediates phosphorylation of SMRT and Histone H3. Proteolytic cleavage of FL-IKKα into p45-IKKα is required to prevent apoptosis of CRC cells in vitro and to sustain tumor growth in vivo. Our finding identify a new potential druggable target for treating patients with advance refractory CRC.

Highlights

A novel truncated active form of IKKα is found in colorectal cancer cells.

Nuclear complex containing p45-IKKα phosphorylates SMRT and histone H3.

Cleavage of IKKα into p45-IKKα is required for cancer cell growth.

Introduction

NF-κB is a transcription factor that regulates innate and acquired immune responses, inflammation and cancer (Hayden and Ghosh, 2004; Hayden et al., 2006; Schulze-Luehrmann and Ghosh, 2006). In the absence of stimulation, NF-κB dimers (such as p65/p50) are primarily cytoplasmic and bound to the inhibitor of κB (IκB). Induction of canonical NF-κB pathway by specific stimuli such as TNFα, bacterial and viral products, or DNA damage, leads to the activation of the IKK (IκB Kinase) complex that phosphorylates IκB inducing its degradation and nuclear translocation of the NF-κB factor. The IKK complex is composed of two catalytic subunits, IKKα/IKK1 and IKKβ/IKK2, and the regulatory IKKγ/NEMO (for NF-κB Essential Modifier). Molecular weight of IKKα and IKKβ is 85 and 87kD respectively, and they share 50% of amino acid identity and 70% of structural similarity. IKKα and β contain an amino-terminal kinase domain (KD), a leuzine zipper (LZ) region involved in protein dimerization and a Helix-loop-helix (HLH) (DiDonato et al., 1997; Zandi et al., 1997). Recently, LZ and HLH regions of IKK have been re-defined based on structural data of IKKβ (Xu et al., 2011). It is established that IKKβ and NEMO are essential mediators of IκB degradation and canonical NF-κB activation. Conversely, activation of IKKα by LTβ, CD40 or BAFF induces processing of p100 into p52. Then, p52/RelB dimers translocate to the nucleus and activate specific gene transcription. This signaling pathway, known as alternative NF-κB, is required for secondary lymphoid organogenesis (Senftleben et al., 2001).

In addition to its cytoplasmic functions, nuclear roles for IKKα have recently been identified such as binding to the chromatin of specific promoter regions to phosphorylate serine10 of histone H3, which affects chromatin condensation and facilitates transcriptional activation of NF-

κB-dependent and independent genes (Anest et al., 2004; Anest et al., 2003; Park et al., 2005; Yamamoto et al., 2003). Nuclear IKKα also regulates cell cycle progression through phosphorylation of the AuroraB kinase (Prajapati et al., 2006) and derepression of 14-3-3σ (Zhu et al., 2007b). In cancer cells, chromatin-bound IKKα activates the metastasis-related gene, *maspin*, through epigenetic modifications (Luo et al., 2007) and associates with other factors such as Notch to regulate specific transcription (Hao et al., 2010; Song et al., 2008; Vilimas et al., 2007). Moreover, IKKα activates *cIAP2* and *IL-8* transcription through phosphorylation of the nuclear corepressor SMRT at serine 2410, which induces its cytoplasmic export and degradation (Hoberg et al., 2004). We previously demonstrated that Colorectal Cancer (CRC) cells contain nuclear IKKα, which phosphorylates SMRT and N-CoR leading to the activation of Notch-target genes *hes1* and *herp2* (Fernandez-Majada et al., 2007a; Fernandez-Majada et al., 2007b). This is important since Notch activity is required for CRC progression (Fre et al., 2005; van Es et al., 2005). Interestingly, inhibition of IKK activity reverted Notch-target gene expression and reduced tumor xenografts growth in nude mice (Fernandez-Majada et al., 2007a; Fernandez-Majada et al., 2007b). However, IKK activity is essential for multiple physiological functions including regulation of immune response, differentiation of lymph nodes, mammary gland and skin and maintenance of liver and gut homeostasis (Luedde et al., 2007; Nenci et al., 2007; Pasparakis et al., 2002), and consequently, cannot be inhibited without producing severe undesirable effects (Chen et al., 2003; Greten et al., 2007; Maeda et al., 2005).

In this work we study the traits that distinguish CRC-related IKK functions from those associated with physiological NF-κB, which has critical implications for further identification of therapeutic druggable targets.

We show that nuclear localization of active IKKα is a common event associated with advanced human CRC, and identify a truncated form of IKKα (referred to as p45-IKKα) that represents the majority of active IKK in this cellular compartment. Truncated IKKα is generated by cathepsin activity, which is increased in CRC. At the biochemical level, active p45-IKKα is in a nuclear complex with NEMO, which specifically phosphorylates SMRT leading to specific gene transcription. Knocking down IKKα prevents growth of CRC cells both in vitro and in vivo, and this effect is rescued by shRNA-resistant IKKα but not by a cleavage-defective mutant. Together our results indicate the possibility to target p45-IKKα generation or activation as a novel strategy for CRC treatment.

Results

CRC cells contain a new nuclear form of IKKα of 45kD.

Activation of nuclear IKKα have been previously associated with human CRC and prostate metastasis in mouse (Fernandez-Majada et al., 2007a; Luo et al., 2007). We here analyze a total of 288 samples (147 adenomas and 141 carcinomas) and the corresponding paired distal normal mucosa from 98 patients and found that more than 60% of the adenomas and 85% of the carcinomas show high P-IKK staining (considering ++ plus +++ intensities) compared with adjacent normal tissues (19% positive), as detected by IHC with two antibodies recognizing phosphorylated serines 180/181 of IKKα and β, respectively (α-P-IKK, Cell Signaling #2681 and Santa Cruz sc-23470) (Figure 1A and 1B). Most of the positive samples showed strong punctuate cytoplasmic staining; in addition 30% of adenomas and 40% of carcinomas contain detectable levels of nuclear P-IKK (Figure 1A, 1C and 1D). Confocal microscopy confirmed the presence of nuclear P-IKK in 11 out of 12 randomly selected samples identified as

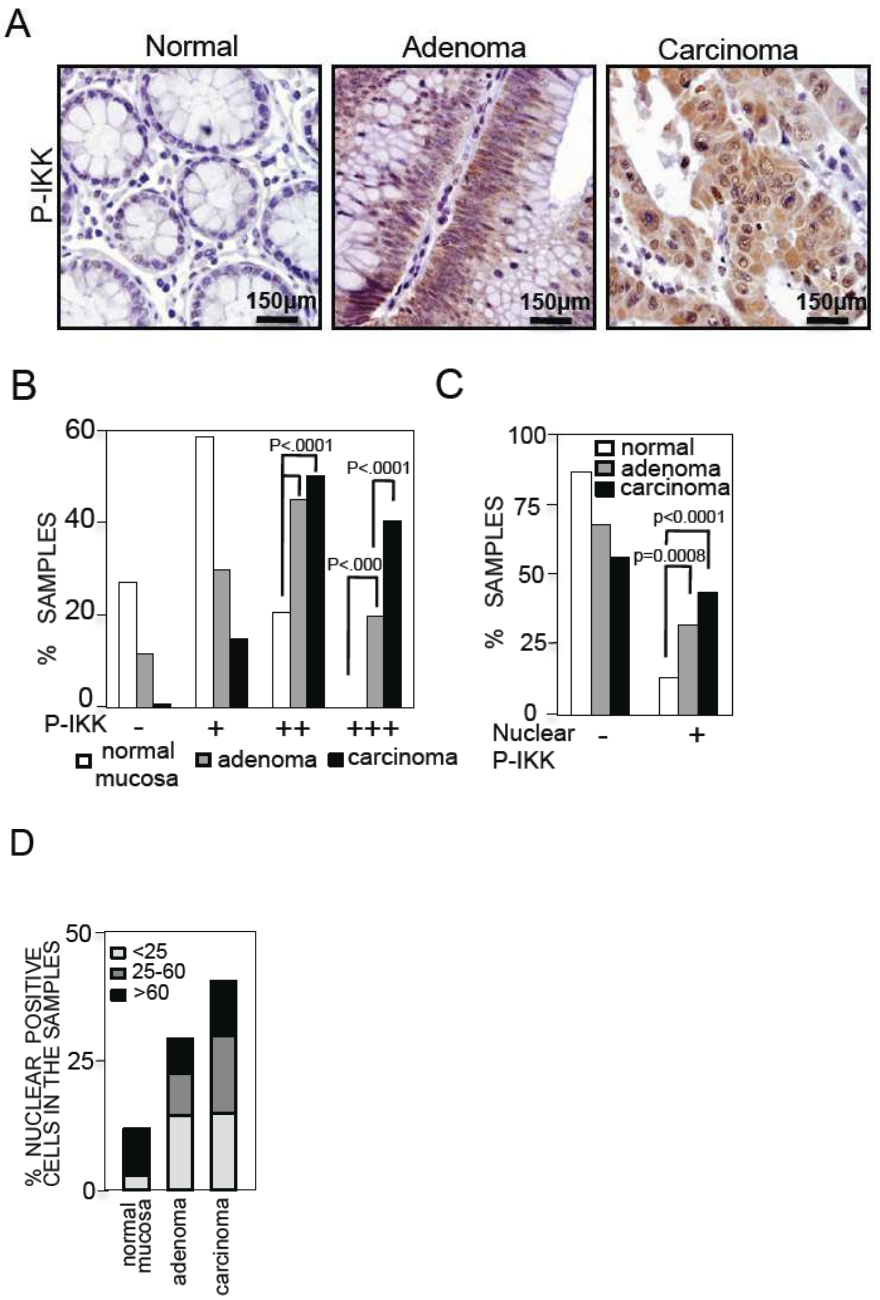


Figure 1. CRC cells express a truncated and active form of IKKα in the nucleus. (A) IHC with α-P-IKKα/βs from normal, adenoma and carcinoma samples (400x) from a human colon tissue micro-array including 288 tumor samples. (B) Normal mucosa, adenoma and CRC samples were classified based on intensity of α-P-IKKα/β staining. (C) Samples were

classified depending on the presence or absence of nuclear α-P-IKKα/β staining. In B and C, Fisher's exact test was used to determine p-values. (D) Percent of positive nuclei in samples with P-IKK staining.

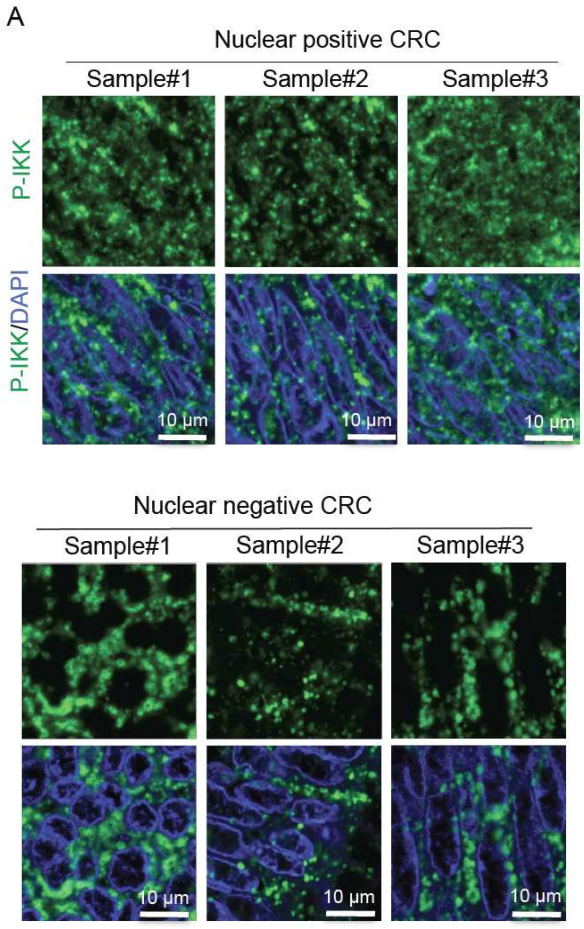


Figure S1. p45-IKK form corresponds to the IKKα homologue, Related to Figure1.

(A) IF with the α-P-IKK antibody (Cell Signaling #2681) visualized by confocal microscopy in three representative samples classified in our screening as nuclear positive or nuclear negative.

nuclear positive in the general screening, and its absence in 12 out of 12 negative samples (96% correspondence). A more detailed analysis revealed that, even in the positive tumors, most of P-IKK was localized in cytoplasmic vesicles, being the nuclear staining restricted to discrete dots (Figure S1A and data not shown). To further investigate the nature of tumor-associated nuclear IKK, we performed subcellular fractionation followed by western blot analysis of fresh CRC samples and normal adjacent mucosa (N) from 4 different patients and 2 human carcinomas grown as xenograft in nude mice (CRCX). We found that most of the active P-IKK corresponding to the expected size of 85-87kD was localized in the cytoplasm of tumor and normal cells. Unexpectedly, the same antibody recognized a double band of around 45kD that was highly enriched in both the cytoplasmic and nuclear fractions of carcinoma cells (from now on p45-IKK) (Figure 1E). Active p45-IKK was also detected in human CRC cell lines using two different α-P-IKK antibodies (Cell Signaling #2681 and #2697) (Figure 1F) and from extracts obtained in the presence of specific protease inhibitors (Figure S1B, lanes 2 and 3) or directly boiled in 1% SDS sample buffer and electrophoresed without further manipulation (Figure S1B, lane 4). α-IKKα antibody, but not α-IKKβ, recognized bands compatible with p45-IKK in all tested CRC cells (Figure 1F) and human CRC samples (Figure 1E), suggesting that p45-IKK was an isoform of IKKα. Of note that non-phosphorylated p45-IKK was also detected in the cytoplasm of non-transformed cells (Figure 1E). To further demonstrate that p45-IKK was a product of IKKα, we performed knockdown experiments in HCT116 and Ls174T cells, followed by western blot analysis. We found that different shRNAs targeting IKKα reduced both the 85kD and the 45kD bands detected with α-IKKα antibody from total (Figure 1G) and nuclear extracts (Figure S1C). In

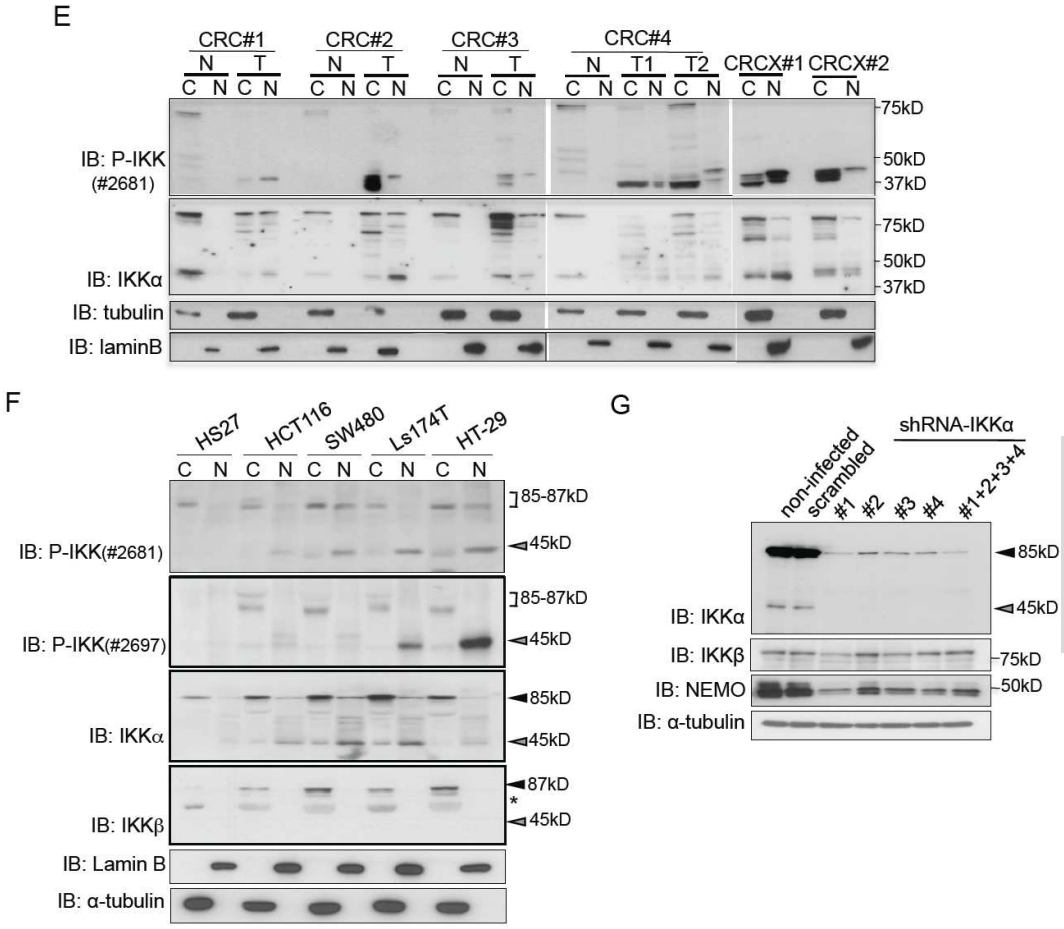


Figure 1. CRC cells express a truncated and active form of IKKα in the nucleus. (E) Western blot analysis of cytoplasmic and nuclear extracts from human CRC samples and their normal adjacent tissue and two human CRC samples grown as xenografts in nude mice. (F) Western blot analysis of cytoplasmic and nuclear extracts from different CRC cell lines (HCT116, SW480, Ls174T, HT-29) and non-transformed cell line HS27. (G) Western blot analysis of total cell extracts from HCT116 cells transduced with shRNA against IKKα or with a scrambled shRNA. IKKβ levels are shown to test isoform specificity. In E, F and G, tubulin and lamin B were used as fractionation and loading controls. Cytoplasmic, C; Nuclear, N.

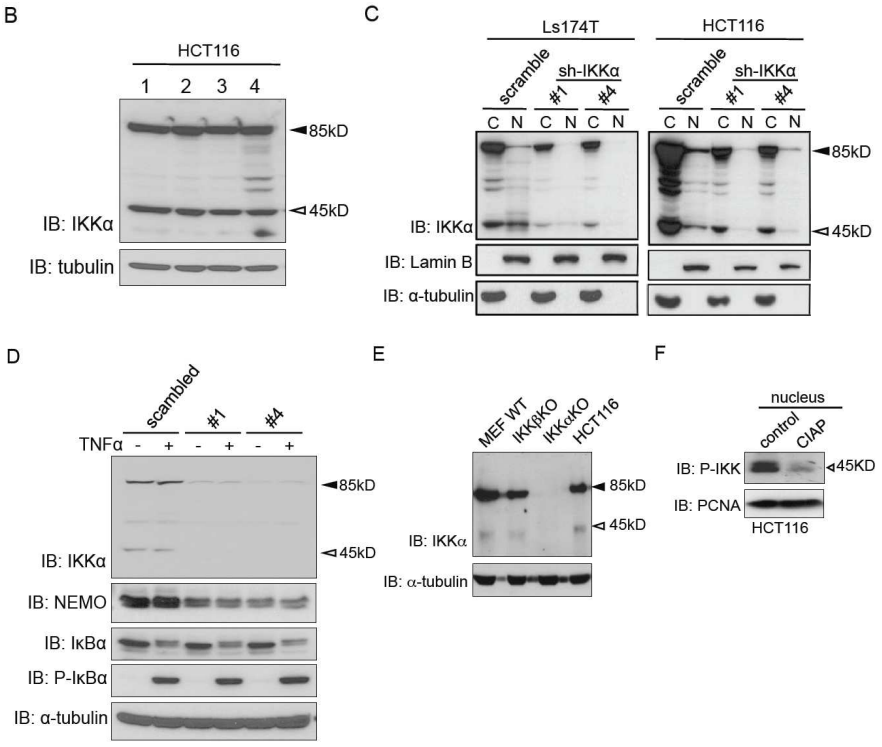


Figure S1. p45-IKK form corresponds to the IKKα homologue, Related to Figure1. (B) HCT116 cells were centrifuged at 1000 rpm and lysed in lysis buffer (lane 1), lysis buffer plus pan caspase inhibitor Z-VAD-FMK (lane 2), plus the Cathepsin inhibitor Z-FA-FMK (lane 3) or directly in 1% SDS-containing Laemmli buffer (lane 4) and analyzed by western blot. (C) Cytoplasmic and nuclear extracts from Ls174T and HCT116 cells transduced with the indicated shRNAs were analyzed by western blot for the presence of IKKα and p45-IKKα. LaminB and tubulin are shown as fractionation and loading controls. (D) Western blot analysis of P-IκBα from HCT166 cells transduced with the indicated shRNAs and treated 20min with TNFα when indicated. (E) Western blot analysis using the α-IKKα antibody (OP133) from wildtype MEFs or MEFs knockout for IKKα or β, run together with cell lysates from HCT116 cells. Of note, IKKα knockout cells lack both the 85kD and the 45kD bands. (F) Lysates from HCT116 cells were incubated with calf intestinal phosphatase (CIAP) during 30 minutes at 30ºC and lysates were analyzed by immunoblot for the expression of P-IKK.

contrast, IKKβ levels were not affected, demonstrating the specificity of the shRNA, although we observed a reduction of NEMO levels (Figure 1G and S1D) that did not affect canonical NF-κB activity as detected by IκBα phosphorylation after TNFα treatment (Figure S1D). Besides, we detected low levels of p45-IKKα in Mouse Embryonic Fibroblasts (MEF) that were absent from IKKα knockout (KO) MEFs (Figure S1E) supporting the IKKα nature of p45. Importantly, antibodies recognizing phosphorylated IKK failed to detect this band (data not shown), as in normal human samples (Figure 1D), suggesting that p45-IKKα is inactive in non-transformed cells. Further demonstrating that p45-IKKα is phosphorylated in CRC, treatment of HCT116 nuclear extracts with calf intestinal alkaline phosphatase (CIAP) abrogated detection of p45-IKK by α-P-IKK (Figure S1F). These results indicate the existence of a new IKKα species with an apparent size of 45kD that represents the majority of active IKK in the nucleus of CRC cells. Conversely, non-transformed cells contain non-phosphorylated p45-IKKα, which is localized in the cytoplasm.

Generation of the nuclear p45-IKKα form present in CRC cells

We tested the possibility that high levels of p45-IKKα found in CRC cells were due to mutations in the IKKα sequence that translate into a truncated protein. Because of its inferred molecular size, we focused on studying the region involving exons 13 to 15 of IKKα, which was found to be mutated in Squamous Cell Carcinomas (Liu et al., 2006). First, we performed Single Strand Conformational Polymorphism (SSCP) analysis of this region, amplified using intronic primers from genomic DNA corresponding to 161 human CRC samples (5 stage I, 52 stage II, 83 stage III and 21 stage IV). In this screening, we did not detect any alteration

beyond a single nucleotide change in an intronic region of sample 515T (Figure S2A and data not shown). By direct sequencing we confirmed the absence of mutation in this region using genomic DNA from HCT116, HT-29 and SW480 cells and 16 human CRC samples. Interestingly, analysis of the cDNA identified a deletion of exon 14 in one of the samples that led to a frame-shift that generates a premature STOP codon at position 520 (data not shown). Together this data indicates that mutation of the IKKα gene is not the main mechanism contributing to p45-IKKα generation.

Next we investigated whether p45-IKKα was produced by FL-IKKα processing. With this objective we transduced HEK-293, HCT116 and HT-29 cells with a retroviral vector codifying for FL-IKKα fused to the myc epitope at the N-terminal end (myc-IKKα). Western blot analysis of the lysates demonstrated the presence of the expected 85kD IKKα protein, in addition to a 45kD that was recognized with the α-myc antibody (Figure 2A). In HEK-293 cells, myc-IKKα generated a pattern of bands that was identical to the endogenous IKKα from CRC cells (Figure S2B), although p45 was more prominent in CRC cells. Identity of the 45kD band was further established by precipitation of HA-IKKα with the α-HA antibody followed by detection with specific α-IKKα antibody generated against its N-terminal end (Figure 2B) and by conventional mass-spectrometry analysis of the tryptic peptides (data not shown). Generation of p45-IKKα from HA-IKKα was not modified by mutation of serines 176 and 180 to alanine (S176/180A) or glutamic acid (S176/180E) indicating its activation status was not necessarily linked with cleavage (Figure S2C). These results indicate that FL-IKKα generates p45-IKKα, which comprises the N-terminal kinase and the Ubiquitin-like domain (ULD) but lacks the regulatory Scaffold/Dimerization domain (SDD) (Figure 2C). Since IKK exhibits a conformation that involves the physical interaction of SDD with

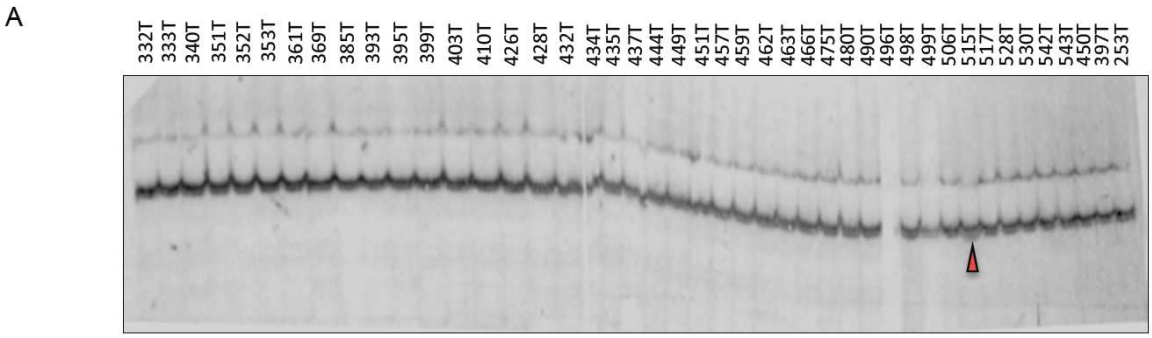


Figure S2. p45-IKK is generated by processing of full-lenght IKKα, Related to Figure2. (A) Presence of point mutations in exons 13-15 of IKKα gene was analyzed by PCR-single-strand. Representative gel including the tumor sample in which intronic mutation was identified (arrow head) conformation polymorphism in three independent reactions using intronic primers (primers and PCR conditions are available upon request). PCR products were diluted 1:16 in formamide-dye loading buffer, incubated for 3 min at 95ºC, cooled on ice and loaded onto 6% polyacrylamide nondenaturing sequencing gel. Electrophoresis was carried out at room temperature under 7 W for 10 to 12 hours. Gels were silver stained and vacuum dried at 85ºC. Shifted single-strand conformation polymorphism bands were excised from the gel, reamplified and sequenced.

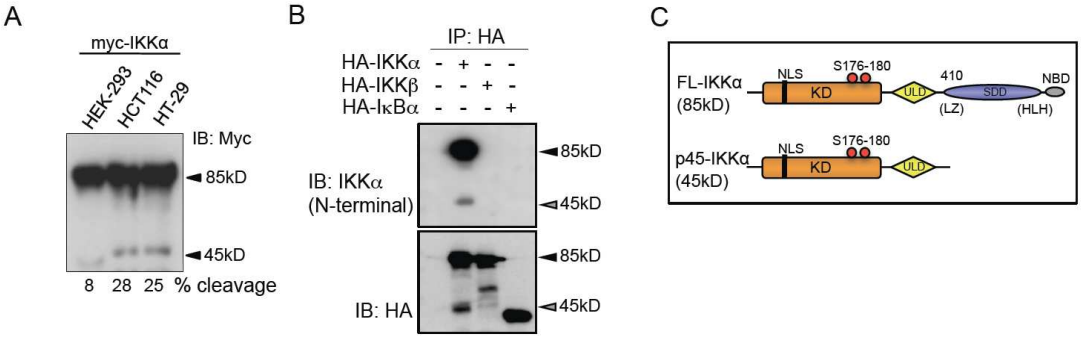


Figure 2. p45-IKKα is generated by proteolytic processing of the full length form and corresponds to the amino-terminal half of the kinase. (A) Cell lines were transduced with a retroviral vector expressing myc-IKKα. Total lysates were analyzed by western blot using an antibody against the myc epitope (9E10 clone). (B) HEK-293T cells were transfected with HA-IKKα, HA-IKKβ and HA-IκBα and 48h after transfection, cell lysates were immunoprecipitated with α-HA antibody and the precipitates analyzed by western blot with α-HA and α-IKKα antibodies. One of three independent experiments performed is shown. (C) Schematic representation of FL-IKKα that contains the kinase domain, the

ubiquitin-like domain (ULD), an the elongated α-helical scaffold/dimerization domain (SDD), including the regions previously characterized as Leuzine Zipper and Helix Loop Helix domains, and the NEMO-binding domain (NBD). Predicted truncated p45-IKKα includes the kinase domain but lacks the SDD and the NBD regions.

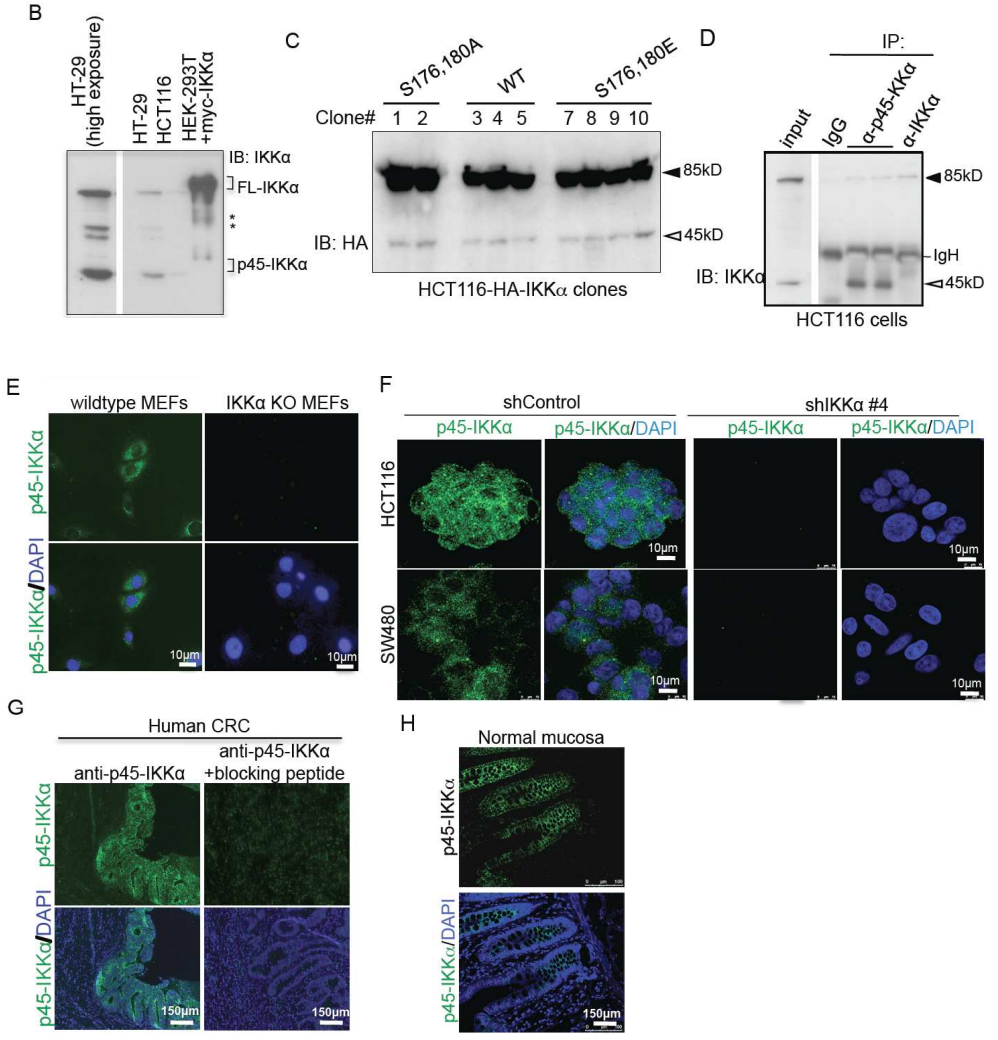
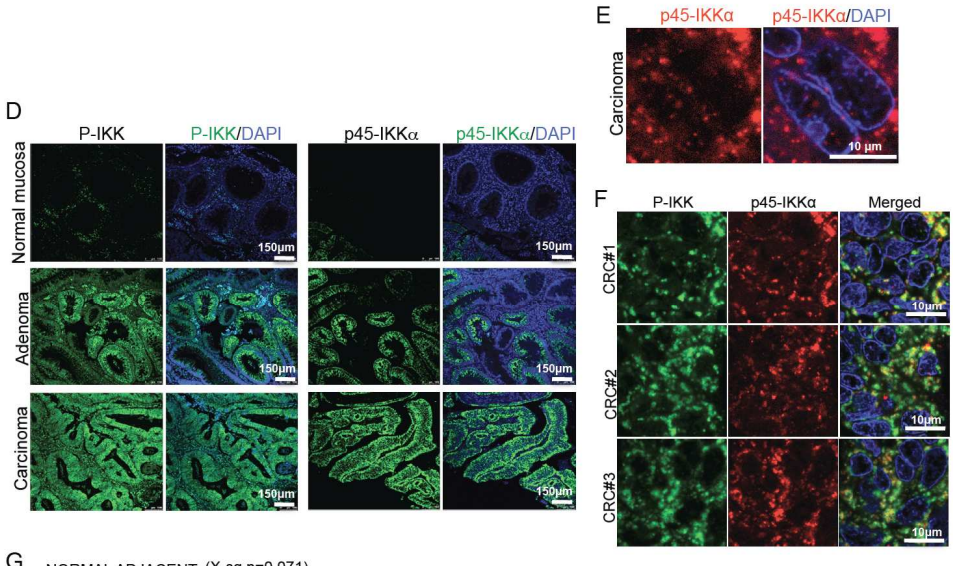


Figure S2. p45-IKK is generated by processing of full-lenght IKKα, Related to Figure2. (B) Similar cleavage pattern of the exogenous myc-IKKα expressed in HEK-293 and endogenous IKKα from HT-29 (higher exposure shown in the left side) and HCT116 cells. (C) Western blot analysis using the α-HA antibody of total extracts from HCT116 cells expressing wildtype HA-IKKα (3-5), a mutant with serines of the activation loop mutated to alanine (1-2) or both serines mutated to glutamate (7-10). (D) HCT116 cell lysates were

precipitated using a general anti-IKKα antibody, the monoclonal antibody anti-p45-IKKα (881H3) or the non-relevant IgG immunoglobulin as a control. Precipitated IKK was detected using the anti-IKKα antibody OP133. (E) Immunofluoresence of wildtype and IKKα knockout fibroblasts using the anti-p45-IKKα antibody 881H3. (F) Immunodetection of p45-IKKα in HCT116 and SW480 cells transduced with the indicated shRNA. (G) Immnostaining with the 881H3 antibody of human CRC samples incubated with or without the blocking peptide (used to generate the antibody). (H) p45-IKKα staining in a normal human colonic mucosa showing a preferential distribution in the bottom of the crypt.



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NORMAL ADJACENT (X-sq p=0.071)

P-IKK \ p45-IKKα	-	+	++	+++
-	27(25)	8(7)	10(12)	2(3)
+	1(3)	0(1)	3(2)	2(0)
++	1(1)	0(0)	1(1)	0(0)
+++	0(0)	0(0)	0(0)	0(0)

TUMOR SAMPLES (X-sq p=0.0001)

P-IKK \ p45-IKKα	-	+	++	+++
-	71(62)	11(9)	23(26)	15(23)
+	14(11)	0(2)	5(5)	3(4)
++	9(17)	3(3)	9(7)	13(7)
+++	0(4)	0(1)	2(2)	5(1)

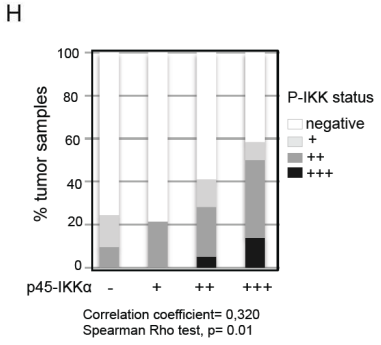


Figure 2. p45-IKKα is generated by proteolytic processing of the full length form and corresponds to the amino-terminal half of the kinase. (D) Serial sections of human intestinal mucosa, adenoma and carcinoma samples were stained with α-P-IKK and anti-p45-IKKα (881H3) antibodies, and visualized by confocal microscopy. (E) Detail of nuclear

and cytoplasmic p45-IKKα staining in one CRC sample. (F) Colocalization of p45-IKKα with P-IKK staining in 3 different CRCs. (G) A group of 183 human colon tumors and 55 normal mucosas, previously characterized for P-IKK, were analyzed by IHC for the presence of p45-IKKα. Tables represent the distribution of p45-IKKα staining in samples categorized as negative or positive (+, ++ and +++) for P-IKK. Statistical analysis demonstrated that P-IKK and p45-IKK expression was distributed randomly in the normal mucosa (X-square test, p=0.07) but strongly associated in the CRC samples (p=0.0001). (H) Spearman Rho test demonstrated a linear positive correlation between P-IKK and p45-IKK levels, which reached statistical significance (p=0.01).

the ULD and kinase domains (Xu et al., 2011), we predicted that p45-IKKα might differentially expose the regions close to the cleavage site compared with FL-IKKα. Based on this, and to further study the distribution and prevalence of p45-IKKα in human samples, we generated monoclonal antibodies against peptide AA241-424 of human IKKα, and tested for their capacity to precipitate p45-IKKα from non-denatured CRC cell lysates. Among these antibodies, we obtained the clone 881H3 that preferentially binds p45-IKKα from tumor cell lysates compared with the full length (FL) non-denatured form (Figure S2D), and generated a specific staining pattern in WT MEF that was not detected in IKKα KO cells (Figure S2E). In CRC cells, staining with the 881H3 antibody was found in the nucleus and cytoplasm and was lost in cells treated with sh-RNA against IKKα (Figure S2F). Thus, we selected 881H3 to study the distribution of p45-IKKα by IHC in human samples. In CRC samples 881H3 generated a specific staining pattern that resembled active P-IKKα including cytoplasmic vesicles and discrete nuclear dots (Figure 2D and 2E). By double IHC and confocal microscopy analysis we detected a substantial colocalization of p45-IKKα with P-IKK in CRC (Figure 2F). As a control, staining with 881H3 in CRC was lost after incubation with the blocking peptide (Figure S2G). Positive staining for p45-IKKα was also detected in normal colonic mucosas previously categorized as negative

for P-IKK, mainly restricted to the proliferative basal regions (Figure S2H). By crossing our data from active IKK (P-IKK) and p45-IKKα expression, we found that detection of p45-IKKα levels was independent of P-IKK status in the normal mucosa but it was associated in the CRC group of samples (Figure 2D and 2G). A more detailed study of the correlation between p45-IKKα and P-IKK staining demonstrated that p45-IKKα levels significantly and positively correlated with P-IKK reactivity in CRC samples (Figure 2H).

Cathepsin-mediated processing generates p45-IKKα in vitro.

To identify putative proteases that mediate IKKα processing, we created a Python script and queried for candidates using the information in the MEROPS peptidase database (Rawlings et al., 2010). This search was performed using the region including exons 13-14 as a target sequence (AA300-450). We identified three putative cleavage sites for caspase 3/6/7, cathepsin B/L and cathepsin K that could account for the generation of p45-IKKα (Figure 3A). To test whether these sites were functional, we designed an expression vector containing the myc-tag fused to the AA300-450 IKKα fragment that includes all three sites. We found that this IKKα fragment expressed in HEK-293 cells generated a 25kD band when incubated at acid pH (optimal for cathepsin activity), which was further increased after incubation with nuclear extracts from Ls174T CRC cells (Figure 3B). Treatment with z-FA-FMK, a specific inhibitor of cathepsin B and L (Sillence and Allan, 1997) but not with the caspase inhibitor z-VAD (not shown), significantly reduced IKKα processing in these experiments (Figure 3C). Next, we generated point mutants of the FL-IKKα construct to disrupt each of the identified

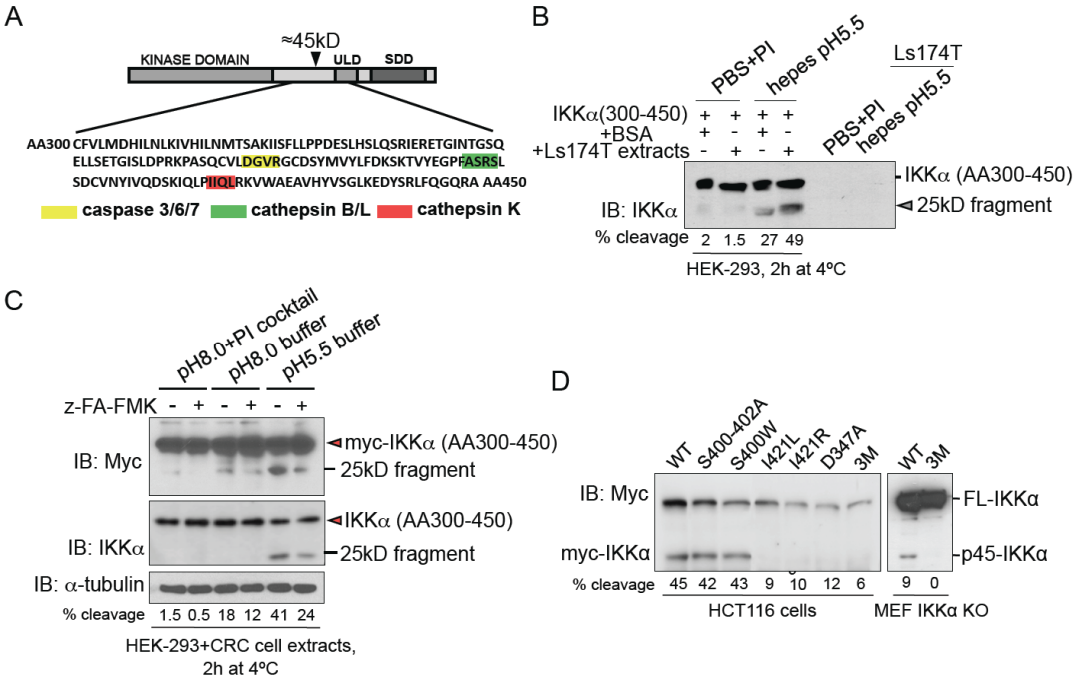


Figure 3. p45-IKKα is generated in vitro by cathepsin-mediated processing. (A) Bioinformatic analysis of the region including AA 300 to 450 of IKKα revealed the presence of putative protease cleavage sites for caspase 3/6/7, cathepsin B/L and cathepsin K. (B) HEK293T cells expressing the MT-IKKα 300-450 construct were lysed in PBS plus protease inhibitor cocktail (pH 8.0) or 10mM HEPES (pH 5.5). Cell lysates were incubated with two volumes of 100mg/mL BSA or Ls174T lysates for 2h at 4ºC and western blot analysis was performed. (C) HEK293T cells expressing MT-IKKα 300-450 WT were lysed in pH 8.0 plus protease inhibitor cocktail buffer, pH 8.0 buffer or pH 5.5 buffer with or without 20mM z-FA-FMK as indicated, in the presence of LS174T lysates, incubated for 2 hours at 4ºC and analyzed by western blot. (D) HCT116 cells (left panel) or IKKα KO MEFs (right panel) expressing myc-IKKα WT or the indicated mutants were lysed in buffer containing protease inhibitors and analyzed by western blot. All the experiments were repeated a minimum of three times with comparable results. Percentage of cleaved IKKα relative to the FL or the AA300-450 fragment (determined by densitometry from a representative experiment) is shown at the bottom of each lane.

protease recognition sites individually and in combination. As shown in Figure 2A, we found that myc-IKKα expressed in HCT116 cells was efficiently processed into p45-IKKα, however single mutations in cathepsin or caspase sites, and the triple mutation of all three protease recognition sites (3M) reduced IKKα cleavage (from 45% in the WT to 9-10%, 12% and 6% in the mutants, respectively). Similarly, the IKKα-3M mutant failed to be processed in non-transformed cells (Figure 3D). These results suggest that all three sites might contribute to some extent to p45-IKKα generation. To further define the cleavage site for p45-IKKα generation, we performed a more detailed analysis of the mass spectrometry data obtained from the precipitated HA-p45-IKKα fragment. In these experiments we failed to detect most of the central region of the IKKα protein (were cleavage is predicted to occur) but we identified a single peak (extracted ion chromatogram) compatible with the expected end-terminal sequence generated from Cathepsin B/L cleaved IKKα protein after trypsin digestion (peptide TVYEGPFAS). In contrast, we did not detect any tryptic peptide with the characteristics of a Caspase- or Cathepsin K-processed IKKα fragment (not shown). Together these results indicate that p45-IKKα is generated by specific protease activity, most likely through cathepsins.

p45-IKKα is generated by cathepsin-dependent processing in vivo

To investigate the putative involvement of cathepsins in generating p45-IKKα in vivo, we ectopically expressed FL-IKKα alone or together with cathepsin B, L, K or caspase 3 in HEK-293 cells. We found that expression of either cathepsin homologue increased generation of p45-IKKα compared with cells transfected with control vector (Figure 4A) or caspase 3 (Figure S3A). In agreement with the possibility that cathepsins

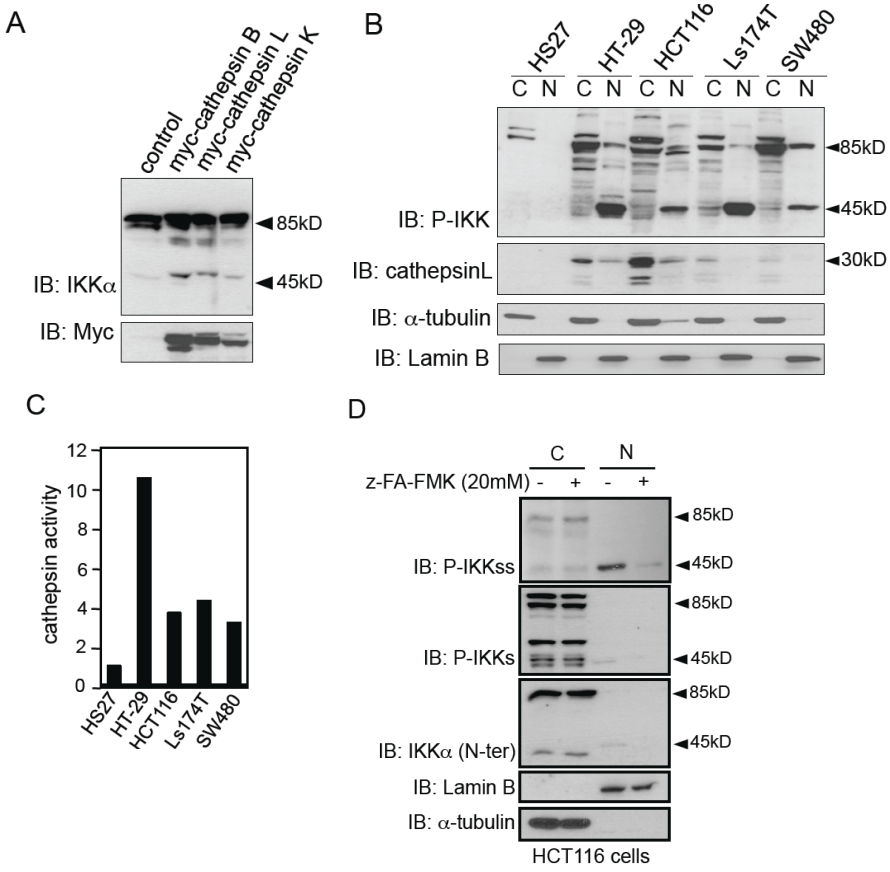


Figure 4. Cathepsin-B and L promote the formation of p45-IKKα *in vivo*. (A) 293T cells expressing MT-IKKα were cotransfected with control vector, MT-cathepsin B, MT-cathepsin L or MT-cathepsin K. 48h after transfection, cell lysates were obtained and analyzed by western blot to determine the levels of p45-IKKα. (B) Western blot analysis of nuclear and cytoplasmic extracts to determine Cathepsin L levels from the indicated cell lines. (C) Cathepsin activity from the indicated CRC cell lines was determined compared with the non-transformed HS27 cells. (D) HCT116 cell cultures were incubated for 16 hours with or without z-FA-FMK (20mM). Cytoplasmic and nuclear cell extracts were obtained and analyzed by western blot. In B and D, α-Lamin B and α-tubulin were used as fractionation and loading controls.

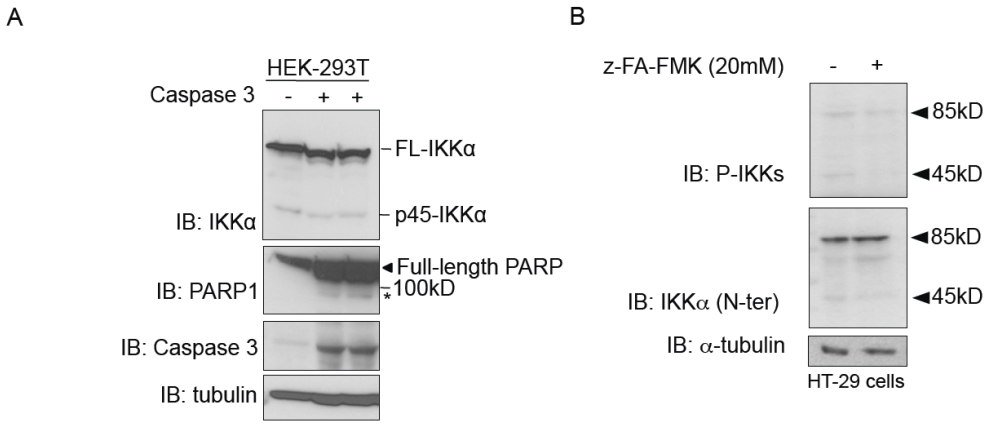


Figure S3. Processing of full-lenght IKKα is medaited by Cathepsins, Related to Figure4. (A) HEK-293T cells transduced with an expression vector for Caspase3 and analyzed by western blot for IKKα cleavage. Truncation of PARP1 (89kD band) is shown as a control for Caspase3 activity and indicated with and asterisk. (B) Western blot analysis of HT-29 cell cultures untreated or treated with the cathepsin inhibitor z-FA-FMK for 48h.

mediate IKKα cleavage in CRC cells, we found increased levels of cathepsin L (Figure 4B) and B (data not shown) in both nuclear and cytoplasmic lysates, associated with high cathepsin B/L activity (Figure 4C) in all tested CRC cell lines compared with non-transformed HS27 cells. However, cathepsin B/L activity does not strictly correlate with levels of active p45-IKKα in each individual cell line, indicating that cathepsin-mediated processing is a tightly regulated process. Most important, abrogation of cathepsin B/L activity by the pharmacological inhibitor z-FA-FMK mostly abolished formation of endogenous nuclear p45-IKKα in CRC cells (Figures 4D and S3B). By IF followed by confocal microscopy analysis, we detected high levels of cathepsin B and L in human primary CRC tumors that colocalized with P-IKK in specific ring-shaped cytoplasmic vesicles reminiscent of lysosomal or endosomal structures (Figure 4E and Figure S3C). In these vesicles P-IKK appeared

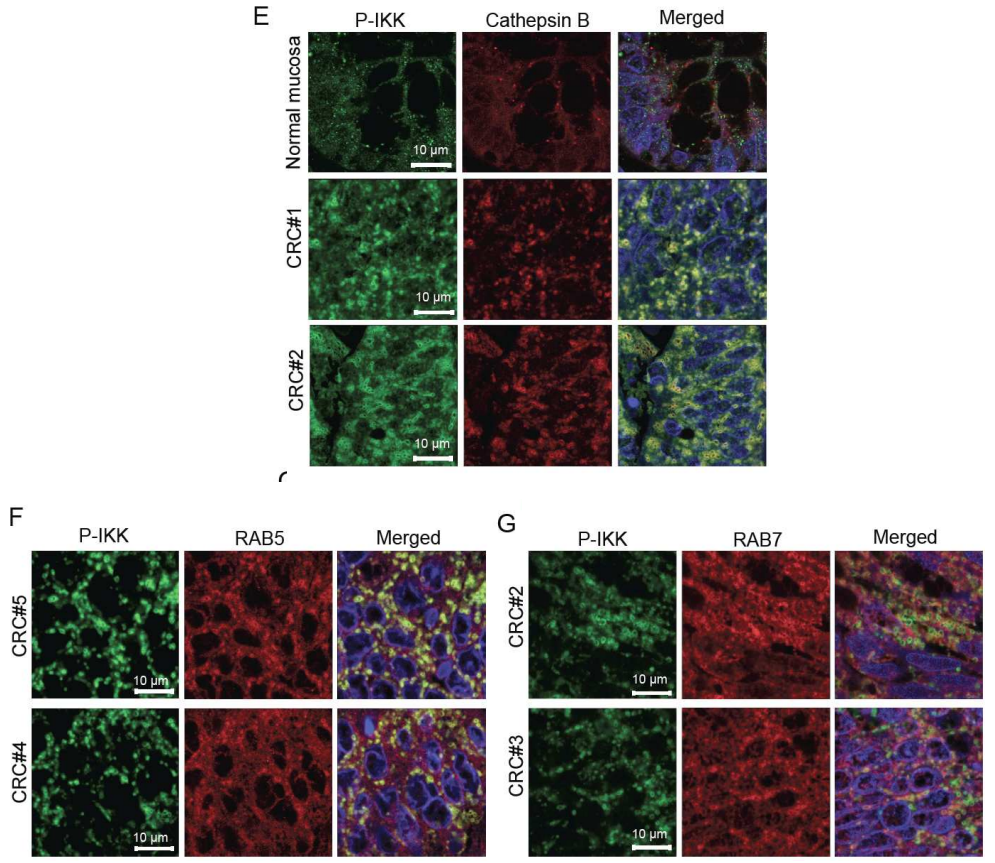
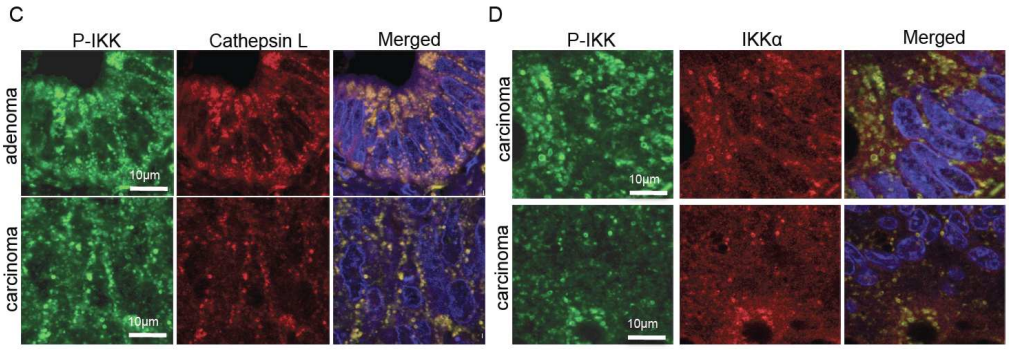


Figure 4. Cathepsin-B and L promote the formation of p45-IKKα in vivo. (E) Confocal images of double staining for P-IKK and cathepsin B in CRC samples. (F and G) Confocal images of double staining for P-IKK and the endosomal markers RAB5 (F) and RAB7 (G). Representative images were selected to illustrate the degree of colocalization between different proteins in CRC. Scale bars, 10 µM. All experiments were performed in triplicates.



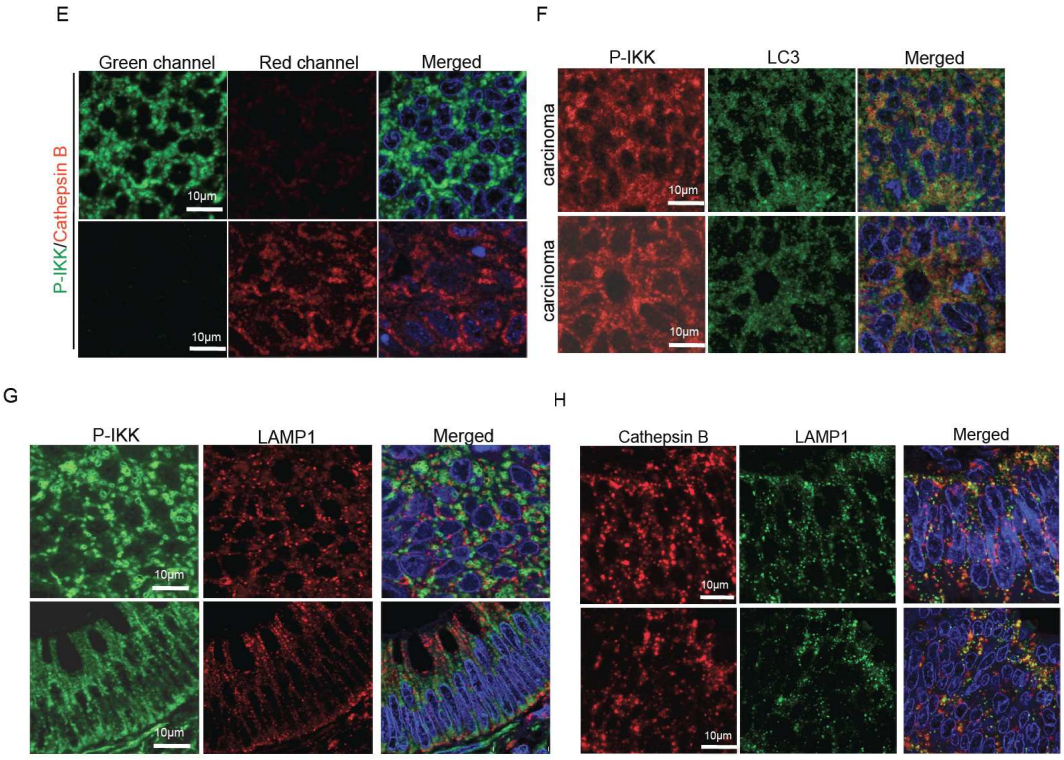


Figure S3. Processing of full-lenght IKKα is mediated by Cathepsins, Related to Figure4. (C) IF followed by Confocal Microscopy analysis of P-IKKα/β and Cathepsin L expression in two different tumor samples. (D) Confocal images showing the partial overlap between P-IKK staining and total IKKα. (E) Two different controls are shown to illustrate the absence of contamination between green and red channel in our analysis. Specifically, in the upper panels sample was processed as in Figure 4E without adding the α-cathepsin B antibody, whereas in the lower panels what is lacking is the α-P-IKKα/β antibody. (F-H) Double IF followed by confocal microscopy analysis of (F) P-IKKα/β and LC3, (G) P-IKK and LAMP1 and (H) Cathepsin B and LAMP1. All images were captured in the same conditions. Scale bars represent 10µm.

restricted to the membrane rings where it co-stains with the specific α-IKKα antibody (Figure S3D), when compared with the more central localization of cathepsins B or L. Different controls for cross-reaction of antibodies or cross-contamination of fluorochromes were performed (Figure S3E). Further characterization of these structures demonstrated that they contained RAB5, a small GTPase protein involved in trafficking of early endosomes (Poteryaev et al., 2010) (Figure 4F) and we found colocalization between P-IKK and some vesicles positive for the late endosomal marker RAB7 (Figure 4G). In contrast, P-IKK detection absolutely diverged from staining with the autophagosomal marker LC3 (Figure S3F) and the lysosomal marker LAMP1 (Figure S3G), excluding the possibility that in cancer cells active p45-IKKα is part of a degradation product. Of note that in these samples cathepsin B was only partially distributed in the lysosomal particles (Figure S3H).

Together these results indicate that cathepsin activity is mainly responsible for generating p45-IKKα in CRC cells and suggest that the interaction between IKKα and cathepsins takes place in specific endosomal vesicles. Sorting of IKKα into these vesicles might contribute to regulate IKKα processing.

Truncated IKKα displays specific biochemical properties

To study the biochemical characteristics of p45-IKKα, we performed gel filtration experiments in Superdex S200 columns from HCT116 (Figure 5A) and SW480 nuclear extracts (Figure S4A) and determined the distribution of active IKKα in the different fractions. We found that active/phosphorylated p45-IKKα co-eluted with the non-active FL-IKKα and NEMO in the high molecular weight (HMW) fractions (#19-25) (larger than 210kD) whereas FL-active IKKα was recovered in low molecular

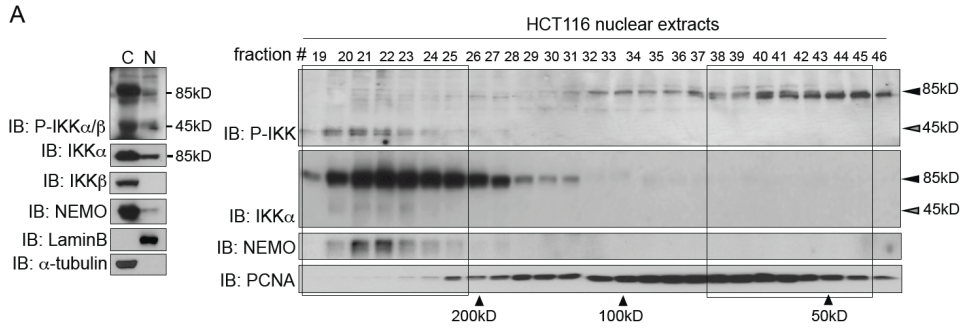


Figure 5. p45-IKKα associates with nuclear NEMO in CRC cells. (A) Western blot analysis of cytoplasmic and nuclear extracts from HCT116 cells using the indicated antibodies (left panel). 100µL of HCT116 nuclear extracts were loaded on a Superdex200 column. One drop (40µL approximately) per fraction was collected and analyzed by western blot with the indicated antibodies (right panel).

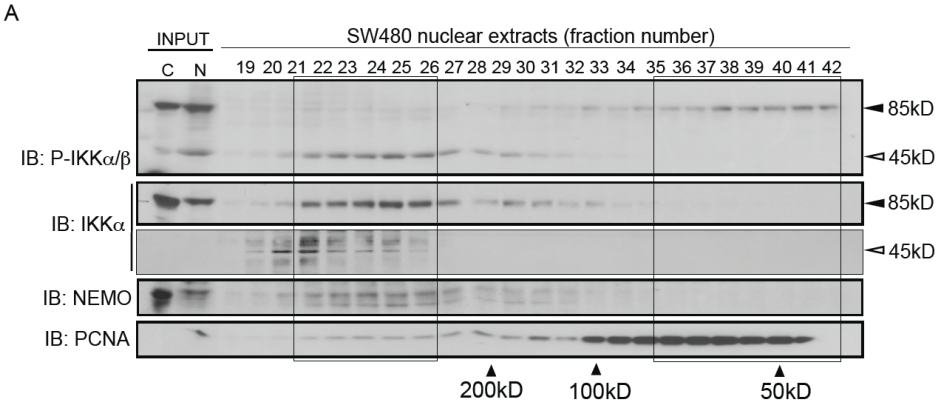


Figure S4. p45 forms a complex with NEMO and full-length IKKα in CRC cells, Related to Figure5. (A) Western blot analysis of SW480 nuclear fractions separated in a Superdex 200 column. Western blot analysis for P-IKK, IKKα and NEMO is shown. This experiment was repeated at least three times with identical results.

weight (LMW) fractions 38-45, likely corresponding to monomeric IKKα (Figure 5A and Figure S4A). Further suggesting that p45-IKKα was in a HMW complex with non-active FL-IKKα and NEMO, p45-IKKα expressed in IKKα KO MEFs eluted in intermediate molecular weight fractions 26-36 (Figure 5B, left panels) and partially shifted to HMW fractions 19-24

when FL-IKKα was re-introduced, co-eluting with exogenous IKKα and NEMO (Figure 5B, right panels). Existence of this complex was confirmed by precipitation of endogenous NEMO from fractionated nuclear HCT116 extracts (fractions #20-25) (Figure 5C) and total cell lysates (Figure S4B) and detection of both p45 and FL-IKKα in the precipitates. Different amounts of NEMO, FL-IKKα and p45-IKKα in the precipitates from the fractions suggest the existence of specific complexes with diverse stoichiometries. To study whether IKKβ participates in this complex we precipitated myc-p45-IKKα expressed in HEK-293T cells together with HA-IKKα or HA-IKKβ. We found that HA-IKKα but not HA-IKKβ associated with p45-IKKα in these conditions (Figure 5D).

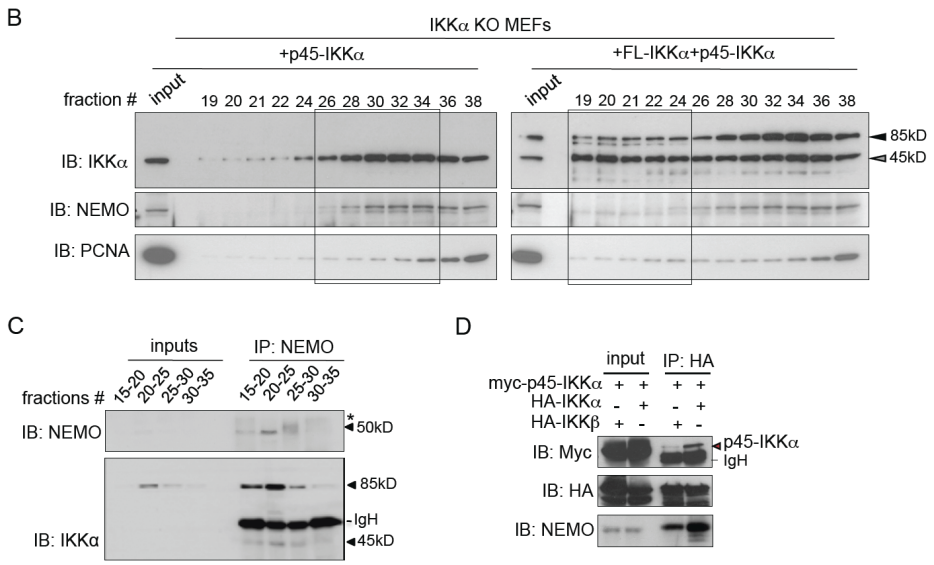


Figure 5. P45-IKKα associates with nuclear NEMO in CRC cells. (B) IKKα KO Mouse Embryonic Fibroblasts (MEF) were transfected with p45-IKKα (left panels) or p45-IKKα plus FL-IKKα (right panels). 72h after puromycin selection, whole cell extracts were obtained and lysates were loaded on a Superdex200 column. One drop per fraction was collected and analyzed by western blot with the indicated antibodies. (C) HCT166 nuclear extracts were fractionated in Superdex200 column and the indicated fractions were precipitated with the α-NEMO antibody. Western blot analysis demonstrated the

presence of endogenous FL-KKα (85kD) and p45-IKKα in the precipitates. The asterisk denoted a non-specific band in the western blot for NEMO. (D) 293T cells were transfected with HA-IKKα or HA-IKKβ and MT-p45-IKKα. 48h after transfection cell lysates were immunoprecipitated with α-HA. Western blot analysis showed that MT-p45-IKKα precipitates with HA-IKKα but not with HA-IKKβ.

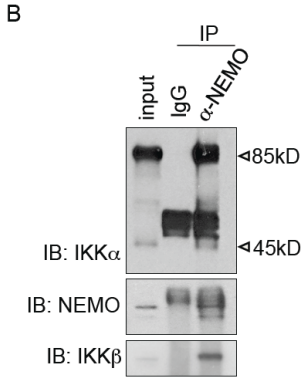


Figure S4. p45 forms a complex with NEMO and full-length IKKα in CRC cells, Related to Figure5. (B) Whole cell extracts from SW480 cells were immunoprecipitated with the α-IKKγ/NEMO antibody or the control IgG and tested for the presence of full-length or p45-IKKα in the precipitates.

Nuclear IKKα has been shown to be associated with the chromatin through histone H3 (Zhu et al., 2007a). By pull-down assays, we found that FL-IKKα but not p45-IKKα alone associates with histone H3 (Figure 5E), however p45-IKKα can bind histone H3 in the presence of FL-IKKα (Figure 5E). In agreement with these results we found that IKKα associates with histone H3 through its C-terminal region (Figure S4C). Importantly, ectopically expressed p45-IKKα localized essentially in the cytoplasm (although it retains the NLS sequence, see figure 2C) in the absence of endogenous IKKα, and was re-distributed into the nuclear and chromatin compartments in the presence of ectopic FL-IKKα, as shown by western blot from IKKα deficient MEFs (Figure 5F). However, treatment of these cells with the nuclear export inhibitor Leptomycin B resulted in

the accumulation of both FL-IKKα and p45-IKKα in the nucleus (Figure S4D), indicating that NLS is functional in p45-IKKα but its chromatin binding and nuclear retention require the participation of FL-IKKα.

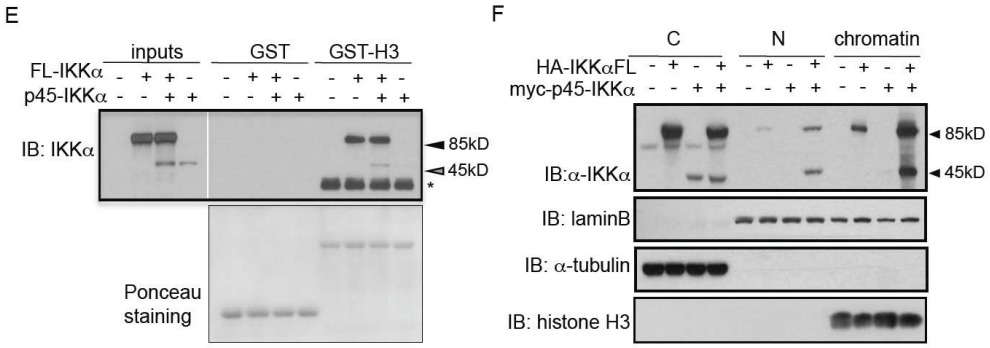


Figure 5. P45-IKKα associates with nuclear NEMO in CRC cells. (E) Pull-down assay with GST-H3 and cell lysates from 293T cells transfected with HA-FL-IKKα, HA-FL-IKKα plus MT-p45-IKKα, or MT-p45-IKKα alone. The presence of IKKα or p45-IKKα in the precipitates was determined by western blot. Ponceau staining of GST proteins is shown. Inputs represent 10% of the lysate. The asterisk indicates a non-specific band corresponding to GST-H3 that was detected with α-IKKα antibody. (F) IKKα deficient MEFs were transfected with control vector, HA-IKKα, MT-p45-IKKα or both constructs. Cytoplasmic, nuclear and chromatin fractions were obtained and analyzed by western blot with α-IKKα antibody. Levels of tubulin, laminB and histone H3 are shown as fractionation and loading controls. All experiments were repeated at least three times with comparable results.

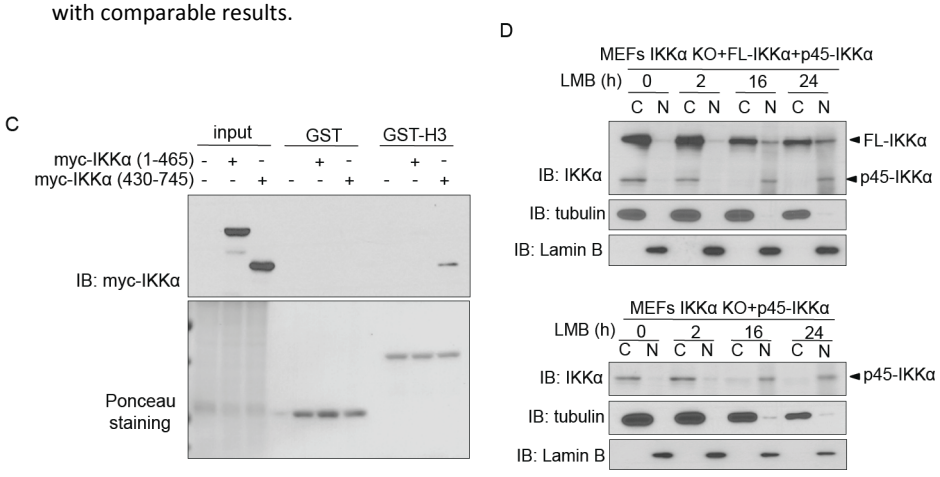


Figure S4. p45 forms a complex with NEMO and full-length IKKα in CRC cells, Related to Figure5.. (C) Pull down of the N-terminal or C-terminal half of IKKα using GST-H3 as bait. (D) Cell fractionation and western blot analysis of IKKα knockout MEFs reconstituted with the indicated IKKα constructs treated with LMB at the indicated time points. C, cytoplasmic; N, nuclear.

p45-IKKα promotes phosphorylation of specific substrates both in vitro and vivo.

We have previously shown that SMRT and N-CoR corepressors are substrates for IKKα kinase in CRC cells (Fernandez-Majada et al., 2007a; Fernandez-Majada et al., 2007b). To identify the nuclear fraction that contains this kinase activity, we performed immunoprecipitations of Superdex S200 fractions 19-25 (HMW) and 38-45 (LMW) from HCT116 nuclear extracts with either α-IKKα or α-P-IKK antibodies and assayed the capacity of the precipitates to phosphorylate GST-N-CoR or GST-SMRT fusion proteins. Precipitates obtained from the HMW fractions (19-25) containing active p45-IKKα, but not precipitates from fractions 38-45 (LMW) including active FL-IKKα (Figure 6A), phosphorylated both N-CoR (Figure 6B) and SMRT (Figure S5A) *in vitro.*

To further test whether p45-IKKα was involved in phosphorylating nuclear substrates *in vivo*, we transfected HA-FL-IKKα, truncated active MT-p45-IKKα or both constructs in IKKα KO MEFs and measured their effect on different substrates by western blot analysis. In agreement with the *in vitro* data, active p45-IKKα induced SMRT phosphorylation in

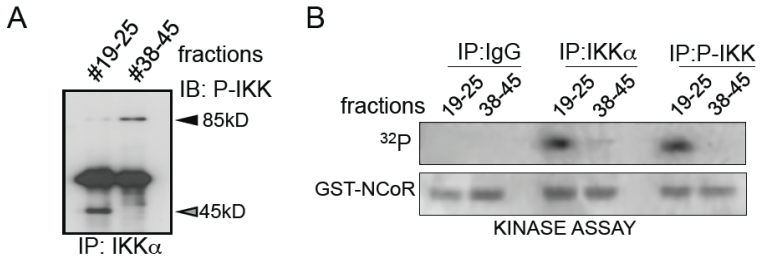


Figure 6. p45-IKKα promotes phosphorylation of specific substrates both in vitro and vivo. (A) α-IKKα precipitates from fractions 19-25 and 38-45 and western blot analysis to determine the presence of FL or truncated active IKKα. (B) Kinase activity of the α-IKKα and α-P-IKKα/β precipitates from Superdex200 fractions was assayed on GST-NCoR (amino acids 2256-2452) and detected by $_{32}$P incorporation. Total levels of GST-N-CoR protein are shown.

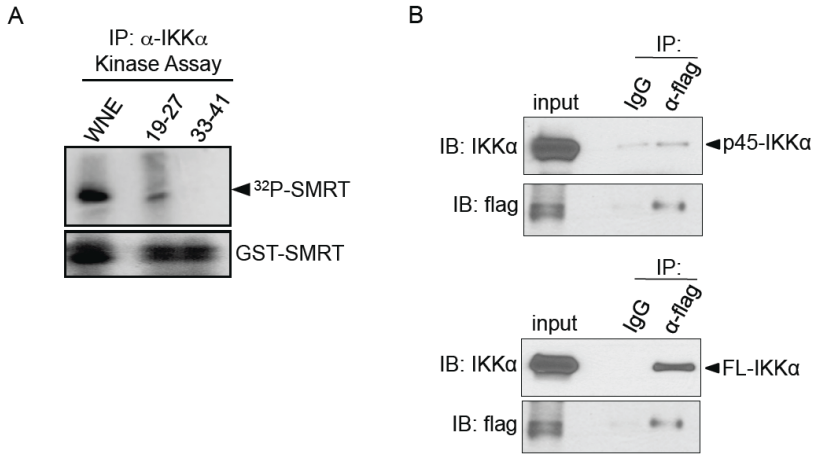


Figure S5. The p45-IKKα complex phosphorylates SMRT in vitro, Related to Figure6. (A) Kinase activity assay of immunoprecipitated IKKα from fractions 19-27 and 33-41 on a GST-SMRT detected by 32P incorporation. Precipitation of whole nuclear extracts (WNE) is shown as a control. Coomassie Blue staining shows total levels of GST-SMRT. (B) Coprecipitation of flag-SMRT with FL-IKKα or p45-IKKα. (C) Coprecipitation of HA-IKKβ with IKKα wildtype or IKKα-3M mutant.

serine 2410 and this effect was enhanced by FL-IKKα. In addition, combination of active p45-IKKα and FL-IKKα induced phosphorylation of histone H3 (Figure 6C). These results indicate that p45-IKKα is required for specific nuclear IKKα kinase activities, although p45-IKKα by itself was unable to associate (Figure S5B) or phosphorylate SMRT or NCoR in vitro (not shown). We also measured the transcriptional effects of re-introducing FL- or p45-IKKα in IKKα KO cells. As shown in Figure 6D, ectopic expression of FL-IKKα induced the activation of several genes known to be repressed by SMRT such as *Hes1*, *Herp2* and *Hes5*. This effect was potentiated by p45-IKKα co-expression whereas p45-IKKα alone did not have any transcriptional effect.

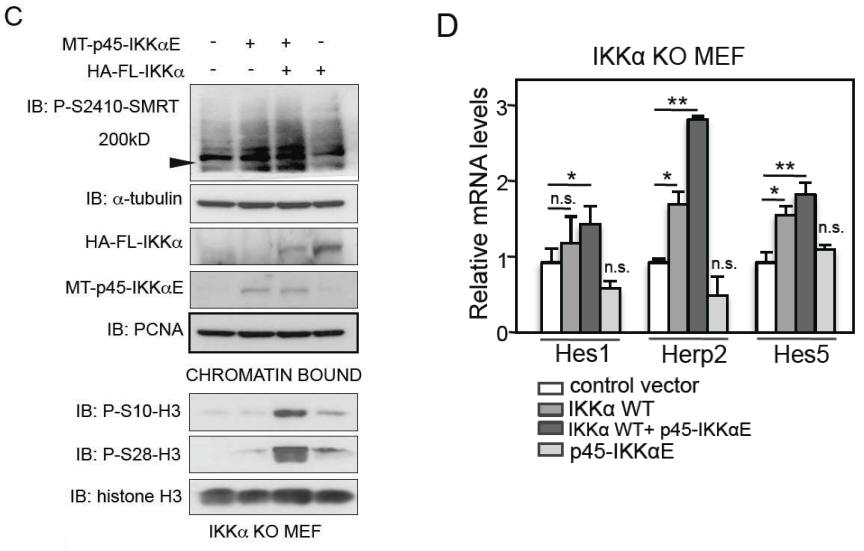


Figure 6. p45-IKKα promotes phosphorylation of specific substrates both in vitro and vivo. (C) IKKα KO MEFs were transfected with the indicated plasmids and selected for 72 hours with puromycin. Whole cell extracts or chromatin extracts were obtained and analyzed by western blot with the indicated antibodies. P-SMRT antibody detected different isoforms ranging from 150 to 300 kD. Levels of α-tubulin, PCNA and histone H3 are shown as loading controls. (D) qRT-PCR showing the expression levels of different SMRT-repressed genes in the IKKα KO MEFs reconstituted as described.

Next, we investigated whether phosphorylation of nuclear IKKα targets and transcription of SMRT-repressed genes in CRC cells depends on p45-IKKα, To do this, we transduced HCT116 or HT-29 cells with retroviral vectors containing an IRES-GFP and codifying for WT IKKα, the IKKα 3M mutant alone or 3M together with p45-IKKα. Next, we sorted the GFP positive cells and knocked down endogenous IKKα using shRNA that targets its 3'UTR (shRNA#4, Figure 1G and S1C), which does not affect the IKK constructs. Efficiency of endogenous IKKα depletion and expression of the different IKKα constructs was confirmed by western blot of sorted cells (Figure 6E). We found that WT IKKα, but not the IKKα-3M mutant, restored or even increased phosphorylation of SMRT and histone H3 in IKKα knocked down CRC cells. Similar effects were observed when p45-IKKα was co-expressed with non-cleavable IKKα (Figure 6E). In contrast, IKKα-3M efficiently binds to IKKβ (Figure S5C), induces IκBα phosphorylation (Figure 6F) and was activated by the IKK kinase TAK1 (Figure 6G). Most importantly, changes in SMRT phosphorylation induced by IKKα reconstitution correlated with changes in the transcriptional activity of specific SMRT targets, including the anti-apoptotic gene cIAP2 (Figure 6H).

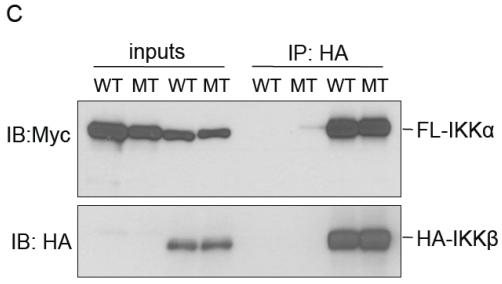


Figure S5. The p45-IKKα complex phosphorylates SMRT in vitro, Related to Figure6. (C) Coprecipitation of HA-IKKβ with IKKα wildtype or IKKα-3M mutant.

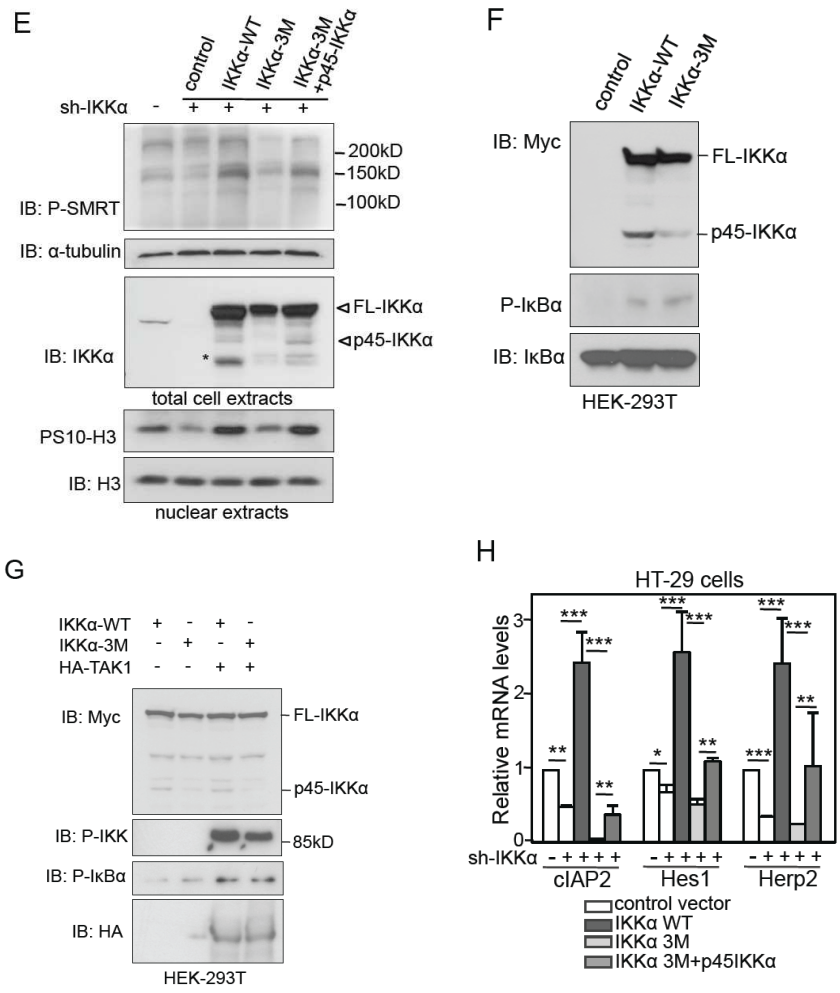


Figure 6. p45-IKKα promotes phosphorylation of specific substrates both in vitro and vivo. (E) Phosphorylation of SMRT and histone H3 was analyzed by western blot analysis in IKKα depleted HCT116 cells transduced with the indicated IKKα constructs. In the α-IKKα blot the asterisk indicates the p45 fragment generated from FL-IKKα and arrows indicate the unprocessed proteins codified by exogenous FL-IKKα and p45-IKKα constructs. (F) Western blot showing the levels of IκBα phosphorylation in cells transduced with the indicated constructs. (G) Western blot showing phosphorylation of exogenous myc-IKKα (WT and 3M) and IκBα induced by TAK1. (H) Expression levels of different SMRT-repressed genes in the indicated cell pools as determined by qRT-PCR. In D and H, statistically significance was determined using T-test (* $p<0.05$; ** $p<0.01$, *** $p<0.001$). All experiments were performed a minimum of three times with comparable results.

Truncated P-IKKα is required to prevent apoptosis and support the growth of CRC cells

Finally, we measured the contribution of IKKα and p45-IKKα activities to CRC. Indicative of their functional relevance, knocking down IKKα significantly inhibits the growing capacity of all tested CRC cell lines (Figure 7A). Using the same strategy as before (transducing WT IKKα or the 3M mutant or the IKKα mutant plus p45-IKKα, followed by endogenous IKKα knockdown), we tested whether p45-IKKα was required to revert IKKα depletion in CRC cells. We found that the effects of IKKα knockdown in CRC cell growth were specifically rescued by WT IKKα or by the IKKα 3M mutant plus p45-IKKα but not by IKKα 3M alone (Figure 7B). Flow cytometry analysis demonstrated that only IKKα or 3M plus p45-IKKα protected CRC cells from apoptosis, as measured by annexin V binding (Figure 7C) and induced a slight, but significant, increase in cell proliferation (Figure 7D). Prosurvival effects of p45-IKKα can be explained, at least in part, by regulation of CIAP2 (Deveraux et al., 1998). We next determined the capacity of HCT116 and HT-29 cells depleted from endogenous IKKα and reconstituted with WT or the non-cleavable IKKα mutant to grow as tumor xenografts in nude mice. CRC cells expressing WT IKKα generated significantly larger tumors than cells expressing the IKKα mutant (Figures 7E, 7F and 7G and S6), indicating the pathological relevance of p45-IKKα.

In summary, we have identified a cathepsin-dependent mechanism that generates truncated IKKα, which is found in the nucleus of tumor cells in its active form, where it is responsible for specific kinase activities that directly impinge on cancer cell growth both in vitro and in vivo.

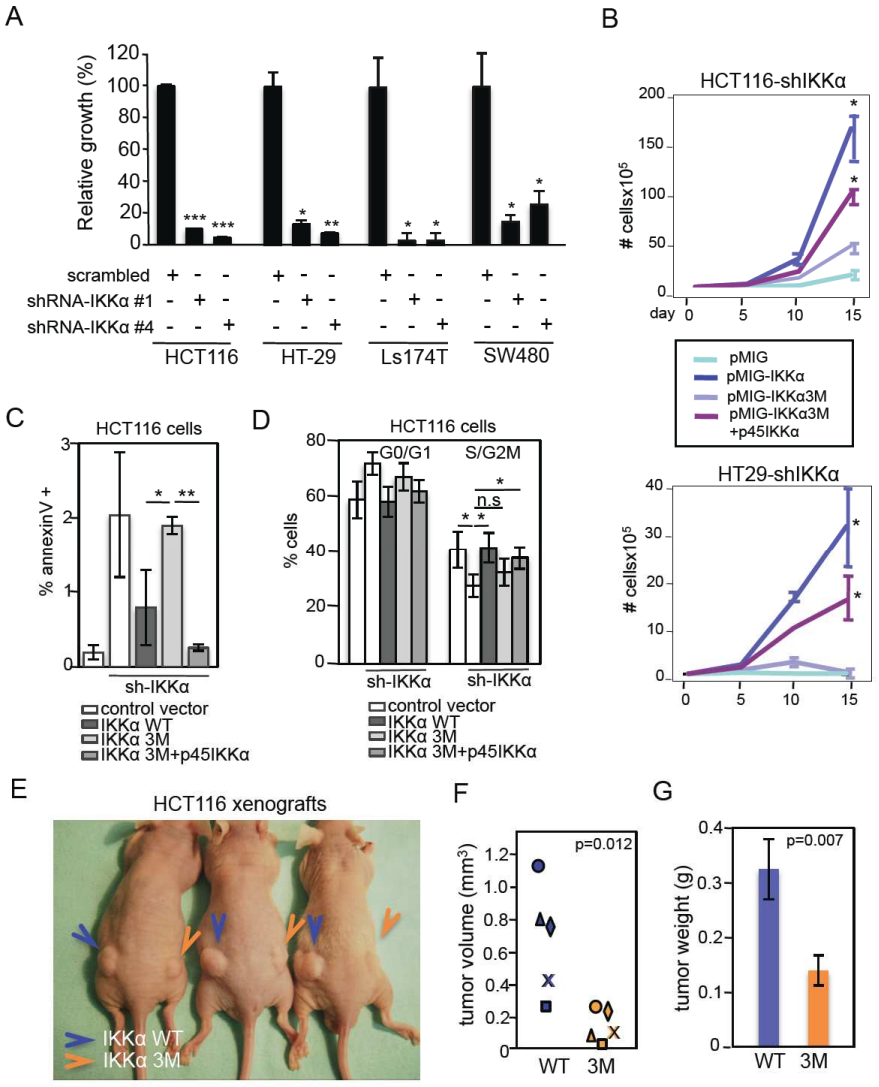
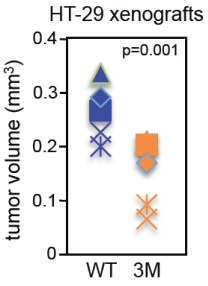


Figure 7. Generation of p45-IKKα is required to maintain CRC cell growth in vitro and in vivo. (A) Cells transduced with the indicated shRNAs were seeded at 10^3 cells per well (in 6 well plates) and quantified after one week. (B) Effects of IKKα depletion and reintroduction of the different IKKα constructs in the proliferation ratio of HCT116 and HT-29 cells. (C, D) Flow cytometry analysis of AnnexinV binding and cell cycle in HCT116 cells transduced with the indicated constructs. (E) Generation of tumor xenografts from HCT116 cells expressing WT or the non-cleavable IKKα 3M mutant. Three representative from five animals included in this experiment are shown. (F-G) Measurement of tumor

size (F) and weight (G) 3 weeks after injection. Statistical significance was determined using T-test (* p<0.05; ** p<0.01 and no significance, n.s.).



Figures S6. Cleavage of IKK into p45 is essential for the tumorigenic activity of IKKα in CRC cells, Related to Figure7. HT29 cells depleted from endogenous IKKα (by shIKKα) and reconstituted with IKKα wildtype or 3M were injected subcutaneously in nude mice. Graph represents tumor measures two weeks after injection. Statistical significance of the differences was calculated using T-test.

Discussion

Our results indicate that human CRC tumors display constitutive nuclear IKKα phosphorylation, associated with increased tumor grade. In tumor cells, we found that IKKα is proteolytically processed into a 45kD fragment, in a cathepsin-dependent manner. Moreover, p45-IKKα and P-IKK levels showed a significant correlation in CRC samples. At the functional level, we found that nuclear active p45-IKKα co-elutes and interacts with non-phosphorylated FL-IKKα and NEMO and is capable of phosphorylating SMRT and N-CoR corepressors and histone H3. Cleavage of IKKα into p45-IKKα is required for tumor growth in vitro and in vivo, although we detected some p45-IKKα and P-IKK staining in few normal samples mainly restricted to the proliferative compartment. The physiological significance of p45-IKKα and the mechanisms regulating its generation, activation and nuclear translocation are currently being investigated.

Although IKKα is mostly found in the cytoplasm associated with IKKβ and NEMO, we here demonstrate that truncated p45-IKKα translocates to the nuclear compartment in CRC cells where it interacts with FL-IKKα and NEMO. Which are the mechanisms regulating formation of this IKK complex remain unknown, but they might include availability of IKK components (i.e. low amounts of nuclear IKKβ compared with nuclear IKKα and intermediate levels of NEMO), post-translational modifications of specific elements and the participation of adaptor proteins.

On the other hand, it is known that IKKα phosphorylates specific nuclear targets such as histone H3 and nuclear corepressors (Anest et al., 2003; Hoberg et al., 2004; Yamamoto et al., 2003). Here, we show that p45-IKKα is required for SMRT and histone H3 phosphorylation. By analysis of the elution fractions from Superdex S200 columns, we detected that all the FL-P-IKKα eluted in a LMW fraction compatible with monomeric IKK, which is unable to phosphorylate SMRT. Which are the substrates of nuclear FL-P-IKK kinase remains to be elucidated. On the other hand, FL-IKKα is required for chromatin binding of p45-IKKα association with SMRT and phosphorylation of SMRT and histone H3 by p45-IKKα, although it retains the kinase domain and NLS sequences. In this sense, it has been recently demonstrated the importance of the SDD domain of IKKβ (that is conserved in IKKα) not only for substrate recognition but also for kinase activation (Xu et al., 2011). We propose that p45-IKKα uses the SDD and NBD domains from FL-IKKα to achieve their functions.

Mechanisms that lead to the formation of p45-IKKα may not be unique and are likely context dependent. Examples include frameshift mutations generating premature STOP codons that are present in Squamous Cell Carcinoma (Liu et al., 2006) and splicing variants that codify for proteins lacking the SDD as it was found in T-lymphocytes and in the brain

(McKenzie et al., 2000). However, we did not find such mutations in around 200 samples analyzed, and identified alternative splicing of exon14 (leading to truncated IKKα) in only one CRC sample. Thus, we propose that the main source of p45-IKKα in CRC cells is proteolytic processing of IKKα by cathepsins. This is consistent with increased cathepsin activity found in CRC that can be associated with the enhanced aerobic glycolysis, previously described by Warburg (Swietach et al., 2007), but also with other cancer-related pathways such as erbB2-K-RAS (Fehrenbacher et al., 2008; Kim et al., 1998), JAK-STAT (Kreuzaler et al., 2011) or vitamin D-cystatin (Alvarez-Diaz et al., 2009). In addition, we found that mutations in a caspase consensus site of IKKα also reduced p45-IKKα generation, which might suggest that the presence of adjacent protease binding sites might facilitate cathepsin recognition or that the caspase site is functional under specific conditions. Whether cathepsin-mediated processing is differentially required to generate cytoplasmic or nuclear p45-IKKα is somewhat puzzling and requires further investigation. Most important, in CRC cells cathepsin B and L colocalized with P-IKK in cytoplasmic ring-shape structures, corresponding to endosomal vesicles that express RAB5, suggesting that IKKα processing and activation occurs previous to its nuclear translocation. Similar mechanisms of endosomal-mediated processing have been shown to regulate ligand-independent activation of Notch (Wilkin et al., 2008), activation of interferon response through TLR4 (Kagan et al., 2008; Tseng et al., 2010), death signaling induced by the TNFα receptor (Schneider-Brachert et al., 2004) and dorso-ventral specification in Drosophila (Lund et al., 2010).

Interestingly, our results indicate that p45-IKKα is not restricted to CRC since non-phosphorylated forms are consistently found in the cytoplasm

of non-transformed MEF and human colonic mucosa. However, homozygous mutations of human IKKα leading to a premature STOP codon at position 422 results in a lethal syndrome due to severe fetal malformation defects (Lahtela et al., 2010), indicating the functional requirement of one FL allele. Further work, including generation of new animal models should decipher the physiological and pathological contribution of p45-IKKα. However, we found that cancer cells lacking IKKα cannot form tumors *in vivo* when reconstituted with a non-cleavable IKKα mutant.

The relevance of this work resides in the characterization of p45-IKKα which function is not directly related with NF-κB but holds important tumorigenic potential. This finding opens the possibility of designing new anticancer treatments targeting IKKα cleavage that should restrict the negative effects of inhibiting general IKK activity and thus NF-κB. In addition, we have generated a novel antibody that specifically recognizes p45-IKKα by IHC, IF and IP of CRC samples, which in the near future will be applicable, likely in combination with P-IKK detection, for analysis of human tumors, stratification of CRC patients and other clinical-related applications.

Experimental Procedures

Human Colorectal Samples

Samples from patients were obtained from the archives of the Tumor Bank of Hospital del Mar. All patients gave written consent to donate the tumor specimen. The Ethics Committee of our institution approved the study.

Production of Monoclonal antibodies against p45-IKKα

These antibodies were generated by Abyntek (Spain) using the peptide AA241-424 of human IKKα as immunogen.

Gel filtration assay on Superdex200 column

100μL of HCT116 nuclear extracts were lysed in PBS containing 0.5% Triton X-100, 1mM EDTA, 100mM Na-orthovanadate, 0.25mM PMSF and complete protease inhibitor cocktail (Roche, Basel, Switzerland), centrifuged and loaded on Superdex200 gel filtration column (GE Healthcare). One drop (40μL) per fraction was collected and analyzed by western blot.

Protein Kinase assays

Nuclear fractions 19-27 and 36-45 from Superdex200 column were pre-cleared and incubated with α-IKKα or α-P-IKKα/β overnight at 4°C. Precipitates were captured with ProteinA-Sepharose, washed and assayed for their kinase activity on GST fusion proteins. Kinase reaction was performed at 30ºC in 20mM Tris pH7.5, 5mM $MgCl_2$ and 1mM DTT.

Statistical Methods

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 P-IKK data by applying a rank transformation on the dependent variable. Analysis was performed using SAS version 9.1.3 software (SAS Institute Inc., Cary, NC), and level of significance was established at 0.05 (two sided).

Tumor xenografts

HCT116 and HT-29 cells were transduced with different IKKα-retroviral vectors and sorted based on YFP expression. Then cells were transduced with shRNA vectors, selected for 3 days with puromycin and tested for the expression of the target proteins. $2x10^4$ cells were suspended in matrigel, injected subcutaneously in nude mice and after 3 weeks, visible

tumors were measured and photographed. Animals were kept under pathogen-free conditions and all procedures approved by the Animal Care Committee.

Extended Supplementary Methods

Plasmids

Expression vector for HA-IKKα, HA-IKKα_{AA}, HA-IKKα_{EE}, and HA-IKKβ were a gift from Michael Karin Lab. The expression plasmids MT-IKKα and MT-IKKα300-450 were constructed by inserting the PCR-amplified corresponding region, using a HA-IKKα construct as a template, into the mammalian expression vector pCS2-MT (Invitrogen). Additional information regarding histone H3, cathepsin and IKK mutant constructs is included as Supplementary data. Specific shRNAs against IKKα were from sigma (x-2811c1, x-1194s1c1, x-2274s1c1 and x-2866s1c1). For the infection experiments, IKKα constructs were cloned in the retroviral vector MSCV-IRES-GFP and the viral particles were produced using the standard techniques. MT-IKKα S400-402A, S400W, I421L, I421R, D347A and S400W/I421R, and the corresponding mutations in MT-IKKα300-450, were constructed by using the QuikChange™ Site-Directed Mutagenesis Kit from Stratagene and according to manufacturer instructions. The expression plasmids MT-cathepsin B, MT-cathepsin L and MT-cathepsin K were constructed by inserting the PCR-amplified corresponding region, using HS27 cDNA as a template, into the mammalian expression vector pcS2-MT. The expression plasmid MT-p45-IKKα and MT-p45-IKKα_E were constructed by inserting the PCR-amplified corresponding region, using a HA-IKKα and HA-IKKα_E respectively as a template, into the mammalian expression vector pcS2-MT. GST-H3, GST-N-CoR and GST-SMRT were generated by PCR and cloned in-frame into a PGEX-5.1 vector (Pharmacia). The expression plasmids HA-IKKαΔH and MT-IKKαΔLH were

constructed by inserting the PCR-amplified corresponding region, using a HA-IKKα and MT-IKKα respectively as a template, into the mammalian expression vector pcS2-MT. Caspase 3 was amplified by PCR and inserted into the pcDNA.3 expression vector.

Cell lines and culture reagents.

HEK-293, HS27, HCT116, SW480, Ls174, HT-29 and MEF were grown in DMEM plus 10% FBS. Human TNFα from Preprotech was used at 40 ng/ml. Recombinant lentivirus and retrovirus were produced by transient transfection of HEK-293T cells according to Tronolab protocols (http://tronolab.epfl.ch/page58122.html). 20 μg of transfer vector, 15 μg of packaging plasmid (psPAX2), and 6μg of envelope plasmid (pMD2.G) were used. After 3 days, supernatant was ultracentrifuged and viral pellet resuspended in 100 μl of PBS. 20μl of fresh viral suspension was used per infection**.**

Antibodies

α-IκBα (sc-1643), α-p65 (sc-109), α-IKKβ (sc-7330), α-HDAC1 (sc-7872), α-NEMO (sc-8330), α-PCNA (sc-56), α-cathepsin B (sc-6493), α-cathepsin L (sc-6500), α-Rab5 (sc-46692), α-Rab7 (sc-10767), α-LC3α (sc-134226) and α-LAMP1 (sc-17768) were purchased from Santa Cruz Biotechnology. α-IKKα (OP-133) from Oncogen. α-P-IKKα-Ser180/IKKβ-Ser181 (2681), α-P-IKKα-Ser176,180/IKKβ-Ser177,181 (2697S), α-N-terminal IKKα (2682), α-P-IκBα$_{32-36}$ (92465), α-p65 (pS536) (3031S), α-PARP1 (9542) from Cell Signaling. α-SMRT (06-891), α-Caspase-3 (06-735) from Upstate. α-α-tubulin from Sigma. α-HA (12CA5) from Covance. α-MT antibody was the 9E10 hybridoma. The phosphospecific SMRT antibody α-pS2410 has been previously described (Hoberg et al., 2004).

Cell fractionation

Nuclei were isolated in 0.1% NP-40/PBS for 5min on ice, followed by centrifugation at 1900 rpm and then lysed in 50mM Tris-HCl pH 7.5, 150mM NaCl, 1% Nonidet P-40, 5mM EGTA, 5mM EDTA, 20mM NaF and complete protease inhibitor cocktail (Roche). Supernatants were recovered as the cytoplasmic fraction. For cytoplasm/nuclear/chromatin separations, cells were lysed in 10mM HEPES, 1.5mM MgCl2, 10mM KCl, 0.05%NP40 at pH 7.9, 10min on ice and centrifuged at 13.000 rpm. Supernatants were recovered as cytoplasmic fraction and the pellets lysed in 5mM HEPES, 1.5mM MgCl2, 0.2 mM EDTA, 0.5mM DTT and 26% glycerol and sonicated 5' three times to recover the soluble nuclear fractions. Pellet included the chromatin fraction. Lysates were run in SDS/PAGE and transferred to immobilon-P transfer membranes (Millipore) for western blotting.

Western blot analysis and immunoprecipitation assays

Cells were lysed 30min at 4ºC in 300µl PBS plus 0.5% Triton X-100, 1mM EDTA, 100 mM Na-orthovanadate, 0.25 mM PMSF and complete protease inhibitor cocktail (Roche). For immunoprecipitation, supernatants were pre-cleared 2h with 1% of BSA, 1µg IgGs and 50µL SPA beads. Precleared lysates were incubated O/N with 3µg of indicated antibodies. Antibody-Protein complexes were captured with 30µL SPA beads for 2h. After washing, precipitates were analyzed by western blot.

Immunofluorescence and Confocal Microscopy Analysis

P-IKKα/β was detected with HRP-linked secondary antibody and developed with the TSA^{TM} Plus Cyanine3/Fluorescein System (PerkinElmer) whereas the other antibody was visualized directly using Alexa Fluor 568 (donkey anti-mouse or donkey anti-goat IgG). For double detection of P-IKKα/β and Rab7, samples were incubated overnight with

P-IKKα/β antibody and developed using TSA™ Plus Cyanine3/Fluorescein System. Samples were treated with 2% H_2O_2 for 30min and then incubated with α-Rab7 antibody overnight that was detected with HRP-linked secondary antibody and developed with TSA™ Plus Cyanine3/Fluorescein System. As negative controls of the experiment, we followed the same protocol in the absence of P-IKKα/βs or Rab7 antibody. Confocal microscopy analysis was performed using a Leica TCS SP5 CFS microscope.

SSCP analysis

Presence of point mutations in exons 13 to 15 of IKKα gene was analyzed by PCR-single-strand conformation polymorphism in three independent reactions using intronic primers (primers and PCR conditions are available upon request). PCR products were diluted 1:16 in formamide-dye loading buffer, incubated for 3 min at 95ºC, cooled on ice and loaded onto 6% polyacrylamide nondenaturing sequencing gel. Electrophoresis was carried out at room temperature under 7 W for 10 to 12 hours. Gels were silver stained and vacuum dried at 85ºC. Shifted single-strand conformation polymorphism bands were excised from the gel, reamplified and sequenced.

Detection of Mutations of *IKKα* from cDNA

PCR primers (5'-GTAAAGTGTGGGCTGAAGCAGTG-'3 and 5'-AATGGCACGCTGTTCCAGAG-'3) flanking intron 14 and 15 of the human *IKKα* gene were used to generate a genomic DNA fragment. An expanded high-fidelity Taq-polymerase (*pfu*Turbo, from Stratagene), was used for the PCR. The PCR products were subcloned into PGEM-T vectors (Promega) and sequenced.

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. Multiple areas of invasive carcinoma, adenomatous lesions from the same surgical sample, and normal adjacent mucosa were identified on corresponding hematoxylin-eosin-stained slides. Tissue blocks were transferred to a recipient "master" block using a Tissue Microarrayer. Each core was 0.6-mm wide spaced 0.7–0.8mm apart. Paraffin sections of 4μm were hydrated, permeabilized and antigen retrieval was achieved by incubating with 10mM citrate buffer overnight at 80ºC. Primary antibodies were incubated overnight and then developed with the DAB System (DAKO).

Quantitative RT-PCR

Total RNA was obtained using the RNeasy kit from Qiagen. RNA quality was assessed on agarose gels and quantified by Nano-Drop1000 (Nano-Drop, Wimington, DE). cDNA was synthesized with the RT-First Strand cDNA Synthesis kit (Amersham Pharmacia Biotech) following manufacture instructions. Real-time polymerase chain reaction (PCR) was performed in triplicates on the Light Cycler 480 (Roche) and SYBR-Green (Applied Biosystems) was used to detect gene expression. Expression of individual genes was normalized to β-actin expression.

Flow cytometry analysis and Cell Sorting

Apoptosis of 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). Cell sorting was performed on a FACSVantage or Aria (Becton & Dickinson). All data were analyzed with the FlowJo software (Tree Star, Inc).

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RESULTS: PART 2

p45-IKKα is a potential therapeutic target for KRAS/BRAF mutated cancer.

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Running title: Specific inhibition of IKKα prevents tumor formation

Abstract

Tumor cells carrying KRAS mutations can, by different mechanisms, stimulate the NF-κB pathway, which in turn provides them with specific properties. Here, we show that mutant BRAF exerted a minor impact on NF-κB but induced a strong activation of p45-IKKα, a proteolytic product of full-length IKKα that is generated in the endosomal compartment of Colorectal Cancer (CRC) cells. Mechanistically, BRAF promotes polyubiquitination of TRAF6 leading to the recruitment of active TAK1 to the p45-IKKα complex in the endosomes. We found that p45-IKKα is required for KRAS/BRAF-mediated cell transformation, and inhibitors of the endosomal function abolished TAK1 and p45-IKKα activities, and induced apoptosis of BRAF mutant cells. Using a human orthotopic xenograft model we demonstrated the efficacy of these drugs in preventing the metastatic capacity of primary CRC cells from a patient with acquired resistance to standard chemotherapy.

Introduction

Colorectal Cancer (CRC) is the second leading cause of cancer death in Western populations. Progression from adenoma to invasive CRC requires a number of well-characterized genetic alterations including those of APC, SMAD4 or KRAS [1]. Activating mutations of KRAS are present in about 40% of advanced CRC tumors and about an additional 15% contain mutations in its downstream effector BRAF. Clinically relevant is the fact that RAS and RAF mutations are difficult to target and preclude the use of EGFR inhibitors such as *Cetuximab* or *Panitumumab* in advanced metastatic colorectal cancer [2,3]. RAS signaling cascade initiates in the plasma membrane in response to EGFR activation, being the MAP kinase pathway its major downstream target. The first component of this pathway is the kinase RAF, which phosphorylates MEK1/2 that activates ERK1/2 and finally, 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 pointed out the possibility to develop and use RAF inhibitors for therapeutic purposes. Unfortunately, different RAF inhibitors that blocked proliferation of BRAF mutant cells were ineffective against RAS mutant cells, and even activated the MAPK pathway in the presence of wild-type (WT) RAF[4-8]. In addition, efficacy of BRAF inhibitors can also be suppressed by the action of HGF secreted by tumor microenvironment, and most of the tumors that initially respond to RAF inhibition tend to develop resistance through different mechanisms[9]. Better results are now being obtained using combinations of BRAF and MEK inhibitors, although specific MEK inhibitors show different efficacy against KRAS or BRAF mutated tumors [8,9]. Nevertheless, the identification of new druggable elements downstream of RAS is one of the primary lines of research in the cancer field.

NF-κB pathway is a key regulator of innate and acquired immunity [10-12], which activity is required for cancer maintenance and progression in multiple systems [13-16]. Activation of NF-κB pathway lays downstream of the Inhibitor of κB Kinase (IKK) complex that is composed of two catalytic subunits, IKKα and IKKβ, and the regulatory element IKKγ/NEMO (for NF-κB Essential Modulator). Phosphorylation-mediated degradation of IκB (for Inhibitor of κB), which is accomplished by the activity of IKKβ, results in the nuclear entrance of the NF-κB factor (mostly the p65/p50 heterodimer) leading to canonical NF-κB activation. TAK1 is the principal kinase that phosphorylates and activates IKKβ[17,18] and recruitment of TAK1 to the IKK complex is induced by K63-linked polyubiquitination of TRAF and NEMO, which acts as a bait for the TAK1 adaptors TAB2/3 [18] (reviewed in [19]). On the other hand, alternative NF-κB signaling is IKKβ, NEMO and TAK1 independent and mostly relies on the activation of IKKα by NIK. Then, IKKα phosphorylates p100 and induces its proteolytic processing into p52, which translocates to the nucleus associated with RelB to activate specific gene transcription [20]. Additional roles for the IKKα kinase have recently been identified including phosphorylation of serine10 of histone H3 at both NF-κB dependent and independent gene promoters [21-24], cell cycle control through phosphorylation of AuroraB kinase [25] and transcriptional activation of 14-3-3σ [26], or inactivating phosphorylation of the nuclear corepressor SMRT [27]. IKKα also plays a predominant role in particular cancer systems such as squamous cell carcinoma, where it induces *Hox* and *Irx* genes[28] following chromatin release of SUMOylated IκBα, prostate cancer cells where it regulates transcription of the metastasis-related gene *Maspin* in cells [29], and CRC

though the activation of Notch-targets *Hes1* and *Herp2* and the anti-apoptotic gene *cIAP2* [30,31].

Oncogenic KRAS is known to induce canonical NF-κB by different mechanism leading to the apoptosis inhibition in cancer cells [32-38]. In lung cancer, KRAS through PKC and p62 facilitates TRAF6 activation and NEMO polyubiquitination, leading to TAK1-dependent phosphorylation of IKKβ[39,40]. A paracrine loop involving IL1β and p62 activates NF-κB in pancreatic cancer[36]. Importantly, inhibition of TAK1[41] or other NF-κB elements[16,42] reverts cancer progression in several in vivo systems including tumors carrying KRAS/BRAF mutations, pointing out the possible use of NF-κB inhibitors for specific anti-cancer therapies. However, general inhibition of NF-κB or IKK activity results in a severe toxicity, and can either suppress or promote cancer in a context dependent manner [43], which denotes the need of studying the individual contribution of NF-κB elements to specific tumor types.

Recently, we identified a proteolytic fragment of IKKα that we called p45-IKKα, which is generated by cathepsin-mediated cleavage of the full-length protein in the early endosomes of CRC cells. Active p45-IKKα bound non-active full-length IKKα and NEMO to form a complex that does not induce NF-κB but is responsible to phosphorylate SMRT and histone H3, leading to enhanced transcription of the Notch target genes *Hes1* and *Herp2,* and apoptosis inhibition [44]. Here, we report that p45-IKKα is downstream of oncogenic RAS and RAF being required for their transformation activity, and show that inhibition of p45-IKKα activation can provide a therapeutic opportunity for cancer patients carrying such mutations.

Results

KRAS signaling through BRAF induces p45-IKKα activity in CRC cells.

We first tested whether oncogenic KRAS favors the activation of specific IKK subunits. We found that the constitutively active KRASG12V induced phosphorylation of IKKα/β (85-87 kD band) in NIH-3T3 cells, as predicted. Interestingly, mutant KRAS had a more pronounced effect on the levels of active p45-IKKα (Figure 1A). Consistently, KRASG12V had a minor effect on either canonical or alternative NF-κB signaling as indicated by the similar levels of IκBα and p52 in control and transduced cells. Activation of p45-IKKα by KRASG12V was prevented by treatment with BRAF inhibitors, suggesting that it occurred downstream of the BRAF kinase, whereas PI3K inhibitors had no measurable effects (Figure 1A). Consistent with these results, constitutively active BRAFV600 mutant transduced in NIH-3T3 cells resulted in p45-IKKα activation and augmented phosphorylation of the non-canonical IKK targets SMRT and histone H3, but had a discrete effect on full-length P-IKKα/β, or p100/p52 and IκBα levels. As a control, TNFα treatment led to IκBα phosphorylation and degradation in both control and BRAF transduced cells (Figure 1B). Of note that TNFα induced a slight and temporary phosphorylation of p45-IKKα suggesting that physiological levels of active p45-IKKα might have some effect on canonical NF-κB signaling (Figure 1B). Further demonstrating its IKKα identity, the anti-P-IKK antibody does not detect any 45kDa band in the lysates from IKKα knockout cells transduced with BRAFV600E (Figure S1A). Pharmacologic inhibition of BRAF activity but not of MEK1/2 prevented phosphorylation of p45-IKKα by BRAFV600E (Figure 1C), indicating that p45-IKKα activation lays downstream of BRAF but is independent of MEK. In these same experiments, we did not detect significant changes in the total levels of

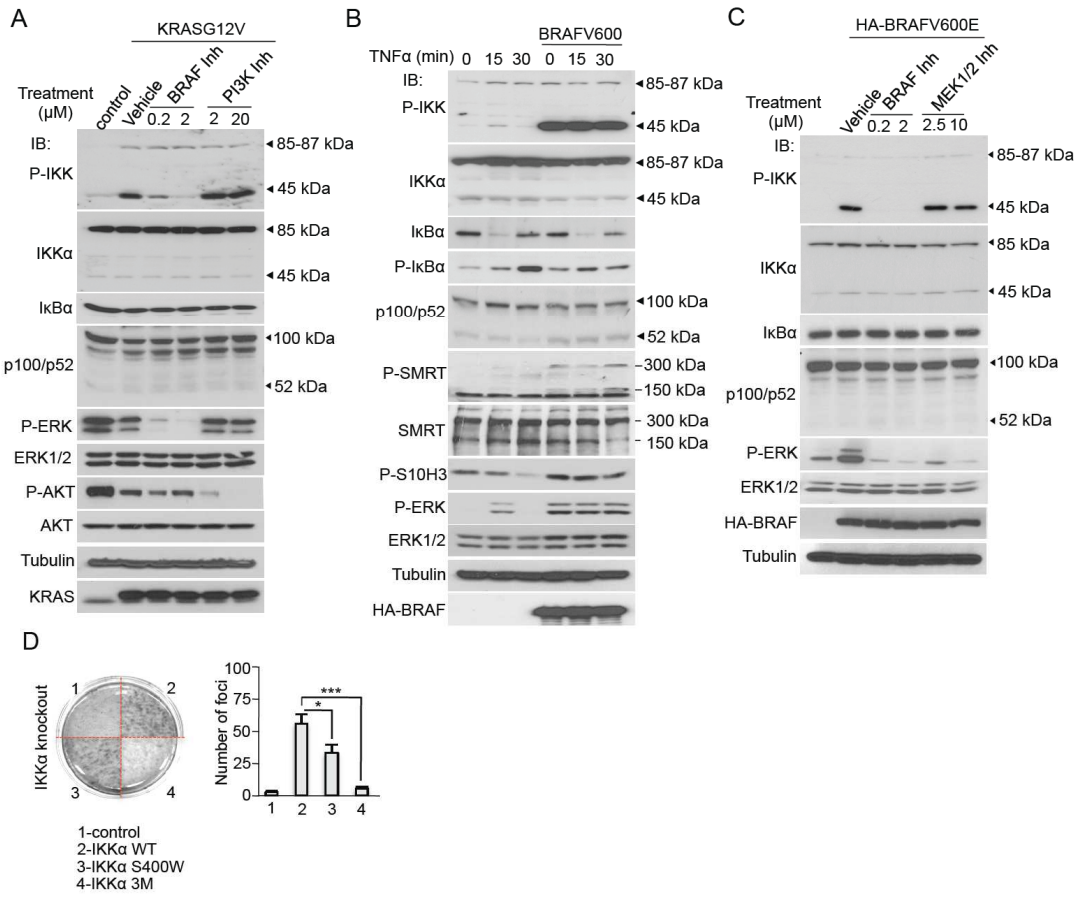


Figure 1. Oncogenic KRAS through BRAF modulates and depends on IKKα activity. (A) NIH-3T3 cells were infected with a control vector or with KRASG12V. Two days after the infection cells were treated with BRAF or PI3K inhibitors at the indicated concentrations for 30 minutes. Whole cell extracts were obtained and analyzed by western blot analysis with the indicated antibodies. (B) NIH-3T3 cells were infected with a control vector or with a BRAF-V600E vector. Two days after the infection, TNFα treatment was added at the indicated times and whole cell extracts were obtained and analyzed by western blot analysis with the indicated antibodies. (C) NIH-3T3 cells were infected with a control vector or with BRAF-V600E. Two days after the infection cells were treated with BRAF or MEK inhibitors at the indicated concentrations for 30 minutes. Whole cell extracts were obtained and analyzed by western blot analysis with the indicated antibodies. (D) Foci formation assay in IKKα knockout MEFs. MEFs were transfected with BRAFV600E vector and with empty vector (pcDNA3.1) or with a combination of the indicated IKKα constructs.

Cells were selected in G418-containing medium for two weeks, and two months after transfection, cell cultures were washed with PBS, fixed and stained with methylene blue. Representative images for each condition are shown, as well as the quantification of the number of foci obtained.

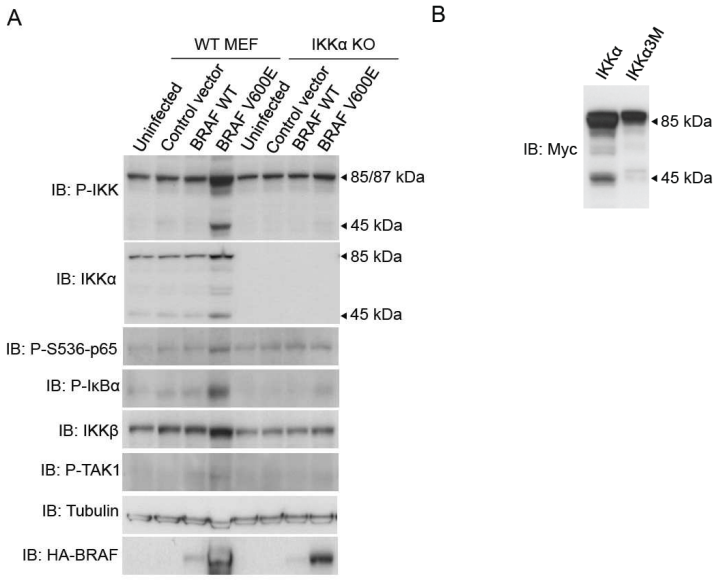


Figure S1. Related to Figure 1. (A) WT or IKKα KO MEFs were infected with the indicated lentiviral vectors. Two days after the infection whole cell extracts were obtained and analyzed by western blot. (B) NIH-3T3 cells were infected with MT-IKKα or with MT-IKKα3M. Two days after the infection whole cell extracts were obtained and analyzed by western blot analysis with the anti-myc tag antibody.

IKKα and p45-IKKα, or in elements of the canonical or alternative NF-κB pathways. Equally importantly, BRAFV600E failed to induce cell transformation in IKKα deficient fibroblasts (Figure 1D), but this capacity was restored by ectopic expression of WT IKKα. In contrast the IKKα3M mutant that is defective for p45-IKKα processing (Figure S1B) did not support BRAFV600E-mediated transformation, whereas an intermediate phenotype was obtained using the S400W point mutant that shows a reduced capacity to be processed into p45-IKKα [44].

We then tested whether this same mechanism operates in CRC cells carrying activating BRAF mutations. As shown in Figures 1E and S1C, HT29 and WiDr cells contain high levels of active p45-IKKα that were significantly reduced in the presence of the BRAF inhibitor, but remained unaffected after treatment with other inhibitors of the MAPK pathway (such as MEK, PI3K or the p38 inhibitors). In these experiments, none of the treatments modified IκBα and p100/p52 protein levels, indicating that BRAF and p45-IKKα activities do not regulate NF-κB in these CRC cells. In contrast, BRAF inhibition led to a significant decrease on histone H3 and SMRT phosphorylation, and to a reduction in 14-3-3σ levels, that were partially recovered once BRAF inhibition was relieved concomitant with p45-IKKα reactivation (Figure 1F). Importantly, changes in phosphorylated histone H3 levels (that is also a proliferation marker) were not directly connected with cell cycle variations, as denoted by ki67 detection. Moreover, inhibition of BRAF imposed the transcriptional repression of specific IKKα target genes such as *Hes1, Hes5*, *Herp2*, *cIAP2*, *Maspin* and *14-3-3σ*, but had a variable impact on the double IKKα/NF-κB target IL8 and *Nfkbia* and no effect on the canonical NF-κB target *TNFAIP3/A20*, as determined by q-RTPCR (Figure 1G). Indicating that in cells carrying mutant BRAF, p45-IKKα activation was not mediated by a paracrine loop involving secreted factors that directly impact on NF-κB, we only detected small amounts of phosphorylated p45-IKKα in LIM1215 cells (that does not contain mutations in KRAS or BRAF), either untreated or incubated with the conditioned-media of HT29 or WiDr cells. Most importantly, p45-IKKα activation in cells treated with these supernatants was totally abrogated after incubation with the BRAF inhibitor (Figure 1H). Comparable results were obtained using conditioned media from NIH-3T3 cells infected with BRAFV600 (Figure S1D).

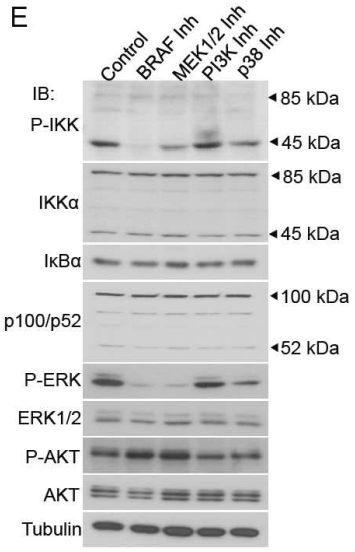


Figure 1. Oncogenic KRAS through BRAF modulates and depends on IKKα activity (E) HT29 cells were treated for 30 minutes with vehicle, BRAF inhibitor (2 μM), MEK1/2 inhibitor (10 μM), PI3K inhibitor (20 μM) or p38 inhibitor (10 μM). Whole cell extracts were obtained and analyzed by western blot analysis with the indicated antibodies.

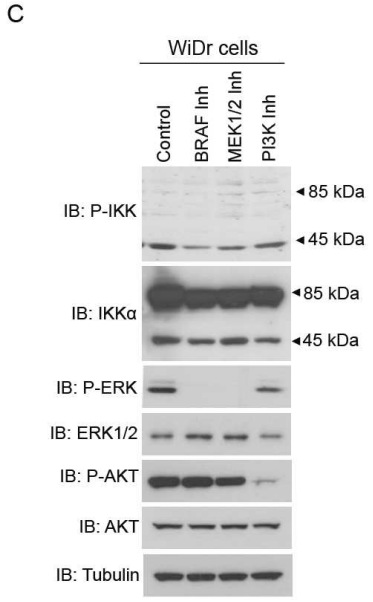


Figure S1. Related to Figure 1. (C) WiDr cells were treated for 30 minutes with vehicle, BRAF inhibitor (2 μM), MEK1/2 inhibitor (10 μM) or PI3K inhibitor (20 μM). Whole cell extracts were obtained and analyzed by western blot with the indicated antibodies.

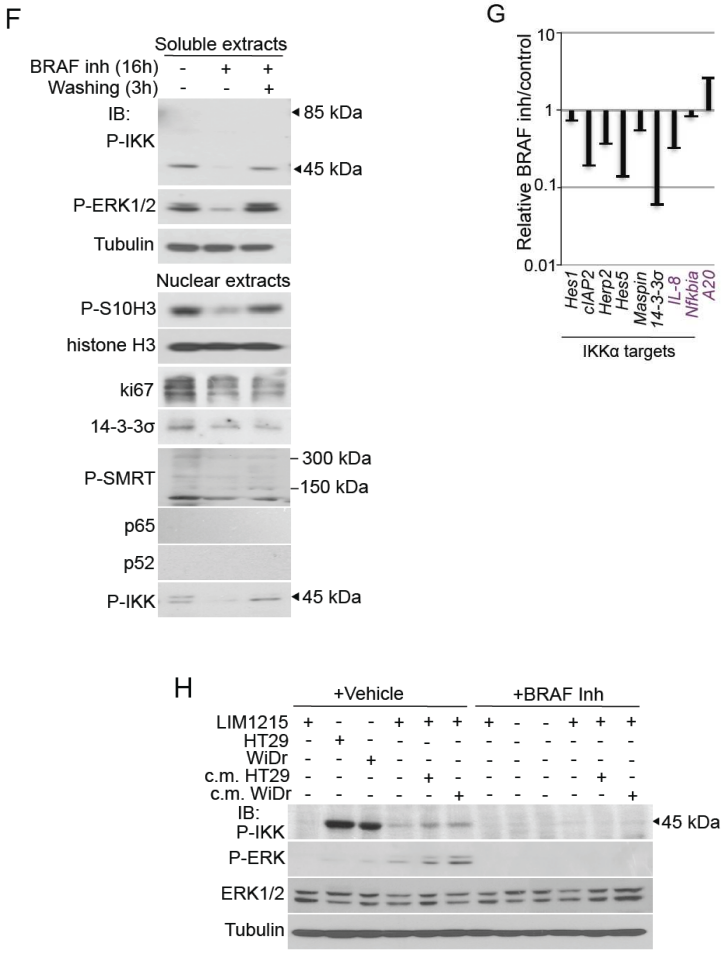


Figure 1. Oncogenic KRAS through BRAF modulates and depends on IKKα activity (F) HT29 cells were treated with vehicle or 2 μM BRAF inhibitor for 16 hours. In the indicated case, the inhibitor was removed and fresh media was added for 3 hours. Cytoplasmic and nuclear extracts were obtained and analyzed by western blot analysis with the indicated antibodies. (G) qRT-PCR showing the expression levels of different IKKα and NFκB target genes in HT29 cells treated with vehicle or 2 μM BRAF inhibitor for 16 hours. (H) LIM1215, HT29 and WiDr cells were seeded and two days after the seeding the media from each case was added to LIM1215 cells as indicated. The day after, cells were treated with vehicle or 2 μM BRAF inhibitor for 30 minutes. Whole cell extracts were obtained and analyzed by western blot analysis with the indicated antibodies.

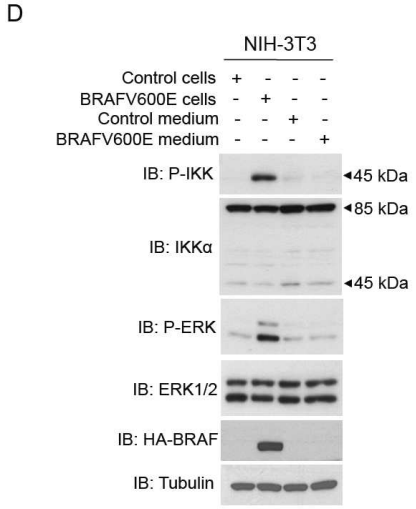


Figure S1. Related to Figure 1. (D) NIH-3T3 cells were infected with a control vector or with BRAF-V600E. Two days after the infection the media from both cases was added to non-infected NIH-3T3 cells as indicated. This was repeated for three days in a row. Whole cell extracts were obtained and analyzed by western blot analysis with the indicated antibodies.

These results demonstrate that BRAF induces p45-IKKα activity in a cell autonomous manner, with minor impact on the canonical and alternative NF-κB pathways.

Activation of p45-IKKα by BRAF is mediated by TAK1

TAK1 and NIK are the major kinases involved in IKK complex activation, being the latter induced after NIK stabilization. We did not detect any change in NIK levels following BRAFV600 transduction (not shown), and ectopic expression of NIK failed to promote p45-IKKα phosphorylation in HEK293T cells, while enhanced p100 processing into p52 (not shown). Thus, we tested whether TAK1 mediates p45-IKKα activation downstream of oncogenic BRAF. We found that knocking down TAK1 with two different shRNAs significantly reduced active p45-IKKα in HT29

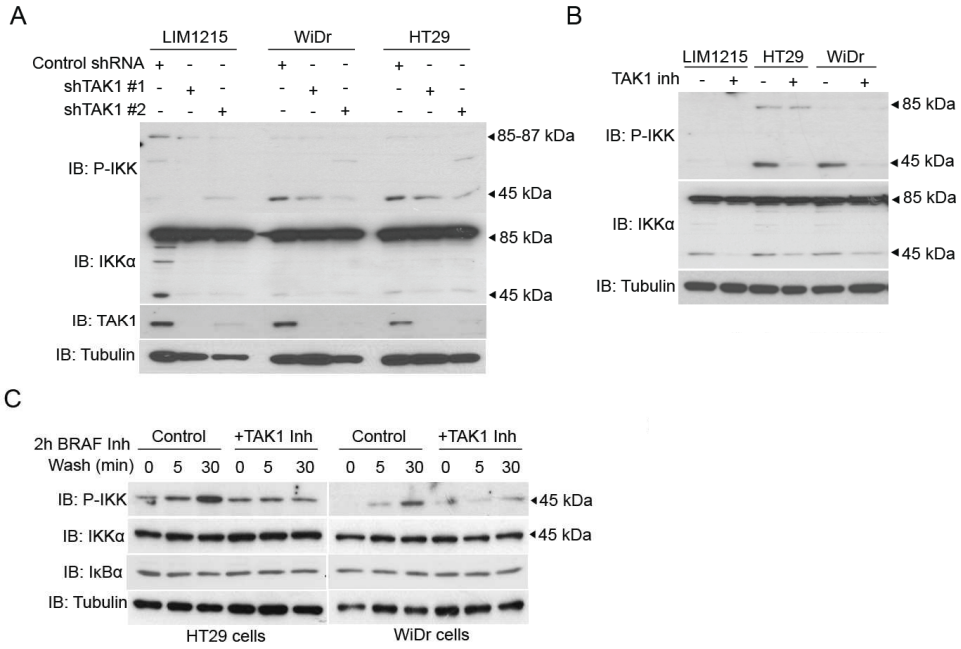


Figure 2. BRAF promotes TAK1-mediated activation of p45-IKKα. (A) Western blot analysis of total cell extracts from LIM1215, HT29 and WiDr cells transduced with different shRNA against TAK1 or with a scrambled shRNA. (B) LIM1215, HT-29 and WiDr cells were treated for 2 hours with vehicle or TAK1 inhibitor (10 μM). Whole cell extracts were obtained and analyzed by western blot analysis with the indicated antibodies. (C) HT29 and WiDr cells were treated for 2 hours with BRAF inhibitor (2 μM). Inhibitor was then removed and fresh media or fresh media with TAK1 inhibitor (10 μM) was added. Whole cell extracts were obtained at the indicated time points and analyzed by western blot analysis with the indicated antibodies.

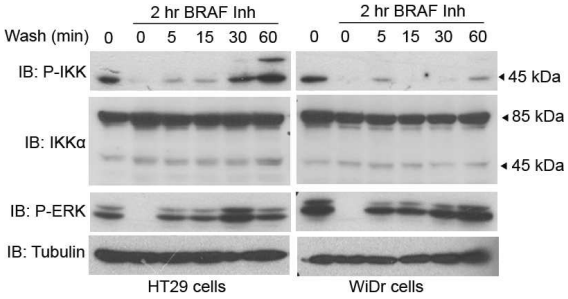


Figure S2. Related to Figure 2 HT29 and WiDr cells were treated for 2 hours with BRAF inhibitor (2 μM). Cells were then washed with PBS and fresh media was added. Whole cell extracts were obtained at the different time points and analyzed by western blot with the indicated antibodies.

and WiDr cells, at comparable levels to the LIM1215 cell line (Figure 2A). Similarly, treating BRAF mutant cells with a pharmacologic TAK1 inhibitor (2 hours of treatment) completely blocked p45-IKKα phosphorylation, associated with a slight reduction in the total levels of p45-IKKα (Figure 2B). We refined these experiments by characterizing the dynamics of p45-IKKα phosphorylation after BRAF inhibition and re-activation in CRC cells, in the absence or presence of the TAK1 inhibitor. Following 2 hours of treatment with the inhibitor, 5 minutes of washing were sufficient to detect some p45-IKKα reactivation, which reached a maximum after 30-60 minutes (Figure 2C and S2). Most importantly, activation of p45-IKKα was precluded in the presence of TAK1 inhibitor (Figure 2C), whereas TAK1 manipulation does not significantly affect IκBα and p100/p52 levels in these experiments.

To gain some understanding on the molecular mechanism that mediates p45-IKKα activation by TAK1 downstream of BRAF, we measured the levels of TRAF6 ubiquitination in HT29 cells untreated or treated with BRAF inhibitor. By precipitation of endogenous TRAF6 followed by WB analysis, we found detectable levels of polyubiquitinated TRAF6 in HT29 cells that were abolished after BRAF inhibitor treatment (Figure 2D). Consequently, BRAF inhibition also prevented the association of TAK1 and IKKα with TRAF6, and reduced the levels of polyubiquitinated NEMO in this complex (Figure 2D). Similarly, BRAF inhibition resulted in a significant reduction in the levels of p45-IKKα and NEMO that precipitated with TAK1, which was reverted after washing (Figure 2E). Of note that IKKβ was consistently absent from the TAK1 precipitates in these cancer cells. In parallel, and taking advantage of a monoclonal antibody (881H3) that preferentially recognizes the p45-IKKα complex in

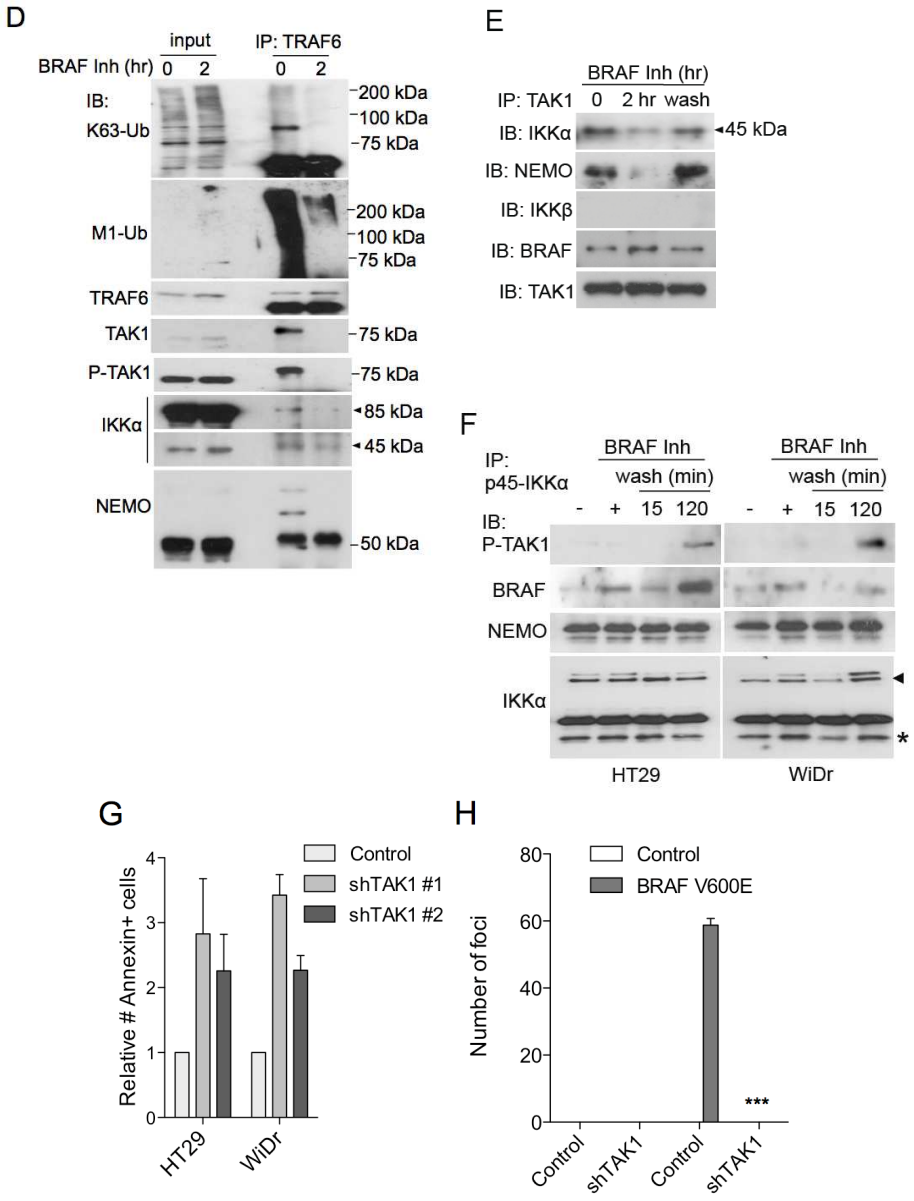


Figure 2. BRAF promotes TAK1-mediated activation of p45-IKKα. (D) HT29 cells were treated for 2 hours with BRAF inhibitor (2 μM). Whole cell extracts were obtained and immunoprecipitated with TRAF6 antibody. Samples were analyzed by western blot with the indicated antibodies. (E) HT29 cells were treated for 2 hours with BRAF inhibitor (2 μM). Inhibitor was then removed and fresh media was added. Whole cell extracts were

obtained at the indicated time points and immunoprecipitated with TAK1 antibody. Samples were analyzed by western blot with the indicated antibodies. (F) HT29 and WiDr cells were treated for 2 hours with BRAF inhibitor (2 μM). Inhibitor was then removed and fresh media was added. Whole cell extracts were obtained at the indicated time points and immunoprecipitated with p45-IKKα antibody. Samples were analyzed by western blot with the indicated antibodies. (G) HT29 and WiDr cells were transduced with different shRNA against TAK1 or with a scrambled shRNA and the relative number of apoptotic cells was determined by flow cytometry analysis of AnnexinV binding. (H) NIH-3T3 cells were infected with the indicated vectors. Two weeks after infection, culture dishes were washed with PBS, fixed with 4% PFA and stained with methylene blue. Quantification of the number of foci is shown. Statistic analysis was performed using T-test (***p<0.001).

its native form[44], we precipitated p45-IKKα from HT29 and WiDr cells following BRAF manipulation. We found small amounts of TAK1 bound to p45-IKKα in both untreated and BRAF-inhibited cells (Figure 2F). Importantly, a strong association between TAK1 and p45-IKKα was observed after releasing BRAF inhibition, indicative of a highly dynamic interaction that is modulated by BRAF.

Supporting the functional relevance of TAK1 downstream of mutant BRAF, transduction of shRNA against TAK1 induced apoptotic cell death of HT29 and WiDr cells as determined by AnnexinV binding (Figure 2G), and prevented the BRAFV600-mediated transformation of NIH-3T3 cells (Figure 2H).

Our results indicate that BRAF activity regulates TRAF6 polyubiquitination in CRC cells, thus facilitating association of active TAK1 with the p45-IKKα complex.

Endosome acidification inhibitors revert BRAF-mediated transformation and cancer cell growth in a p45-IKKα dependent manner.

We previously found that p45-IKKα activation was linked to the endosomal compartment of CRC cells [44]. We here used a specific protocol that allows the characterization of endosomal-associated proteins[45] to test the effect of BRAF activation on different IKK-related elements in this cellular compartment. In NIH-3T3 cells, ectopic BRAFV600 expression increased the levels of polyubiquitinated NEMO in the endosomal compartment (lanes 2) associated with an accumulation of active TAK1 and its adaptor TAB2 (Figure 3A). Interestingly, BRAFV600 was also located in the endosomes. In HT29 and WiDr CRC cells, mutant BRAF and several IKKα species, likely corresponding to post-translational modifications or partial proteolytic fragments of full-length IKKα, and whose identity was again confirmed in IKKα deficient MEFs (Figure S3A), colocalized in the endosomal compartment together with polyubiquitinated NEMO and TAK1 (Figure 3B). Clinically relevant, inhibitors of the endosomal V-ATPase (responsible for endosome acidification) such as chloroquine or the antibiotic bafilomycin A1 abrogated TAK1 phosphorylation and p45-IKKα activation (Figure 3C). In these experiments, we also found a decrease in the total levels of active β-catenin after treatment that can be explained by the reduction in TAK1 activity, as previously shown [41]. Therefore, we proposed that endosomal inhibitors that are currently used in patients for other applications (chloroquine is used for malaria prevention and treatment, and bafilomycin A1 as antibacterial, antifungal, and immunosuppressive agent) might target BRAF mutated cancer cells by interfering with p45-IKKα activity, but also with other proteins carrying oncogenic activity such as β-catenin. As predicted, chloroquine or bafilomycin A1 treatment

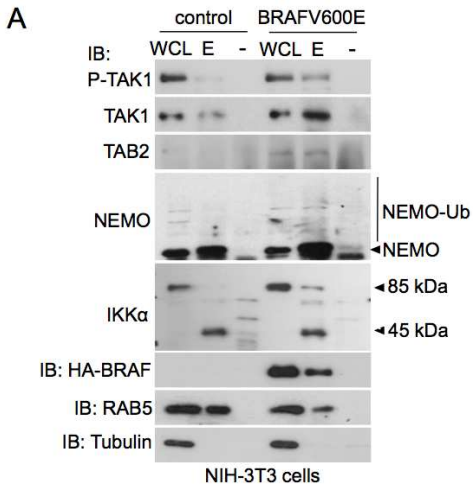


Figure 3. BRAF-dependent p45-IKKα activation, transformation, and CRC cell growth is reverted by endosome acidification inhibitors. (A) NIH-3T3 cells were infected with a control vector or with BRAF-V600E. Two days after the infection cells were digitonin-permeabilized, isolated and left untreated (WCL) or treated with proteinase K in the absence (E) or presence of Triton X-100 (-). Samples were analyzed by western blot with the indicated antibodies. Rab5 is used as a control of endosomal purity.

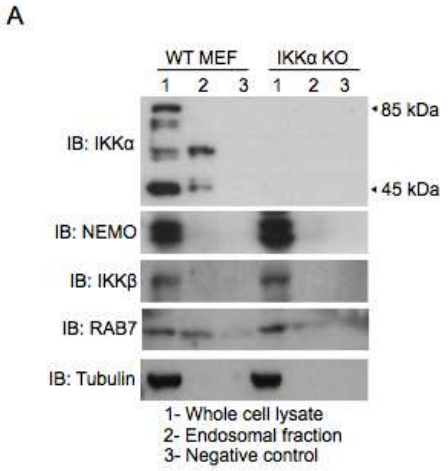
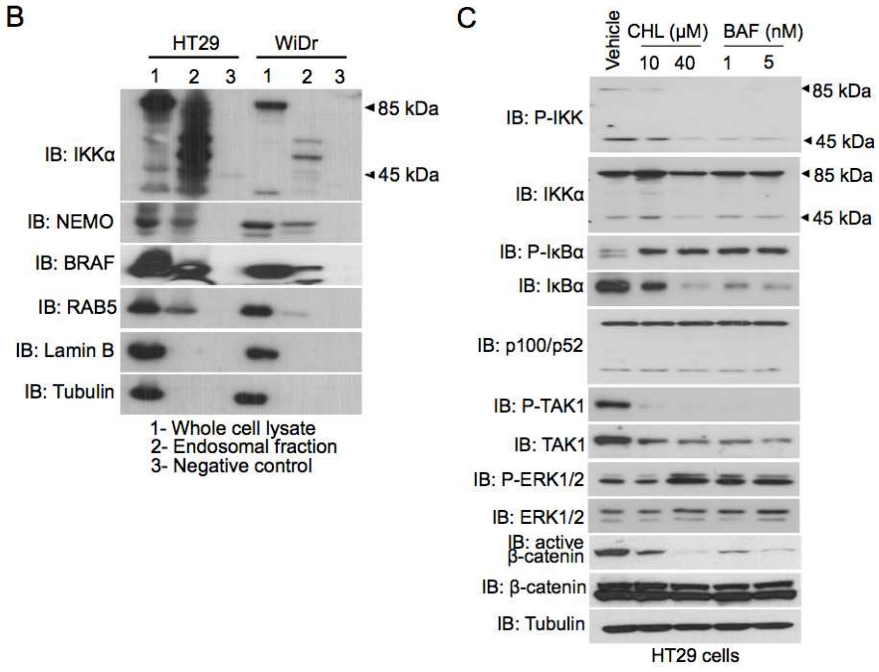
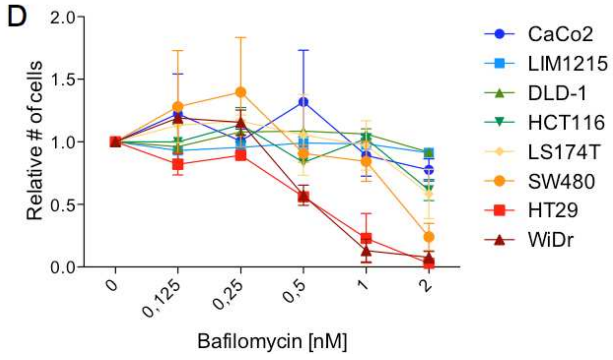


Figure S3. Related to Figure 3 (A) WT and IKKα KO MEFs were permeabilized in medium containing digitonin (see methods), isolated by centrifugation at 1000 rpm and left untreated (1) or treated with proteinase K in the absence (2) or presence of Triton X-100 (3). Samples were analyzed by western blot with the indicated antibodies.


B
HT29
WiDr
1 2 3 1 2 3
IB: IKKα
IB: NEMO
IB: BRAF
IB: RAB5
IB: Lamin B
IB: Tubulin
85 kDa
45 kDa
1- Whole cell lysate
2- Endosomal fraction
3- Negative control
C
Vehicle
CHL (μM)
10 40
BAF (nM)
1 5
IB: P-IKK
IB: IKKα
IB: P-IκBα
IB: IκBα
IB: p100/p52
IB: P-TAK1
IB: TAK1
IB: P-ERK1/2
IB: ERK1/2
IB: active β-catenin
IB: β-catenin
IB: Tubulin
85 kDa
45 kDa
HT29 cells


D
Relative # of cells
2.0
1.5
1.0
0.5
0.0
0
0,125
0,25
0,5
1
2
Bafilomycin [nM]
CaCo2
LIM1215
DLD-1
HCT116
LS174T
SW480
HT29
WiDr

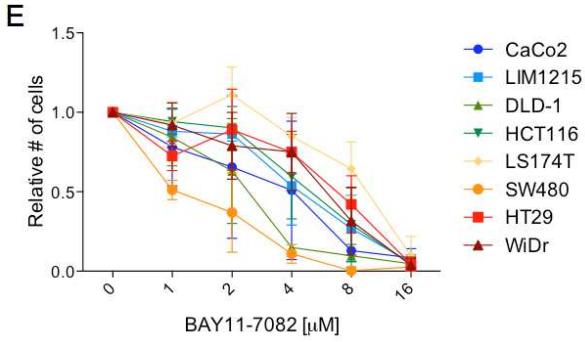

E
Relative # of cells
1.5
1.0
0.5
0.0
0
1
2
4
8
16
BAY11-7082 [μM]
CaCo2
LIM1215
DLD-1
HCT116
LS174T
SW480
HT29
WiDr

Figure 3. BRAF-dependent p45-IKKα activation, transformation, and CRC cell growth is reverted by endosome acidification inhibitors. (B) HT29 and WiDr cells were digitonin-permeabilized, isolated and left untreated (1) or treated with proteinase K in the absence (2) or presence of Triton X-100 (3). Samples were analyzed by western blot with the indicated antibodies. Rab5 is used as a control of endosomal loading and purity. (C) HT29 cells were treated with chloroquine or bafilomycin A1 for 16 hours at the indicated concentrations. Whole cell extracts were obtained and analyzed by western blot analysis with the indicated antibodies. (D and E) LIM1215 (WT for KRAS and BRAF), Caco2 (WT for KRAS and BRAF), DLD-1 (KRAS G13D), HCT116 (KRAS G13D), LS174T (KRAS G12D), SW480 (KRAS G12V), HT29 (BRAF V600E) and WiDr (BRAF V600E) cells were treated for 72 hours with bafilomycin A1 (D) or with BAY11-7082 (E) at the indicated concentrations. Quantification of the number of cells after the treatment is shown compared to the control.

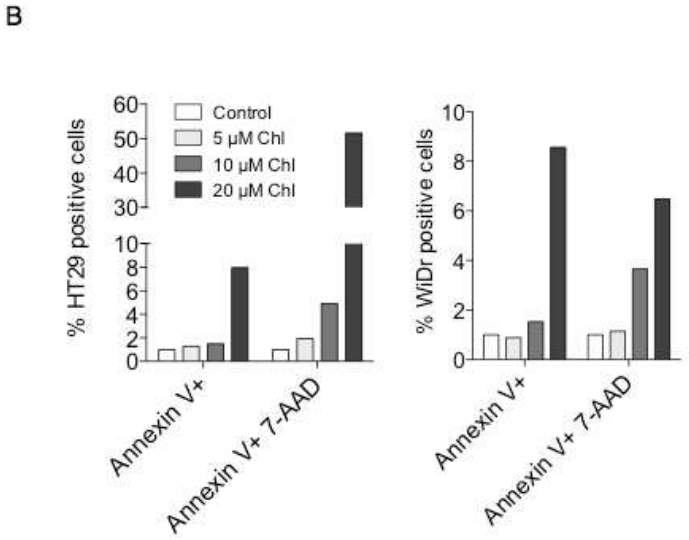


Figure S3. Related to Figure 3 (B) HT29 and WiDr cells were treated with chloroquine for 48 hours at the indicated concentrations. Apoptosis was determined by flow cytometry analysis of AnnexinV binding.

reduced cell growth in CRC cells, being cells carrying BRAF mutations (HT29 and WiDr) the most sensitive to the drugs followed by cells with mutant KRAS (HCT116, Ls174T and SW480) as determined by cell counting (Figure 3D). We also found a positive correlation between drug sensitivity and the levels of active p45-IKKα in CRC cell lines (data not shown), with the exception of the Caco2 cell line, but not with the presence of mutations in APC or β-catenin. Interestingly, general IKK inhibition with BAY11-7082 led to CRC cell death in a dose-dependent manner that was independent of KRAS and BRAF mutations or active p45-IKKα levels (Figure 3E), likely reflecting the essential requirement of most cell lines for NF-κB. Reduced cell growth induced by endosomal inhibitors was linked to increased apoptotic rate of as determined by annexinV binding (Figure S3B), with no significant changes in the cell cycle profiles generated by propidium iodide incorporation (data not shown). To determine the relative contribution of p45-IKKα activity to the effects of chloroquine in BRAF mutated cells, we transduced them with a constitutively active form of p45-IKKα (denoted as p45-IKKα_E in which serine 176 was changed to glutamic acid). Ectopic expression of p45-IKKα_E significantly reduced the sensitivity of HT29 and WiDr cells to chloroquine (Figure 3F), although they both stopped growing at high drug concentrations (not shown). Furthermore, chloroquine treatment reduced the transformation capacity of BRAF-V600E in NIH-3T3 cells, in a dose-dependent manner (Figure 3G), which was significantly reversed by the expression of constitutively active p45-IKKα (Figure 3H).

These data indicate that p45-IKKα activation in BRAF mutant CRC cells takes place in the endosomal compartment and can be efficiently blocked by V-ATPase inhibitors. Consequently, BRAF mutant cells are

particularly sensitive to these inhibitors, which could be clinically relevant.

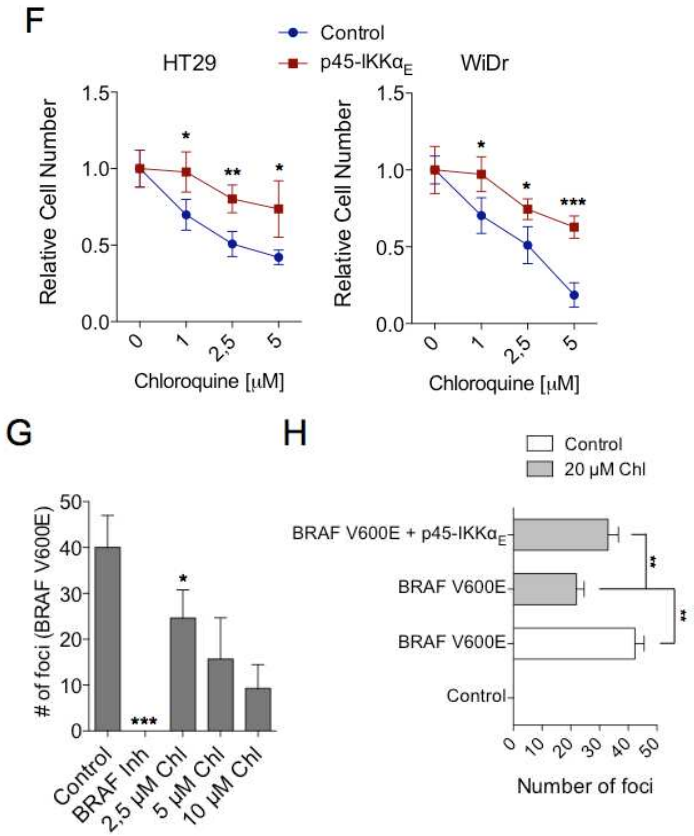


Figure 3. BRAF-dependent p45-IKKα activation, transformation, and CRC cell growth is reverted by endosome acidification inhibitors. (F) HT29 and WiDr cells were transduced with a control vector or with a p45-IKKαE and selected for 72 hours in G418. Next, cells were replated and treated for 72 hours with chloroquine at the indicated concentrations. Quantification of the number of cells after the treatment is shown compared with the control. (G) NIH-3T3 cells were infected with BRAF-V600E. Two days after the infection cells were treated with vehicle, BRAF inhibitor (2 μM) or chloroquine at the indicated concentrations. Inhibitors were freshly added every two days. One week after the treatment, culture dishes were washed with PBS, fixed and stained with methylene blue. Quantification of the number of foci obtained is shown in the graph. (H) NIH-3T3 cells were transduced with the indicated vectors. Cells were

then selected with G418 for 72 hours for the transduction. After this cells were treated with chloroquine. Chloroquine was freshly added every two days. One week after the treatment culture dishes were washed with PBS and fixed and stained with methylene blue. Quantification of the foci obtained is shown. In all panels, statistic analysis was performed using T-test (*p<0.05; **p<0.01; ***p<0.001).

Targeting p45-IKKα prevents tumor initiation and CRC metastasis in vivo.

We previously demonstrated that p45-IKKα was active in the nucleus of human CRC cells associated with advanced disease[44]. Based on our new data we predict that active IKKα levels might correlate with activation of the BRAF/MAPK pathway in CRC samples. By IHC analysis of 184 adenoma and carcinoma samples and the corresponding paired distal normal mucosa from 98 of these patients, we found that 80% of samples with high levels of nuclear P-IKK were positive for P-ERK1/2 staining, compared with 20% of P-ERK positivity in those samples that were negative for IKK activation (p<0.001) (Figures 4A and 4B). In a subset of 93 samples that had been analyzed for the presence of KRAS, BRAF and PI3K mutations, we found that tumors with high levels of nuclear P-IKK include 60% of tumors carrying the BRAFV600E mutation (9 out of 16; p<0.001) (Figure S4), suggestive of an association between p45-IKKα activity and the BRAF pathway in CRC.

Because endosomal inhibitors induced apoptosis of CRC cells carrying BRAF or KRAS mutations and high levels of active p45-IKKα while they exhibit low toxicity in non-mutated epithelial cells, we studied the possibility to exploit the use of endosomal-targeting drugs for treating human CRC tumors. For this study, we selected a human tumor carrying the KRASG12V mutation that contained huge amounts of active p45-IKKα and P-ERK1/2 (Figures 4C and 4D). Importantly, this particular tumor had

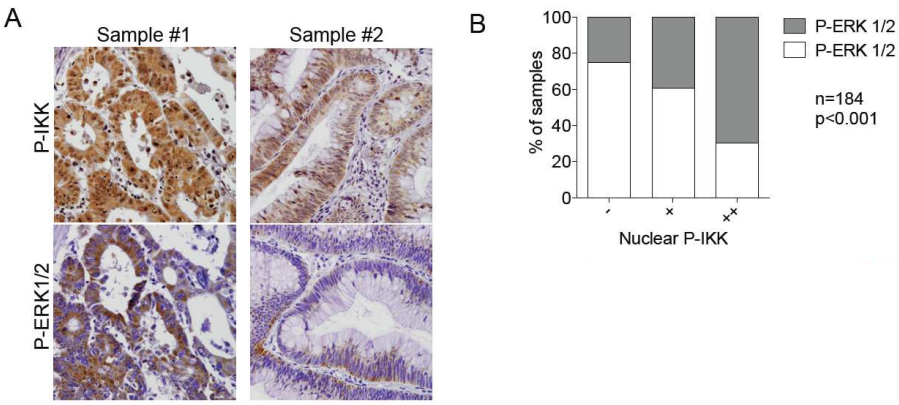
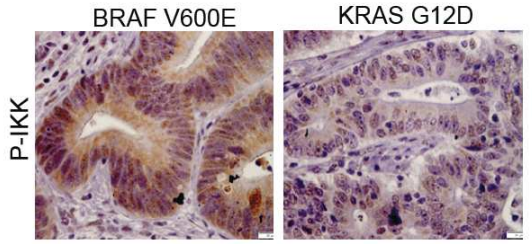


Figure 4. Endosome inhibitors prevent metastatic tumor initiation in vivo. (A) IHC with α-P-IKKα/βs and α-P-Erk1/2 from serial sections of human in inal adenoma and carcinoma samples (400x) from a human colon tissue micro-array including 184 tumor samples. (B) Adenoma and carcinoma samples were classified depending on the intensity of nuclear α-P-IKKα/β staining and P-Erk1/2 staining. Fisher's exact test was used to determine the p-value.



Nuclear P-IKK	negative	low	high	TOTAL
WT	5 (6.2)	68 (62.6)	9 (13.2)	82
Mutant	2 (0.8)	3 (8.4)	6 (1.8)	11
TOTAL	7	71	15	93

p<0.001

Figure S4. Related to Figure 4. IHC with α-P-IKKα/βs in cancer samples with the BRAFV600E mutation and KRASG12 or KRASG13. Tables represent the distribution of the P-IKKα/βs nuclear staining pattern in the samples categorized as WT or mutant for BRAF status. Statistical analysis demonstrated that P-IKK levels strongly associated with the BRAF mutations in CRC samples (X-square test, p=0.0001).

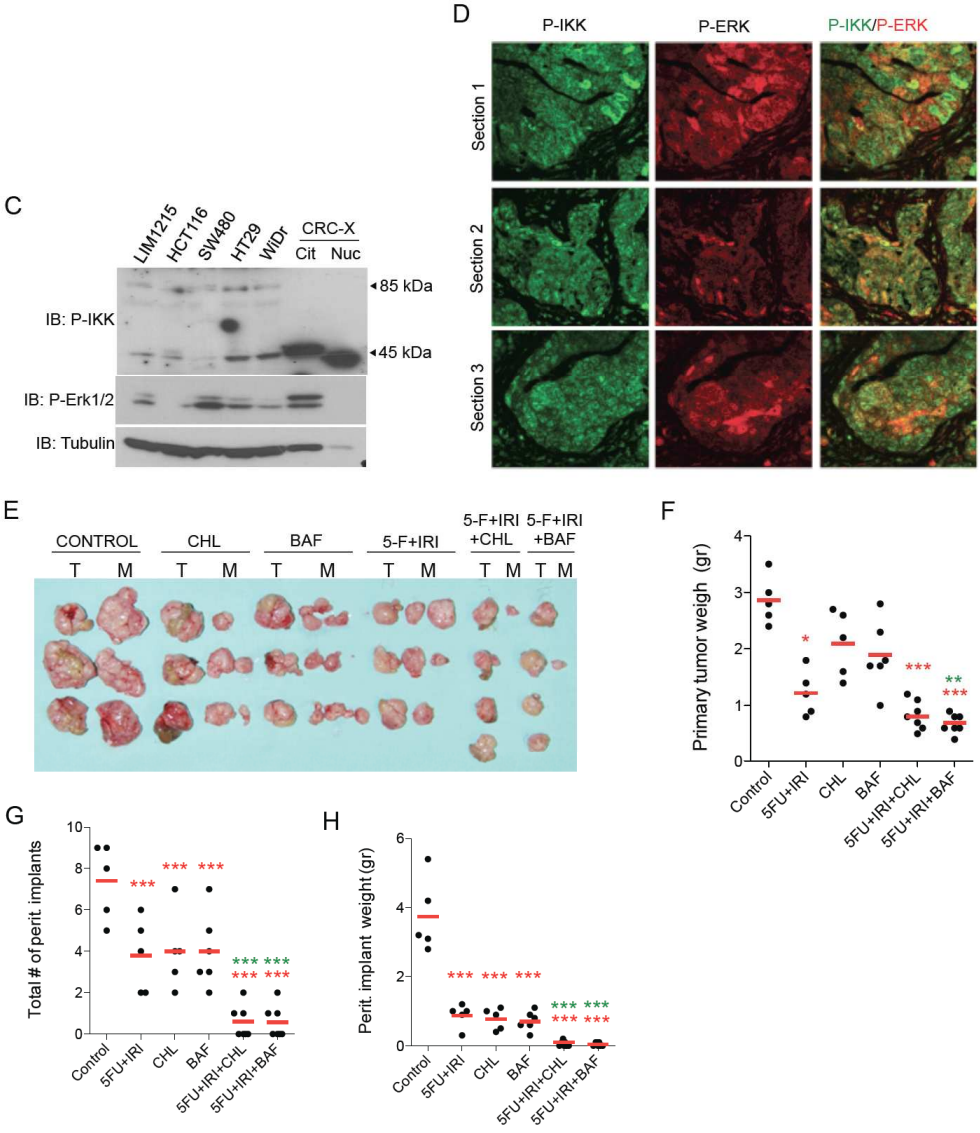


Figure 4. Endosome inhibitors prevent metastatic tumor initiation in vivo. (C) Protein extracts from the primary human CRC tumor used in the in vivo experiments were analyzed by western blot in comparison with a number of CRC cell lines: LIM1215 (WT for KRAS and BRAF), HCT116 (KRAS G13D), SW480 (KRAS G12V), HT29 and WiDr (BRAF V600E). (D) Confocal images of different sections from the implanted tumor double stained for P-IKK and P-ERK1/2. (E) Photograph of the tumors recovered at the end of the experiment with the indicated treatments. T refers to the primary tumor and M to

the peritoneal implants. (F, G and H) Tumor weight (F), total number of peritoneal implants (G) and the total weigh of the peritoneal implants (H) obtained with the different treatments. Statistical analysis was performed by T-test comparing the different treatments with control animals (red asterisks) or with animals treated with 5-FU+Irinotecan (green asterisks); *p<0.05; **p<0.01; ***p<0.001.

become refractory to 5-fluorouracyl (5-FU) and Irinotecan chemotherapeutic agents, and had developed intra-peritoneal metastases in the patient. A fragment of the tumor was first expanded subcutaneously in nude mice and after that, comparable fragments of the tumor were orthotopically implanted in the wall of the cecum of 30 immune-compromised animals. Mice were then separated in the different groups of treatment (5 animals per group), and treatments started concurrently when tumors were detectable by palpation. 21 days after initiating the treatments, animals were euthanized and analyzed for the presence of tumors both in the intestine or associated to the wall of the peritoneum. We found that untreated tumors greatly resembled the original human tumor, and developed multiple implants in the peritoneum of the mice that we referred to as metastases. Treatment with 5-FU plus Irinotecan had a significant therapeutic effect in this model leading to the reduction in the size of the primary tumor (Figures 4E and 4F), and the number and size of intra-peritoneal metastases (Figures 4E, 4G and 4H), associated with a significant toxicity in the animals (not shown). Treatment with chloroquine and bafilomycin A1 alone led to a moderate decrease in the size of the primary tumor and a significant decrease in the metastatic load, that was comparable to the effects of 5-FU plus Irinotecan (Figures 4E, 4F, 4G and 4H), but without signs of toxicity at the administered doses. Most importantly, combination of the standard treatment with either chloroquine or

bafilomycin A1 resulted in the total elimination of intraperitoneal implants in 8 out of 10 animals (Figures 4E, 4G and 4H), without increasing the general toxicity of the chemotherapeutic treatment.

These results support previous data indicating that endosomal inhibitors can potentiate the effectiveness of conventional anti-cancer cocktails in different types of tumor [46-49], but further provides a mechanism-driven therapeutic opportunity for CRC patients carrying KRAS/BRAF mutations. Most importantly, it can offer an alternative treatment for patients carrying these mutations that have developed resistance to the standard treatments.

Discussion

Tumors carrying mutant KRAS or BRAF usually associate with worse prognosis, since these mutations preclude the use of anti-EGFR inhibitors. Moreover, KRAS inhibitors are extremely toxic and the recently developed BRAF and MEK inhibitors only showed a partial anti-tumoral effect due to the acquisition of drug resistance. In this context it is of crucial 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 revert TAK1 activity, and prevent phosphorylation of p45-IKKα in CRC cells thus potentiating the anti-tumoral and anti-metastatic effect of conventional chemotherapy in vivo. This is a mechanism-driven observation, since we demonstrate that p45-IKKα activation requires the activity of the early endosomal compartment[44], and demonstrate how activated BRAF is capable of inducing accumulation of polyubiquitinated NEMO in the endosomes,

which is a mark for TAB2/TAK1 recruitment (Figures 3A, 3B and 3C). Whether this mechanism for TAK1 sorting into the endosomes through TAB2 and NEMO is linked with specific post-translational modifications of other upstream elements, including RAS mono-ubiquitination (that takes place in the endosomal compartment) and is a pre-requisite for subsequent RAF activation[50], needs to be further investigated.

Beyond the identification of the mechanism linking BRAF and p45-IKKα activation, we have here demonstrated that BRAF and p45-IKKα activities do not primarily affect canonical or alternative NF-κB but mostly impacts on specific IKKα substrates such as histone H3 or the SMRT co-repressor. Consistent with this observation, BRAF inhibitors resulted in the transcriptional repression of IKKα and/or SMRT target genes *cIAP2*, *Hes1 and Herp2*, known to regulate specific tumor features as well as the pro-metastatic gene *Maspin*, but does not significantly affect the NF-κB targets *Nfkbia* and *TNFAIP3*/*A20*. Thus, the possibility to discriminate between oncogenic IKKα functions and the IKK-mediated activation of NF-κB is of essential relevance from a therapeutic point of view since NF-κB is essential for cell survival and its general inhibition results in undesirable and unpredictable side effects [30,51]. Thus, we propose that inhibitors of the endosomal function can be used in combination with standard therapies for treating patients carrying KRAS or BRAF mutated tumors and to prevent tumor metastasis. Although our current goal is to identify novel and more effective inhibitors of the endosomal function with higher specificity towards preventing TAK1 and p45-IKKα activation, the finding that available and clinically approved compounds can be used as adjuvant treatments to current chemotherapy provides the possibility of a more immediate translation to CRC patients. The fact that inhibitors of endosomal acidification are not exclusively blocking p45-IKKα activity

can also have an advantageous effect for CRC treatment. In this sense, these endosome inhibitors have been previously found to modulate β-catenin activation [52] but also Notch activation [53]. Interestingly, both pathways are pathologically activated in most types of CRC, independent of KRAS/BRAF mutations. Our results show a prevalent effect of chloroquine or bafilomycin A1 on cells that carry such mutations; however its putative effect on Notch or β-catenin activity may provide additional advantages when considering tumor heterogeneity and the importance of this pathway on cancer initiating cells.

In summary, we have identified a new signaling cascade downstream of KRAS/BRAF that involves trafficking of several NF-κB and IKK regulators to the endosomes and results in the processing and activation of p45-IKKα. Inhibition of this endosomal activity provides a previously unexpected way of curing cancer.

Experimental Procedures

Plasmids

The expression plasmid MT-IKKα and MT-p45-IKKα$_E$ were constructed by inserting the PCR-amplified corresponding region, using a HA-IKKα and HA-IKKα$_E$ respectively as a template, into the mammalian expression vector pcS2-MT. MT-IKKα S400W and MT-IKKα-3M were constructed by using the QuikChange™ Site-Directed Mutagenesis Kit from Stratagene and according to manufacturer instructions. Specific shRNAs against IKKα (x-2811c1 and x-2866s1c1) and TAK1 (x-2844s1c1 and x-601s1c1) were from sigma. For the infection experiments, lentiviral expression vectors for BRAF-WT and BRAF-V600E were a gift from Mario Encinas lab. The lentiviral plasmid for KRASG12V was constructed by inserting the PCR-amplified corresponding region, using and HA-KRASG12V as a template, into the lentiviral vector MSCV-IRES-GFP. Viral particles were produced using the standard techniques.

Antibodies and Inhibitors

α-IκBα (sc-1643), α-NEMO (sc-8330), α-Rab5 (sc-46692), α-P-IKKα-Ser180/IKKβ-Ser181 (sc-23470) and LaminB (sc-6216) were purchased from Santa Cruz Biotechnology. α-IKKα (OP-133) from Oncogen. α-P-IKKα-Ser176,180/IKKβ-Ser177,181 (2697S), α-P-IκBα$_{32-36}$ (92465), α-P-Erk1/2 (4370), α-B-Raf (9433), α-TAK1 (4505), α-P-TAK1-Ser412 (9339), α-AKT (9272) and α-P-AKT (9275) from Cell Signaling. α-P-Histone H3-Ser10 (06-570), α-Histone H3 (06-755), α-Pan-Ras(Ab3)(OP40) and α-Active–β-catenin clone 8E7 (05-665) from Millipore. α-α-tubulin and α-β-catenin (c2206) from Sigma. α-HA (12CA5) from Covance. α-MT antibody was the 9E10 hybridoma. Monoclonal antibodies against human p45-IKKα have been described previously [44]. 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 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 µg of transfer vector, 15 µg of packaging plasmid (psPAX2), and 6µg of envelope plasmid (pMD2.G) were used. After 3 days, supernatant was ultracentrifuged and viral pellet resuspended in 100 µl of PBS. 20µl of fresh viral suspension was used per infection.

Western blot analysis and immunoprecipitation assays

Cells were lysed 30min at 4ºC in 300µl PBS plus 0.5% Triton X-100, 1mM EDTA, 100 mM Na-orthovanadate, 0.25 mM PMSF and complete protease inhibitor cocktail (Roche). For immunoprecipitation, supernatants were pre-cleared 2h with 1% of BSA, 1µg IgGs and 50µL SPA beads. Precleared lysates were incubated O/N with 3µg of indicated antibodies. Antibody-Protein complexes were captured with 30µL SPA beads for 2h. After washing, precipitates were analyzed by western blot.

Cell fractionation

Nuclei were isolated in 0.1% NP-40/PBS for 5min on ice, followed by centrifugation at 1900 rpm and then lysed in 50mM Tris-HCl pH 7.5, 150mM NaCl, 1% Nonidet P-40, 5mM EGTA, 5mM EDTA, 20mM NaF and

complete protease inhibitor cocktail (Roche). Supernatants were recovered as the cytoplasmic fraction. For cytoplasm/nuclear/chromatin separations, cells were lysed in 10mM HEPES, 1.5mM MgCl2, 10mM KCl, 0.05%NP40 at pH 7.9, 10min on ice and centrifuged at 13.000 rpm. Supernatants were recovered as cytoplasmic fraction and the pellets lysed in 5mM HEPES, 1.5mM MgCl2, 0.2 mM EDTA, 0.5mM DTT and 26% glycerol and sonicated 5' three times to recover the soluble nuclear fractions. Pellet included the chromatin fraction. Lysates were run in SDS/PAGE and transferred to immobilon-P transfer membranes (Millipore) for western blotting.

Statistical Methods

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 P-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., Cary, NC), and the level of significance was established at 0.05 (two sided).

Treatment of orthotopic xenografts with endosome acidification inhibitors

Human tumors were perpetuated (>5 passages mice to mice) and kept crio-preserved for further experiments. In these experiments, equivalent pieces of individual tumors were implanted orthotopically in the wall of the cecum. After 2-3 weeks tumors were detectable by palpation, and the animals were separated in groups of 5 mice each and treated with the indicated drugs. After 21 days of treatment, mice were euthanized and the tumors collected, measured and processed for IHC analysis. Animals were kept under pathogen-free conditions, and the Animal Care Committee at the Catalan Institute of Oncology approved all procedures.

Immunofluorescence and Confocal Microscopy Analysis

For the double detection of P-IKK and P-ERK1/2, samples were incubated overnight with P-IKKα/β antibody, detected with the Universal Staining Kit from DAKO and developed using the TSA™ Plus Cyanine3/Fluorescein System. Samples were treated with 2% H_2O_2 for 2 hours and then incubated with α-P-Erk1/2 antibody overnight that was detected with HRP-linked secondary antibody and developed with TSA™ Plus Cyanine3/Fluorescein System. As negative controls of the experiment, we followed the same protocol in the absence of P-IKKα/βS180/181 or P-ERK1/2 antibody. Confocal microscopy analysis was performed using a Leica TCS SP5 CFS microscope.

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 0.7–0.8mm apart. Paraffin sections of 4μm were hydrated, permeabilized and antigen retrieval was achieved by incubating with 10mM citrate buffer overnight at 80ºC. Primary antibodies were incubated overnight and then developed with the DAB System from DAKO.

Quantitative RT-PCR

Total RNA was obtained using the RNeasy kit from Qiagen. RNA quality was assessed on agarose gels and quantified by Nano-Drop1000 (Nano-Drop, Wimington, DE). cDNA was synthesized with the RT-First Strand cDNA Synthesis kit (Amersham Pharmacia Biotech) following

manufacture instructions. Real-time polymerase chain reaction (PCR) was performed in triplicates on the Light Cycler 480 (Roche) and SYBR-Green (Applied Biosystems) was used to detect gene expression. Expression of individual genes was normalized to β-actin expression.

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 protein 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 6.5µg/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 the reaction was stopped by adding 20 mM PMSF preheated in loading buffer, and samples used for WB analysis.

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CONCLUSIONS

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Part 1:

1. Adenoma and carcinoma samples show increased levels of total and nuclear active IKK compared with the normal adjacent tissue.
2. The majority of nuclear active IKK found in cell lines and human CRC samples correspond to a 45kD isoform of IKKα.
3. p45-IKKα is generated by cathepsin B/L-dependent proteolytic processing of full-length IKKα and can be detected with an specific antibody against the cleavage site.
4. Active p45-IKKα is found in a complex with NEMO and non-phosphorylated full length IKKα in the nucleus of CRC cells that is able to phosphorylate specific nuclear substrates.
5. p45-IKKα is required for CRC cell survival and tumor formation.

Part 2:

1. Oncogenic KRAS through BRAF modulates and depends on IKKα activity.
2. BRAF induces phosphorylation of p45-IKKα through TRAF6 poly-ubiquitination, TAK1 activation and association with the p45-IKKα complex.
3. Inhibitors of endosome acidification block BRAF-mediated phosphorylation of p45-IKKα and CRC cell growth in a p45-IKKα-dependent manner.
4. Inhibitors of endosome acidification reduced tumor growth and peritoneal implants in an orthotopic model of human CRC.

DISCUSSION

DISCUSSION

Our results indicated that human CRC tumors display constitutive nuclear IKKα phosphorylation, which is significantly associated with increased tumor grade. We have shown that active nuclear IKKα predominately correspond to a proteolytically processed fragment 45kD fragment whose generation is cathepsin-dependent. Increased p45-IKKα levels correlated with increased P-IKK expression in CRC samples. Moreover, increase in nuclear active IKKα correlates with elevated p45-IKKα levels, likely as a result of increased cathepsin activity in advanced tumors. Interestingly, we identified the non-NF-κB targets SMRT, N-CoR and histone H3 as substrates for nuclear IKKα in tumor cells. Importantly, we found that cleavage of IKKα into p45-IKKα is required for tumor growth in vitro and in vivo (Figure 1).

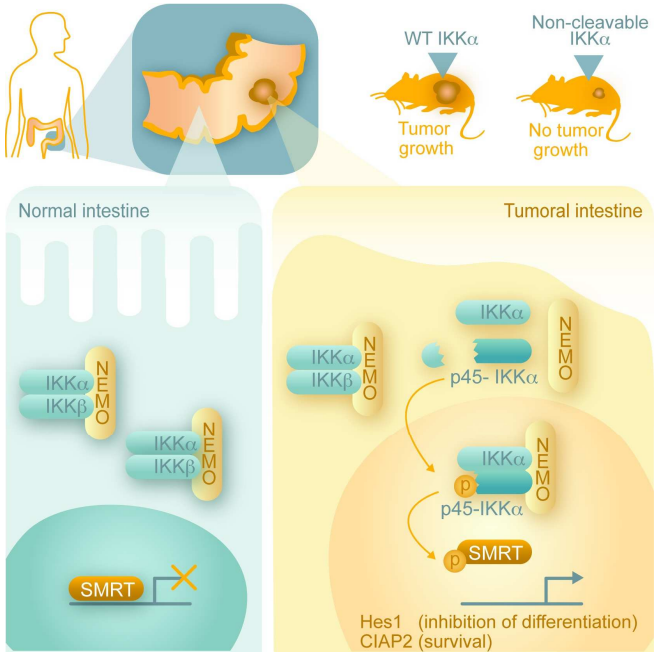

Figure 1. p45-IKKα is responsible for specific nuclear IKK activity in colorectal cancer.

Non-active IKKα is mostly distributed in the cytoplasm associated with IKKβ and NEMO, however, it was demonstrated that IKKα can translocate to the nucleus following TNFα induction and in tumor cells. We have now discovered that it is a truncated active p45-IKKα form that translocates to the nuclear compartment where it interacts with non-active FL-IKKα and NEMO. Which are the mechanisms regulating the formation of this specific IKK complex remain unknown, but they might include differential availability of IKK components (i.e. low amounts of nuclear IKKβ compared with the high levels of nuclear IKKα and the intermediate levels of NEMO), post-translational modifications of specific elements of the complex and participation of specific adaptor proteins that potentiate complex assembly. Equally important, we found that previously identified IKKα nuclear targets such as histone H3 or nuclear corepressors[1–3] are in fact dependent on p45-IKKα. Unexpectedly, analysis of the eluted fractions from Superdex S200 columns demonstrated that active FL-IKKα eluted in the LMW fractions likely corresponding to a monomeric form, which is not able to phosphorylate nuclear corepressors or histone H3. On the other hand, FL-IKKα is required for chromatin binding of p45-IKKα and for nuclear phosphorylation of SMRT and histone H3. We have demonstrated that the regulatory domains lacking in p45-IKKα (including SDD and NBD) are important for regulating its interaction with specific substrates although it retains the kinase domain and NLS sequences. In this sense, it has been recently demonstrated the importance of the SDD domain of IKKβ (that is conserved in IKKα) not only for substrate recognition but also for kinase activation[4]. We propose that p45-IKKα uses the SDD and NBD domains from FL-IKKα to achieve its function.

On the other hand, other and our results suggest that the mechanisms leading to p45-IKKα generation may not be unique and it is likely cell type dependent. Different possibilities include frameshift mutations leading to premature STOP codons[5]. These nucleotide substitutions can cause missense and nonsense mutations in IKKα, and deletions can cause frameshifts for IKKα. We have analyzed exons 13-15 in around 200 human CRC tumors and have not found this type of mutations. Another mechanism could be alternative splicing at specific exons. In this sense, splicing variants of the IKKα mRNA that codify for proteins lacking their regulatory Helix-Loop-Helix (HLH) regions, or both their HLH and their Leuzine Zipper regions, now re-defined as SDD[4], have previously been found in T-lymphocytes and in the brain[6]. In fact, in one of our CRC samples we identified an alternative IKKα splice variant lacking exon14 that resulted in a predicted truncated protein comparable to p45-IKKα. However, this is not a general mechanism in the more than 200 tumor samples and cell lines analyzed. Instead, our results suggest that the main source of p45-IKKα in CRC cells is the proteolytic processing of FL-IKKα by cathepsins. This is consistent with the fact that tumor cells display increased cathepsin activity as a result of enhanced aerobic glycolysis, also known as Warburg effect[7].

Moreover, human CRC samples showed increased levels of cathepsin B and L that colocalized with P-IKK in cytoplasmic ring-shape structures, corresponding to endosomal vesicles (as determined by RAB5 and RAB7 expression), suggesting that processing and activation of IKKα is previous to its nuclear translocation. Our prediction is that the higher levels of IKKα processing in cancer cells is in part the consequence of increased cathepsin levels/activity induced downstream of specific cancer-

associated pathways such as KRAS[8], ErbB2[9], the JAK-STAT[10] and the vitamin D pathway[11] in different types of cancers, including colon, breast and liver cancers. On the other hand, sorting of IKKα into the endosomal compartment is also crucial for its subsequent processing but the mechanisms involved are totally unknown. However, similar mechanisms of endosomal internalization leading to specific signaling have been demonstrated for several NF-κB related elements. For example, TLR4 induces TIRAP-MyD88 signaling at the plasma membrane and then it is endocytosed to activate TRAM-TRIF signaling in the early endosomes, leading to the production of interferons[12]. In this same context, it seems that TRIF-dependent signaling triggers noncanonical TRAF3 self-ubiquitination that activates the interferon response[13]. Similarly, TNF-R1 is internalized into the endosomes where it recruits a specific signalosome called the "death-inducing signaling complex" (DISC)[14]. In Drosophila, NF-κB-dependent dorso-ventral specification requires Toll receptor recruitment to the early endosomes to mediate NF-κB signaling[15].

Notch, a relatively unconnected signaling pathway, can also experience a ligand-independent activation following Deltex-induced internalization into the endosomal compartment. However, if endosomal Notch transits into the lysosome lumen, it is degraded; if Notch is delivered to the limiting membrane of the lysosome, it is activated by partial degradation of its extracellular domain[16].

Interestingly, our results indicate that the presence of p45-IKKα is not restricted to tumor cells since the non-phosphorylated form (that by definition is non active) is consistently found in the cytoplasm of non-transformed MEF and human normal colonic mucosa, thus suggestive of

a physiologic role for the p45-IKKα form. Because homozygous mutations of human IKKα leading to a premature STOP codon at position 422 results in a lethal syndrome associated with severe fetal malformation defects[17], we predict that physiological p45-IKKα functions might require the presence of the FL allele. Further work, including generation of new animal models should decipher the physiological and pathological contribution of p45-IKKα and to better discriminate between the effects of FL-IKKα and p45-IKKα under physiologic and pathologic situation. By now we are generating double mutants carrying the *Apc*^{Min} allele and the intestinal specific *Ikkα* deficiency. The analysis of these animals will tell us about the possibility and the interest of obtaining specific knockin animals carrying mutant IKKα forms that cannot be cleaved, and thus cannot generate p45-IKKα. This model would permit studying the requirement of p45-IKKα for pathway activation and for the generation of adenomas in the *Apc*$^{Min/+}$ mouse model.

The relevance of this work greatly resides in the characterization of a new form of IKKα which function is not required for the activation of canonical NF-κB or alternative NF-κB pathways but holds important tumorigenic potential. As we have mentioned before, it is of crucial importance for future therapeutic applications to distinguish between oncogenic functions and functions required for the normal development of a cell. We believe that our finding opens the possibility of designing new therapeutical anticancer treatments targeting IKKα cleavage that should restrict the negative effects of inhibiting general IKK activity and thus NF-κB. In addition, we have generated a novel antibody (*881H3)* that specifically recognizes p45-IKKα by IHC, IF and IP in human tumor samples, which is currently commercialized by Millipore

(http://www.millipore.com/catalogue/item/MABF222). We are currently testing the use of 881H3, likely in combination with anti-P-IKK antibodies for stratification of CRC patients (to identify those that would benefit from specific anti-IKKα therapies) and other clinical related applications. However, we need to be cautious since over-expression of a specific pathway or elements is not always related with its usefulness as therapeutic target. For example, several anti-EGFR monoclonal antibodies for the extracellular domain of the EGFR were developed to serve as predictive biomarkers for cetuximab therapy response, based on the clinical paradigm of breast cancer, where women with highly HER2 receptor-expressing tumors are more likely to respond to trastuzumab treatment. Unfortunately, early clinical studies did not confirm a correlation between EGFR expression levels and response to EGFR inhibitor therapy, and in fact several CRC patients showed good response despite the absence of measurable EGFR (Chung et al., 2005). In contrast, increased *EGFR* copy number is significantly associated with tumor response to cetuximab treatment, being the presence of activating KRAS mutations (that operate downstream and independent of EGFR activation) predictive of resistance to cetuximab[18]. A different study also identified *KRAS* mutations as a predictive response factor in cetuximab plus chemotherapy treatments[19]. Conversely, detection of the KRAS wildtype alleles is a strong predictor of increased overall survival in patients with irinotecan-refractory metastatic CRC treated with cetuximab[20]. All these studies have led the American Society of Clinical Oncology (ASCO) to propose that patients with metastatic CRC who are candidates for anti-EGFR antibody therapy should be tested for the presence of *KRAS* mutations, and when *KRAS* is mutated in codons 12 or 13, patients should not receive anti-EGFR as part of their treatment[21]. On

the other hand the expression levels of EGFR ligands, such as epiregulin or amphiregulin, are also good predictors of cetuximab response in patients with metastatic CRC and wildtype KRAS[22,23]. We propose that inhibitors of the oncogenic effects of active p45-IKKα, will be useful future tools for treating CRC patients carrying high levels of p45-IKKα and P-IKK.

Tumors carrying KRAS or BRAF mutations usually associate with worse prognosis, and there are not many therapeutical options. Moreover, KRAS inhibitors are extremely toxic, which led to the development of specific RAF kinase inhibitors. Thus, several small molecule inhibitors of RAF kinase activity are currently available that have been tested for specific applications. Of note, most of the kinase inhibitor are pan-RAF inhibitors, which could reflect the highly conservation that is present in RAF kinase domains[24]. Some of these inhibitors have demonstrated a dramatic effect on reducing tumor load, but after this initial response tumors develop resistance through different mechanisms[25–32]. As mentioned, the tyrosine kinase receptors of the EGFR family have received most of the attention as therapeutic targets in the case of tumors that are WT for KRAS and BRAF. In particular, tumors that overexpress EGFR and ErbB2 can be treated with specific inhibitors of these receptors, but this strategy has also demonstrated some efficacy in tumors that carry activating mutations in RAS, which then produce large amount of EGF-related factors that could act in an autocrine-paracrine manner in other cells of the tumor that are KRAS WT[33]. From these specific examples and many others that arise from clinical trials using gefitinib and erlotinib, the general conclusion is that identifying the population that would benefit from a specific therapy is crucial not only

for patient treatment but also for the better design of the clinical trials. In this sense, it was important the identification of a subset of lung cancer patients with mutations in the tyrosine kinase domain of the EGFR that improved the selection of patients for lung cancer trials who are most likely to take benefit from EGFR TKI treatment[34–36]. These same studies led to the discovery of a specific mutation in the EGFR that conferred resistance to lung tumors from patients receiving chronic gefitinib treatment[37–39]. Different clinical trials testing EGFR TKI therapies have also included patients based on the presence of documented EGFR mutations[40–46]. In these trials it was demonstrated that EGFR TKI therapy shows a response rate of about 50-70% with an excellent progression-free survival (PFS). In addition, they are well-tolerated compared with the conventional platinum-based chemotherapy treatments.

Another important topic is to understand why some drugs are more efficient in some cancer types that in others that carry the same mutation. For instance, vemurafenib effectively kills BRAFV600E mutant melanoma cells but have limited therapeutic effect in BRAFV600E mutant colon tumors, whereas in the latter blockade of the EGFR shows a strong synergy with BRAFV600E inhibition[47]. The most likely explanation is that BRAFV600E inhibition in CRC causes a rapid feedback activation of the EGFR, which accounts for the continued tumor proliferation through other RAF homologues (that is prevented by the EGFR inhibitor). In this context, the ideal therapeutic drug would be one that specifically inhibits oncogenic BRAF without inducing CRAF or other oncogenic pathway. Interestingly, a recent work has demonstrated that vemurafenib-resistant melanomas become drug-addicted and cessation of drug administration leads to the regression of the established drug-resistant

tumors[48]. In this specific scenario, the authors propose that a discontinuous-treatment strategy, which exploits the fitness disadvantage showed by drug-resistant cells in the absence of the drug, would preclude the onset of a lethal drug-resistant disease.

Because of the importance of the EGFR-KRAS-BRAF axis in cancer generation and progression it is of crucial importance to identify novel druggable targets downstream of BRAF with special attention on putative molecules that are currently used in the clinics for other therapeutic applications. For this purpose, the better characterization of the pathways and elements that are specifically induced downstream of mutant RAS or RAF is essential.

Our work identified IKKα as a downstream effector of RAS in cancer, and this specific IKKα function seems to be only required for cancer cells, suggesting that its inhibition should have reduced toxicity for the normal cells and for the patients in general. Thus we propose that, for example, IKKα inhibitors could be combined with BRAF inhibitors in tumors with increased EGFR-RAF activity mediated by the WT allele[27,28,31]. In addition, the molecules we identified as inhibitors of oncogenic IKKα activity are molecules that have been widely used in the clinics for many years. Chloroquine has been long used for the treatment and prevention of malaria, whereas bafilomycin A1 has been shown to have antibacterial an antifungal effects, as well as antimalarial effects. These drugs are then ideal candidates to be tested in cancer patients, since they are already approved for their use in humans and the secondary-adverse effects at the therapeutic doses used have been studied, which significantly shortens the period between target discovery and the approval of a new

therapeutic indication. Our results clearly indicate that both bafilomycin A1 and chloroquine (targeting the endosomal activity of the cells) can synergize with the more conventional anti-cancer cocktails, leading to either the elimination of the metastatic properties of cancer cells or preventing tumor initiation after cell dissemination, which needs to be further investigated. Moreover, it is important to remark that targeting the endosomal activity does not increase the toxicity of the treatments in our animal models, suggesting that they preferentially kill tumor cells, which is in agreement with our *in vitro* data. The possibility to combine these inhibitors with the conventional chemotherapic treatments should allow reducing the dose of the latter, thus attenuating the general toxic effects that they cause in the patients.

Mechanistically (Figure 2), we found that BRAF activity enhances the ubiquitination of TRAF6, which result in the co-recruitment of the TAB2/3-TAK1 complex and the NEMO/p45-IKKα complex inside the endosomes. This facilitates TAK1 activation and ends up in a TAK1-dependent phosphorylation of p45-IKKα. We are currently investigating how BRAF induces the ubiquitination of TRAF6. On the other hand, we are still analyzing what are the signals that induce IKKα sorting into the endosomes. By now, we have found that several of the elements that are required for p45-IKKα activation such as BRAF, TAK1 or NEMO are localized inside the endosomes, suggesting that they are all internalized

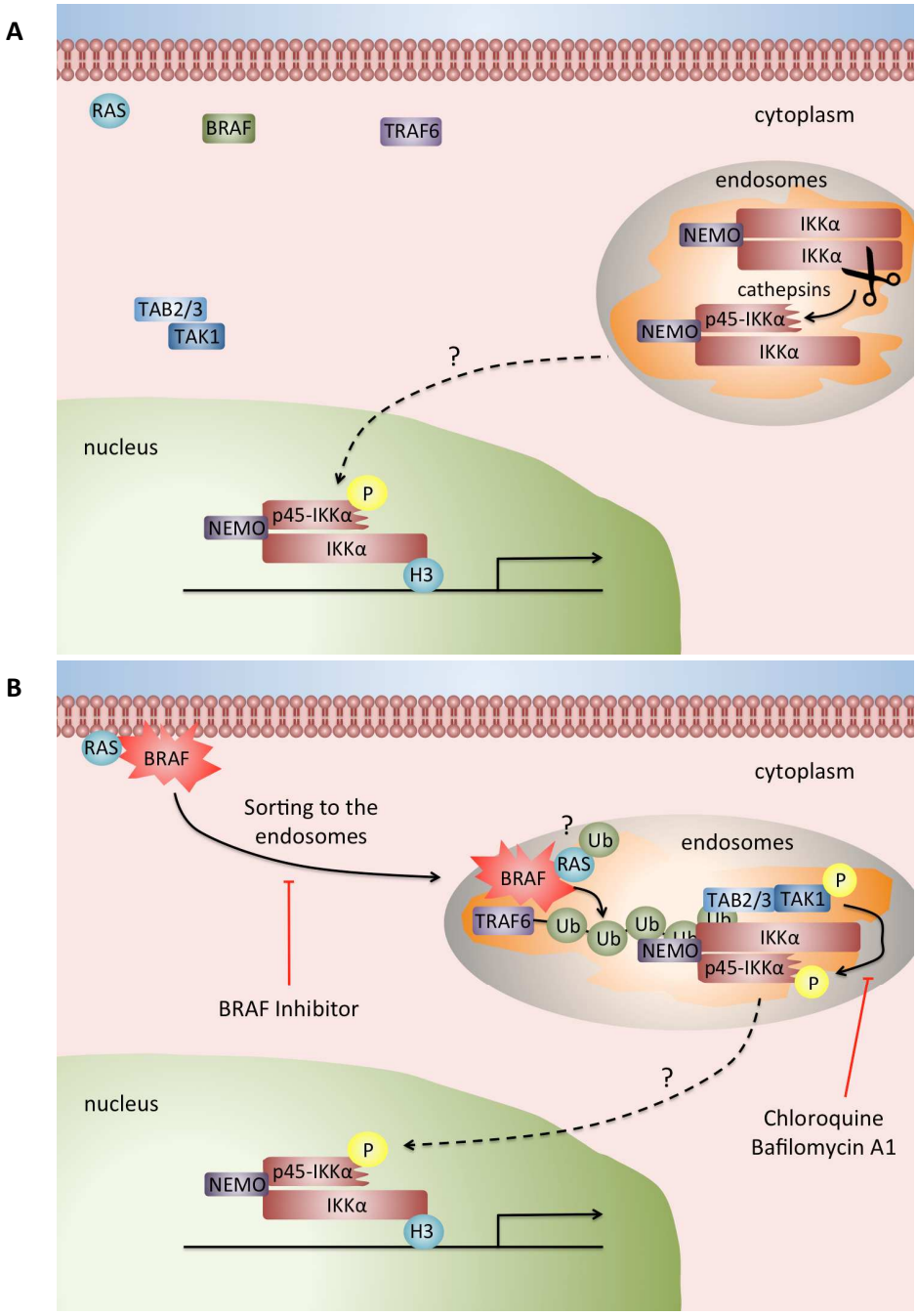

Figure 2. A. p45-IKKα generation in the endosomes in the absence of BRAF activation. B. BRAF activity enhances TRAF6 ubiquitination, which results in TAK1 and p45-IKKα recruitment, allowing TAK1-dependent activation of p45-IKKα

in a complex, in a similar fashion to what was described for other NF-κB regulatory elements such as TNF-R1[14] or TLR4[12]. Our first hypothesis is that ubiquitination and/or sumoylation of NEMO (that is part of the p45-IKKα complex) could impose its preferential endosomal distribution. However, it is also possible that TAB2 or TAB3 specifically bind TAK1 in a BRAF-dependent manner leading to the cytosolic or endosomal TAK1 distribution, which then activates either the canonical NF-κB pathway (through IKKβ) or p45-IKKα. Moreover, activation of the IKK complex by BRAF does not affect canonical or alternative NF-κB but mostly impacts on nuclear IKKα substrates such as histone H3 or the SMRT co-repressor. In agreement with this, we found that BRAF inhibitors led to the transcriptional repression of specific IKKα target genes such as *cIAP2* or *Hes1* but does not affect the NF-κB targets *Il6* or *Nfkbia*. This is again an indication that BRAF inhibitors and inhibition of oncogenic IKKα should have a minor effect on physiologic NF-κB activity. The possibility to discriminate between oncogenic IKKα functions and the IKK-mediated activation of NF-κB is of essential relevance from a therapeutic point of view since NF-κB is essential for cell and organism survival and its general inhibition results in severe undesirable side effects[49,50].

Using all the information we have presented here, we are now in the way of designing new combination of drugs that improved the effectiveness of conventional chemotherapy on preventing tumor spreading. Our current goal is to discover novel and more effective inhibitors of the endosomal function with higher specificity towards preventing p45-IKKα activation, which will open a previously unexpected way of treating tumors carrying KRAS/BRAF activation. We are also working on the identification of new small molecules that target specifically the

oncogenic effects of IKKα, without affecting not only normal NF-κB activation, preferably with no effect on normal IKKα activity. As a first approach, we will focused on molecules that interfere the interaction between p45-IKKα and TAK1, which is supposed to be exclusive of cancer cells, and not required for the activation on botch the canonical and the alternative NF-κB pathways. Another possibility would take into account the structural differences between full length IKKα and p45-IKKα. As mentioned in the text, p45-IKKα lacks the SDD domain located in the C-terminal end of IKKα, and as a consequence the central region of IKKα is exposed in the p45-IKKα but hidden in the full-length form. We propose to target this differentially-exposed region to specifically interfere with p45-IKKα activity. Related to this, it is important to remark the impossibility of targeting the kinase domain of p45-IKKα without targeting the full-length IKKα kinase but also the IKKβ kinase since both are extremely similar. A different strategy would take advantage of the fact that p45-IKKα binds full length IKKα, which is required for its activation and chromatin association. Yet another strategy would require the identification of the posttranslational modifications on IKKα that makes it to be recruited to the endosomes. We could identify the enzymes responsible and pharmacologically interfere with their activity.

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ABBREVIATIONS

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APC: Adenomatous Polyposis Coli

BAFF: B cell-activating factor

BCR: B cell receptor

CAC: Colitis-Associated Colon Cancer

CBP: CREB-binding protein

CDK: Cyclin-Dependent Kinase

CRC: Colorectal Cancer

DD: Death Domain

DEN: Diethyl Nitrosamine

DLBCL: Diffuse Large B-Cell Lymphoma

DSBs: Double-Strand Breaks

EDI: Ectodermal Dysplasia with Immune deficiency

EGF: Epidermal Growth Factor

EGFR: EGF receptor

ERK: Extracellular signal-Regulated Kinase

FAP: Familial Adenomatous Polyposis

GAPs: GTPase-Activating Proteins

GEF: Guanine Exchange Factor

GRB2: Growth factor Receptor-Bound protein 2

HCC: Hepatocellular Carcinoma

HGF: Hepatocyte Growth Factor

HLH: Helix-Loop-Helix

HNPCC: Hereditary Nonpolyposis Colon Cancer

hnRNP-U: Heterogeneous Ribonucleoprotein U

IECs: Intestinal Epithelial Cells

IFN: Interferon

IGF1-R: Insulin-like growth factor 1 Receptor

IRAK: IL-1R Associated Kinase

IκB: Inhibitor of κB proteins

LPS: Lipopolysaccharide

LT: Lymphotoxin

MEK: Mitogen-Activated Protein Kinase ERK Kinase

MMR: Mismatch Repair

MSI: MicroSatellite Instability

MyD88: Myeloid Differentiation primary response gene 88

NBD: NEMO-binding domain

NEMO: NF-κB Essential Modulator

NF1: Neurofibromin 1

NLS: Nuclear Localization Sequence

NSAIDs: Non Steroidal Anti-Inflammatory Drugs

NSCLC: Non Small-Cell Lung Cancer

PAMPs: Pathogen Associated Molecular Patterns

PAR: Poly(ADP-Ribose)

PARP-1: Poly(ADP-ribose)-Polymerase-1

PI3Ks: Phosphatidylinositol 3-Kinases

RANK: Receptor Activator of Nuclear Factor κB

RHD: Rel Homology Domain

RIP1: Receptor Interacting Protein 1

RTKs: Receptor Tyrosine Kinases

S1P: Sphingosine-1-Phosphate

SCC: Squamous Cell Carcinoma

SCF: Skp1-Cullin-F-box

SCLC: Small-Cell Lung Cancer

SDD: Scaffold/Dimerization Domain

SMRT: Silencing Mediator for Retinoic Acid and Thyroid hormone receptor

SUMO: Small Ubiquitin-related Modifier

TAB: TAK1 Binding Protein

TAK1: TGFβ–activated Kinase 1

TCR: T cell receptor

TGFβ-RII: TGF-β receptor II

TLR: Toll-like receptor

TNF: Tumor Necrosis Factor

TNFR: TNF receptor

TRADD: TNFR1-associated Death Domain

TRAFs: TNFR-associated factors

ULD: Ubiquitin-Like Domain

VEGF-R: Vascular Endothelial Growth Factor Receptor