



PAK1 modulates a PPAR γ /NF- κ B cascade in intestinal inflammation



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ABSTRACT

P21-activated kinases (PAKs) are multifunctional effectors of Rho GTPases with both kinase and scaffolding activity. Here, we investigated the effects of inflammation on PAK1 signaling and its role in colitis-driven carcinogenesis. PAK1 and p-PAK1 (Thr423) were assessed by immunohistochemistry, immunofluorescence, and Western blot. C57BL6/J wildtype mice were treated with a single intraperitoneal TNF α injection. Small intestinal organoids from these mice and from PAK1-KO mice were cultured with TNF α . NF- κ B and PPAR γ were analyzed upon PAK1 overexpression and silencing for transcriptional/translational regulation. PAK1 expression and activation was increased on the luminal intestinal epithelial surface in inflammatory bowel disease and colitis-associated cancer. PAK1 was phosphorylated upon treatment with IFN γ , IL-1 β , and TNF α . In vivo, mice administered with TNF α showed increased p-PAK1 in intestinal villi, which was associated with nuclear p65 and NF- κ B activation. p65 nuclear translocation downstream of TNF α was strongly inhibited in PAK1-KO small intestinal organoids. PAK1 overexpression induced a PAK1-p65 interaction as visualized by co-immunoprecipitation, nuclear translocation, and increased NF- κ B transactivation, all of which were impeded by kinase-dead PAK1. Moreover, PAK1 overexpression downregulated PPAR γ and mesalamine recovered PPAR γ through PAK1 inhibition. On the other hand PAK1 silencing inhibited NF- κ B, which was recovered using BADGE, a PPAR γ antagonist. Altogether these data demonstrate that PAK1 overexpression and activation in inflammation and colitis-associated cancer promote NF- κ B activity via suppression of PPAR γ in intestinal epithelial cells.

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1. Introduction

Inflammatory bowel diseases (IBD) such as Crohn's disease (CD) and ulcerative colitis (UC) are associated with an increased risk of developing colorectal cancer (CRC). Mesalamine, 5-aminosalicylic acid (5-ASA), is an anti-inflammatory drug used to treat UC, and epidemiological evidence suggests that it has chemopreventive effects [1]. We previously identified p-21 activated kinase-1 (PAK1) as a 5-ASA target [2]. PAK1 is a serine/threonine kinase effector of the small Rho GTPases Rac1/Cdc42 [3], which regulates cytoskeletal dynamics and epithelial

cell (IEC) migration and homeostasis. Recently, we demonstrated that PAK1 is overexpressed in IBD and CAC and promotes cell survival pathways [4–6]. However, the exact cause and consequence of PAK1 overexpression in intestinal inflammation have yet to be defined.

Several studies support the notion that canonical NF- κ B activation promotes intestinal tumorigenesis through the upregulation of pro-inflammatory cytokines, proliferation, and cell survival [7–10]. NF- κ B activation is regulated by the transcription factor RelA (p65). At basal levels, p65 is sequestered in the cytoplasm by its inhibitors IKK α / β and I κ B. Upon pathway activation, I κ B is degraded, and p65 translocates into the nucleus [11]. NF- κ B signaling in immune cells drives the expression of pro-inflammatory cytokines such as TNF α or IL-1 β , which subsequently activate NF- κ B in IECs thereby promoting cell survival [12]. In support of this, TNF α -administered NF- κ B1^{-/-} mice show increased IEC apoptosis [12]. PAK1 was previously reported to stimulate NF- κ B [13], albeit its exact mechanism within the canonical pathway is unknown.

Here, we have investigated the effect of PAK1 activation in IECs upon inflammation and its relevance for NF- κ B signaling. We observed that

Abbreviations: PAK1, p-21 activated kinase 1; PPAR γ , peroxisome proliferator associated receptor gamma; IBD, inflammatory bowel disease; NF- κ B, nuclear factor-kappa B; CRC, colorectal cancer; UC, ulcerative colitis; CD, Crohn's disease; CAC, colitis-associated cancer; EV, empty vector; WT, wild type; KD, kinase dead; KO, knock out; SIO, small intestinal organoids; SB, small bowel; LB, large bowel; IEC, intestinal epithelial cells; Rosi, Rosiglitazone

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several cytokines including TNF α can activate PAK1 and induce its nuclear localization. In IECs, such PAK1 activation induces a p65 interaction and assists its nuclear translocation through inhibition of PPAR γ .

2. Materials and methods

2.1. Animal experiments

9 week old, C57BL/6 mice were provided by Charles River (Margate, UK) and housed at the University of Liverpool. All procedures were performed according to UK Home Office license guidelines. Mice were euthanized 1.5 h after receiving an intraperitoneal injection of TNF α at 0.33 mg/kg body weight. The large (LB) and small bowel (SB) were dissected and paraffin embedded as previously described.

2.2. Cell culture, isolation of small intestinal epithelial organoids, and reagents

Primary human colon epithelial cells (HCEC-1CT), a generous gift from Dr. Jerry W. Shay, were cultured as described previously [14]. HCT116 cells were purchased from ATCC. Small intestinal organoids (SIO) were isolated, from C57BL/6 wild type (WT) and PAK1^{-/-} (PAK1 KO) mice (purchased from MMRRC UNC, Chapel Hills, NC), and maintained as previously described [15]. All cells were serum starved in incomplete medium for 12 h prior to treatment with recombinant protein. Reagents (Supplementary Table S2) were applied 2–12 h prior to inflammatory cytokines. Cells were harvested at 60–70% confluency.

2.3. Co-culture

Polymorphonuclear cells (PMN) were isolated, activated, and cultured using a previously established co-culture system [16]. Briefly, isolated PMNs were activated using 50 ng/mL phorbol 12-myristate 13-acetate (PMA) at 37 °C for 30 min. PMNs were washed twice with Ca/Mg free-Hank's buffer salt solution (HBSS), and applied to the upper chamber of a 0.45 μ m Transwell insert in a 1:50 epithelial/PMN ratio. Following treatment, PMNs were discarded, and colonic epithelial cells were harvested for western blot analysis.

2.4. Immunohistochemical analysis

Immunohistochemistry (IHC) was performed on 3–5 μ m tissue sections from human samples, and mouse SB or LB [6]. The human samples were obtained from the Clinical Institute of Pathology at the Medical University of Vienna. The study was approved by the local ethics committee. Samples were selected from endoscopic biopsies or surgical specimens of active IBD and CAC patients. Control specimens were taken from normal colon tissue. CD patients displayed active colonic disease, and CAC included both adenoma and invasive tumors.

2.5. Immunoreactivity score

An immunoreactivity score (IRS) was generated by two blinded investigators. Separate IRSs were generated for the small bowel (SB) and the large bowel (LB). The SB included villus and crypt epithelial staining, while the LB included surface and crypt epithelial staining. The intensity of staining included a score from 0–3, 0 (no staining), 1 (low), 2 (medium), 3 (high). The percentage of positively stained

epithelial cells was evaluated on a scale from 0–4, 0 (<1%), 1 (1–10%), 2 (10–50%), 3 (51–80%), and 4 (>80%) in 4 separate fields of view. The mean intensity and percentage of positively stained epithelial cells was multiplied to generate the IRS, and the highest score attainable was 12. Data shown represent the mean of the 2 scorers' findings.

2.6. Quantitative real-time PCR

RNA was isolated using TRIzol reagent (Life Technologies). cDNA synthesis was carried out using ThermoScript RT-PCR System (Invitrogen) according to manufacturer's protocol. Quantitative RT-PCR was carried out in triplicate using Fast SYBR Green Master Mix with primers (Supplementary Table S3). All data were normalized to the endogenous control 36b4. Relative quantification of transcripts were calculated using the comparative CT method.

2.7. Western blotting (WB) and co-immunoprecipitation (co-IP)

Cells were washed in PBS and harvested in RIPA buffer for whole cell lysates. Nuclear and cytoplasmic extracts were collected as previously described [17]. SIO were released from matrigel in ice cold PBS, and centrifuged at 1000 RPM for 5 min. The matrigel was discarded and the organoids were sonicated for 2–3 s in RIPA buffer. All lysates were harvested for 30 min on ice and centrifuged at 4 °C for 30 min. Protein concentration was determined by Bradford assay and measured on an (Anthos 2010) photometer. For co-immunoprecipitation (co-IP), 750 μ g of protein were incubated with 2 μ g rabbit or mouse IgG or primary antibody shaking at 4 °C overnight followed by incubation with A/G agarose beads for 2 h at room temp. Samples were washed 5 \times with PBS and added to SDS sample buffer. For WB, 25–50 μ g protein were added to SDS sample buffer for 10 min at 95 °C. Protein samples were separated using SDS-PAGE and transferred on a PVDF membrane. Membranes were blocked in Western blocker solution (Sigma) for 1 h, and incubated with primary antibodies (Supplementary Table S1) overnight at 4 °C. Protein bands were visualized using IRDye coupled secondary antibodies and imaged on an Odyssey infrared imaging system (LI-COR). Images were obtained and densitometry was calculated using Odyssey application software version 3.0.21.

2.8. Electroporation, PAK1 overexpression, and RNA interference

Transient transfection in HCEC-1CT was achieved via electroporation using Amaxa basic nucleofector 2B kit and device for primary mammalian epithelial cells (Lonza) [17]. PAK1 was overexpressed with 5 μ g of pCMV6M-PAK1 (WT-PAK1), a generous gift from Dr. Jonathan Chernoff, pCMV6M-PAK1 K299R (KD-PAK1) (Addgene) or a pCMV6M empty vector (EV) (Addgene) for 72 h. PAK1 knockdown using RNA interference was achieved using 100 nM siPAK1 (Dharmacon) or 100 nM scrambled siRNA (Santa Cruz) for 48 h.

2.9. Immunofluorescence

HCEC-1CT and SIO were fixed in 4% paraformaldehyde at room temperature for 30 min and blocked in 0.1% Triton-X-100/2% horse serum for 1 h. Samples were incubated with primary antibody in 1% BSA/PBS overnight at 4 °C, then washed and incubated with fluorescent secondary antibody for 1 h at room temperature. Samples were washed, dried, and mounted in medium containing DAPI (Vector Laboratories), and imaged on an Olympus IX81 or BX51 microscope.

Fig. 1. (A–D). PAK1 is overexpressed and activated in IBD. (A) Human colonic tissue stained for PAK1. PAK1 expression increases in comparison to normal mucosa at the luminal epithelial surface and crypts in both Crohn's disease (CD), ulcerative colitis (UC), and in tumors of colitis associated cancer (CAC). (B) Box plots compare mean PAK1 immunoreactivity scores (IRS) in normal mucosa (n = 6), CD (n = 7), and UC (n = 6) at the luminal surface and crypts. PAK1 is overexpressed in CAC (n = 8). The circle is the outlier from the boxplot. (C) Human colonic tissue stained for p-PAK1. In CD and UC, PAK1 activation in epithelial cells is found at the membrane, specifically at the luminal surface, but not in the crypts. In CAC, PAK1 phosphorylation is found within the nuclei and cytoplasm of tumor cells. (D) Inflammatory cytokines activate PAK1 in HCEC-1CT. WB of HCEC-1CT RIPA whole cell lysates using 50 μ g protein. Cells were either untreated (Con) or treated with IFN γ , IL-1 β , or TNF α for 5–60 min and analyzed for PAK1 activation with a p-PAK1 Thr 423 antibody. TNF α induced the most profound effect in PAK1 activation at 30 min. α -Tubulin was utilized as a loading control.

2.10. Luciferase assay

HCEC-1CT cells were transiently transfected with a TkpGL3 NF- κ B or PPAR luciferase response element, or co-transfected with scrRNA, siPAK1, EV, WT-PAK1, or KD-PAK1 and seeded into 24 well plates. Cells were lysed with a lysis buffer containing 4% Triton X-100, 100 mM Glycyl-glycine, 100 mM MgSO₄, and 250 mM EGTA for 20 min on a shaker at room temperature. 20 μ L of lysate were added into a 96 well plate and analyzed on a Plate Chameleon V photometer (Hidex) in which 100 μ L of luciferase activity buffer containing 100 mM Glycyl-glycine, 100 mM MgSO₄, 20 mM ATP (Sigma), and 2.5 mM D-luciferin (Sigma) were injected per well. All measurements were performed using technical triplicates.

2.11. Statistics

Statistical analysis was performed using SPSS (version 21.0). Metric outcome variables were compared using univariate analysis of variance (ANOVA) and Tukey HSD post-hoc tests. RT-PCR data and immunoreactivity scores were analyzed using a 2-tailed *t*-test. *p*-Values less than 0.05 were considered significant (**p* < 0.05, ***p* < 0.01, ****p* < 0.001). All data are expressed as mean \pm standard deviation.

3. Results

3.1. Pro-inflammatory cytokines promote PAK1 activation in IEC

PAK1 is associated with gut tumorigenesis [5,18,19] and we recently demonstrated its overexpression in IBD and colitis-associated cancer (CAC) [6]. Here we investigated PAK1 activation and signaling in IBD and CAC. Normal intestinal mucosa, CD, UC, and CAC samples were stained with both a PAK1 and p-PAK1 antibody that detects phosphorylation at Thr-423, a residue that is essential for PAK1 activation [20]. In comparison to normal mucosa, total and nuclear PAK1 was overexpressed at the luminal epithelial surface and crypts in CD and UC, as well as in tumors of CAC (Fig. 1A–B). PAK1 activation was absent within the normal mucosa, and cytoplasmic p-PAK1 levels increased at the luminal intestinal epithelial surface in both UC and CD, but not within the crypts. CAC tumor samples displayed increased PAK1 phosphorylation in both the cytoplasm and nucleus (Fig. 1C, Supplementary Fig. S1A).

IFN γ , IL-1 β , and TNF α are pro-inflammatory cytokines which contribute to gut inflammation in IBD [21]. We investigated their effect on PAK1 activation in HCEC-1CT cells. IFN γ , IL-1 β , and TNF α induced phosphorylation of PAK1 at Thr-423 within 10 to 30 min (Fig. 1D). Immunofluorescence of p-PAK1 revealed cytoplasmic levels of p-PAK1 increased upon TNF α stimulation, and this effect was abolished upon PAK1 kinase inhibition using IPA-3 (Supplementary Fig. S1B). We further verified the specificity of our p-PAK1 antibody using IPA-3 upon WT-PAK1 overexpression in the presence of TNF α (Supplementary Fig. S1C). PAK1 overexpression also resulted in PAK1 auto-phosphorylation. IFN γ , IL-1 β , and TNF α also induced p-PAK1 in the colorectal cancer cell line HCT116 as early as 5 min peaking within the 30–60 min range (Supplementary Fig. S1D). Accumulation of polymorphonuclear leukocytes (PMNs) within colonic crypts is a key feature of UC [16]. HCEC-1CT cells

were co-cultured with freshly isolated PMNs using Transwell inserts. Total PAK1 expression increased more than two-fold at 60 min (Supplementary Fig. S1E). Taken together, these data demonstrate that pro-inflammatory cytokines and activated PMNs regulate PAK1 activation and expression in human colonic epithelial cells.

3.2. PAK1 regulates NF- κ B activation downstream of TNF α

Our data showed that TNF α , which plays a key role in the pathogenesis of IBD [21], and activates canonical NF- κ B signaling, induced PAK1 activation. Next, we investigated the role of PAK1 in NF- κ B signaling. Control (EV), WT-PAK1, and KD-PAK1 vectors were overexpressed in HCEC-1CT and Western blot analysis was performed on cytoplasmic and nuclear fractions. WT-PAK1 overexpression induced p65 nuclear translocation in the absence of TNF α , whereas KD-PAK1 did not (Fig. 2A). Co-immunoprecipitation showed a direct interaction of p65 and PAK1 upon WT-PAK1 overexpression which was reduced upon KD-PAK1 indicating the importance of auto-phosphorylation of PAK1 for interaction with p65 (Fig. 2B). In the absence of TNF α , immunofluorescence staining of p-PAK1 and p65 revealed localization of both proteins in the cytoplasm. TNF α induced translocation and co-localization of p-PAK1 and p65 in the nucleus (Fig. 2C). Immunofluorescence in HCEC-1CT revealed that TNF α activated nuclear translocation of p65 was blocked upon PAK1 kinase inhibition using IPA-3 (Fig. 2D). P-PAK1 inhibition by IPA-3 was confirmed by Western blot (Fig. 2D). Western blot analysis supported these observations and demonstrated that KD-PAK1 abrogated the effect of TNF α on p65 nuclear translocation (Supplementary Fig. S2A). Densitometry of nuclear p65 revealed that WT-PAK1 overexpressing cells had increased nuclear p65 and were more sensitive to TNF α in comparison to EV or KD-PAK1 (Supplementary Fig. S2A). Further, the effect of PAK1 overexpression and the dependence of PAK1 kinase activity on NF- κ B transactivation were confirmed using an NF- κ B luciferase reporter assay (Fig. 2E). These data demonstrate that PAK1 overexpression or its activation by TNF α results in a p-PAK1–p65 interaction, nuclear translocation, and transcriptional activation of NF- κ B.

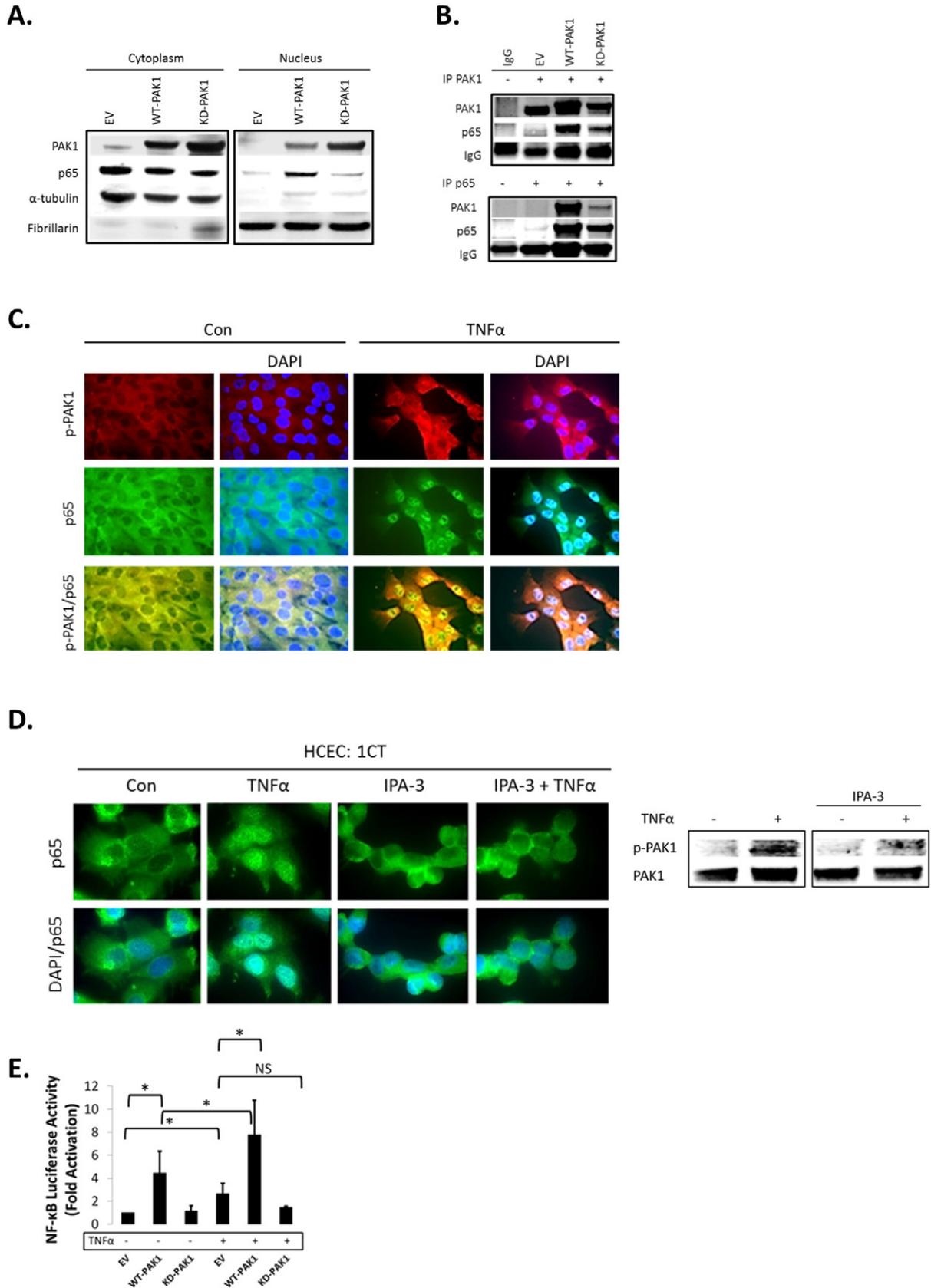
As the findings above indicate a concerted action of PAK1 and p65, we further assessed the distribution of such a PAK1–p65 interaction in the mouse intestine. PAK1 and p-PAK1 were expressed in the cytoplasm within cells of small bowel (SB) villi and of the large bowel (LB) luminal surface (Fig. 2F). P65 was also cytoplasmic and its localization resembled PAK1 and p-PAK1 in the LB (Fig. 2F). Less than 1% of IECs had nuclear p65. These data suggest that without inflammation, PAK1 and p65 are co-expressed in differentiated IEC of SB villi and LB luminal surface and canonical NF- κ B signaling is not active.

Considering that PAK1 and p65 expression was associated throughout the mouse intestine, we investigated the effect of TNF α on PAK1 expression and activation *in vivo*. Mice were *i.p.* injected with TNF α 1.5 h before sacrificing and the SB and LB were collected for immunohistochemistry. Total PAK1 expression was not altered upon TNF α treatment (data not shown). We observed a modest increase in p-PAK1 at the LB epithelial surface (Supplementary Fig. S2B). Nuclear p65, which was not present in control mice, also increased upon TNF α treatment at the luminal surface (Supplementary Fig. S2B).

Fig. 2. (A–H). PAK1 overexpression regulates NF- κ B activation downstream of TNF α in HCEC-1CT. (A) WB of HCEC-1CT cytoplasmic and nuclear lysates. Cells were transfected with EV, WT-PAK1 or KD-PAK1 and probed for PAK1 and p65. WT-PAK1 but not KD-PAK1 overexpression increased nuclear translocation of p65. Loading controls included α -tubulin (cytoplasm) and Fibrillarlin (nucleus). (B) Co-IP of PAK1 (top) and p65 (bottom) probed for PAK1 and p65 demonstrating a PAK1–p65 interaction. (C) Immunofluorescence (IF) of p-PAK1 and p65 co-staining in untreated (Con) and TNF α treated cells. Upper panel p-PAK1, middle panel p65, and lower panel merged p-PAK1/p65 images with or without merged DAPI channel. PAK1 and p65 colocalized upon TNF α . (D) IF of p65 or merged DAPI/p65 in HCEC-1CT. Cells were pre-treated with 10 μ M IPA-3 (2 h) followed by 10 ng/mL TNF α for 30 min. Nuclear accumulation of p65 by TNF α is impeded by p-PAK1 inhibition. The WB verified p-PAK1 activation by TNF α and its inhibition by IPA-3 in HCEC-1CT. (E) NF- κ B luciferase assay of EV, WT-PAK1, and KD-PAK1 overexpression in untreated and TNF α treated cells. Data are representative of 3 independent experiments, ANOVA, Tukey HSD; **p* < 0.05. (F) Immunohistochemistry of large bowel (LB) or small bowel (SB) mouse tissue sections stained for PAK1, p-PAK1, or p65. Box plots are mean PAK1 immunoreactivity scores (IRS) comparing SB crypt and villi expression, and LB crypt and surface expression in control mice (*n* = 3) mice (*t*-test, 2 tailed; ****p* < 0.001). (G) IHC of p-PAK1 and p65 within small intestinal tissue in TNF α injected mice. TNF α increases p-PAK1 expression and p65 (black arrow) nuclear translocation within villi, but not in crypts. PAK1 KO mice were stained for p-PAK1 as a negative control. Graphs show mean villi p-PAK1 and p65 IRSs (\pm SD) (*n* = 3) mice per group (*t*-test, 2 tailed; ****p* < 0.001) (H) IF of p65 or merged DAPI/p65 in wild type (WT) and PAK1 KO mouse small intestinal organoids (SIO) with or without TNF α . WB of PAK1 protein expression in WT or PAK1 KO SIO.

PAK1 activation upon TNF α treatment within the SB was even more profound (Fig. 2G). This effect was marked towards the villus tip with an observable gradient along the villus axis. In parallel with PAK1 activation, a robust accumulation of nuclear p65 was observed, which was absent in untreated mice. Ex vivo immunofluorescence in small

intestinal organoids (SIO) from WT and PAK1 KO mice confirmed that PAK1 is required for nuclear p65 translocation following TNF α treatment (Fig. 2H). Complete deletion of PAK1 in PAK1 KO SIO was verified by Western blot (Fig. 2H). Inhibition of PAK1 kinase activity using IPA-3 also impeded nuclear accumulation of p65 on TNF α in SIO (data not



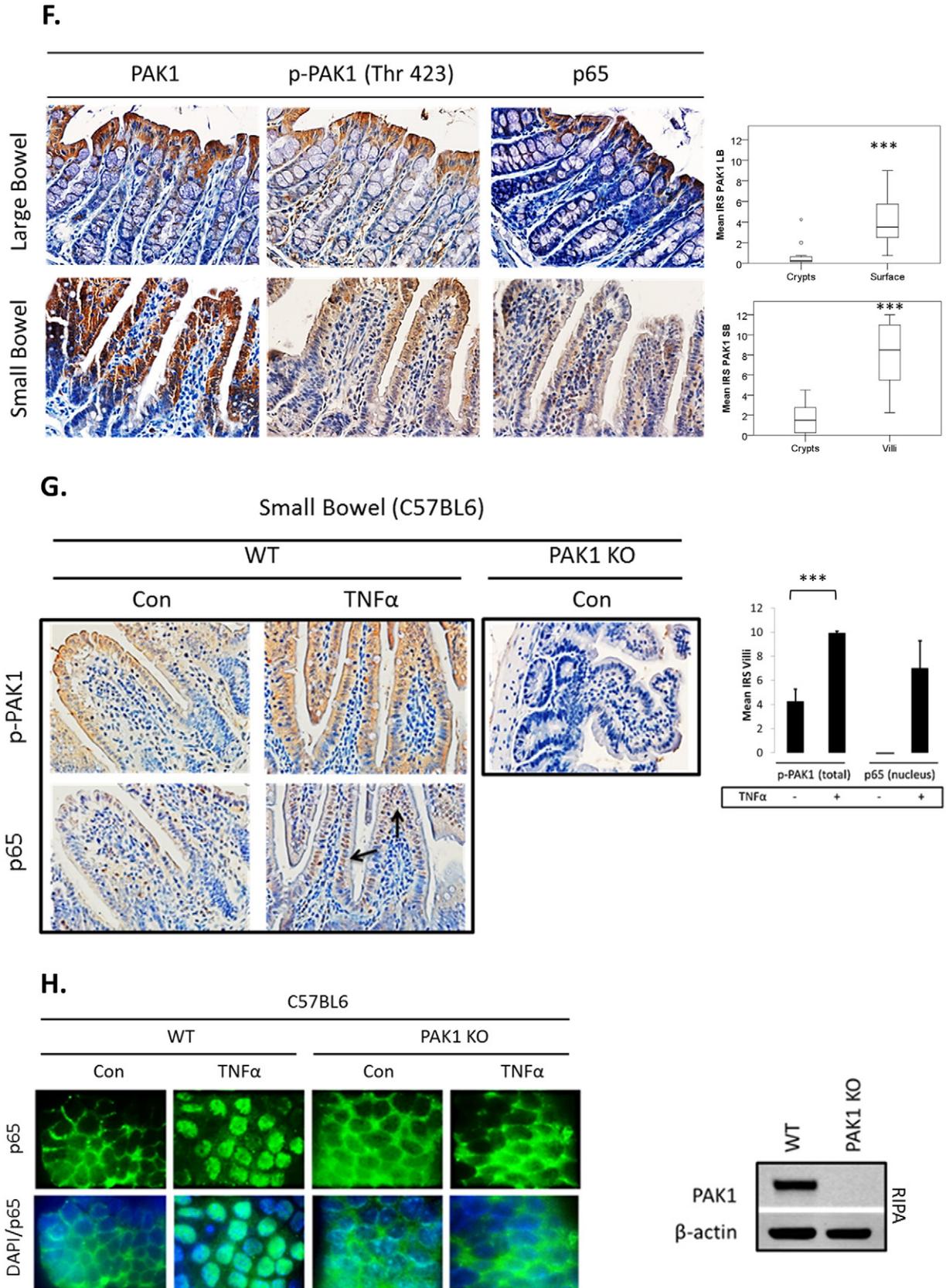


Fig. 2 (continued).

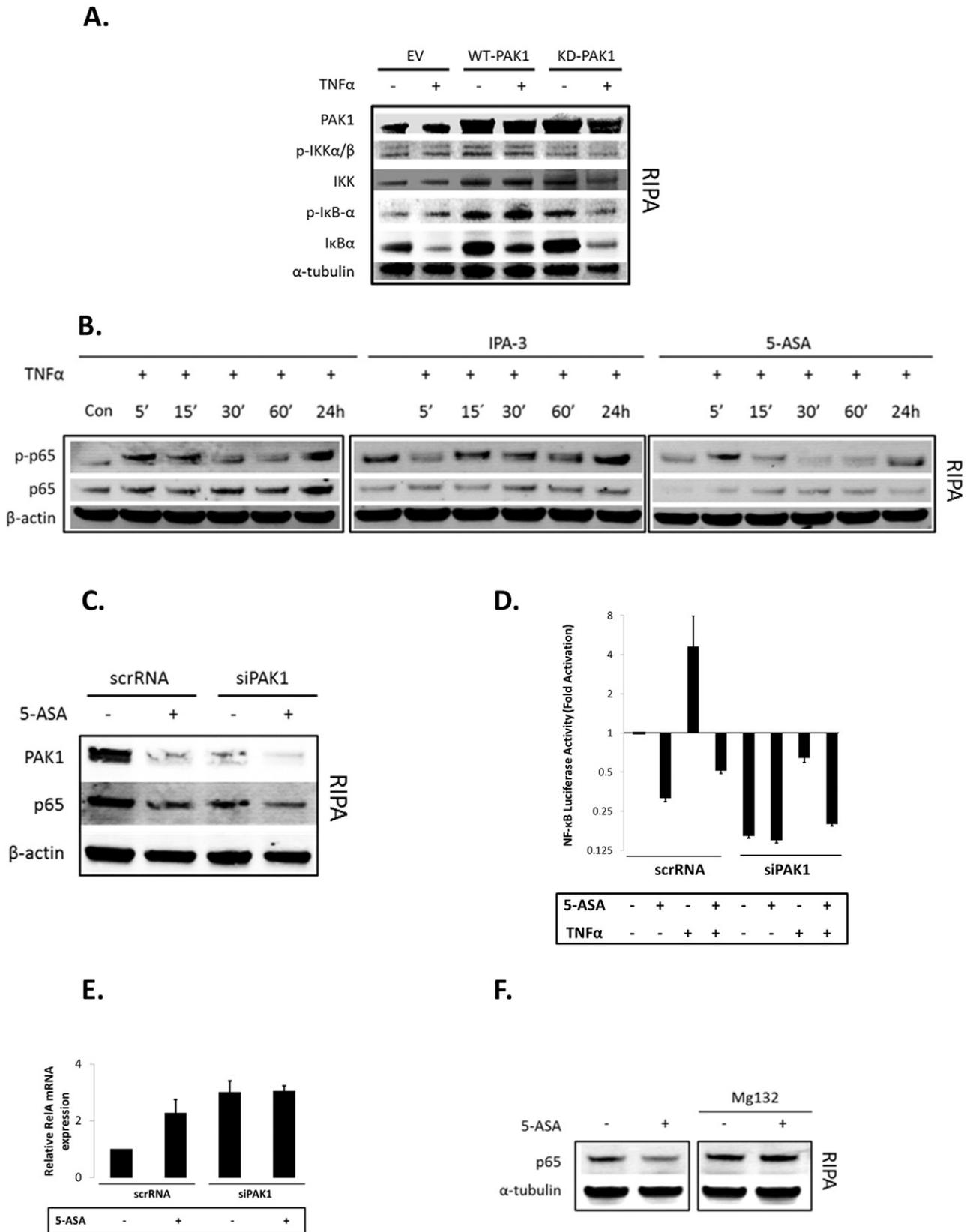
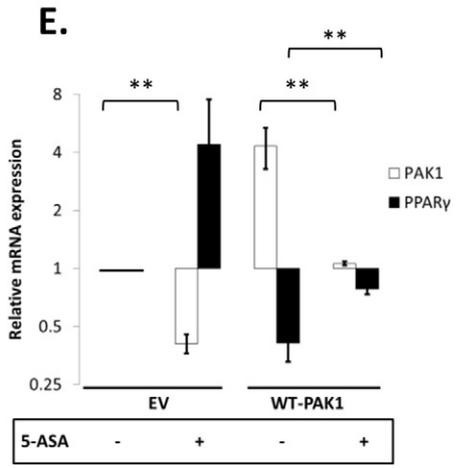
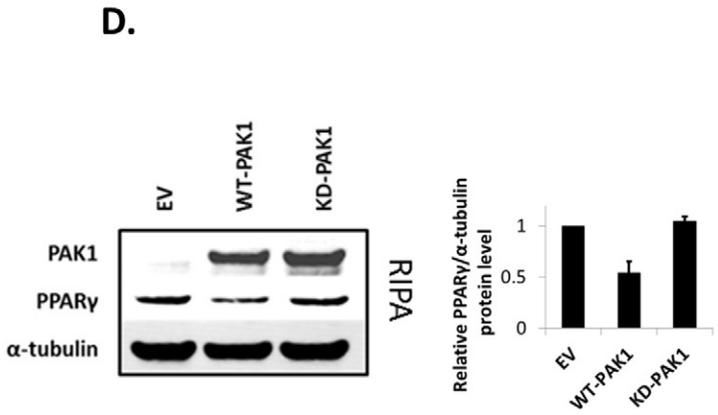
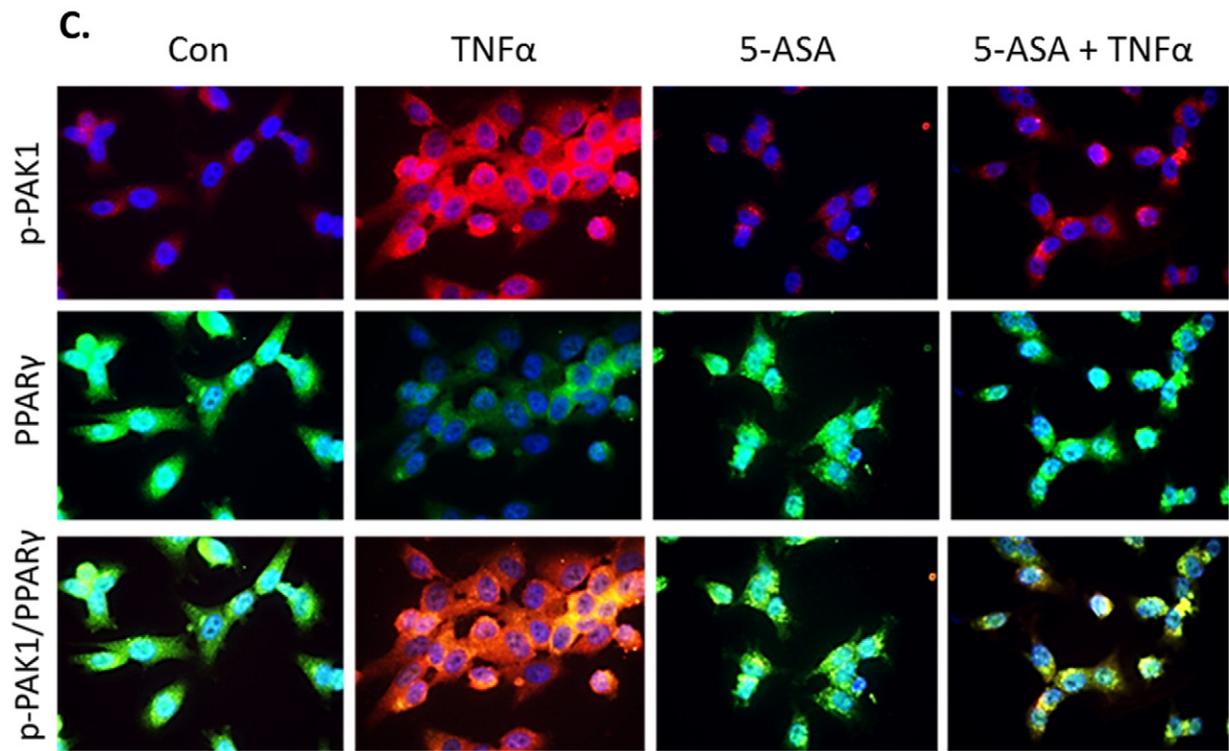
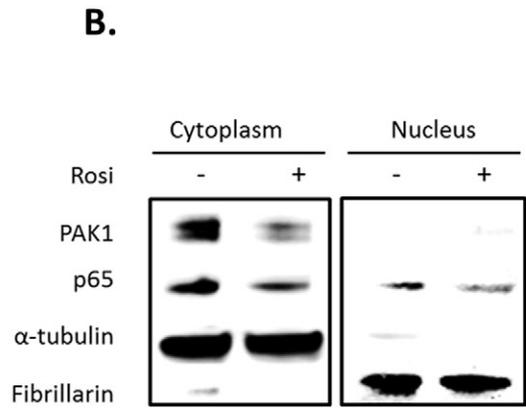
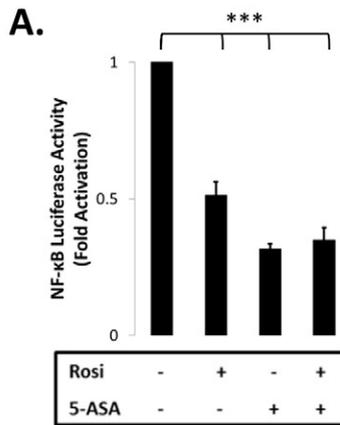


Fig. 3. (A–F). PAK1 is required for complete activation of NF- κ B downstream of I κ B in HCEC-1CT. (A) EV, WT-PAK1, or KD-PAK1 overexpressing cells were treated with 10 ng/mL TNF α (30 min). RIPA lysates were analyzed by WB for PAK1, p-IKK α/β , IKK α , p-I κ B- α , and I κ B α expression. WT or KD-PAK1 overexpression did not interfere with the degradation of I κ B following TNF α treatment. (B) WB of RIPA lysates analyzed for p-p65 and p65. Cells were treated with TNF α or pretreated (12 h) with 10 μ M IPA-3 or 20 mM 5-ASA. Pretreatment with IPA-3 or 5-ASA blocked the effect of TNF α on p65. (C) WB of whole cell (RIPA) lysates probed for PAK1, p65, and β -actin. PAK1 knock down by both 5-ASA and siPAK1 blocked total p65 protein levels. (D) Cells were co-transfected with a NF- κ B luciferase reporter and siPAK1 or scrRNA. Pretreatment with 5-ASA and PAK1 knockdown impeded the effect of TNF α on NF- κ B transcriptional activation. Data are representative of 3 independent experiments. (E) Relative mRNA expression of RelA upon siPAK1 or scrRNA with or without 5-ASA treatment. PAK1 inhibition increased transcription of RelA. (F) HCEC-1CT cells were treated with 20 mM 5-ASA (24 h) with or without the proteasomal inhibitor MG132 (6 h). RIPA lysates were analyzed for p65 and α -tubulin by WB. 5-ASA inhibits p65 but not upon pretreatment with MG132. All data are representative of 3 independent experiments.



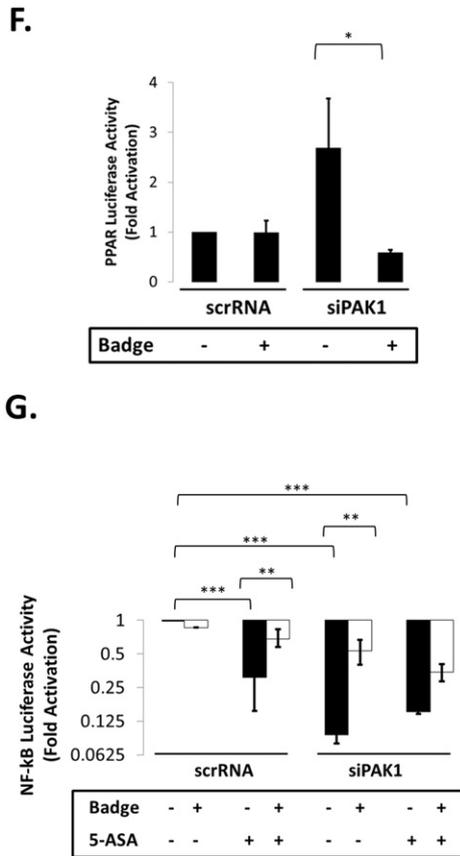


Fig. 4. (A–G). PAK1 modulates a PPAR γ /p65 cascade. (A) HCEC-1CT cells were transfected with a NF- κ B luciferase reporter and treated with 1 μ M Rosi \pm 20 mM 5-ASA (24 h). Rosi and 5-ASA blocked NF- κ B transcriptional activity ANOVA, Tukey HSD; *** p < 0.001. (B) Rosi treated cells were fractionated and analyzed by WB for PAK1 and p65. Rosi blocked PAK1 and p65 expression. (C) IF of p-PAK1 and PPAR γ upon TNF α (30 min) with or without 20 mM 5-ASA pretreatment (24 h). TNF α resulted in p-PAK1 activation and PPAR γ nuclear export and downregulation which was blocked by 5-ASA. (D) The effect of EV, WT-PAK1, and KD-PAK1 overexpression on PPAR γ was analyzed by WB. α -Tubulin was used as a loading control. Densitometry of PPAR γ levels normalized to α -tubulin, data are mean and SD from 2 independent experiments. (E) RTPCR of PAK1 and PPAR γ after EV or WT-PAK1 overexpression with or without 5-ASA treatment (24 h). PAK1 overexpression blocked PPAR γ at the mRNA level, an effect that was recovered by 5-ASA. Data are representative of 3 independent experiments (t -test, 2 tailed; ** p < 0.01). (F) PPAR luciferase reporter assay in PAK1 knock down cells using scrRNA or siPAK1. siPAK1 activated PPAR transcription, and this effect was recovered upon 0.1 μ M BADGE pretreatment (12 h), ANOVA, Tukey HSD; * p < 0.05. (G) NF- κ B luciferase assay in PAK1 knock down cells using scrRNA or siPAK1. Cells were pretreated with BADGE (12 h) with or without 5-ASA (24 h). BADGE recovered the effect of 5-ASA or siPAK1 on NF- κ B. All data are representative of 3 independent experiments ANOVA, Tukey HSD; ** p < 0.01, *** p < 0.001.

shown). These data support a role for PAK1 in NF- κ B signaling downstream of TNF α in IECs.

3.3. PAK1 is required for complete activation of NF- κ B downstream of I κ B in IECs

In order to delineate PAK1's role within canonical NF- κ B signaling, we further investigated the IKK α / β -I κ B cascade in HCEC-1CT cells. If PAK1 was upstream of IKK α / β or I κ B, PAK1 overexpression would modulate I κ B degradation. Western blot analysis revealed that neither WT-PAK1 nor KD-PAK1 mediated TNF α 's effect on I κ B degradation (Fig. 3A). PAK1 knockdown (siPAK1) reduced p65 nuclear translocation upon TNF α treatment, but did not affect I κ B phosphorylation or degradation further suggesting that PAK1 acts downstream of I κ B (Supplementary Fig. S3A).

Considering that PAK1 associates with p65 upon TNF α stimulation, we investigated its effect on p65 stability. PAK1 inhibitors IPA-3 as

well as 5-ASA reduced total p65 protein levels with and without TNF α (Fig. 3B), although 5-ASA but not IPA-3 inhibited p65 phosphorylation. Compared to TNF- α treatment, 5-ASA treatment reduced p65 levels over time upon stimulation with TNF- α and this effect was more prominent at 24 h. IPA-3 however, inhibited total p65 levels but did not reduce p65 phosphorylation. This suggested that the effect of PAK1 inhibition on p65 is a result of p65 stability, but not phosphorylation. Mechanistically, 5-ASA reduced PAK1 expression to a similar extent as siPAK1 and both resulted in reduction of p65 total protein levels (Fig. 3C) and p65 transcriptional activity (Fig. 3D). 5-ASA did not display an additive effect to siPAK1 alone suggesting that 5-ASA utilizes PAK1 to reduce p65. TNF α transactivation of NF- κ B was blocked upon 5-ASA, siPAK1, and their combination. NF- κ B transactivation by TNF α was also blocked by IPA-3 (Supplementary Fig. S3B).

We further investigated whether siPAK1 or 5-ASA reduced p65 at the transcriptional level. Surprisingly, both siPAK1 and 5-ASA increased RelA mRNA (Fig. 3E). Inhibition of the proteasome with MG132, however, blocked 5-ASA's effect on p65 suggesting that PAK1's role within the canonical NF- κ B pathway is rather posttranslational (Fig. 3F). These data demonstrate that (1) PAK1 stabilizes p65 protein downstream of I κ B, (2) PAK1 is required for complete activation of NF- κ B by TNF α , and (3) 5-ASA inhibits NF- κ B transcriptional activity via inhibition of total PAK1 in IECs.

3.4. PAK1 modulates a PPAR γ /p65 cascade within IECs

Our data indicated that 5-ASA blocks NF- κ B in IECs through inhibition of PAK1. 5-ASA is also a known agonist of PPAR gamma (PPAR γ) [22] and PPAR γ polyubiquitinates and degrades p65 [23]. PPAR γ is highly expressed in the gut, and regulates IEC differentiation and migration within SB villi [24]. Within the LB, PPAR γ activation has anti-inflammatory and chemopreventive effects [22,25].

First, we confirmed that both Rosiglitazone (Rosi; a PPAR γ agonist) and 5-ASA – to a lesser degree – increased PPAR transcriptional activity (data not shown). Both also blocked NF- κ B signaling in our system (Fig. 4A). Interestingly, Rosi did not add to 5-ASA's effect on NF- κ B inhibition, suggesting that both agonists utilize PPAR γ activation to inhibit NF- κ B (Fig. 4A). These data were confirmed at the protein level in which PPAR γ activation by Rosi reduced p65 levels in both cytoplasm and nucleus (Fig. 4B). Rosi also reduced PAK1 protein abundance.

Pro-inflammatory cytokines, such as TNF α , result in PPAR γ degradation [26], therefore we investigated the TNF α -PAK1-PPAR γ cascade via immunofluorescence. Control HCEC-1CT cells had low levels of p-PAK1, and PPAR γ was present in the nucleus and cytoplasm. TNF α treatment resulted in PAK1 phosphorylation concomitant with an apparent reduction of nuclear and cytoplasmic PPAR γ expression (Fig. 4C). 5-ASA blocked activation of PAK1 and retained PPAR γ levels in the presence of TNF α . These data suggest that TNF α results in activation of PAK1 and in reduction of PPAR γ , both of which can be blocked by 5-ASA.

To better understand the interaction between PAK1 and PPAR γ , we overexpressed PAK1 in HCEC-1CT. Total PPAR γ protein levels were reduced upon WT-PAK1 but not upon KD-PAK1 transfection (Fig. 4D). At the transcriptional level, 5-ASA inhibited PAK1 while increasing PPAR γ mRNA levels (Fig. 4E). WT-PAK1 overexpression decreased PPAR γ mRNA levels and 5-ASA recovered PPAR γ levels. These data demonstrate that PAK1 activation and overexpression downregulate PPAR γ both at the protein and mRNA levels, which can be recovered by 5-ASA.

WT-PAK1 overexpression increased the effect of TNF α on NF- κ B transactivation (as shown in Fig. 2E). If this was a result of PPAR γ inhibition, PPAR γ activation should recover this effect. NF- κ B luciferase assay showed that in control cells (EV), Rosi blocked TNF α transactivation of NF- κ B (Supplementary Fig. S4A). Rosi also blocked the effect of WT-PAK1 on NF- κ B transactivation. The synergistic effect of WT-PAK1 and TNF α on NF- κ B transactivation was also reduced upon PPAR γ activation by Rosi. These data were further verified by Western blot in which Rosi inhibited nuclear PAK1 and p65 upon TNF α in WT-PAK1 overexpressing

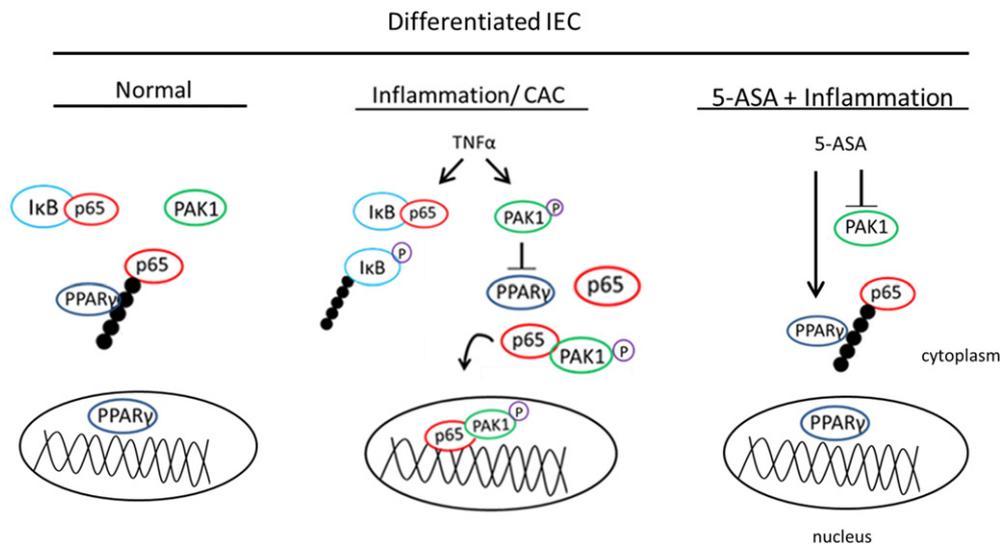


Fig. 5. PAK1 modulates a PPAR γ /p65 cascade. (Left) Within normal differentiated IEC, PAK1 activity and expression is low while PPAR γ is present in both the cytoplasm and nucleus. Cytoplasmic NF- κ B is maintained in an inactive state via the sequestration of p65 by I κ B. PPAR γ further regulates free p65 through E3 ligase activity and proteasomal degradation in the cytoplasm. (Middle) In chronic inflammation and colitis associated cancer (CAC) PAK1 is hyperactivated while PPAR γ is downregulated. Pro-inflammatory cytokines such as TNF α induce PAK1 phosphorylation, activation, and nuclear colocalization with p65. Activated PAK1 also blocks PPAR γ , further increasing the nuclear accumulation of p65 independently of I κ B. (Right) The anti-inflammatory drug 5-ASA inhibits PAK1 thereby restoring PPAR γ expression, and increasing its activity in inhibiting free p65 in the cytoplasm.

HCEC-1CT cells (Supplementary Fig. S4B). Rosi treatment of cells with siPAK1 did not further block transactivation of NF- κ B (Supplementary Fig. S4C). These data support that PAK1 activation and overexpression activates NF- κ B through inhibition of PPAR γ .

WT-PAK1 overexpression suppressed PPAR γ (Fig. 4D&E), which was recovered upon 5-ASA treatment. If inhibition of PAK1 is required for PPAR γ activation, PAK1 knock down should result in PPAR γ activation. In fact, siPAK1 increased PPAR γ mRNA and its downstream target CD36 (Supplementary Fig. S4D). Moreover, PPAR γ and CD36 expression was increased five-fold in PAK1-KO SIO (Supplementary Fig. S4E). siPAK1 also induced PPAR transactivation and the PPAR γ antagonist BADGE blocked this effect (Fig. 4F). These data demonstrate that PAK1 inhibition itself is sufficient to activate PPAR γ transcriptional activity.

If PAK1 regulates NF- κ B signaling through modulation of PPAR γ , then PPAR γ inhibition should impede the effect of PAK1 inhibition in reducing NF- κ B transactivation. As shown in Fig. 3D, 5-ASA, siPAK1, and 5-ASA in combination with siPAK1 inhibited NF- κ B transcriptional activity. Inhibition of PPAR γ with BADGE reduced the effect of 5-ASA and siPAK1 on NF- κ B transactivation (Fig. 4G). Overall these data demonstrate that (1) PAK1 activates NF- κ B through suppression of PPAR γ , and (2) activation of PPAR γ by 5-ASA (PAK1 inhibition) or PAK1 silencing downregulate NF- κ B.

4. Discussion

Chronic gut inflammation triggers the development of CAC. Here we demonstrate that in colitis PAK1 is overexpressed in IECs although PAK1 activation is limited to the luminal surface. With advancement to CAC, PAK1 is further overexpressed and phosphorylated throughout the tumor. PAK1 activation by pro-inflammatory cytokines contributes to canonical NF- κ B signaling through inhibition of PPAR γ . Mechanistically, PAK1 increases p65 stability downstream of I κ B, and is required for complete activation of NF- κ B by TNF α . 5-ASA, commonly used in the treatment of IBD, downregulated NF- κ B transcriptional activity via PAK1–p65 inhibition. Indeed, 5-ASA has been shown to activate PPAR γ [22,25] and to inhibit PAK1 [27] and its regular intake reduces the risk of CAC in UC [28]. Altogether, our data reveal a novel role of PAK1 for IEC homeostasis during gut inflammation.

Pro-inflammatory cytokine secretion from immune cells drives inflammation in IBD [21]. Inflammatory cytokines such as TNF α were

previously reported to activate PAK1 in fibroblasts, keratinocytes, macrophages, and endothelial cells [13,29–31]. Here we also demonstrate phosphorylation of PAK1 by IL-1 β and IFN γ in IEC. Although, we did not investigate the mechanism of PAK1 activation by these cytokines, upstream activators of PAK1 such as the Rho-GTPases Rac1/Cdc42 may be involved [32–34].

In vivo, in the absence of inflammation, PAK1 expression was more robust at the luminal surface epithelium and in small intestinal villi than in the crypts. This was surprising as PAK1 phosphorylates β -catenin, which is predominately located in the proliferative zone at the base of the crypts [35,36]. Interestingly, p65 expression was also localized at the luminal surface, supporting that expression of PAK1 and p65 are associated throughout the intestine. PAK1 expression was more pronounced within SB villi in comparison to the surface of the LB epithelium, which may provide a physiological advantage in modulating cytoskeletal rearrangements required for enterocyte migration along the villus axis. During inflammation, PAK1 triggers p65 nuclear translocation thereby preventing cells from physiological shedding likely through inhibition of apoptosis [12]. This would assist in the restoration of intestinal barrier integrity after intestinal infections. In CAC, PAK1 is both overexpressed and activated. We modeled PAK1 overexpression via WT-PAK1 overexpression in HCEC-1CT, which induced a PAK1–p65 interaction as well as nuclear translocation of p65, and NF- κ B transactivation in the absence of TNF α . Such activation of p65 has been shown to delay apoptosis and promote carcinogenesis [8]. Moreover, TNF α -activated NF- κ B transactivation was enriched upon overexpression of WT-PAK1 but impeded upon KD-PAK1, suggesting a role for PAK1 kinase activity in TNF α signaling. We observed subtle differences between the transcriptional assay and protein data which may be an effect of the specificity of the two readouts, and a PAK1 specific role for NF- κ B activation in the nucleus [37]. Regarding the protein data, KD-PAK1 overexpression could not fully impede nuclear p65 translocation upon TNF-stimulation, possibly due to PAK1 independent pathways. Therefore, it is likely that overexpression of KD-PAK1 reduces NF- κ B transactivation more effectively than reducing p65 protein levels. One limitation to our study is that we did not treat PAK1-KO mice with TNF α . However, we utilized SIO from PAK1-KO mice which blocked nuclear translocation of p65 downstream of TNF α . Together, our data support PAK1 as a critical mediator of NF- κ B activation downstream of TNF α within IEC and suggest that PAK1 overexpression, activation,

and nuclear localization may contribute to tumor development by sustaining NF- κ B activity.

TGF β 3 activated kinase (TAK1) is required for canonical NF- κ B activation primarily through phosphorylation of IKK α and through inducing I κ B degradation [38]. Neither PAK1 overexpression nor knockdown interfered with the upstream IKK α /I κ B cascade, suggesting that PAK1's effect on p65 is through an alternative pathway separate from TAK1 and downstream of I κ B.

Besides its role in fatty acid oxidation/transport, and increasing insulin sensitivity [39], PPAR γ was recently reported to be an E3 ubiquitin ligase, which induces p65 degradation [23]. 5-ASA acts as a PPAR γ agonist and also inhibits PAK1 and NF- κ B [22,27,40]. Here we demonstrate that 5-ASA induced degradation of p65 protein (Fig. 3C) indicative of PPAR γ 's E3 ubiquitin ligase activity. Thus, it was imperative to investigate the PAK1/PPAR γ axis. In fact, upon pro-inflammatory signals (as observed in IBD), PAK1 activation suppresses PPAR γ and contributes to NF- κ B signaling by stabilizing p65. This notion is supported by our observation that total p65 levels are reduced by both 5-ASA and PAK1 inhibition (IPA-3, siPAK1). TNF α initiates PPAR γ recruitment and degradation of p65 in mouse embryonic fibroblasts [23]. In our system, TNF α increased PAK1 activation concurrent with PPAR γ downregulation (Fig. 4C). As PAK1 overexpression inhibits PPAR γ at the protein and transcriptional levels, it is likely that PAK1 itself, or its downstream targets (JNK or ERK) could phosphorylate PPAR γ leading to its nuclear export and proteasomal degradation [41]. Moreover, PPAR γ is known to regulate its own expression [42], therefore down regulation of PPAR γ at the protein level would result in reduced transcription, which we also observed in our system (Fig. 4D&E). PAK1 activation or overexpression could inhibit PPAR γ transcription through multiple pathways. In pluripotent mesenchymal stem cells, TAK1 suppresses PPAR γ via a NF- κ B interacting kinase (NIK) cascade [26]. PAK1 activation of NIK was previously described [43] and may result in PPAR γ inhibition. It is plausible that PAK1 activation and subsequent phosphorylation of SMART [44], Snail [45], or the estrogen receptor [46] may downregulate PPAR γ [47–49]. Nevertheless, our data implicate PAK1 in modulating a PPAR γ /p65 cascade, which has not previously been reported.

We did not study the effect of PAK1 on other PPARs as 5-ASA is a specific agonist for PPAR γ [22], and only PPAR γ but not PPAR α or δ induces p65 degradation [23]. Interestingly, both 5-ASA and Rosiglitazone inhibited PAK1 (Figs. 3C and 4B). One explanation may be upon ligand activation, PPAR γ may not only mark p65 but also a PAK1–p65 complex for proteasomal degradation. 5-ASA also inhibits PAK1 at the mRNA level which is suggestive of an additional mechanism independent of PPAR γ ligand activation.

We propose that PAK1 is another player in PPAR γ /NF- κ B cascade in intestinal epithelial cells (Fig. 5). In differentiated normal IECs, PPAR γ regulates NF- κ B through proteasomal degradation of p65. However, in the presence of inflammation or CAC, PAK1 is overexpressed and activated, whereas PPAR γ is suppressed [50]. PAK1 overexpression or activation blocks PPAR γ leading to increased NF- κ B transcriptional activation. In addition we further demonstrate that inhibition of PAK1 by 5-ASA also leads to activation of PPAR γ 's E3 ligase activity.

Overall, these findings support a role for PAK1 in epithelial cell homeostasis in IBD and CAC. Pharmacological inhibition of PAK1 overexpression may recover PPAR γ levels and impair the inflammatory response such as NF- κ B activation in IEC.

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Disclosure of potential conflicts of interest

CG has a research collaboration with Shire Pharmaceuticals and received research support, lecturing or consulting honoraria from Ferring and Dr. Falk Pharma.

Author contributions

KD: conception and design, acquisition of data, analysis and interpretation of data, writing of the manuscript, VK: conception and design, acquisition of data, analysis and interpretation of data and revision of manuscript, ML: analysis and interpretation of data, TC: interpretation of data, proof-reading of the manuscript, development of methodology, FH: analysis and interpretation of data, NG: development of methodology, RE: statistical analysis, JW: acquisition of data, analysis and interpretation of data, MP: interpretation of data, revision of the manuscript, AW: obtained funding, revision of manuscript, CG: obtained funding, conception and design, study supervision, revision of the manuscript. All authors read and approved the final manuscript.

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