

Research Article

# IKK $\alpha$ plays a major role in canonical NF- $\kappa$ B signalling in colorectal cells

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Inhibitor of kappa B (I $\kappa$ B) kinase  $\beta$  (IKK $\beta$ ) has long been viewed as the dominant IKK in the canonical nuclear factor- $\kappa$ B (NF- $\kappa$ B) signalling pathway, with IKK $\alpha$  being more important in non-canonical NF- $\kappa$ B activation. Here we have investigated the role of IKK $\alpha$  and IKK $\beta$  in canonical NF- $\kappa$ B activation in colorectal cells using CRISPR–Cas9 knock-out cell lines, siRNA and selective IKK $\beta$  inhibitors. IKK $\alpha$  and IKK $\beta$  were redundant for I $\kappa$ B $\alpha$  phosphorylation and turnover since loss of IKK $\alpha$  or IKK $\beta$  alone had little (SW620 cells) or no (HCT116 cells) effect. However, in HCT116 cells IKK $\alpha$  was the dominant IKK required for basal phosphorylation of p65 at S536, stimulated phosphorylation of p65 at S468, nuclear translocation of p65 and the NF- $\kappa$ B-dependent transcriptional response to both TNF $\alpha$  and IL-1 $\alpha$ . In these cells, IKK $\beta$  was far less efficient at compensating for the loss of IKK $\alpha$  than IKK $\alpha$  was able to compensate for the loss of IKK $\beta$ . This was confirmed when siRNA was used to knock-down the non-targeted kinase in single KO cells. Critically, the selective IKK $\beta$  inhibitor BIX02514 confirmed these observations in WT cells and similar results were seen in SW620 cells. Notably, whilst IKK $\alpha$  loss strongly inhibited TNF $\alpha$ -dependent p65 nuclear translocation, IKK $\alpha$  and IKK $\beta$  contributed equally to c-Rel nuclear translocation indicating that different NF- $\kappa$ B subunits exhibit different dependencies on these IKKs. These results demonstrate a major role for IKK $\alpha$  in canonical NF- $\kappa$ B signalling in colorectal cells and may be relevant to efforts to design IKK inhibitors, which have focused largely on IKK $\beta$  to date.

## Introduction

The nuclear factor- $\kappa$ B (NF- $\kappa$ B) transcription factors (homo- and hetero-dimers of p65/RelA, RelB, c-Rel, NF- $\kappa$ B1/p50 and NF- $\kappa$ B2/p52) are sequestered in the cytoplasm through their binding to inhibitor of kappa B (I $\kappa$ B) proteins. In response to cytokines and stress signals, the I $\kappa$ B proteins are rapidly phosphorylated by the I $\kappa$ B kinases (IKKs) [1], triggering their proteasomal degradation and allowing NF- $\kappa$ B to enter the nucleus to drive gene expression. In the canonical NF- $\kappa$ B pathway tumour necrosis factor (TNF $\alpha$ ) or interleukin-1 (IL-1) receptor signalling activates TAK1, which phosphorylates and activates IKK $\alpha$  and IKK $\beta$  when they are bound to dimers of a regulatory subunit, NEMO (NF- $\kappa$ B essential modifier).

Early studies revealed distinct and non-redundant functions for IKK $\alpha$  and IKK $\beta$ . Loss of IKK $\beta$  was embryonic lethal in mice [2,3] and IKK $\beta$ -null mouse embryonic fibroblasts (MEFs) exhibited striking defects in phosphorylation and degradation of I $\kappa$ B $\alpha$  and NF- $\kappa$ B activity following treatment with TNF $\alpha$  or IL-1 [4,5]. Similarly, conditional knockouts showed the importance of IKK $\beta$  in macrophages [6], osteoclasts [7] and the gut epithelium [8,9]. In contrast, IKK $\alpha$ -null mice survived a month post-birth, but developed defects in epidermal and skeletal development [3,10,11] that were independent of IKK $\alpha$  kinase activity and NF- $\kappa$ B [12]. A similar, more severe phenotype was seen in patients with autosomal recessive loss-of-function mutations in *CHUK* (encoding human IKK $\alpha$ ) [13]. An essential role for IKK $\alpha$  in non-canonical NF- $\kappa$ B activation in developing lymphoid organs and B cell maturation was

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†This paper is dedicated to our friend and former PhD student Dr Jack Prescott.

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subsequently demonstrated [14–17]. Whilst IKK $\alpha$ -null MEFs showed some defects in p65 phosphorylation and NF- $\kappa$ B activity in response to TNF $\alpha$  or IL-1, IKK $\beta$  homodimers were sufficient for canonical I $\kappa$ B $\alpha$  phosphorylation and NF- $\kappa$ B activation [2,3,18]. Furthermore, binding between NEMO and IKK $\alpha$  was weaker than with IKK $\beta$  [19,20] and IKK $\beta$  was a more effective I $\kappa$ B $\alpha$  kinase *in vitro* [21].

From these studies, a consensus emerged that IKK $\beta$  is the critical NEMO-dependent IKK in the canonical NF- $\kappa$ B pathway, with IKK $\alpha$  acting in the noncanonical pathway, independent of NEMO. As such, IKK $\beta$  has been the focus of drug discovery efforts [22,23]; development of IKK $\alpha$  inhibitors has lagged behind, partly reflecting the difficulty in targeting the IKK $\alpha$  catalytic site [24]. Despite this consensus, reports continue to suggest IKK $\alpha$  is important in canonical NF- $\kappa$ B signalling. For example, TNF $\alpha$ - or IL-1-stimulated, NF- $\kappa$ B-dependent gene expression persists in IKK $\beta$ -null MEFs [2,4]. Furthermore, in contrast with the developing liver, mature hepatocytes are not sensitised to TNF $\alpha$ -induced apoptosis following IKK $\beta$  deletion, suggesting that IKK $\alpha$  can compensate in adult mice [25]. IKK $\alpha$  is also obligatory for RANK-induced canonical NF- $\kappa$ B activation in mammary epithelial cells [12]. In contrast with earlier studies, IL-1, but not TNF $\alpha$ , promotes NEMO-dependent I $\kappa$ B $\alpha$  degradation and NF- $\kappa$ B activation in IKK $\beta$ -null MEFs [26]. The same laboratory demonstrated that TNF- $\alpha$  or IL-1 induced NF- $\kappa$ B activity was completely defective in IKK $\alpha$ -null MEFs and suggested IKK $\alpha$  activates canonical NF- $\kappa$ B independently of NEMO [27], possibly through direct phosphorylation of p65.

In contrast with MEFs, both IKK $\alpha$  and IKK $\beta$  contribute to TNF $\alpha$ -induced NF- $\kappa$ B activation in HeLa cells [28]. Furthermore, patients with homozygous null mutations of *IKBKB* exhibit less severe phenotypes than IKK $\beta$ -null mice [29,30]; whilst they go on to develop severe combined immunodeficiency, they are normal at birth and exhibit no liver damage or developmental defects seen in IKK $\beta$ -null mice, suggesting that IKK $\alpha$  can compensate within the canonical NF- $\kappa$ B pathway in human cells. Finally, whilst primary skin fibroblasts from these patients exhibited drastically reduced NF- $\kappa$ B activation in response to TNF $\alpha$ ; the response to IL-1 $\beta$  was marginally affected [29]. Overall, it seems the role of IKK $\alpha$  and IKK $\beta$  in canonical NF- $\kappa$ B signalling varies with cell- or tissue-type, developmental stage, stimulus and even species, making it critical to assess the role of the IKKs in physiologically and pathologically relevant systems rather than relying on studies in MEFs.

We are interested in activation of NF- $\kappa$ B and inflammation in the colon where inflammatory bowel disease (IBD) incidence increases with age and may predispose to cancer. This is well established in colorectal cancer (CRC) where NF- $\kappa$ B promotes progression of ulcerative colitis and Crohn's disease, to colitis-associated cancer (CAC) [31]. Anti-TNF $\alpha$  therapy is employed in both colitis and Crohn's and NF- $\kappa$ B is constitutively active in human tumour samples from both CAC and sporadic CRC and is associated with poor prognosis [32–34]. Constitutive NF- $\kappa$ B activation is also observed in CRC cell lines, suggesting that cell-autonomous mechanisms may lead to aberrant pathway activation, in addition to inflammatory conditions within the tumour micro-environment [35,36]. Indeed, intestinal epithelial cell-specific deletion of IKK $\beta$  decreased tumour incidence in a mouse model of CAC [8]. Given the importance of inflammatory NF- $\kappa$ B signalling in IBD and CRC and the debate on the relative role of IKK $\alpha$  and IKK $\beta$  and we generated IKK $\alpha$ , IKK $\beta$  and IKK $\alpha/\beta$  double knockouts in colorectal cancer cell lines. Here, our initial characterisation of these cells reveals an unexpected and prominent role for IKK $\alpha$  in canonical NF- $\kappa$ B signalling.

## Materials and methods

### Cell culture, transfections and plasmids

HCT116 cells were cultured at 37°C in a humidified incubator with 95% air and 5% CO<sub>2</sub> in DMEM (Life Technologies) supplemented with 10% (v/v) foetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin. Cells were passaged at ~80% confluence every 3–4 days by enzymatic detachment with 0.05% trypsin/EDTA solution. SW620 cell were cultured at 37°C in a humidified incubator with 95% air and 5% CO<sub>2</sub> in Liebowitz's L-15 media (Life Technologies) also supplemented with 10% (v/v) foetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin and 0.075% (v/v) sodium bicarbonate. Cells were passaged at ~80% confluence every 3–4 days by enzymatic detachment with 0.05% trypsin/EDTA solution. Transfections were performed using Lipofectamine 2000 according to the manufacturer's instructions. pGL4.32[luc2P/NF- $\kappa$ B-RE] was a kind gift from Prof. Neil Perkins (Institute for Cell and Molecular Biosciences, University of Newcastle, U.K.). *Wild-type* FLAG-IKK $\alpha$  and kinase inactive FLAG-IKK $\alpha$  were kind gifts from Prof. Lienhard Schmitz (Department of Biochemistry, Justus-Liebig-Universität Gießen, Germany). *Wild-type*

pCMV2TAG-IKK $\beta$  and kinase inactive pCMV2TAG-IKK $\beta$  (K44M) were purchased from Addgene (Plasmid #11103 and 11104, respectively). pD1301-AD Cas9 expression vector was supplied by Horizon Discovery<sup>TM</sup>.

## RT-qPCR gene expression analysis

Total RNA was extracted from cells using the RNeasy Kit (QIAGEN), QIAshredders (QIAGEN) and RNase-Free DNase Set (QIAGEN) according to manufacturer's instructions. An amount of 1  $\mu$ g RNA was reverse transcribed to cDNA using the Quantitech Reverse Transcription kit according to manufacturer's instructions. cDNA was diluted 1 : 20 in RNase-free water and stored at  $-20^{\circ}\text{C}$ . RT-qPCR was performed using SYBR Green (QIAGEN) and primers with the following sequences: IKK $\alpha$ : F 5'-TGTACACAGAGTTCTG CCG-3', R 5'-TGAAGTCTCCCATCTTGAGG 3'; IKK $\beta$ : F 5'-CTTGGGACAGGGGATTTGG-3', R 5'-ATT GGGGTGGGTCAGCCTTC-3'; YWHAZ: F 5'-TGATCCCCAATGCTTCACAAG-3', R 5'-GCCAAGTAACGG TAGTAATCTCC-3'. This was performed in a 96-well plate format using a CFX96 Real-Time PCR Detection System (Bio-Rad). Data were normalised to the geometric mean of the reference gene expression and relative gene expression change calculated using the Pfaffl method. Statistical analysis was performed with the REST 2009 software package using an applied two-sided pair-wise fixed reallocation randomisation test.

## CRISPR genome editing

Target guide RNA (gRNA) sequences targeting either *CHUK* (IKK $\alpha$ ) or *IKBKB* (IKK $\beta$ ) genes were designed using the gGUIDEbook<sup>TM</sup> software (Desktop Genetics). The sequences used to target *CHUK* were 5'-AGCCGC TCCCGCATCTCCCA-3', 5'-ACAGACGTTCCCGAAGCCGC-3' and 5'-TCTTCATAATCTGGATTTCA-3', and for *IKBKB*, 5'-AGCCGCTCCCGCATCTCCCA-3', 5'-TGTACCAGCATCGGGTGAGG-3' and 5'-AGGCCTTT ACAACATTGGC-3'. The sequences were cloned into pD1301-AD mammalian Cas9 vector containing a GFP reporter. HCT116 or SW620 cells were transfected by electroporation with 3.75  $\mu$ g of either the IKK $\alpha$ -targeted Cas9 plasmid DNA, the IKK $\beta$ -targeted Cas9 plasmid DNA or empty-vector GFP Cas9 plasmid DNA before being combined and incubated at  $37^{\circ}\text{C}$ , 5% (v/v)  $\text{CO}_2$  for 48 h. For the double knockout of IKK $\alpha$  and IKK $\beta$ , 1.875  $\mu$ g of each plasmid was transfected. Electroporation was performed with Nucleofector<sup>TM</sup> Kit V (Lonza) using programme D-032 on a Nucleofector<sup>TM</sup> device (Lonza). After 48 h, cells were harvested by trypsinisation, centrifuged and resuspended in 2% (v/v) FBS in PBS. The cell suspension was filtered through a 40  $\mu\text{m}$  cell strainer (CellTrics). GFP-positive cells were single-cell sorted into 96-well plates containing 100  $\mu\text{l}$  complete growth media containing 25% conditioned media using a FACS Aria III (BD) configured with an 85  $\mu\text{m}$  nozzle. Single cell colonies were allowed to grow over a 10–14-day period and media replaced with fresh every 4 days. Upon confluency, cells were split into two 96-well plates: one for genotyping and one for clonal expansion. Genomic DNA was extracted from cells as described previously and used as a template to PCR amplify a 1 kb region spanning the gRNA target using Phusion High-Fidelity DNA polymerase (Invitrogen) according to manufacturer's instructions. Purified PCR product was cloned into pCR2.1 TOPO vector and transformed into XL1 Blue competent cells (Agilent). Multiple individual bacterial colonies were picked and sent for Sanger sequencing. Candidate clones were expanded and knockout was verified by Western blot.

## Western blot analysis

Whole-cell lysates were generated from cells suspended in tris-glycine (TG) lysis buffer containing 20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 1 mM EGTA, 1% v/v Triton X-100, 10% v/v glycerol and 1.5 mM  $\text{MgCl}_2$  and freshly supplemented with 1 mM  $\text{Na}_3\text{VO}_4$ , 1 mM PMSF, 10  $\mu\text{g}/\text{ml}$  aprotinin, 10  $\mu\text{g}/\text{ml}$  leupeptin and 50 mM NaF. Protein concentration of lysates was measured using a Bradford assay (Bio-Rad). Samples were prepared for Western blotting by boiling in 4X Laemmli sample buffer for 5 min at  $95^{\circ}\text{C}$ . Denatured and normalised samples were resolved by SDS-PAGE running at 25 mA per gel, transferred to methanol-activated Immobilon P PVDF membrane at 300 mA for 90 min using the Mini Trans-Blot Cell wet blotting system. Membranes were blocked in 5% (w/v) powdered milk/TBS-Tween 20 or 5% (w/v) BSA/TBST for 30 min and probed with specific primary antibodies (see below) at  $4^{\circ}\text{C}$  overnight with gentle agitation. Membranes were washed three times in TBST for 5 min and probed with appropriate HRP-conjugated secondary antibody in 5% (w/v) powdered milk/TBST for 1 h at RT. Following another three TBST washes, antibody-antigen complexes were detected by the enhanced chemiluminescence (ECL) system coupled with an autoradiographic film developer. Antibodies used were as follows: Anti-N-IKK $\alpha$  (CST #2682), anti-C-IKK $\alpha$  (Abcam #ab109749), anti-N-IKK $\beta$  (Abcam #ab124957), anti-C-IKK $\beta$  (CST 2684), anti-p-IKK $\alpha/\beta$  (S176/180) (CST 2697), anti-I $\kappa$ B $\alpha$  (CST #9242), anti-p-I $\kappa$ B $\alpha$  (S32/36) (CST #9246), anti-p65 (CST #8242), anti-p-p65 (S468) (CST #3039), anti

p-p65 (S536) (CST #3033), anti-JNK1 (Santa-Cruz sc-819), anti-p-JNK1/2 (CST #9255), anti-p38 (in-house), anti-p-p38 (T180/Y182) (CST #9211), anti-PARP (CST #9542), anti-FLAG (Sigma–Aldrich F-3165), anti-c-Rel (CST #4727), anti- $\beta$ -actin (Sigma A5441), anti- $\beta$ -tubulin (Abcam ab6046), anti-Lamin A/C (Santa-Cruz sc-7292), anti-HSP90 (CST #4874).

### Subcellular fractionation

Cells were harvested by gently scraping in PBS-EDTA (10 mM). Cells were then pelleted and washed with PBS. The washed pellet was then gently resuspended in five pellet volumes of ice-cold Buffer A (10 mM Hepes pH 7.9, 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 0.1 mM EDTA), supplemented with 1 mM DDT and protease (complete, mini, EDTA-free protease inhibitor cocktail Roche) and phosphatase (PhosSTOP, Roche) inhibitors. Cells were incubated on ice for 15 min then 0.1% (v/v) NP-40 added to release the cytoplasmic contents. A 1 ml dounce homogeniser was used to ensure complete cell lysis. Nuclei were subsequently pelleted at 800 g for 10 min at 4°C and the cytoplasmic fraction (supernatant) transferred to a fresh tube. This supernatant was centrifuged again at 10 000g for 5 min to pellet any contaminating nuclei. NaCl and glycerol were added to the cytoplasmic fraction to final concentrations of 137 mM and 10% (v/v), respectively. The nuclei pellet was washed at least three times in ice-cold Buffer A, prior to addition of 0.7 pellet volumes of RIPA buffer (Sigma), supplemented with >250 U benzonase nuclease (Sigma–Aldrich). Samples were incubated at 4°C for 4 h with end-over-end inversion, with occasional vortexing. The nuclear fraction was subsequently cleared by centrifugation.

### Analysis of cells with sub-G1 DNA content by flow cytometry

Following harvest, cells were pelleted by centrifugation for 5 min (1500 rpm, 4°C), washed in PBS and resuspended in 0.25 ml PBS containing 25  $\mu$ g RNase and 12.5  $\mu$ g propidium iodide (PI). Following 30 min incubation at 37°C, samples were passed through a 25-gauge needle to reduce cell clumping. Cell cycle profiles were acquired with an LSRII flow cytometer (BD), using a 488 nm excitation laser line and a 670 nm long pass emission filter to measure binding of PI to DNA, and counting 10 000 cells per sample. Data was analysed using FlowJo v10.

### NF- $\kappa$ B luciferase assays

In 96-well tissue culture plates with opaque sides (Thistle Scientific), HCT116 or SW620 cells were transfected with 0.1  $\mu$ g/well pGL4.32[luc2P/NF- $\kappa$ B-RE] luciferase experimental-reporter plasmid DNA and 0.01  $\mu$ g/well constitutively expressed pCMV renilla plasmid DNA (used for normalisation). The following day the relevant treatments were performed. Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega), and on a MicroLumatPlus LB96V reader, according to manufacturer's instructions.

### High-content confocal image acquisition and analysis

Cells were seeded in triplicate in CellCarrier 96-well plates (PerkinElmer) for 48 h prior to fixation with 4% v/v paraformaldehyde/PBS for 15 min at RT. Following three washes in PBS, cells were permeabilised with 0.1% v/v Triton X-100/PBS for 10 min. Cells were washed once in PBS and blocked for 1 h in blocking buffer (1 $\times$  PBS, 5% v/v goat serum, 0.2% Tween 20, 0.02% w/v sodium azide) at RT. Cells were incubated with primary antibody diluted in antibody dilution buffer at 4°C overnight. Cells were washed three times in PBS and then incubated with secondary antibody diluted in blocking buffer for 1 h at RT in the dark. Cells were washed a final three times in PBS. High-content image acquisition was performed using an In Cell Analyzer 6000 (GE Healthcare). Confocal images were analysed using the CellProfiler software package.

### Inhibitor compounds and cell culture treatments

Initial stocks of BIX02514 were kindly supplied by Prof. Sir Philip Cohen (University of Dundee) and subsequently purchased from Tocris). Stock solutions of drugs were diluted in cell culture medium as appropriate to yield the final drug concentration. All treatments were adjusted to contain the same volume of vehicle. Vehicle-only containing medium was used as a control. Treated cells were incubated at 37°C, 5% CO<sub>2</sub>/95% air for the length of time indicated in the figure legend.

### Statistical analysis

Results, unless otherwise stated, are presented as a mean  $\pm$  standard deviation (SD) and originate from three independent experiments. Statistical significance was determined by two-way ANOVA (repeated measures)

with Tukey or Dunnett post-hoc test using GraphPad 7 where stated. Significance values were given in the figures and set at  $P > 0.05$  (ns) and  $P \leq 0.0001$  (\*\*\*)

## Results

### Generation of IKK $\alpha$ KO, IKK $\beta$ KO and IKK $\alpha/\beta$ DKO HCT116 cells

HCT116 cells are an epithelial colorectal cancer cell line established in 1981 from an adult male patient. HCT116 cells are wild type for TP53 but harbour a KRAS<sup>G13D</sup> and PIK3CA<sup>H1047R</sup> mutation as well as inactivating mutations in CTNNB1, CDKN2A/p14ARF, MLH1. IKK $\alpha$  or IKK $\beta$  knockout (KO) and IKK $\alpha/\beta$  double knockout (DKO) HCT116 cell lines were generated by CRISPR–Cas9 gene editing and single-cell cloning (see Materials and Methods). Clones A3, C4, A8 and E10 were confirmed as WT. Clones F6 and C7, derived from cells expressing gRNA 24 and clone A2, derived from cells expressing gRNA 25, were confirmed as IKK $\alpha$  KO. Clones G9 and A7, derived from cells expressing gRNA 87 and clone A4, derived from cells expressing gRNA 76, were confirmed as IKK $\beta$  KO. Clones C8 and G1, derived from cells expressing both gRNA 25 and gRNA 87, and clone E9, derived from cells expressing both gRNA 24 and gRNA 87, were confirmed as IKK $\alpha/\beta$  DKO. Sanger sequencing confirmed the presence of homozygous frameshift mutations and likely premature stop codons (Supplementary Figures S1 and S2). Immunoblotting with N-terminal or C-terminal antibodies against IKK $\alpha$  or IKK $\beta$  confirmed that each KO clone was null for full-length protein (Figure 1A and Supplementary Figure S3); no compensatory up-regulation of the remaining IKK subunit was observed in single KO clones. IKK $\alpha$  KO and DKO clones exhibited a greater than 2-fold reduction in IKK $\alpha$  mRNA; a similar reduction in IKK $\beta$  mRNA was seen in the IKK $\beta$  KO and DKO clones (Supplementary Figure S4A,B).

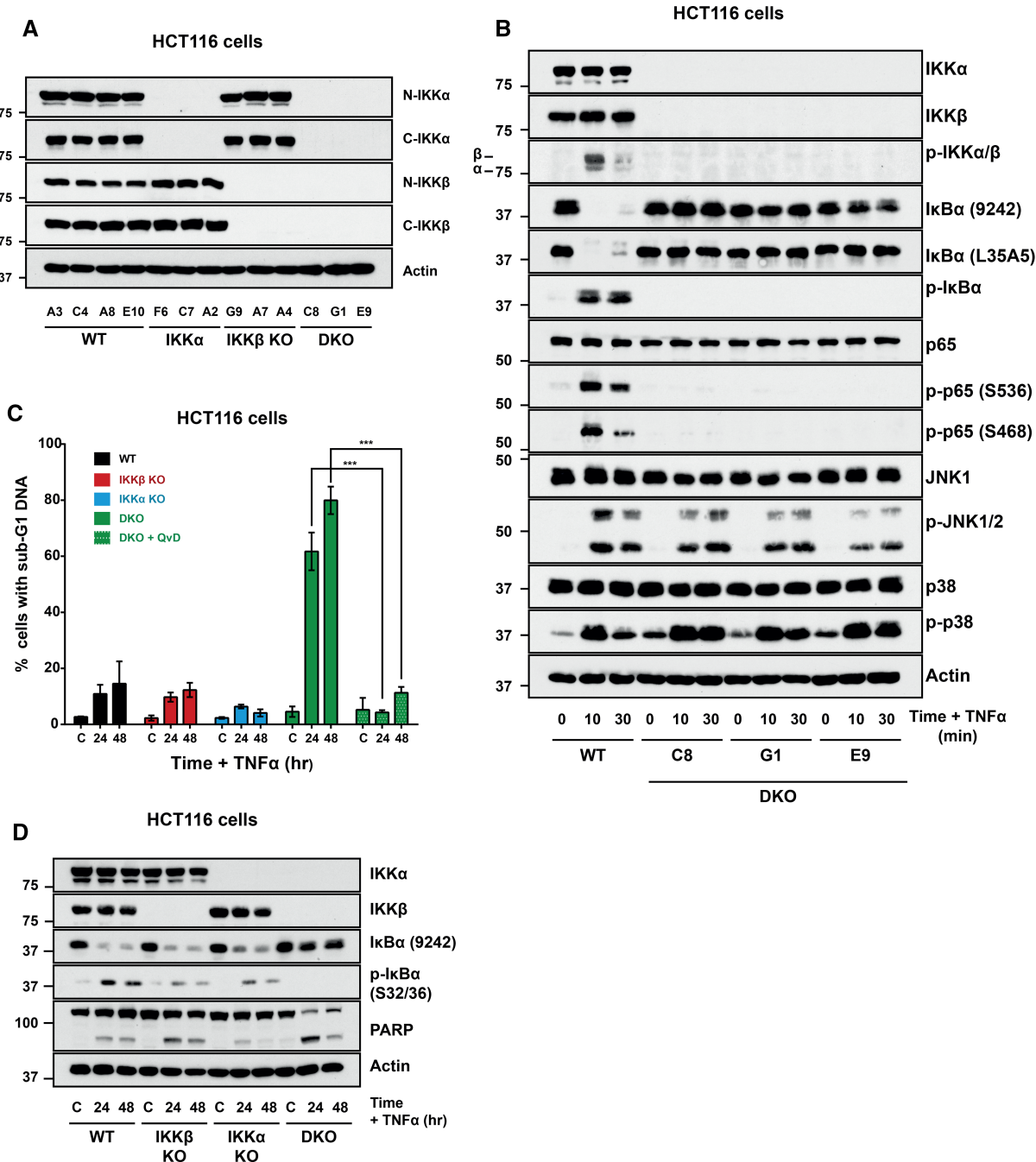
To assess the consequences of IKK knockout we examined the signalling response to TNF $\alpha$ , focusing first on the IKK $\alpha/\beta$  DKO cells. All three IKK $\alpha/\beta$  DKO clones lacked TNF $\alpha$ -inducible IKK activity, judged by phosphorylation of known substrates I $\kappa$ B $\alpha$  and p65 (Figure 1B). The absence of p65 phosphorylation at serine 536 (S536) and S468 in DKO clones indicated that in the absence of IKK $\alpha$  and IKK $\beta$  these sites cannot be phosphorylated in response to TNF $\alpha$  by other kinases, such as TBK1 and IKK $\epsilon$  [37]. The IKK $\alpha/\beta$  DKO clones also exhibited a complete loss of TNF $\alpha$ -inducible NF- $\kappa$ B transcriptional activity (see below). IKK $\alpha/\beta$  DKO clones exhibited normal phosphorylation of JNK at T183/Y185 in response to TNF $\alpha$ . However, the DKO clones exhibited increased basal p38 phosphorylation at T180/Y182, and TNF $\alpha$ -stimulated p38 phosphorylation was more persistent (Figure 1B).

Activation of NF- $\kappa$ B is cytoprotective during TNF $\alpha$  stimulation [38,39]. Consistent with this, IKK $\alpha/\beta$  DKO cells exhibited a striking caspase-dependent cell death following TNF $\alpha$  stimulation (Figure 1C) that was accompanied by caspase activation and PARP cleavage (Figure 1D), consistent with cell death by apoptosis. Thus, as expected, IKK $\alpha/\beta$  DKO cells were completely defective for TNF $\alpha$ -dependent activation of IKK and were sensitised to TNF $\alpha$ -driven apoptosis.

### A prominent role for IKK $\alpha$ in canonical NF- $\kappa$ B signalling downstream of TNF $\alpha$

We next examined TNF $\alpha$  responses in IKK $\alpha$  or IKK $\beta$  KO cells, comparing with WT cells (Figure 2A,B). IKK $\alpha$  and IKK $\beta$  were phosphorylated on their activation loops after 10 minutes of TNF $\alpha$  treatment; the antibody used (p-S176/S180) detects IKK $\alpha$  only when phosphorylated at S176/180 and IKK $\beta$  only when phosphorylated at S177/181. The KO clones highlighted the specificity of this antibody; the upper band being IKK $\beta$  (S177/181) and the lower band IKK $\alpha$  (S176/180) (Figure 2A,B). Interestingly, IKK $\beta$  S177/181 phosphorylation was enhanced in IKK $\alpha$  KO cells relative to WT whilst IKK $\alpha$  S176/180 phosphorylation was also enhanced in the IKK $\beta$  KO clones relative to WT cells. This was also seen over longer time courses, where IKK $\beta$  S177/181 and IKK $\alpha$  S176/180 phosphorylation was both enhanced and more sustained in the IKK $\alpha$  KO and IKK $\beta$  KO cells, respectively (Supplementary Figure S5A,B). In WT cells I $\kappa$ B $\alpha$  was phosphorylated at S32/36 and degraded within 10 min of TNF $\alpha$  treatment and I $\kappa$ B $\alpha$  protein levels were restored by 60 min, reflecting the NF- $\kappa$ B-driven negative feedback loop [40]. Both the IKK $\alpha$  KO and IKK $\beta$  KO cells exhibited a similar, modest delay in I $\kappa$ B $\alpha$  degradation relative to WT cells after 10 and 30 min TNF $\alpha$  treatment, but I $\kappa$ B $\alpha$  levels were restored after 60 min in both IKK $\alpha$  KO and IKK $\beta$  KO cells. These results indicate that IKK $\alpha$  and IKK $\beta$  are functionally redundant for I $\kappa$ B $\alpha$  phosphorylation in HCT116 cells, each compensating for the other.

p65 NF- $\kappa$ B was rapidly phosphorylated at S468 and S536 following TNF $\alpha$  treatment; phosphorylation of S536 was unaffected by IKK $\alpha$  KO or IKK $\beta$  KO, suggesting that IKK $\alpha$  and IKK $\beta$  are fully redundant for phosphorylating S536, consistent with both being able to phosphorylate S536 [5,41,42]. Interestingly, basal



**Figure 1. Characterisation of CRISPR–Cas9 IKK knockout HCT116 cells.**

Part 1 of 2

(A) Best candidate clones were expanded and whole-cell lysates generated for independent, single-cell derived WT, IKK $\alpha$  KO, IKK $\beta$  KO and IKK $\alpha/\beta$  DKO CRISPR clones. Lysates were fractionated by SDS–PAGE and Western blotted with the indicated antibodies to confirm absence of expressed protein corresponding to targeted gene. Three clones each of IKK $\alpha$  KO (F6, C7, A2), IKK $\beta$  KO (G9, A7, A4) and IKK $\alpha/\beta$  DKO (C8, G1, E9) were selected for validation and characterisation. In parallel, we derived four wild-type clones (A3, C4, A8, E10) that had been transfected with Cas9 but no guide. Data are from a single experiment representative of three independent Western blots giving similar results. (B) WT clone (A3) and three independent IKK $\alpha/\beta$  DKO clones were seeded in their normal growth medium for 48 h, prior to treatment with 10 ng/ml recombinant TNF $\alpha$  for the indicated timepoints. Whole-cell extracts were prepared, fractionated by SDS–PAGE and Western blotted with the indicated antibodies. Data are from a single experiment. p, phospho-. (C) WT (A3), IKK $\alpha$  KO (F6), IKK $\beta$  KO (G9) and IKK $\alpha/\beta$  DKO (C8) HCT116 cells were seeded in triplicate in normal growth medium for 24 h prior to treatment with 10 ng/ml TNF $\alpha$  for the indicated timepoints (24 and 48 h). Caspase-dependent cell death was blocked by pre-treating cells for 30 min with 10  $\mu$ M of the pan-caspase inhibitor, QVD-Oph. Cells that were sub confluent at the point of

**Figure 1. Characterisation of CRISPR–Cas9 IKK knockout HCT116 cells.**

Part 2 of 2

harvest, were fixed, stained with propidium iodide and percentage sub-G1 population (as a crude marker of cell death) assessed by flow cytometry. Results are mean  $\pm$  SD of the three independent experiments. Significance testing was performed using two-way ANOVA (repeated measures) with Tukey post-hoc testing.  $P < 0.0001$  (\*\*\*). (D) The same clones were treated as in (C) and whole lysates prepared and Western blotted with the indicated antibodies. Data are from a single experiment representative of two showing similar results.

phosphorylation of S536 was absent from IKK $\alpha$  KO clones but was unaffected in IKK $\beta$  KO clones, suggesting that IKK $\alpha$  might contribute to basal phosphorylation of S536. Notably, TNF $\alpha$ -driven p65 S468 phosphorylation was strongly diminished in IKK $\alpha$  KO clones, but largely unaffected by IKK $\beta$  KO, suggesting that IKK $\alpha$ , not IKK $\beta$ , is critical for phosphorylation at this site in response to TNF $\alpha$ . This is in direct contrast with the literature where IKK $\beta$  has been suggested to be the dominant kinase phosphorylating S468 in response to TNF $\alpha$  and IL-1, with little if any contribution from IKK $\alpha$  [43]. To examine the effect of IKK KO on NF- $\kappa$ B transcriptional activity cells were transfected with a tandem NF- $\kappa$ B-dependent luciferase reporter (NF- $\kappa$ B:Luc) (Figure 2C,D). Both basal and especially TNF $\alpha$ -induced NF- $\kappa$ B activity was substantially reduced in IKK $\alpha$  KO clones relative to WT (Figure 2C,D) but unaffected by IKK $\beta$  KO. IKK $\alpha$ / $\beta$  DKO clones were completely defective for TNF $\alpha$ -induced NF- $\kappa$ B activity (Figure 2C,D), consistent with the complete absence of IKK activity (Figure 1D).

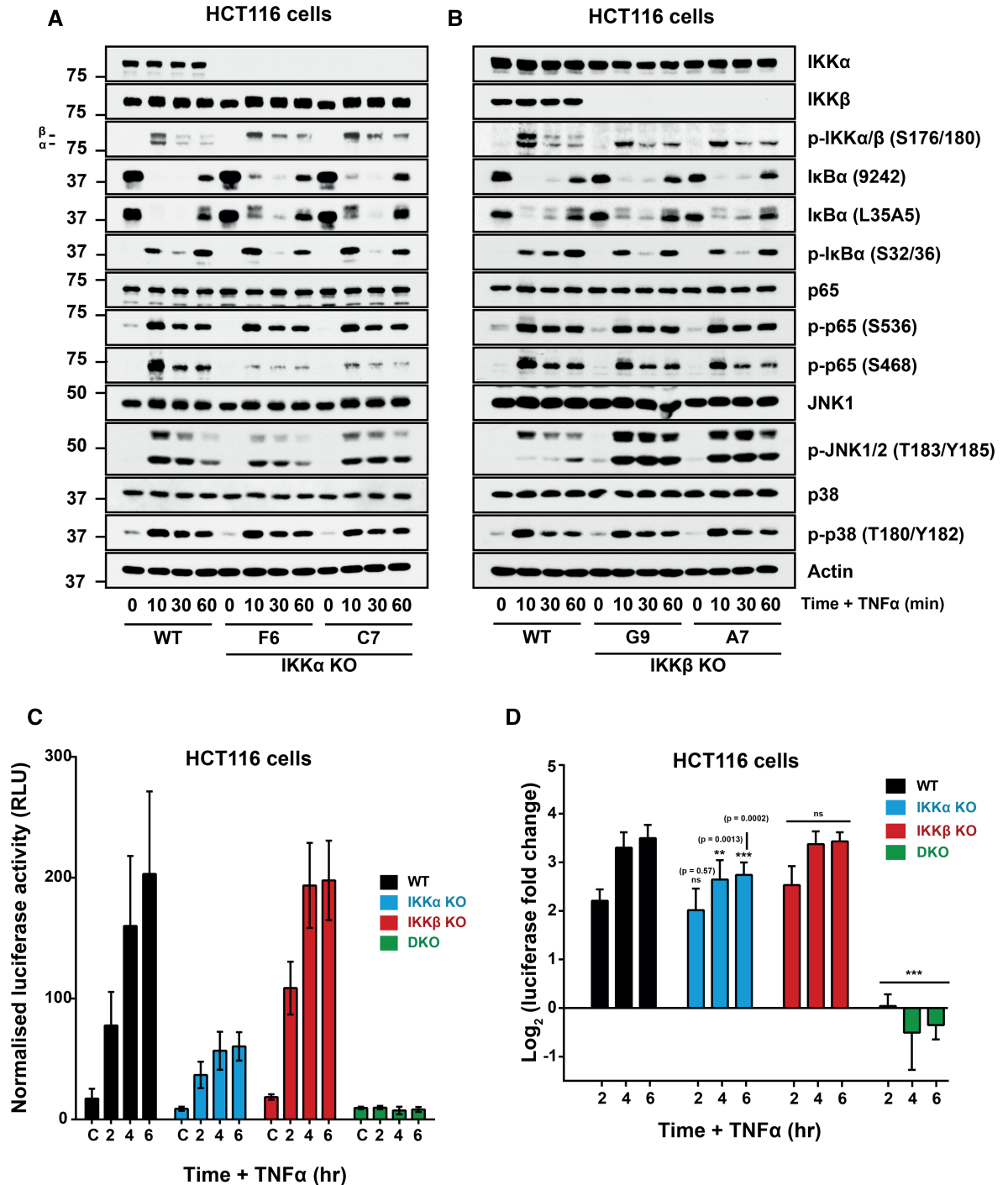
Phosphorylation of p38 was unaffected by knockout of either IKK $\alpha$  or IKK $\beta$  (Figure 2A,B), suggesting that the enhanced p38 phosphorylation observed in DKO clones (Figure 1B) reflected the complete loss of IKK kinase activity and/or NF- $\kappa$ B activity. Activating phosphorylation of JNK1/2 in response to TNF $\alpha$  was unaffected by IKK $\alpha$  KO but greatly enhanced by IKK $\beta$  KO.

### siRNA or selective IKK $\beta$ inhibition in IKK KO cells confirms a prominent role for IKK $\alpha$ in canonical NF- $\kappa$ B signalling

The preceding results suggested a prominent role for IKK $\alpha$  in canonical NF- $\kappa$ B activation. To confirm these results by orthogonal approaches IKK $\alpha$  KO or IKK $\beta$  KO clones were treated with siRNA targeting the remaining IKK, knocking down IKK $\beta$  in IKK $\alpha$  KO cells or vice versa (Figure 3A,B). This was sufficient to almost completely block the degradation of I $\kappa$ B $\alpha$  after 10 min TNF $\alpha$  treatment in the IKK $\alpha$  or IKK $\beta$  KO clones (Figure 3A); thus, the residual IKK activity observed in these KO clones was due to the remaining IKK kinase confirming that IKK $\alpha$  and IKK $\beta$  are functionally redundant in TNF $\alpha$  dependent degradation of I $\kappa$ B $\alpha$ . Phosphorylation of p65 at S536 and S468 was also diminished following the knockdown of the remaining IKK subunit in both the IKK $\alpha$  and IKK $\beta$  KO clones but IKK $\alpha$  KO or IKK $\alpha$  knockdown had the strongest effect on S468 phosphorylation. NF- $\kappa$ B:Luc assays also demonstrated that knocking down the remaining IKK subunit caused a significant reduction in TNF $\alpha$ -induced NF- $\kappa$ B activity (Figure 3B).

To complement this siRNA approach, we used the IKK $\beta$  inhibitor BIX02514, which is  $\sim$ 300-fold selective for IKK $\beta$  (*in vitro* IC<sub>50</sub> of 300 nM) versus IKK $\alpha$  (*in vitro* IC<sub>50</sub> of 100  $\mu$ M) (reviewed in 23). BIX02514 had only a modest effect on TNF $\alpha$ -induced degradation of I $\kappa$ B $\alpha$  in WT cells but this resulted in a significant 2-fold decrease in the TNF $\alpha$ -induced NF- $\kappa$ B activity suggesting that IKK $\alpha$  and IKK $\beta$  both contribute to induction of NF- $\kappa$ B in wild-type cells (Figure 4A,B). BIX02514 had no effect on the phosphorylation of p65 at S536 or S468 in WT cells, suggesting that IKK $\alpha$  phosphorylates these sites in response to TNF $\alpha$ . In IKK $\alpha$  KO cells BIX02514 completely inhibited I $\kappa$ B $\alpha$  phosphorylation, I $\kappa$ B $\alpha$  degradation, phosphorylation of p65 at S536, the residual phosphorylation of S468 and TNF $\alpha$ -induced NF- $\kappa$ B:Luc activity (Figure 4A,B). These results strongly suggest that the IKK activity observed in IKK $\alpha$  KO cells is IKK $\beta$  and that both kinases normally contribute to TNF $\alpha$ -stimulated NF- $\kappa$ B activation. BIX02514 had no effect on TNF $\alpha$ -induced phosphorylation and degradation of I $\kappa$ B $\alpha$ , or the phosphorylation of p65 in IKK $\beta$  KO cells, confirming that IKK activity in these cells is not due to residual IKK $\beta$  activity but rather due to IKK $\alpha$ . Indeed, BIX02514 had no significant effect on the induction of NF- $\kappa$ B:Luc in IKK $\beta$  KO cells, again indicating that IKK $\alpha$  could fully compensate for IKK $\beta$  loss (Figure 4B).

TNF $\alpha$ -driven NF- $\kappa$ B transcriptional activity could be restored to IKK $\alpha$ / $\beta$  DKO cells by transiently re-expressing WT IKK $\alpha$  or WT IKK $\beta$  constructs but not their kinase dead (KD) counterparts (Supplementary Figure S6A). Notably IKK $\alpha$  was more effective at restoring NF- $\kappa$ B:Luc than IKK $\beta$ , despite IKK $\beta$  being



**Figure 2. Redundant and non-redundant functions for IKKα and IKKβ in NF-κB activation.**

Part 1 of 2

(A) WT clone (A3) and two independent IKKα KO clones or (B) WT clone (A3) and two independent IKKβ KO clones were seeded in their normal growth medium for 48 h, prior to treatment with 10 ng/ml recombinant TNFα for the indicated timepoints. Whole-cell extracts were prepared, fractionated by SDS-PAGE and Western blotted with the indicated antibodies. Similar results were seen for the two other WT clones (A8 and E10) and the other IKKα (A2) and IKKβ (A4) KO clones (data not shown). (C and D) WT, IKKα KO, IKKβ KO and IKKα/β DKO HCT116 cells were seeded in antibiotic-free growth medium overnight prior to transient transfection with NF-κB-RE firefly luciferase and renilla luciferase plasmids. The following day, cells were treated with 10 ng/ml recombinant TNFα for the indicated time periods. Firefly luciferase was normalised to renilla luciferase and data expressed as log<sub>2</sub>(fold change in TNFα-induced luciferase activity relative to the relevant, matched



**Figure 2. Redundant and non-redundant functions for IKK $\alpha$  and IKK $\beta$  in NF- $\kappa$ B activation.**

Part 2 of 2

untreated condition). Results are mean  $\pm$  SD of three independent experiments, each of which was performed with three independent clones, seeded in triplicate (WT – A3, A8 and E10. IKK $\alpha$  KO – F6, C7, A2. IKK $\beta$  KO – G9, A7, A4 and IKK $\alpha/\beta$  DKO – C8, G1, E9). Significance testing was performed using two-way ANOVA (repeated measures) with Tukey post-hoc test. *P* values relate to comparison with corresponding timepoint(s) of WT control. *P* < 0.0001 (\*\*\*)

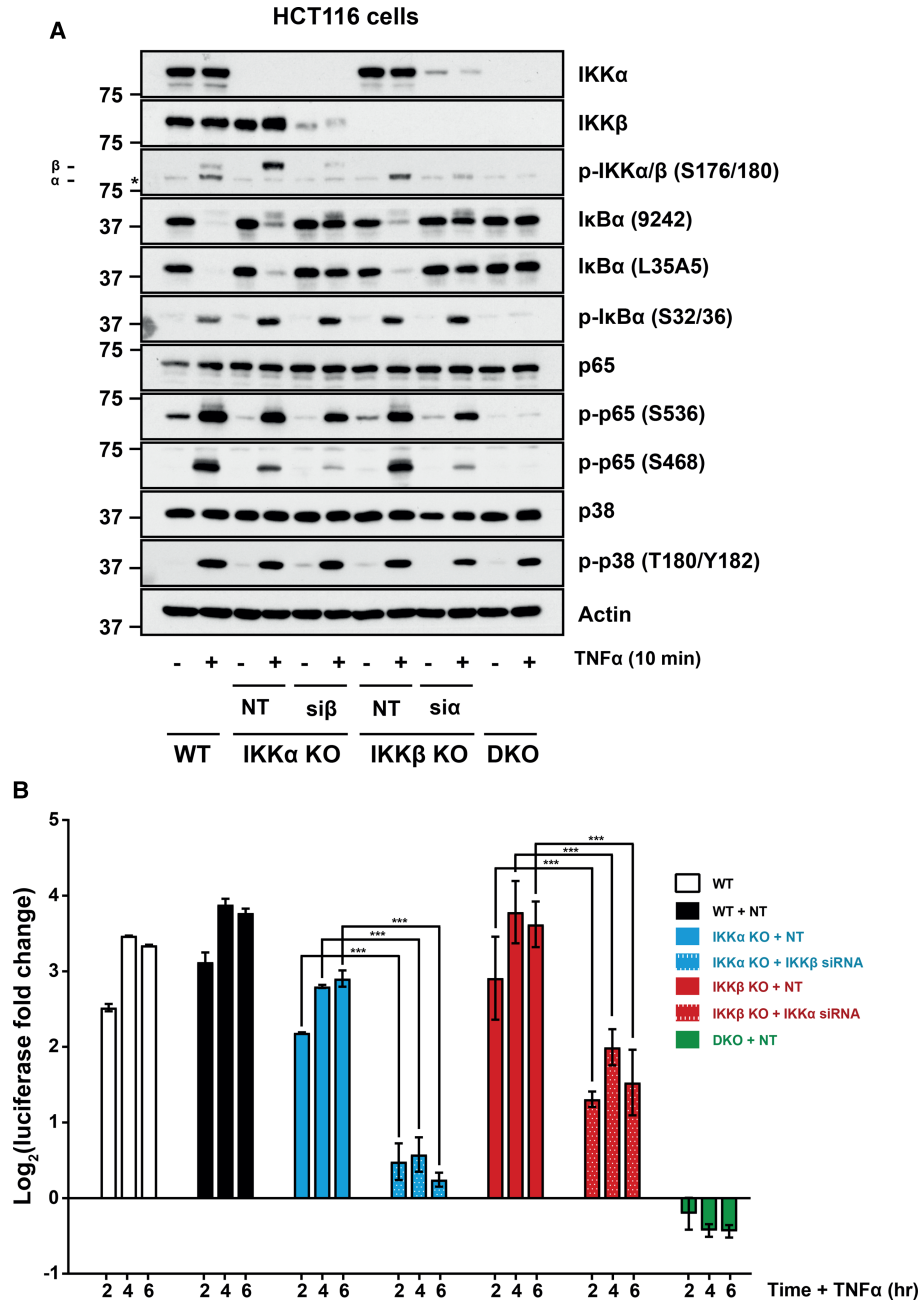
expressed at slightly higher levels (Supplementary Figure S6A,B); re-expression of both WT IKK $\alpha$  and IKK $\beta$  had an additive effect. WT IKK $\alpha$  re-expression in IKK $\alpha$  KO cells rescued TNF $\alpha$ -induced NF- $\kappa$ B activation, whereas overexpression of WT IKK $\beta$  had no effect (Supplementary Figure S6B,C). This further highlights the importance of IKK $\alpha$  in the canonical NF- $\kappa$ B response to TNF $\alpha$  in these cells. KD IKK $\beta$  strongly inhibited TNF $\alpha$ -induced NF- $\kappa$ B activation in WT cells and IKK $\beta$  KO cells, and inhibited the restored TNF $\alpha$ -inducible NF- $\kappa$ B activation in DKO cells when co-expressed with WT IKK $\alpha$ . KD IKK $\beta$  also inhibited the induction of NF- $\kappa$ B in IKK $\alpha$  KO cells, indicating that KD IKK $\beta$  acts in a dominant negative fashion towards both WT IKK $\beta$  and IKK $\alpha$  consistent with previous studies [28]. KD IKK $\alpha$  also inhibited the induction of NF- $\kappa$ B in WT cells, albeit not to the same extent as KD IKK $\beta$ , and also inhibited the induction of NF- $\kappa$ B in IKK $\alpha$  and IKK $\beta$  KO cells, indicating that kinase inactive IKK $\alpha$  also acts as a dominant negative to inhibit both IKK $\alpha$  and IKK $\beta$ .

### IKK $\alpha$ KO cells exhibit a far greater defect in TNF $\alpha$ -induced p65 nuclear translocation than IKK $\beta$ KO cells

IKK $\alpha$  and IKK $\beta$  were redundant for I $\kappa$ B $\alpha$  phosphorylation and degradation (Figure 2A,B) but only IKK $\alpha$  KO cells exhibited a significant reduction in TNF $\alpha$ -induced NF- $\kappa$ B:Luc. Consequently, we examined the nuclear translocation of NF- $\kappa$ B subunits by immunostaining for p65 (Figure 5A,B). In WT HCT116 cells TNF $\alpha$  stimulated p65 accumulation within the nucleus, with a peak after 30 min, followed by a progressive decline. Consistent with the NF- $\kappa$ B:Luc results, IKK $\alpha$  KO cells exhibited a striking reduction in p65 nuclear localisation at all time points examined whereas IKK $\beta$  KO cells exhibited a similar p65 nuclear localisation response to WT cells; IKK $\alpha/\beta$  DKO cells exhibited no p65 nuclear localisation in response to TNF $\alpha$  (Figure 5A). These observations were quantified in three independent clones of each of the four genotypes (WT, IKK $\alpha$  KO, IKK $\beta$  KO and IKK $\alpha/\beta$  DKO) through high-content confocal image analysis (Figure 5B) and confirmed that IKK $\alpha$  KO clones exhibited a strong, statistically significant reduction in TNF $\alpha$ -induced nuclear localisation of p65, whereas IKK $\beta$  KO clones exhibited a far more modest reduction in nuclear p65. Interestingly, when we analysed another NF- $\kappa$ B subunit, c-Rel, IKK $\alpha$  KO and IKK $\beta$  KO clones exhibited very similar defects in TNF $\alpha$ -induced nuclear localisation relative to WT suggesting each IKK made a similar contribution to c-Rel nuclear translocation (Figure 6A,B); IKK $\alpha/\beta$  DKO cells exhibited no c-Rel nuclear localisation in response to TNF $\alpha$ .

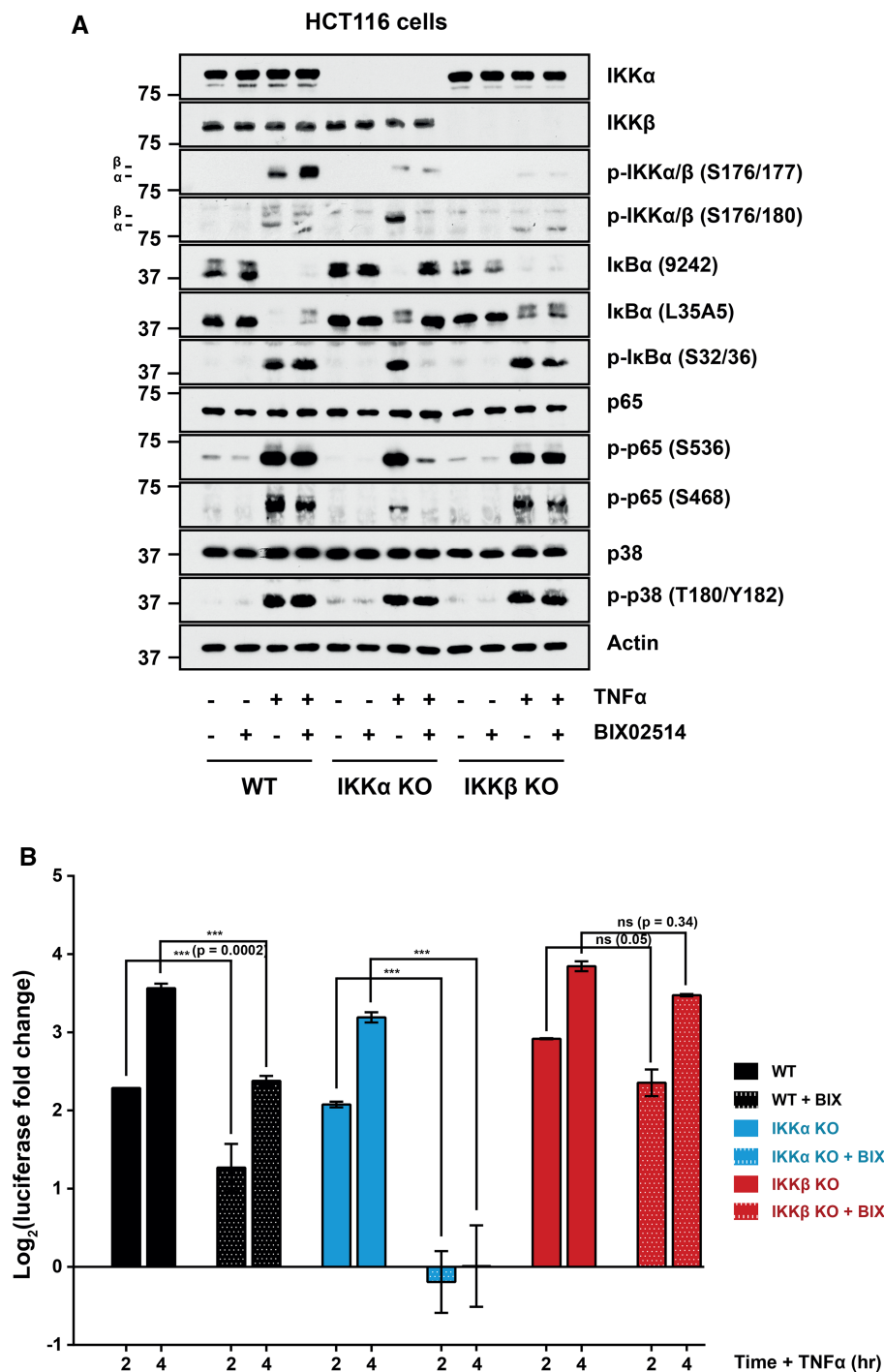
We confirmed this analysis using subcellular fractionation as an orthogonal assay; fractions were well resolved as judged by  $\beta$ -tubulin and Lamin A/C (Supplementary Figure S7A,B). Consistent with the immunofluorescence analysis, TNF $\alpha$ -induced nuclear translocation of p65 was greatly reduced in IKK $\alpha$  KO cells compared with WT whereas p65 translocation was only slightly reduced in IKK $\beta$  KO cells. In contrast, IKK $\alpha$  KO and IKK $\beta$  KO cells exhibited similar defects in the TNF $\alpha$ -induced nuclear translocation of c-Rel relative to WT cells, again confirming the immunofluorescence results. The greater defect in p65 nuclear translocation in IKK $\alpha$  KO cells relative to IKK $\beta$  KO cells was not a consequence of a greater defect in TNF $\alpha$ -induced I $\kappa$ B $\alpha$  degradation as this was comparable between IKK $\alpha$  KO and IKK $\beta$  KO cells. Previous studies have reported a significant proportion of full-length IKK $\alpha$  localised to the nucleus of HCT116 cells [44], while others have reported TNF $\alpha$ -inducible nuclear translocation of IKK $\alpha$  [45]. A proportion of full-length IKK $\alpha$  was detected in the nucleus of WT HCT116 cells, but only after over exposure of the blot (Supplementary Figure S7C); this nuclear localisation was confirmed, however, through immunofluorescence staining (Supplementary Figure S7D). However, no increase in nuclear translocation of IKK $\alpha$  after TNF $\alpha$  treatment was observed (Supplementary Figure S7C).

Collectively, these results demonstrate that IKK $\alpha$  KO and IKK $\beta$  KO cells are similarly defective in TNF $\alpha$ -induced nuclear translocation of c-Rel, whereas IKK $\alpha$  KO cells exhibit a major defect in p65 nuclear translocation which is not seen in IKK $\beta$  KO cells. These subunit-selective differences are not explained by differences in I $\kappa$ B $\alpha$  degradation but are likely to be a major factor accounting for the reduced TNF $\alpha$ -inducible NF- $\kappa$ B transcriptional activation observed in IKK $\alpha$  KO cells.



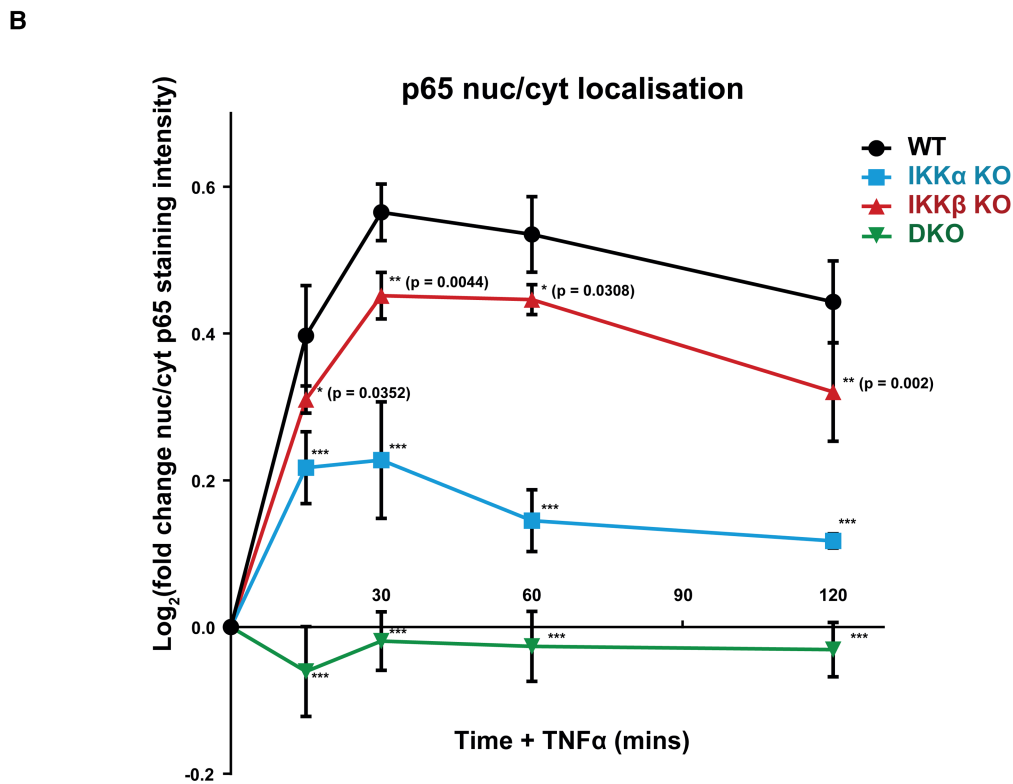
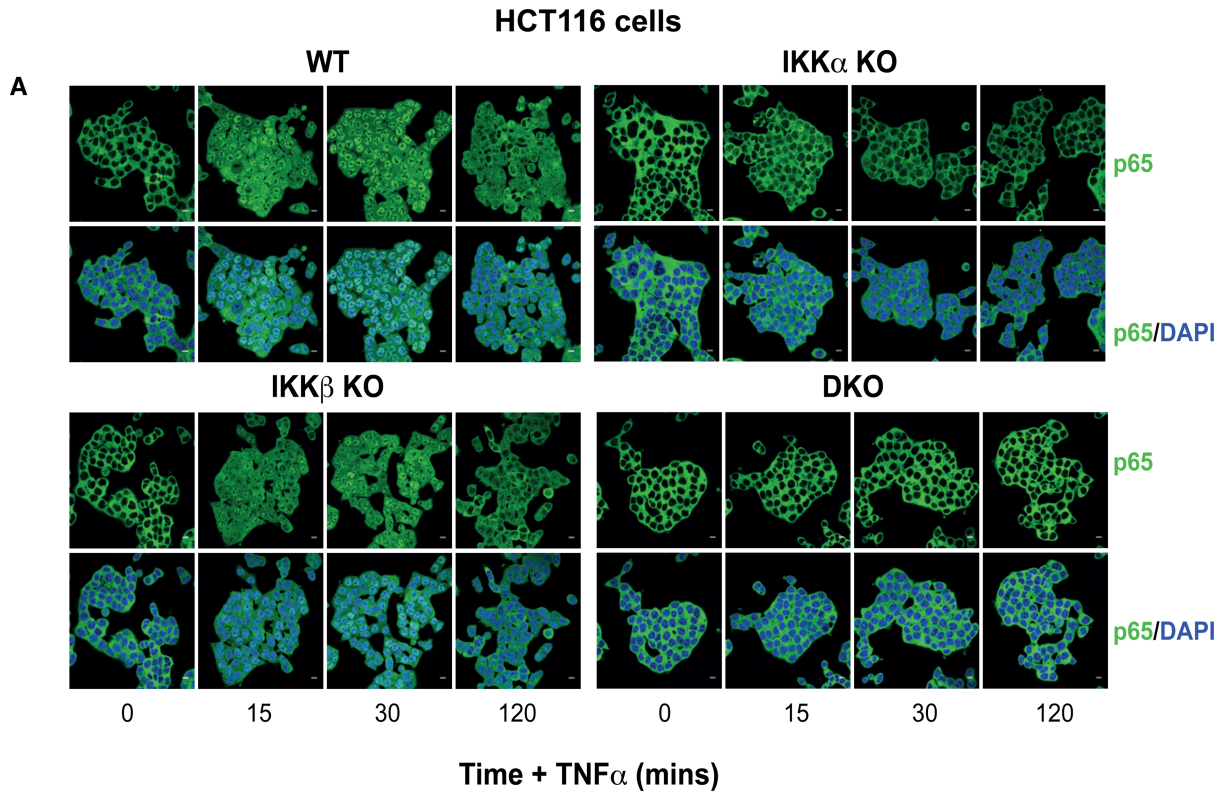
**Figure 3. Knocking down the remaining IKK subunit or IKK $\beta$  inhibition in single KO clones confirms redundant functions of IKKs.**

(A) WT (A3), IKK $\alpha$  KO (F6), IKK $\beta$  KO (G9) and IKK $\alpha$ / $\beta$  DKO (C8) HCT116 cells were transfected with non-targeting (NT) siRNA, IKK $\alpha$ -specific siRNA (si $\alpha$ ) or IKK $\beta$ -specific siRNA (si $\beta$ ). Forty-eight hours later, cells were treated with 10 ng/ml TNF $\alpha$  for 10 min and whole-cell lysates were prepared and Western blotted with the indicated antibodies. Data are from a single experiment representative of two giving similar results. \*Faint non-specific band running at similar molecular weight to P-IKK $\alpha$ . (B) WT, IKK $\alpha$  KO, IKK $\beta$  KO and IKK $\alpha$ / $\beta$  DKO HCT116 cells were transfected as above. Twenty-four hours later, cells were transiently transfected with NF- $\kappa$ B-RE firefly luciferase and renilla luciferase plasmids. The following day, cells were treated with 10 ng/ml TNF $\alpha$  for 2, 4 or 6 h and firefly luciferase normalised to renilla luciferase luminescence and expressed as log<sub>2</sub>(fold change in TNF $\alpha$ -induced luciferase activity relative to the relevant, matched untreated condition). Results are mean  $\pm$  SD of two independent experiments using the same KO clones as in (A), each performed in technical triplicate. Significance testing was performed using two-way ANOVA (repeated measures) with Tukey post-hoc test.  $P < 0.0001$  (\*\*\*).



**Figure 4. Selective IKK $\beta$  inhibition confirms a prominent role for IKK $\alpha$  in canonical NF- $\kappa$ B signalling.**

(A) WT, IKK $\alpha$  KO and IKK $\beta$  KO HCT116 cells were seeded for 48 h prior to treatment with 10  $\mu$ M BIX02514 or DMSO vehicle control for 30 min. Cells were then treated with TNF $\alpha$  (10 min) and processed for western blot. (B) WT, IKK $\alpha$  KO and IKK $\beta$  KO HCT116 cells were transiently transfected with reporter plasmids. The following day, cells were treated with BIX02514 or DMSO vehicle control for 30 min, prior to treatment with TNF $\alpha$  for 2 or 4 h. Firefly luciferase luminescence was normalised as above and expressed as log<sub>2</sub>(fold change in TNF $\alpha$ -induced luciferase activity relative to the relevant, matched untreated condition). Results are mean  $\pm$  SD of two independent experiments using the same KO clones as in (A) each performed in technical triplicate. Significance testing was performed using two-way ANOVA (repeated measures) with Tukey post-hoc test.  $P < 0.0001$  (\*\*\*). BIX, BIX02514.



**Figure 5. A critical role for IKK $\alpha$  in TNF $\alpha$ -induced p65 nuclear translocation.**

Part 1 of 2

WT, IKK $\alpha$  KO, IKK $\beta$  KO and IKK $\alpha$ / $\beta$  DKO HCT116 cells were seeded in normal growth medium for 48 h, prior to treatment with 10 ng/ml TNF $\alpha$  for the indicated times. Immunofluorescence staining of cells was performed with anti-p65 antibody (green) and nuclei were stained with DAPI (blue).

**Figure 5. A critical role for IKK $\alpha$  in TNF $\alpha$ -induced p65 nuclear translocation.**

Part 2 of 2

(A) Representative images are shown for time zero, 15, 30 and 120 min of TNF $\alpha$  stimulation. (B) Cells were seeded in 96 well plates in normal growth medium for 48 h, prior to treatment with 10 ng/ml TNF $\alpha$  for the indicated timepoints. Cells were stained with anti-p65 and DAPI and confocal images captured using the In Cell Analyzer 6000 high-content imaging system (20 $\times$  objective). p65 staining intensity analysis was performed using CellProfiler software. Data is presented as the log<sub>2</sub> transformed fold change in nuclear:cytoplasmic p65 staining intensity. Data are mean  $\pm$  SD of three independent CRISPR–Cas9 KO clones in each case. Significance testing relative to WT control cells performed using two-way ANOVA (repeated measures) with Dunnett post-hoc test.  $P < 0.0001$  (\*\*\*). Scale bar, 10  $\mu$ m.

## Loss of IKK $\alpha$ is more detrimental to IL-1 $\alpha$ induced NF- $\kappa$ B signalling than loss of IKK $\beta$

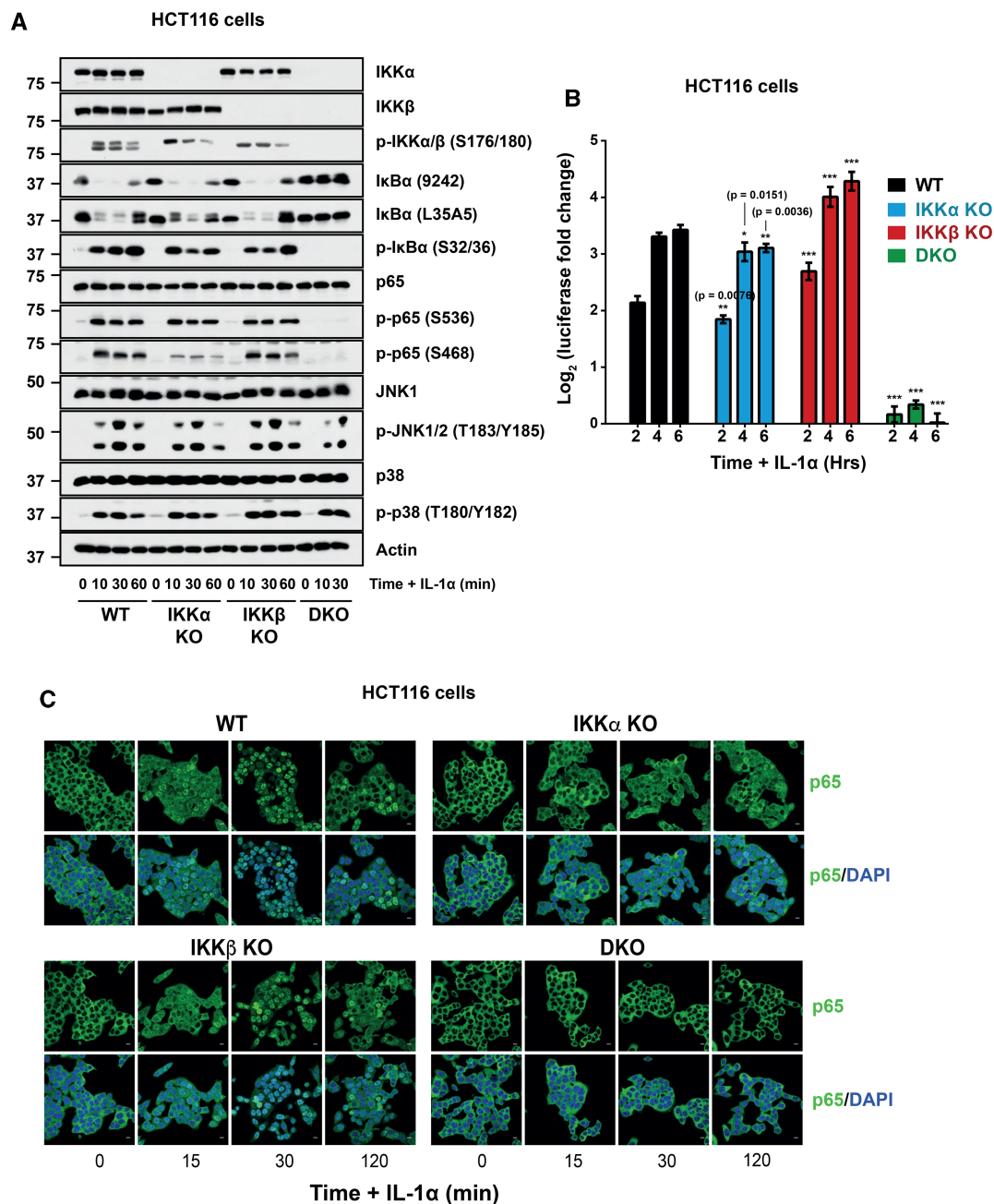
The importance of IKK $\alpha$  or IKK $\beta$  in canonical NF- $\kappa$ B signalling can depend on the stimulus [5,26] so we also examined responses to IL-1 $\alpha$  (Figure 7A). As with TNF $\alpha$ , both IKK $\alpha$  and IKK $\beta$  KO cells, but not IKK $\alpha$ / $\beta$  DKO, cells responded to IL-1 $\alpha$  to activate the canonical NF- $\kappa$ B pathway. The degradation of I $\kappa$ B $\alpha$  in response to IL-1 $\alpha$  was similar in IKK $\alpha$  KO and IKK $\beta$  KO cells, indicating redundant roles for both kinases. Similar to TNF- $\alpha$ , the IL-1 $\alpha$ -induced phosphorylation of p65 at S536 was largely unaffected in IKK $\alpha$  KO or IKK $\beta$  KO cells, but basal phosphorylation at this site was again reduced in IKK $\alpha$  KO cells. Furthermore, IL-1 $\alpha$ -induced p65 S468 phosphorylation was greatly reduced in IKK $\alpha$  KO cells, while IKK $\beta$  KO cells were largely unaffected. Unlike TNF $\alpha$ , however, IKK KO had no effect on the phosphorylation of JNK or p38 in response to IL-1 $\alpha$ . In common with the response to TNF $\alpha$ , IKK $\alpha$  KO cells exhibited a significantly lower IL-1 $\alpha$ -induced NF- $\kappa$ B:Luc response relative to WT; this was not observed in IKK $\beta$  KO cells where the transcriptional response to IL-1 $\alpha$  was slightly enhanced relative to WT (Figure 7B). As expected, IKK $\alpha$ / $\beta$  DKO cells exhibited no response to IL-1 $\alpha$  stimulation. Consistent with these results, IKK $\beta$  KO cells exhibited no defect in IL-1 $\alpha$  induced p65 nuclear translocation relative to WT cells, whereas IKK $\alpha$  KO cells exhibited a strong defect (Figure 7C). Collectively, these results indicate that HCT116 cells are more dependent upon IKK $\alpha$  than IKK $\beta$  for nuclear entry of p65 and activation NF- $\kappa$ B:Luc signalling in response to either TNF $\alpha$  or IL-1 $\alpha$ .

## A prominent role for IKK $\alpha$ in canonical NF- $\kappa$ B signalling in SW620 cells

Finally, we used CRISPR–Cas9 gene editing to generate clones lacking IKK $\alpha$  or IKK $\beta$  in a second, independent colorectal adenocarcinoma cell line, SW620, isolated from a metastatic site in the lymph node of a 51 year old Caucasian male. Like HCT116, SW620 cells harbour an activating KRAS mutation (KRAS<sup>G12V</sup>) as well as heterozygous mutations in TP53 (TP53<sup>R273H</sup> and TP53<sup>P309S</sup>) and homozygous mutation of APC (APC<sup>Q1338\*</sup>). Loss of IKK $\alpha$  or IKK $\beta$  delayed but did not abolish TNF $\alpha$ -driven phosphorylation and degradation of I $\kappa$ B $\alpha$ , consistent with both kinases being redundant for I $\kappa$ B $\alpha$  regulation (Figure 8A). IKK $\beta$  S177/181 phosphorylation was more persistent in IKK $\alpha$  KO cells relative to WT and the same was seen for IKK $\alpha$  S176/180 phosphorylation in the IKK $\beta$  KO clones (Figure 8A). TNF $\alpha$ -driven p65 S536 phosphorylation was unaffected by single IKK KO; phosphorylation of S468 was reduced in IKK $\alpha$  KO cells but unaffected by IKK $\beta$  KO. Activating phosphorylation of JNK1/2 in response to TNF $\alpha$  was enhanced and more persistent in both the IKK $\alpha$  or IKK $\beta$  clones; this was a notable difference to HCT116 cells where JNK activation was enhanced in IKK $\beta$  KO cells but not IKK $\alpha$  KO. p38 phosphorylation was largely unaffected by IKK loss. IKK $\alpha$  KO SW620 cells exhibited a far more modest (and non-significant) reduction in TNF $\alpha$ -induced NF- $\kappa$ B:Luc than was seen in HCT116 cells, whereas loss of IKK $\beta$  had no effect on NF- $\kappa$ B:Luc (Figure 8B).

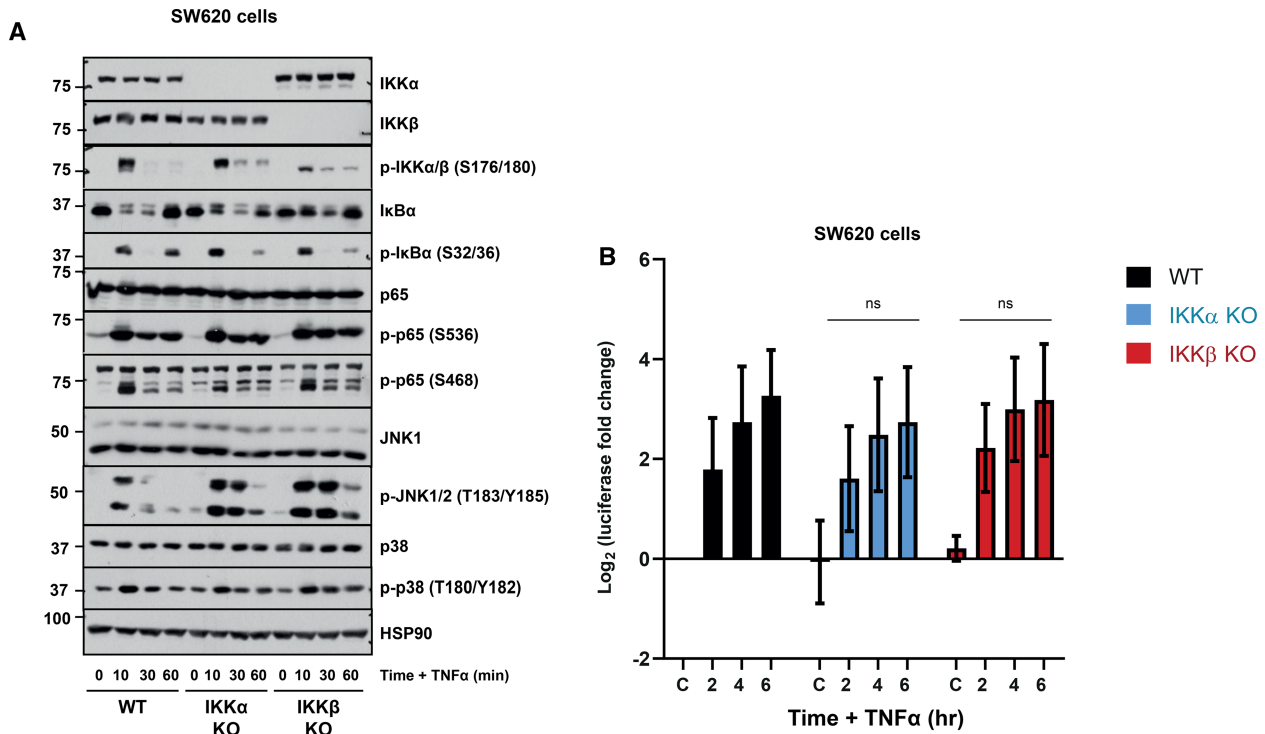
In WT cells a dose of BIX02514 (selective IKK $\beta$  inhibitor) that abolished NF- $\kappa$ B:Luc in IKK $\alpha$  KO cells had modest effects on NF- $\kappa$ B signalling and no effect on TNF $\alpha$ -induced NF- $\kappa$ B:Luc suggesting that loss of IKK $\beta$  activity was largely compensated for by IKK $\alpha$  (Figure 9A,B). In IKK $\alpha$  KO cells BIX02514 completely inhibited TNF $\alpha$ -induced I $\kappa$ B $\alpha$  phosphorylation, I $\kappa$ B $\alpha$  degradation, phosphorylation of p65 at S536 and S468 and abolished TNF $\alpha$ -induced NF- $\kappa$ B:Luc activity (Figure 9A,B). These results strongly suggest that the IKK activity observed in IKK $\alpha$  KO cells is due to IKK $\beta$ . BIX02514 had no effect on TNF $\alpha$ -induced canonical NF- $\kappa$ B signalling or NF- $\kappa$ B:Luc in IKK $\beta$  KO cells, confirming that the IKK activity observed in these cells is not due to residual IKK $\beta$  activity but rather IKK $\alpha$ .





**Figure 7. A major role for IKK $\alpha$  in NF- $\kappa$ B signalling in response to IL-1 $\alpha$ .**

(A) WT (A3), IKK $\alpha$  KO (F6), IKK $\beta$  KO (G9) and IKK $\alpha$ / $\beta$  DKO (C8) HCT116 cells were seeded in their normal growth medium for 48 h, prior to treatment with 25 ng/ml IL-1 $\alpha$  for 10, 30 and 60 min. Whole-cell extracts were prepared, fractionated by SDS-PAGE and Western blotted with the indicated antibodies. (B) The same clones as in (A) were seeded overnight prior to transient transfection with NF- $\kappa$ B-RE firefly luciferase and renilla luciferase plasmids. The following day, cells were treated with 25 ng/ml IL-1 $\alpha$  for 2, 4 and 6 h. Firefly luciferase was normalised to renilla luciferase and data expressed as log<sub>2</sub>(fold change in IL-1 $\alpha$ -induced luciferase activity relative to the relevant, matched untreated condition). Results are mean  $\pm$  SD of three independent experiments, each seeded in technical triplicate. Significance testing was performed using two-way ANOVA (repeated measures) with Tukey post-hoc test. *P* values relate to comparison with corresponding timepoint(s) of WT control. *P* < 0.0001 (\*\*\*). (C) The same clones as in (A) were seeded in normal growth medium for 48 h, prior to treatment with 25 ng/ml IL-1 $\alpha$  for 15, 30 or 120 min. Immunofluorescence staining of cells was performed with anti-p65 (green) and nuclei with DAPI (blue). Scale bar, 10  $\mu$ m.



**Figure 8. Redundant roles for IKK $\alpha$  and IKK $\beta$  in canonical NF- $\kappa$ B signalling in SW620 cells.**

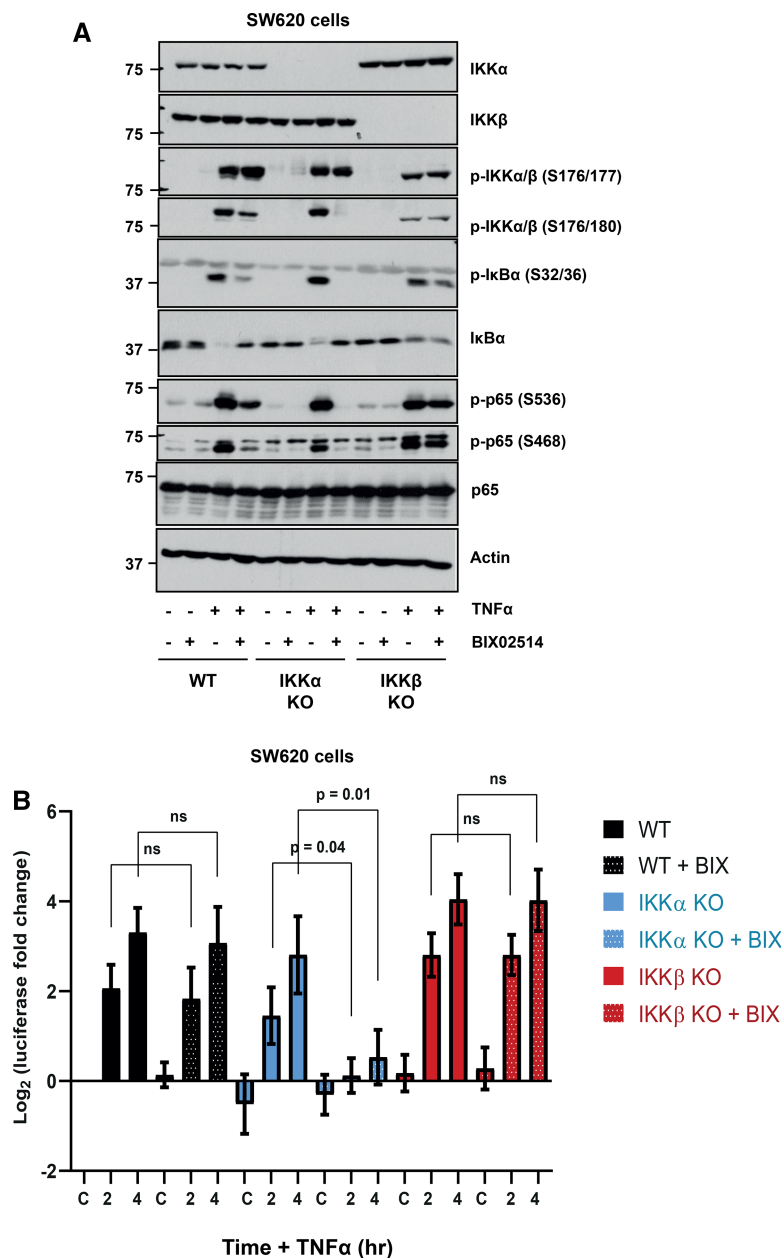
(A) WT, IKK $\alpha$  KO or IKK $\beta$  KO SW620 cells were seeded in their normal growth medium for 48 h, prior to treatment with 10 ng/ml TNF $\alpha$  for 10, 30 and 60 min. Whole-cell extracts were prepared, fractionated by SDS-PAGE and Western blotted with the indicated antibodies. (B) WT, IKK $\alpha$  KO or IKK $\beta$  KO SW620 cells were seeded overnight prior to transient transfection with NF- $\kappa$ B-RE firefly luciferase and renilla luciferase plasmids. The following day, cells were treated with 10 ng/ml TNF $\alpha$  for 2, 4 and 6 h. Firefly luciferase was normalised to renilla luciferase and data expressed as log<sub>2</sub>(fold change in IL-1 $\alpha$ -induced luciferase activity relative to the relevant, matched untreated condition). Results are mean  $\pm$  SD of three independent experiments, each seeded in technical triplicate.

## Discussion

One of the central dogmas of NF- $\kappa$ B signalling is that IKK $\beta$  is the dominant IKK in the canonical pathway, with IKK $\alpha$  playing a more important role in the non-canonical NF- $\kappa$ B activation pathway. However, the literature actually suggests that the role of IKK $\alpha$  and IKK $\beta$  varies with stimulus, cell and tissue type, developmental stage and species. We were interested in inflammatory signalling in colorectal cells so we generated IKK KO in HCT116 and SW620 cells; our results indicate that both IKK $\alpha$  and IKK $\beta$  normally contribute to TNF $\alpha$  dependent NF- $\kappa$ B activation in SW620 cells whereas IKK $\alpha$  plays the dominant role in HCT116 cells.

Despite early reports of a non-redundant role for IKK $\beta$  in I $\kappa$ B $\alpha$  phosphorylation and turnover [43], our results are more consistent with later reports of redundant roles for IKK $\alpha$  and IKK $\beta$  [26,28] since loss of IKK $\alpha$  or IKK $\beta$  alone had little (SW620) or no (HCT116) effect on I $\kappa$ B $\alpha$  phosphorylation and turnover. However, our results provide clear evidence for a major role for IKK $\alpha$  in canonical NF- $\kappa$ B signalling. In HCT116 cells, IKK $\alpha$  was the dominant kinase involved in the basal p65 S536 phosphorylation, stimulated p65 S468 phosphorylation, nuclear translocation of p65 and the NF- $\kappa$ B-dependent transcriptional response to both TNF $\alpha$  and IL-1 $\alpha$ . In these cells IKK $\beta$  homodimers were far less efficient at compensating for the loss of IKK $\alpha$  than IKK $\alpha$  homodimers were able to compensate for the loss of IKK $\beta$ ; this was confirmed when siRNA was used to knock-down the nontargeted kinase in single KO cells. Critically, experiments using the selective IKK $\beta$  inhibitor BIX02514 confirmed that these observations were not specific to IKK $\alpha$  KO cells, and that IKK $\alpha$  also plays a significant role in these responses in WT cells. Some subtle differences were noted in SW620 cells; for example, BIX02514 reduced but did not abolish p65 phosphorylation in WT cells suggesting both IKK $\alpha$  and IKK $\beta$  were contributing to these responses.





**Figure 9. Selective IKK $\beta$  inhibition confirms a prominent role for IKK $\alpha$  in canonical NF- $\kappa$ B signalling in SW620 cells.** (A) WT, IKK $\alpha$  KO or IKK $\beta$  KO SW620 cells were seeded for 48 h prior to treatment with 10  $\mu$ M BIX02514 or DMSO vehicle for 30 min before treating with TNF $\alpha$  (10 min) and processing for western blot. (B) WT, IKK $\alpha$  KO or IKK $\beta$  KO SW620 cells were transiently transfected with reporter plasmids DNA. The following day, cells were treated with BIX02514 or DMSO vehicle control for 30 min, prior to treatment with TNF $\alpha$  for 2 or 4 h. Firefly luciferase luminescence was normalised as above and expressed as log<sub>2</sub>(fold change in TNF $\alpha$ -induced luciferase activity relative to the relevant, matched untreated condition). Results are mean  $\pm$  SD of three independent experiments, each seeded in technical triplicate. Significance testing was performed using two-way ANOVA (repeated measures) with Tukey post-hoc test. *P* values relate to comparison with corresponding timepoint(s) of WT control.

Notably, the level of stimulus-induced activation loop phosphorylation of the remaining IKK subunit was enhanced in the IKK $\alpha$  or IKK $\beta$  KO cells. In both HCT116 and SW620 cells, IKK $\beta$  S177/181 phosphorylation was higher and more sustained in IKK $\alpha$  KO cells relative to WT and the same was seen for IKK $\alpha$  in the IKK $\beta$  KO clones. This higher level of phosphorylation did not reflect a compensatory increase in the expression of

the remaining IKK subunit in the HCT116 cells, whereas we did see a consistent increase in IKK $\alpha$  in the IKK $\beta$  KO SW620 cells. The deubiquitylases CYLD and A20 cleave K63-linked polyubiquitin chains of signalling components upstream of IKK to terminate the NF- $\kappa$ B signalling response and A20 KO MEFs exhibit more sustained TNF $\alpha$  induced IKK activity. Interestingly, IKK $\alpha$  has been shown to play an important role in this feedback inhibition by phosphorylating the regulatory molecule, TAX1BP1 in response to TNF $\alpha$ /IL-1 $\alpha$ ; this promotes the assembly of the A20 ubiquitin-editing complex [46]. However, although A20 is induced upon NF- $\kappa$ B activation [47] we observed no increase in A20 abundance in response to TNF $\alpha$  until later time points (data not shown) so it is unlikely that reduced feedback inhibition explains the higher phosphorylation of the remaining IKK subunits. The enhanced phosphorylation of the remaining IKK subunit in IKK KO cells should be considered in the context of the current model for IKK $\beta$  activation which suggests that TAK1 phosphorylation of IKK $\beta$  at Serine 177 primes the subsequent autophosphorylation of IKK $\beta$  on Serine 181 [48]. Our observations with an IKK $\beta$  selective inhibitor are consistent with this study. The enhanced IKK $\beta$ -dependent autophosphorylation at Serine 181 in IKK $\alpha$  KO cells and IKK $\alpha$  S180 phosphorylation in the IKK $\beta$  KO clones suggests that each IKK is more active in the absence of the other, or more readily activated by TAK1 in the absence of the other (i.e. a stoichiometry or competition effect). Clearly, further work is required to assess the kinetics of TAK1 activation and feedback control [49] in the IKK KO cells.

Serine 468 of p65 has been proposed as an IKK $\beta$ -dependent phosphorylation site since recombinant IKK $\beta$  phosphorylated this site more efficiently than IKK $\alpha$  *in vitro* [43], whilst dominant negative IKK $\beta$ , but not an equivalent IKK $\alpha$  construct, inhibited the TNF $\alpha$  and IL-1 $\alpha$  induced phosphorylation of this site. However, we observed a much greater defect in the TNF $\alpha$  and IL-1 $\alpha$  induced S468 phosphorylation in IKK $\alpha$  KO cells compared with IKK $\beta$  KO cells, suggesting that IKK $\alpha$  homodimers were better able to compensate for the loss of IKK $\beta$  in terms of S468 phosphorylation than IKK $\beta$  homodimers were for the loss of IKK $\alpha$ . The minimal role for IKK $\beta$  in S468 phosphorylation in WT HCT116 cells in response to TNF $\alpha$  was also demonstrated with the IKK $\beta$  inhibitor BIX02514. Unfortunately, although knockout and siRNA-mediated knockdown of IKK $\alpha$  interfered with the phosphorylation of this site, we were unable to convincingly demonstrate sufficiency IKK $\alpha$  re-expression. This could reflect a technical limitation of the transient transfections or transiently transfected FLAG-tagged IKK $\alpha$  and endogenous IKK $\alpha$  may not be functionally equivalent. Alternatively, IKK $\alpha$  may facilitate recruitment of another kinase such as GSK3 $\beta$  and IKK $\epsilon$  [48,49] to phosphorylate S468 in response to TNF $\alpha$ . First-in-class IKK $\alpha$  selective inhibitors have recently been described [50] and may be commercially available in the near future. Such inhibitors would be valuable in resolving the importance of IKK $\alpha$  in S468 phosphorylation in response to TNF $\alpha$  and IL-1 $\alpha$ . It will also be interesting to characterise the function of S468 phosphorylation in more detail in these cells. S468 phosphorylation by IKK $\beta$  occurs in the cytosol while p65 is bound to I $\kappa$ B $\alpha$  and appears to moderately inhibit TNF $\alpha$  and IL-1 $\beta$  induced NF- $\kappa$ B activation of certain genes [43]. This has been proposed to be due to S468 phosphorylation enhancing binding of p65 to the histone deacetylase, GCN5, which in turn recruits a COMMD1-containing E3 ligase complex that targets p65 bound to specific genes for proteasomal degradation [51,52]; thus the reduced S468 phosphorylation may not account for the reduced nuclear translocation of p65 in IKK $\alpha$  KO cells. Clearly, further work is required to resolve the function of S468 phosphorylation.

The marked reduction in TNF $\alpha$ - or IL-1 $\alpha$ -induced nuclear translocation of p65 in IKK $\alpha$  KO HCT116 cells is likely to contribute to the lower NF- $\kappa$ B transcriptional activation in these cells. It is also notable that in the same cells TNF $\alpha$ -induced nuclear translocation of c-Rel was equally dependent on IKK $\alpha$  and IKK $\beta$  and these results were confirmed independently by sub-cellular fractionation. Thus, different NF- $\kappa$ B subunits exhibit different dependencies on IKK $\alpha$  or IKK $\beta$  for their nuclear translocation and these differences cannot be due to differences in I $\kappa$ B $\alpha$  degradation where individual IKK KO has little or no effect. It seems more likely that they are due to differences in IKK-dependent phosphorylation and potentially the sub-cellular location of the IKKs. IKK $\alpha$  has been proposed to have nuclear-specific roles in both the enhancement and termination of NF- $\kappa$ B-dependent gene expression, such as the phosphorylation of Histone H3 and CBP at the promoters of specific genes. The contributions of these proposed nuclear functions of IKK $\alpha$  to the observations made here have yet to be explored. However, other factors could influence the lower NF- $\kappa$ B transcriptional activation observed, and future studies should seek to define the relative DNA binding activities of different NF- $\kappa$ B dimers in IKK $\alpha$  and IKK $\beta$  KO cells and assess promoter occupancy of different NF- $\kappa$ B dimers on specific genes using chromatin immunoprecipitation (ChIP). Furthermore, since the IKK $\alpha$  KO cells exhibit reduced p65 S468 phosphorylation, which has been suggested to promote the proteolytic removal of p65 from the promoters of certain genes, it would be interesting to define the kinetics of promoter occupancy in these cells relative to WT; the overall level of p65-driven expression of certain genes might be lower but perhaps more sustained in these cells for this reason.

One final issue is the role of NEMO and NIK in the single IKK KO cells that we have derived, especially the IKK $\beta$  null cells. It has previously been shown that IKK $\alpha$ -dependent activation of the non-canonical NF- $\kappa$ B pathway requires NIK but is independent of NEMO [53]. It will be interesting to see if the IKK $\alpha$ -dependent NF- $\kappa$ B activation we report here is NEMO-independent and represents NIK stabilisation, and compensatory activation of non-canonical IKK signalling that feeds into the canonical pathway to compensate for loss of IKK $\beta$ . However, the non-canonical pathway is typically slower to elaborate as it requires NIK stabilisation; for example, we see no evidence of NIK stabilisation in IKK $\beta$  KO cells until 24 h after TNF $\alpha$  stimulation (data not shown), suggesting that this may not explain our NF- $\kappa$ B functional reporter assays which were all confined to 2, 4 or 6 h stimulation. These questions will require further investigation.

In summary, this is to our knowledge the first report of IKK $\alpha$  KO, IKK $\beta$  KO and IKK $\alpha/\beta$  DKO in human cells. Our results challenge the prevailing dogma by showing that IKK $\alpha$  is equally (SW620 cells) or more (HCT116 cells) important than IKK $\beta$  for mediating the activation of canonical NF- $\kappa$ B signalling in response to TNF $\alpha$  and IL-1 $\alpha$  in colorectal cells. These results were confirmed by the use of selective IKK siRNA and IKK $\beta$ -selective small-molecule inhibition and should refocus efforts towards the development of IKK $\alpha$  selective inhibitors [50]. These IKK KO cells may prove valuable for future studies into NF- $\kappa$ B-dependent and NF- $\kappa$ B-independent functions of IKK $\alpha$  and IKK $\beta$ .

### Data Availability

All relevant data are contained within the main article and its Supplementary Files.

### Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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### CRedit Author Contribution

**Simon J. Cook:** Conceptualization, Supervision, Funding acquisition, Writing — original draft, Writing — review and editing. **Jack A. Prescott:** Conceptualization, Investigation, Methodology, Writing — original draft. **Kathryn Balmanno:** Validation, Investigation, Methodology. **Jennifer P. Mitchell:** Validation, Investigation, Methodology, Writing — original draft, Writing — review and editing. **Hanneke Okkenhaug:** Data curation, Software, Formal analysis, Methodology.

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### Abbreviations

CAC, colitis-associated cancer; CRC, colorectal cancer; DKO, double knockout; IBD, inflammatory bowel disease; IKK $\beta$ , Inhibitor of kappa B kinase  $\beta$ ; KD, kinase dead; MEFs, mouse embryonic fibroblasts; PI, propidium iodide; TNF, tumour necrosis factor.

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