IKKα is required in the intestinal epithelial cells for tumour stemness

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Background: Colorectal cancer is a common cause of death in developed countries. Progression from adenoma to invasive carcinoma requires accumulation of mutations starting with the Adenomatous Polyposis Coli (Apc) gene. NF-κB signalling is a key element in cancer, mainly related to the activity of IKKβ. IKKα kinase also participates in this process by mechanisms that are primarily unknown.

Methods: We generated a compound mouse model with mutation in Apc and lacking intestinal epithelial IKKα, produced intestinal organoids and tumour spheroids with different genetic backgrounds, and performed immunohistochemistry and RNA-seq analysis.

Results: Deficiency of IKKα prevents adenoma formation, with adenomas lacking IKKα showing reduced proliferation. In contrast, IKKα status did not affect normal intestinal function. The same divergent phenotype was found in the organoid–spheroid model. We also found that epithelial IKKα controls stemness, proliferation and apoptosis-related expression.

Conclusions: IKKα is a potential therapeutic target for Apc mutant colorectal cancer patients.

Keywords: colorectal cancer; IKK; tumour initiation; therapy

The current treatments for advanced CRC include surgery, and a combination of chemotherapies that are ineffective in a proportion of cases. Hence, it is of crucial importance to identify new therapeutic targets that can be exploited to improve CRC treatment and patient survival.

The nuclear factor-κB (NF-κB) is a key transcription factor that not only mediates innate and acquired immune responses but also regulates inflammation and carcinogenesis (Hayden and Ghosh, 2004; Hayden et al, 2006; Schulze-Luehrmann and Ghosh, 2006). Activation of the pathway by different stimuli such as tumour necrosis factor-α (TNFα), bacterial and viral products or DNA damage induces the phosphorylation of the inhibitor of NF-κB (IκB) kinase (IKK) complex downstream of transforming growth factor β-activated kinase 1 (TAK1). This complex is composed by IKKα, IKKβ and IKKγ/NF-κB essential modulator (NEMO) (Ghosh and Karin, 2002) that phosphorylates IκB leading to its ubiquitination and subsequent degradation. Degradation of IκB allows the nuclear translocation of the NF-κB factor, mostly p65/p50 dimers, leading to the activation...
of the canonical pathway. The IKKz subunit also participates in the activation of the alternative NF-κB pathway that is independent of IKKβ, NEMO and TAK1 (Vallabhapurapu and Karin, 2009). Specifically, factors inducing alternative NF-κB lead to the stabilisation of the NF-κB-inducing kinase (NIK) that in turn activates IKKz to phosphorylate p100. This cascade results in the proteolytic processing of p100 into p52 that will then translocate into the nucleus together with RelB to activate specific gene transcription.

Mostly related with its role in inflammation, several studies have demonstrated the association between NF-κB, IKKβ and cancer (Karin and Greten, 2005). In parallel, other reports identified NF-κB-independent functions that are carried out by elements of the pathway, thus contributing to specific tumourigenic capabilities. Some examples are found in squamous cell carcinoma (SCC), where IKKz induces the release of SUMO-1Xb from the chromatin leading to HOX and IRX gene transcription (Mulero et al, 2013). Also in SCC, nuclear IKKz regulates the levels of the metastasis suppressor gene Maspin (Toll et al, 2015), similar to that found in prostate cancer cells (Luo et al, 2007). In CRC, nuclear IKKz regulates the transcription of Notch target genes by phosphorylating the silencing mediator for retinoid and thyroid receptors (SMRT) and the nuclear receptor corepressor (N-CoR) (Fernández-Majada et al, 2007a, b). Moreover, the presence of nuclear active IKKz correlates with cancer progression, and we recently identified a novel IKKz isoform with a predicted molecular weight of 45 kDa (p45-IKKz) that lacks several regulatory regions at the C-terminal end of the kinase (Margalef et al, 2012). Active p45-IKKz localises in the nucleus of CRC cells and forms a complex with non-phosphorylated full-length IKKz and NEMO. Activation of this isoform involves the endosomal compartment and is induced by mutant BRAFV600E through the kinase TAK1 (Margalef et al, 2015). Furthermore, a mouse model deficient in IKKz kinase activity displays reduced Wnt-driven intestinal tumour development associated with an enhanced recruitment of interferon-γ (IFNγ)-expressing M1-like myeloid cells (Göktna et al, 2014). These data demonstrate that IKKz is a key regulatory element of tumourigenesis that acts both in an NF-κB-dependent and -independent manner. In the latter, IKKz might directly regulate essential factors that participate in the oncogenic process. However, to date, there are no conclusive data unveiling the contribution of intestinal epithelial IKKz neither in normal intestinal homeostasis nor in the oncogenic transformation of the intestinal stem and progenitor cells.

We have here generated mice with deletion of IKKz in the intestinal epithelial cells that were crossed with a model of intestinal tumourigenesis driven by APC mutation. We found that intestinal epithelial IKKz is essential for tumour formation while dispensable for normal tissue homeostasis, both in vivo and 3D in vitro cultures. Moreover, by RNA sequencing of wild-type (WT) and IKKz knockout (KO) tumouroids, we uncovered the molecular basis of IKKz addiction in transformed cells.

Conducted according to the guidelines from Generalitat de Catalunya.

**Materials and Methods**

**Animals.** For the generation of intestine-specific IKKz KO animals, the previously described *Ikki*+ mice (Gareus et al, 2007) were crossed with the Villin-Cre (from Jackson Laboratories, Bar Harbor, ME, USA) line, both in C57BL/6 background. *Apc*Min/+ mice (from Jackson Laboratories) were from C57BL/6 background. All mice were genotyped by PCR and animals were kept under pathogen-free conditions. All the animal work was conducted according to the guidelines from the National Institute of Health.

**Immunohistochemistry.** Formalin-fixed Swiss-roll intestinal samples were embedded in paraffin and sectioned at 4 μm. After dewaxing and rehydration, antigen retrieval was achieved using standard methods depending on the antibodies. Endogenous peroxidase activity was quenched by incubating the sections for 20 min with 1.5% H2O2. Primary antibodies Ki67 (Novocastra, Newcastle, UK, MM1), cleaved caspase 3 (Cell Signaling, Danvers, MA, USA, 9661) and β-catenin (Sigma-Aldrich, St Louis, MO, USA, C2026) were diluted in PBS containing 0.05% BSA and incubated overnight at 4 °C. Samples were then incubated with specific horseradish peroxidase (HRP)-labelled polymer (EnVision + System, DAKO, Glostrup, Denmark), and staining was developed using DAB + Substrate (Chromogen System, DAKO).

**Intestinal adenoma isolation and tumouroid culture in Matrigel.** Intestinal crypts and adenomas used in the 3D cultures were isolated as previously described (Sato et al, 2011). For organoid culture, we collected mouse small intestines, sliced them longitudinally and washed in cold PBS. Villi were removed by carefully scraping the surface, and the remaining tissue was cut into 3–5 mm sections. Samples were incubated twice in 2 mM EDTA for 30 min at 4 °C, filtered through a 100 μm pore diameter nylon and centrifuged at 110 g to obtain the crypt-enriched fraction. For tumouroid cultures, adenomas were isolated manually and incubated in 8 mM EDTA for 20 min at 4 °C. The remaining pellet was treated for 20 min at 37 °C in agitation with 0.4 mg ml−1 dispase and subsequently with 1.25 mg ml−1 collagenase each. Cell suspension was centrifuged for 5 min at 1200 r.p.m. and resuspended in 140 nM ROCK inhibitor (Y27632, Sigma) before filtration through 100, 70 and 40 μm pore diameter nylon meshes, consecutively. After that, cells were centrifuged for 5 min at 1200 r.p.m. and seeded in Matrigel (Corning, Corning, NY, USA) drops.

Approximately 10⁴ cells were seeded in 50 μl of Matrigel in 24-well plates. After polymerisation, 500 μl of complete medium ((DMEM/F12, Biological Industries, Kibbutz Beit-Haemek, Israel) with penicillin (100 U ml−1) and streptomycin (100 μg ml−1) (Biological Industries) supplemented with N2 and B27 (Invitrogen, Carlsbad, CA, USA) containing 140 mM ROCK inhibitor (Y27632, Sigma), 100 ng ml−1 Noggin (Peprotech, London, UK), 100 ng ml−1 R-spondin (R&D Systems Minneapolis, MN, USA), 50 ng ml−1 EGF (Sigma) and 20 ng ml−1 basic FGF (Peprotech)) was added and cultures maintained at 37 °C in 5% CO2. Tumouroid measurement was performed using the standard parameters in Fiji-Image (https://fiji.sc).

**Tumouroid immunostaining.** Tumouroids were seeded in Matrigel (Corning) onto a cover glass in a 24-well plate, and maintained in culture for 2 days. For whole-mount immunostaining, tumouroids were fixed with 4% paraformaldehyde for 20 min, permeabilised with 0.5% Triton X-100 (Pierce, Waltham, MA, USA) for 10 min and blocked in 2% BSA and 0.3% Triton X-100 for 1 h. Primary antibody (Kir67, Novoceastra, MM1) was incubated overnight. After extensive washing, secondary antibody donkey-anti-mouse Alexa Fluor 488 (Molecular Probes, Eugene, OR, USA) was incubated for 2 h at room temperature. Slides were mounted in ProLong Diamond Antifade with DAPI (Thermofisher, Waltham, MA, USA).

**Western blot analysis.** Organoids and tumour spheroids were collected, centrifuged at 600 g for 5 min and lysed for 10 min at 4 °C in 100 μl of RIPA buffer supplemented with complete protease inhibitor cocktail (Roche, Basel, Switzerland). Lysates were then sonicated for 10 min and analysed by western blot using standard SDS-polyacrylamide gel electrophoresis (SDS-PAGE) techniques.
In brief, protein samples were boiled in Laemmli buffer, run in 8% polyacrylamide gels and transferred onto polyvinylidene difluoride membranes. The membranes were incubated overnight at 4°C with the appropriate primary antibodies. After washing, membranes were incubated with specific secondary horseradish peroxidase-linked antibodies (DAKO) and visualised using the enhanced chemiluminescence reagent (Biological Industries).

**Image analysis.** The immunohistochemistry (IHC) of intestinal sections was visualised in an Olympus BX61 microscope (Olympus, Shinjuku, Tokyo, Japan), and images were taken using the cellSens Digital Imaging software (Olympus). Measurement of tumour spheroid length (diameter) was performed automatically with ImageJ Software (National Institutes of Health, Bethesda, MD, USA). Heatmaps were generated using R studio (https://www.rstudio.com/) and GSEA, and statistical analysis was performed with publicly available software from the Broad Institute (http://www.broadinstitute.org/gsea/index.jsp).

**RNA-Seq experiment and analysis.** Total RNA from three biological replicates per condition was extracted using RNeasy Micro Kit (Qiagen, Hilden, Germany). The RNA concentration and integrity were determined using Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Libraries were prepared and sequenced at the CRG Genomics unit (Barcelona, Spain) using standard protocols on an Illumina HiSeq2500 (San Diego, CA, USA). Heatmaps were generated using R studio (https://www.rstudio.com/) and GSEA, and statistical analysis was performed with publicly available software from the Broad Institute (http://www.broadinstitute.org/gsea/index.jsp).

**RESULTS AND DISCUSSION**

**IKKα deletion in the intestinal epithelium reduces tumour formation and proliferation.** The IKK/NF-κB signalling pathway has emerged as a key regulator in multiple cancer types, including colon cancer (DiDonato et al, 2012). However, NF-κB also exerts multiple essential physiological functions affecting cell survival that makes its general inhibition extremely toxic (Lam et al, 2005; Gasparian et al, 2009; Wright, 2010).

As IKKα is not required for this general NF-κB function, and previous studies suggested its crucial role in intestinal tumorigenesis, we now studied the specific contribution of intestinal epithelial IKKα to neoplastic transformation. We used a mouse strain carrying Cre recombinase under the control of Villin promoter (Villin-Cre). As Villin is specifically expressed in the epithelial cells of the small and large intestine (Madison et al, 2002), crossing this strain with the one containing loxP site-flanked Ikkα (Gareus et al, 2007) resulted in tissue-specific deletion of the kinase. We then crossed intestinal-specific Ikkα KO mice with ApcMin/+ mice to induce intestinal cell transformation in an IKKα-deficient background (Figure 1A). All different genotypes were born at the expected Mendelian ratios and showed no evident growth defects during the first 3 months of age, indicating that intestinal epithelial IKKα was dispensable for tissue homeostasis (data not shown). Further analysis of 3-month-old animals carrying the ApcMin/+ allele demonstrated that IKKα deficiency imposed a significant decrease in the number of tumours arising in the small intestine when compared with the Ikkα WT littermates or mice carrying one copy of the Ikkα gene (Ikkα+/-) (Figures 1B and C). Next, we studied the possibility that reduced proliferation of transformed cells might contribute to the observed phenotype. By IHC analysis, we found that Ikkα WT adenomas showed a significantly higher percentage of proliferating Ki67-positive cells when compared with the Ikkα KO adenomas (Figures 1D and E). In contrast, we consistently failed to detect any histological difference or changes in the number of Ki67-positive cells in the nontransformed intestinal mucosa of Ikkα WT, heterozygous or KO animals (Figures 1F and G). Because active β-catenin plays a pivotal role in Apc-mutant tumour initiation and maintenance (Van de Wetering et al, 2002) and it was previously identified as a target of IKKα (Albanese et al, 2003), we aimed to determine whether nuclear β-catenin levels were reduced in the Ikkα KO background. By IHC analysis we found that Ikkα KO tumours show a significant decrease in the levels of nuclear β-catenin as compared with the Ikkα control counterparts (Figures 1H and I).

We next explored the possibility that decreased tumour number in the ApcMin/+ Ikkα KO mice were associated to increased apoptosis of the transformed cells. To do so we checked the levels of cleaved caspase 3. By IHC analysis of Ikkα WT and KO ApcMin/+ intestines, we observed very few cleaved caspase 3-positive areas within the tumours of both Ikkα genotypes (Figure 1J). As a positive control, intestines of γ-irradiated mice showed consistent cleaved caspase 3 staining (Figure 1K).

These results indicate that intestinal epithelial Ikkα is essential for tumour initiation and tumour cell proliferation, likely through regulation of β-catenin signalling, but it is dispensable for maintaining normal homeostasis and proliferation of the intestine, a situation that is clinically exploitable. Whether differences on β-catenin activation were due to direct phosphorylation by IKKα need to be further investigated.

**IKKα−/− tumouroids show a decrease in size and proliferation.** The in vivo experiments described above provide strong evidence of the role of Ikkα in tumour formation in the APCMin–/– mouse model. To further investigate the contribution of Ikkα requirement in a pure intestinal epithelial tumour model, we used the ex vivo 3D culture system, in which single primary intestinal cells with tumour-initiating capability generate spheroidal structures (Sato et al, 2009, 2011). We will here refer to these tumour spheroids as tumouroids. Consistent with our in vivo data, Ikkα−/−; ApcMin/+ adenoma cells generated tumouroids that were significantly smaller in size than Ikkα WT counterparts (Figure 2A). This growth-deficient phenotype was much more pronounced in the initial cultures and attenuated after serial passaging of the tumouroid cultures. The significance of this result was confirmed by a careful quantification of sphere diameter (Figure 2B). Similar to that observed in the normal intestine, 3D organoids, which were derived from nontransformed intestinal epithelial cells, showed no differences in their growing capacity when comparing Ikkα+/+ and Ikkα−/− genotypes (Figure 2C). Immunofluorescence analysis of Ki67 expression demonstrated that Ikkα−/− tumouroids were almost depleted from proliferating cells compared with Ikkα+/+ structures (Figures 2D and E). These results identify epithelial Ikkα as an important contributor to intestinal tumour initiation activity ex vivo.

**IKKα governs tumouroid growth by regulating stem cell proliferation and cell cycle-related gene programmes.** We aimed to investigate the mechanisms underlying epithelial Ikkα function in transformed cells by defining the transcriptional programmes that depend on Ikkα in the ApcMin–/– tumouroids. We performed RNA-seq from ApcMin/+ Ikkα WT or Ikkα KO tumouroid cells. Data are accessible at the NCBI GEO database (Edgar et al, 2002), accession GSE101415. Unsupervised cluster analysis demonstrated a clear co-segregation of WT and KO RNA samples (Figure 3A). Importantly, the transcriptional profile of Ikkα KO tumouroids showed a significant decrease in the levels of a previously defined intestinal stem cell (ISC) signature (Muñoz et al, 2012) (Figure 3B), including genes essential for maintaining ISC function such as Notch1 (Riccio et al, 2008; Fre et al, 2011), Cdc6 (Guiu et al, 2014), Lgr5 (Barker et al, 2007), Ephb2 (Batlle et al, 2002; Merlos-Suárez et al, 2011), Ascl2 (van der
Flier et al., 2009) or Lrig1 (Wong et al., 2012), among others. We further confirmed these results by qRT-PCR of several of these genes in a different set of tumouroids from both genotypes (Figure 3C). By Gene Set Enrichment Analysis (GSEA) we identified several pathways that were miss-regulated in the IKKα-deficient adenoma cells compared with the WT. This applies to the apoptosis pathway that was significantly enriched in the absence of IKKα (Figures 3D and F), involving genes such as RIK1, TRADD, FASLG, FAS and CASP8 among others. In contrast, IKKα-deficient adenomas showed a decrease in cell cycle-related genes...
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Figure 1. The IKKα deletion in the intestinal epithelium reduces adenoma formation and proliferation. (A) Schematic representation of the strategy used to generate the compound mice used in our study. (B) Representative images of H&E staining of ApcMin/+;Ikkz+/+ and ApcMin/+;Ikkz−/− intestinal Swiss rolls. Dashed lines delimited two different tumours present in the ApcMin/+;Ikkz+/+ image. (C) Quantification of tumour number in the ApcMin/+;Ikkz+/+ and ApcMin/+;Ikkz−/− intestinal Swiss rolls. (D) Representative images of Ki67 immunohistochemistry (IHC) in ApcMin/+;Ikkz+/+ and ApcMin/+;Ikkz−/− intestines and (E) quantification of the number of Ki67-positive cells per hemi-crypt. Graphs represent the average number of tumours (C) or percentage of cells (E and G) per Swiss roll from animals of each genotype. (H and I) The IHC analysis of β-catenin (H) and quantification of percentage of tumour cells of each genotype showing nuclear β-catenin staining (I). (J and K) The IHC of cleaved caspase 3 in tumours of the indicated genotypes (J) or obtained from mice irradiated 3 h before processing the samples (K). Representative images for (H, J and K) are shown. For statistical analysis, ordinary one-way ANOVA or unpaired t-test was used and the P-values are indicated as ***P<0.001 and ****P<0.0001.

Figure 2. Ikkz−/− tumouroids show a decrease in size and proliferation. (A) Representative images of growing tumouroids from the indicated genotypes. Western blot analysis of IKKα is shown in the right panel. P = passage. (B) Quantification of tumouroid length from three independent experiments performed. (C) Representative images of organoid cultures from nontransformed Ikkz+/+ and Ikkz−/− intestinal stem cells and western blot analysis to demonstrate the efficiency of experiments performed. (D) Immunofluorescence (IF) staining of Ki67 in the ApcMin/+;Ikkz+/+ and ApcMin/+;Ikkz−/− tumouroids from passage 6 grown in Matrigel (Corning). (E) Quantification of the percentage of Ki67-positive cells from three independent experiments that was performed as in (D). The graph shows the average percentage and s.d. of the mean. Magnification of images is indicated. For statistical analysis we used ordinary one-way ANOVA or unpaired t-test. P-values are indicated as *P<0.05, ***P<0.001 and ****P<0.0001.
Figure 3. Ikkα⁻/⁻ tumouroids show altered regulation of intestinal stem cell genes. (A) Unsupervised hierarchical clustering analysis based on Euclidean distances of logged normalised counts between samples from three independent samples per genotype analysed. (B) The RNA-seq heatmap showing differential expression of genes involved in intestinal stemness with scaled (z-score) columns. (C) The q-PCR analysis of the indicated genes in Ikkα⁺/+ (WT) and Ikkα⁻⁻ (KO) tumouroids. The graphs represent the average value and s.d. from two independent tumouroids of each genotype analysed in triplicate. (D) The GSEA plots of genes differentially expressed in Ikkα WT and KO from the apoptosis and cell cycle checkpoint-related gene sets. (E) The GSEA plots of differentially expressed genes from MYC- and NF-κB-related gene sets. The pathways shown were particularly selected from significantly enriched ones to better explain the phenotypes observed. (F) Table showing all signatures from Hallmark gene sets that were significantly enriched (P < 0.01 and FDR < 0.025) in Ikkα⁺/+ or Ikkα⁻⁻ tumouroids. Of note that cell cycle checkpoint-related gene set was identified from Reactome gene sets. P-value and false discovery rate (FDR) are shown. For statistical analysis we used ordinary one-way ANOVA or unpaired t-test. DN = down; KO = knock out; NES = normalised enrichment score; WT = wild-type. P-values are indicated as **P < 0.01 and ***P < 0.001.
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