Investigating the role of a deubiquitinase (USP43) in NF-κB signalling within A375 melanoma cells

Student: Katie Hayward
Supervisors: Ellie Griffiths, Dr. Simon Cook; The Babraham Institute (BI), UK

Background

The NF-κB signalling pathway is crucial for the precise regulation of inflammation and immune responses within organisms and its output is the controlled expression of specific target genes. In disease states such as cancer and auto-inflammatory conditions, this tight control of gene expression can become deregulated.

Figure 1 depicts the canonical pathway for activation of NF-κB signalling. In basal conditions, the transcription factor NF-κB is sequestered within the cytoplasm by IκB. Upon pathway activation by cytokines (such as TNFα), there are several rapid phosphorylation events, including activating phosphorylation of the subunits of the IκB kinases (IKKs). This IKK complex subsequently phosphorylates IκB and targets it for ubiquitination by E3-ligases, resulting in proteasomal degradation of IκB. With this decrease in IκB abundance, the NF-κB heterodimer is no longer sequestered and can instead enter the nucleus to drive target gene transcription.

An unpublished RNAseq screen, completed at the BI, has identified USP43 as a novel NF-κB target gene. This gene encodes a deubiquitinase (DUB), which has previously been found to be mutated in a number of clinical cancer screens, including ovarian and colorectal[1,2]. Additionally, USP43 has been identified as a regulator of HIF1 signalling[3]. This supports a hypothesis of USP43 involvement in NF-kB signalling, as hypoxia is a known activating condition for this pathway. This project aims to explore whether USP43 is expressed as a consequence of NF-κB activation in A375 cells and to investigate any downstream effects.

Aims

1. Validate USP43 CRISPR KO A375 cell line.
2. Investigate the relationship between USP43 expression and the NF-κB signalling.
3. Investigate HA-USP43 cellular localisation.

Methods

Western blot analysis:
Cells were treated with 10 ng/mL TNF (0-24 hrs) and lysed with TG lysis buffer. Total protein per sample was quantified by Bradford assay (Bio-Rad). Lysates were separated by SDS-PAGE using 8% acrylamide gels, transferred by wet transfer to PVDF membranes and blocked in 5% (w/v) powdered milk/TBST. Membranes were incubated overnight with primary antibodies. Membranes were then incubated with fluorescent secondary antibodies and proteins were detected using a LiCOR Odyssey CLx.

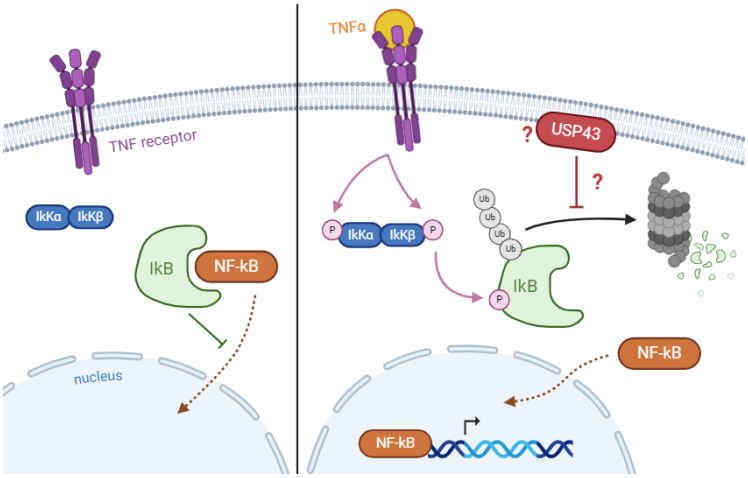

Figure 1. Schematic of canonical NF-kB signalling pathway, under basal conditions (left) and the activated pathway (right). The deubiquitylation targets and cellular localisation of USP43 are not yet well understood.

Confocal Immunofluorescence:
In a 3-well imaging slide (Ibidi), A375 parental cells were seeded. 2 wells were transfected with HA-USP43 dox-inducible plasmid and treated with either 100 ng/mL dox or +DMSO. Cells were fixed with 4% PFA and permeabilised with 0.2% Triton-X 100. After blocking with 5% (v/v) goat serum/PBS, cells were incubated with a HA-tag primary antibody. Following incubation with AlexaFluor-488 secondary antibody and DAPI, the cells were stained with phalloidin and imaged using a Leica Stellaris 8.

NF-κB Luciferase Assay:
Protocol as described by Prescott et. al, 2022.

Results

The initial aim of this project was to investigate the expression of USP43 within A375 cells, which was achieved by Western blotting. Fig. 2A demonstrated that in the parental cell line, the expression of USP43 increased over a time-course of treatment with TNF. This increase in UPS43 was not observed in the KO cell line; USP43 expression was consistently low at all TNF treatment times. The quantification of USP43 bands in this blot (Fig. 2B) supports this conclusion that CRISPR-mediated KO of the gene was successful for the KO cell line. This finding was additionally validated by genomic sequencing that identified base insertions in both alleles of the USP43 gene (Ellie Griffiths, personal communication). This dual conformation of a successful USP43 KO cell line allowed for greater confidence in conclusions determined from subsequent data analysis.

To investigate the relationship between USP43 expression and NF-κB pathway activation, various antibodies against downstream NF- κB components were used to measure changes

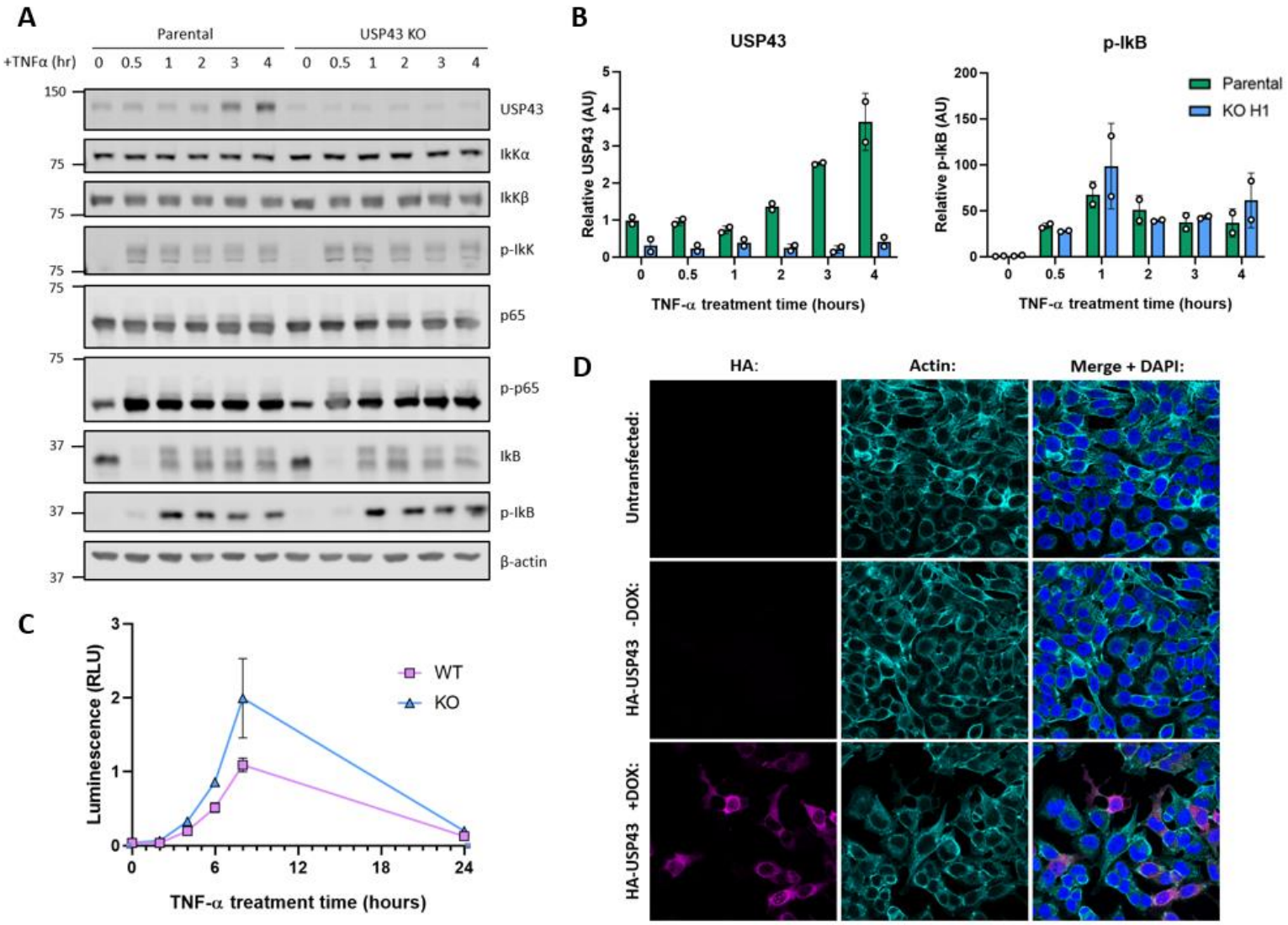

Figure 2. (**A**) Western blot for parental and USP43 KO cell lines treated with TNF between 0-4 hours. Blotted against USP43 and NF-κB components. (**B**) Quantification of (A), n=2. USP43 and p-IκB both normalised against actin. (**C**) Luciferase assay with non-specific CRISPR WT, and the USP43 KO cell line. Results normalised to renilla luciferase (**D**) Confocal IF images measuring cellular localisation of HA-USP43. Actin used as a plasma membrane marker.

in expression levels and phosphorylation following a TNF time-course (Fig. 2A). Notably, the expression of IκB drastically decreased following 30mins +TNF, and the levels of p-IκB increased after 1h. This observation aligns with canonical NF-kB activation, as stimulation by TNF is known to cause IκB degradation and this is also indicated by the increase in phosphorylation levels for both p65 and IKK. The quantification of p-IκB (Fig. 2B) highlights a potential USP43-dependent effect, as within the KO cell line there was a slight increase in phosphorylation within certain conditions. However, additional replicates for statistical analysis are required to be confident of this data. Fig. 2C shows the results of an NF-κB luciferase assay, which is a quantitative measure of NF-κB transcriptional activation. Both WT and KO cell lines were treated with a TNF time course, and an increase in relative luminescence signal between 0-8 hours was observed for both samples. Within the KO cells, this increase in signal was greater than WT cells between 6-8 hours of +TNF and suggests that NF-κB activation is possibly negatively regulated by USP43. However, the 8-hour condition in particular has a large error bar, and therefore until this assay is replicated with statistical analysis, this data must be considered preliminary.

In order to investigate the localisation of USP43 within A375 cells, plasmids expressing a Doxycycline-inducible HA-tagged USP43 construct were transfected, and expression was induced by treatment with Dox and imaged by confocal microscopy (Fig. 2D). This experimental design was selected as there is not yet a commercially available and selective antibody against endogenous USP43 that works for microscopy. Actin was used as a marker of plasma membranes and assisted in the identification that USP43 is typically perinuclear or cytoplasmic within A375 cells. This contradicts previous findings from the Cook group, which indicated that USP43 was associated with the plasma membrane and interacts with E-cadherin in a colorectal cancer cell line (HCT116). However, these results are supported by findings by Pauzaite et al, as this study found that USP43 localised within the cytosol, when overexpressed in HeLa cells. Within Fig. 2D, it was also noted that USP43 formed small nuclear foci in one of the cells. It is not yet clear whether this is a common phenotype of USP43 within these cells; however, this is an interesting potential area of future research for this project.

Future Directions

From the results of this project so far, it can be concluded that USP43 is a TNF-inducible gene within A375 melanoma cells; this adds to similar observations in HCT116 cells. The cellular location of USP43 is not yet clearly understood, as there are contrasting findings within different cell lines. Since USP43 interacts with E-cadherin (a plasma membrane spanning protein) it is possible that the abundance of E-cadherin (which changes under different disease conditions) may influence USP43 location. Additional investigation within a variety of cell lines will be required before a confident validation of the cellular location of USP43 can be made. Specifically, this could be followed up by western blotting of subcellular fractionations. If combined with a TNF treatment time course, any changes in localisation during pathway activation could also be measured. In order to make accurate statistical interpretations of the data collected for this project so far, all of the experiments shown will need to be replicated. Future areas of investigation could also be to identify direct interactions between USP43 and specific deubquitination targets by either pull down or immunoprecipitation assays. Once the function of USP43 is more clearly understood, this protein may be a potential druggable target for cancers and autoinflammatory diseases.

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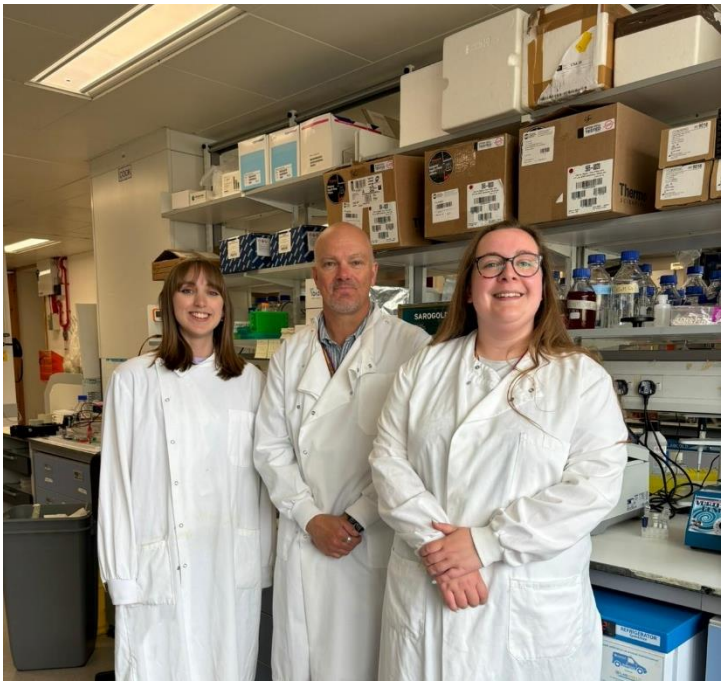
During this experience, I have had the opportunity to learn a wide range of wet lab techniques, as well as deepen my understanding of experimental design and research integrity principles. Additionally, I have received training to use several software tools for data analysis, including GraphPad, ImageJ and ImageStudio 6.0. These are all valuable skills which I hope to carry with me through the rest of my scientific journey.



From left to right: Katie Hayward, Simon Cook, Ellie Griffiths

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