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## Thr-435 Phosphorylation Regulates RelA (p65) NF- $\kappa$ B subunit transactivation

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Page heading title: Regulation of RelA by Thr-435 phosphorylation

## Abstract

Phosphorylation of the RelA (p65) NF- $\kappa$ B subunit has been previously shown to modulate its ability to induce or repress transcription. Here we have investigated the consequences of Thr-435 phosphorylation within the C-terminal transactivation domain of RelA. We confirm that Thr-435 is phosphorylated in cells and is induced by TNF $\alpha$  treatment. Mutational analysis of this site revealed gene specific effects on transcription, with a T435D phosphomimetic mutant significantly enhancing *Cxcl2* mRNA levels in reconstituted *Rela*<sup>-/-</sup> mouse embryonic fibroblasts. Chromatin immunoprecipitation analysis revealed that this mutation results in enhanced levels of histone acetylation, associated with decreased recruitment of the histone deacetylase HDAC1. Moreover, mutation of this site disrupted RelA interaction with HDAC1 *in vitro*. Thr-435 phosphorylation of promoter bound RelA was also detected at NF- $\kappa$ B target genes following TNF $\alpha$  treatment in wild type MEFs. Phosphorylation at this site therefore provides an additional mechanism through which the specificity of NF- $\kappa$ B transcriptional activity can be modulated in cells.

**Keywords:** RelA (p65), *Cxcl2* (MIP2), HDAC1, NF- $\kappa$ B, Histone Acetylation, TNF $\alpha$ .

**Abbreviations:** ANOVA, Analysis of Variance; ChIP, Chromatin Immunoprecipitation; DBD, DNA Binding Domain; DMEM, Dulbecco's Modified Eagle's Medium; ELISA, Enzyme-Linked ImmunoSorbent Assay; EMSA, Electrophoretic Mobility Shift Assay; HAT, Histone Acetyltransferase; HDAC, Histone Deacetylase; I $\kappa$ B, Inhibitor of NF- $\kappa$ B; IKK, I $\kappa$ B Kinase; LPS, Lipopolysaccharide; MEF, Mouse Embryonic Fibroblast; NF- $\kappa$ B, Nuclear Factor  $\kappa$ B; PARP, Poly (ADP-Ribose) Polymerase; PEI, Polyethylenimine; PP, Protein Phosphatase; RHD, Rel Homology Domain; SEM, Standard Error of the Mean; TAD, Transactivation Domain; TAF, TBP Associated Factor; TFIID, Transcription Factor IID; TNF, Tumour Necrosis Factor.

## Introduction

Members of the NF- $\kappa$ B transcription factor family are known to regulate a variety of cellular processes including inflammatory and immune responses, cell survival, cell differentiation and cell proliferation [1]. Furthermore, dysregulation of NF- $\kappa$ B signalling has been implicated in the development and progression of a multitude of diseases, particularly conditions involving chronic inflammation or compromised immunity and cancer [2, 3]. Mammalian NF- $\kappa$ B is a multigene family composed of five members, capable of forming a variety of homo- and heterodimeric complexes through their highly conserved N-terminal Rel homology domain (RHD) [4]. In unstimulated cells NF- $\kappa$ B dimers are primarily found as inactive, cytoplasmic complexes. Classical activation of NF- $\kappa$ B occurs in a well defined, I $\kappa$ B Kinase (IKK) dependent manner, typically culminating in the release of RelA/p50 heterodimers from inhibitory I $\kappa$ B proteins, enabling dimer translocation to the nucleus and transcriptional modulation of NF- $\kappa$ B target genes [5].

The RelA (p65) subunit contains a potent transactivation domain (TAD), which allows recruitment of co-transcriptional regulators and components of the basal transcriptional machinery to gene targets [6]. Numerous post-translational modifications to RelA have been reported and varying effects on transcriptional activity, protein interactions, stability and degradation have been demonstrated [7]. Phosphorylation of sites within RelA's TAD lead to both increased and decreased levels of transcriptional activity, with the precise effect dependent on the context and gene target [8-20]. The effects these modifications exert over protein-protein interactions has not been extensively studied. However, site-specific phospho-dependent increased binding to the co-transcriptional regulators TAFII31 (TATA-box-binding-protein-associated factor 31) and HDAC1 (Histone deacetylase 1) have been shown [9, 11]. Additionally, components of the ubiquitin ligase complex COMMD1 and cullin 2 were found to bind to RelA *via* GCN5, in a phospho site-specific manner, thereby directing RelA ubiquitination and degradation at certain promoters following TNF $\alpha$  stimulation [13, 16].

The mechanisms behind RelA's ability to specifically regulate endogenous target gene expression are continually being uncovered. Activation of certain endogenous target genes was recently shown to occur by two distinct modes; one involving the direct interaction of RelA with the Trap-80 mediator complex subunit and subsequent recruitment RNA Pol II; and the other *via* RelA's ability to regulate promoter occupancy of secondary transcription factors [21]. The murine chemokine *Cxcl2* (MIP2) was found to be regulated in a Trap-80 independent manner and RelA was shown to regulate the recruitment of the secondary transcription factor SP1 to this promoter [21]. Here we present evidence for RelA's ability to induce changes in the acetylation state of histones at the *Cxcl2* promoter. Furthermore, we demonstrate that this effect is influenced by phosphorylation at Thr-435 within RelA's TAD, which modulates RelA's interaction with HDAC1.

## Experimental

### Cell Culture

Human U-2 OS and HEK 293 cells were obtained from the American Type Culture Collection (ATCC). Immortalised mouse embryonic fibroblast (MEF) cells and immortalised *Rela*<sup>-/-</sup> MEFs were provided by Professor Ron Hay (University of Dundee). RelA null MEFs were stably reconstituted with vector alone (Control), wild type RelA (RelA), RelA with Thr-435 mutated to an alanine (T435A) or an aspartic acid (T435D). Stably reconstituted *Rela*<sup>-/-</sup> MEFs were selected with 2 µg/mL puromycin and subsequently maintained in 0.5 µg/mL puromycin.

### Preparation of viral stocks

Viral stocks for lentiviral infections were generated by transfecting 5 µg of viral envelope (pCMV-VSV-G), packaging (pCMVΔR8.91) and vector (PIRESpuro-deNotI and derivatives) plasmids into HEK 293 cells. Media containing the viral stocks was removed after 48 hours. The media was centrifuged at 1200 rpm for 3 minutes and filtered through a 0.45 µm filter to remove cells. Viral stocks were then aliquoted, snap frozen and stored at -80 °C.

### Generation of Stable Cells

Infections were performed by mixing 4 µg/mL polybrene (Sigma) with 30 µL virus stock in a total volume of 3 mL media. This was used to infect a 50 % confluent 10 cm plate of *Rela*<sup>-/-</sup> MEFs by incubating overnight at 37 °C. On the second day the media containing the virus was removed and the plate was washed twice with PBS before the addition of fresh media. On the third day the cells were split 1:5. On the fourth day cells were selected by replacing media with puromycin containing media (2 µg/mL) and when all cells in the mock infection plate had died the puromycin concentration was lowered to 0.5 µg/mL. This concentration was used to maintain stably expressing cell lines. In all experiments pools of reconstituted MEF cells were used.

### Quantitative PCR (qPCR)

QPCR reactions were performed on Rotor-Gene 3000 and 6000 machines (Corbett Research). Ct values were calculated using Rotor-Gene 6 software and all values were calculated relative to GAPDH or Input levels using the Pfaffl method [22].

### Primers

#### RT-PCR

<i>Bcl2l1</i> (Bcl-xl)	FWD: 5'-GATGCAGGTATTGGTGAGTCG-3', REV: 5'-GCTCTCGGCTGCTGCATT-3';
<i>Cxcl1</i> (KC)	FWD: 5'-CTGGGATTACCTCAAGAAC-3', REV: 5'-GAAGCCAGCGTTCACCAGAC-3';
<i>Cxcl2</i> (MIP2)	FWD: 5'-TCAAGGGCGGTCAAAAAGTT-3', REV: 5'-TCCTCCTTTCCAGGTCAGTTA-3';
<i>Cxcl3</i>	FWD: 5'-CCTACCAAGGGTTGATTT-3',

<i>Cxcl4</i> (Pf4)	REV: 5'-CGCTCTTCAGTATCTTCTT-3'; FWD: 5'-GAAAGCGATGGAGATCTTA-3'; REV: 5'-TCTTATATAGGGGTGCTTGC-3';
<i>Cxcl5</i> (GCP-2)	FWD: 5'-CATTTCTGTTGCTGTTCA-3'; REV: 5'-GGGATCACCTCCAAATTA-3';
<i>Cxcl7</i> (Ppbp)	FWD: 5'-CTTCATAACCTCCAGATCTT-3'; REV: 5'-ACACATTACAAAGGGAGATA-3';
<i>Cxcl10</i> (IP-10)	FWD: 5'-CCAAGTGCTGCCGTCATTTTC-3'; REV: 5'-GGCTCGCAGGGATGATTTCAA-3';
<i>Cxcl11</i>	FWD: 5'-AGGAAGGTCACAGCCATAG-3'; REV: 5'-CTCGATCTCTGCCATTTT-3';
<i>Cxcl12</i>	FWD: 5'-CATCTGAAAATCCTCAACACT-3'; REV: 5'-AAGCTTTCTCCAGGTACTCT-3';
<i>Gapdh</i> (GAPDH)	FWD: 5'-GCTACACTGAGGACCAGGTTG-3'; REV: 5'-GCCCCTCCTGTTATTATGGGG-3';
<i>Tnfaip3</i> (A20)	FWD: 5'-GAACAGCGATCAGGCCAGG-3'; REV: 5'-GGACAGTTGGGTGTCTCACATT-3'.
<u>ChIP</u>	
<i>Cxcl1</i>	FWD: 5'-CTAATCCTTGGGAGTGGAG-3'; REV: 5'-CCCTTTTATGCTCGAAAC-3';
<i>Cxcl2</i>	FWD: 5'-CGTGCATAAAAGGAGCTCTC-3'; REV: 5'-GTGCCCCGAGGAAGCTTGT-3';
<i>Tnfaip3</i>	FWD: 5'-CGCTGAGAGAGAGACAAAC-3'; REV: 5'-TGGCCCTGAAGATTAAC-3'.

### Antibodies

Antibodies used were:  $\alpha$ -RelA (sc-372, Santa Cruz);  $\alpha$ -Gal4 (DBD) (sc-577, Santa Cruz);  $\alpha$ - $\beta$ -actin (A5441, Sigma);  $\alpha$ -PARP (9542, Cell Signaling);  $\alpha$ -Pol II (sc-56767, Santa Cruz);  $\alpha$ -SP1 (sc-59, Santa Cruz);  $\alpha$ -Acetylated Histone H3 (06-599, Upstate);  $\alpha$ -Acetylated Histone H4 (ab-1761, Abcam);  $\alpha$ -HDAC1 (06-720, Upstate);  $\alpha$ -PK-TAG/V5-TAG (MCA1360GA, AbD Serotec);  $\alpha$ -HA-Tag (2367, Cell Signaling). The RelA Thr-435 phospho-specific antibody was raised in rabbit by BioGenex GmbH. The peptide used had the sequence TQAGEGT\*LSEALC (phospho-Thr-435 is indicated by \*). The antibody was purified using two-step peptide affinity chromatography with the phospho- and non-phospho-peptides. ELISA analysis confirmed that the purified antibody was specific for the phosphorylated epitope.

### Plasmids

The Gal4 E1B and 3x  $\kappa$ B ConA luciferase reporter plasmids, along with the Gal4 RelA-TAD, RSV-RelA and HA-HDAC1 expression plasmids have been reported previously [18, 23]. The viral envelope (pCMV-VSV-G), packaging (pCMV $\Delta$ R8.91) and vector (PIRESpuro-deNotI) plasmids were obtained from Professor Ron Hay (University of Dundee) and Professor Mary Collins (University College London). The *Cxcl2* (MIP2) luciferase reporter plasmid was obtained from Professor Michael Hottiger (University of Zurich) [24]. PK-

Tagged RelA was generated by inserting a N-Terminal PK-TAG, in a pcDNA3.1 vector. All point mutations were generated by PCR overlap extension, except for the Gal4 fusion mutants, which were generated in a single PCR step, and were sequenced prior to use.

#### *Other Assays*

Chromatin-immunoprecipitation, co-immunoprecipitation, reporter gene assays, western blot and EMSA analysis were performed essentially as described previously [18, 25, 26].

## **Results**

### **RelA Thr-435 is phosphorylated following calyculin A and TNF $\alpha$ treatment.**

Protein Phosphatase 4 (PP4) mediated Thr-435 dephosphorylation was previously proposed to enhance RelA-mediated activation following cisplatin treatment, implying that phosphorylation at this site has a negative effect on RelA's activity [27]. In order to determine the phosphorylation status of Thr-435 in cells, a phospho-specific antibody against this site was raised and ELISA analysis confirmed that the purified antibody was specific for this phosphorylated epitope (Data not shown). Treating U-2 OS osteosarcoma cells with the serine/threonine phosphatase inhibitor calyculin A, resulted in increasing levels of phosphorylation at this site, masked by background bands in direct western but clearly visible after immunoprecipitation with a RelA antibody (Fig. 1A). It was subsequently found that there was weak induction of phosphorylation at this site in U-2 OS cells following stimulation with the well-characterised NF- $\kappa$ B activator, TNF $\alpha$  (Figure 1B). However, rapid, Thr-435 phosphorylation was detected in mouse embryonic fibroblast (MEF) cells following TNF $\alpha$  stimulation (Fig. 1C).

### **Role of Thr-435 on RelA transcriptional activity.**

To investigate potential effects on RelA's transcriptional activity, mediated through the Thr-435 residue, both phospho-null (T435A) and phospho-mimicking (T435D) mutations of this site were created. These mutants were initially generated as TAD fusion proteins with the DNA binding domain (DBD) of Gal4, thereby allowing only TAD-dependent effects to be investigated. Significantly, when transfected into U-2 OS cells, the T435A

stably expressing wild type and mutant forms of RelA at equivalent levels were generated (Fig. 2A).

It is known that deletion of *Rela* results in embryonic lethality from extensive foetal hepatocyte apoptosis, due to increased sensitivity to TNF $\alpha$  [28]. Loss of RelA also sensitises MEFs to TNF $\alpha$ -induced cell death and this can be rescued by stably expressing RelA [29]. Both the wild type and Thr-435 mutant RelA reconstituted cells were rescued from TNF $\alpha$ -induced apoptotic cell death, as revealed by the absence of Poly (ADP-ribose) polymerase (PARP) cleavage, an indicator of caspase mediated cell death (Fig. 2B), indicating that phosphorylation at this site is unlikely to affect this important function of RelA [30].

Stimulation with TNF $\alpha$  induces the activation of the canonical NF- $\kappa$ B pathway, resulting in the rapid translocation of RelA complexes to the nucleus of the cell and enhanced DNA binding to  $\kappa$ B sites in responsive genes, such as *IL8* [1, 31]. DNA binding in response to canonical pathway activation in the reconstituted cells were investigated by EMSA analyses. Although a low level of basal NF- $\kappa$ B DNA binding to an *IL8*  $\kappa$ B oligo could be seen in unstimulated RelA reconstituted cells, this was significantly induced following TNF $\alpha$  stimulation (Fig. 2C). Furthermore, mutating Thr-435 did not affect TNF $\alpha$ -induced DNA binding. Therefore, these reconstituted cell lines contain functional, TNF $\alpha$  inducible RelA, capable of regulating the expression of the anti-apoptotic genes necessary for rescue from TNF $\alpha$  induced cell death.

### Regulation of NF- $\kappa$ B gene targets

To analyze the potential effects, mediated through Thr-435 on known NF- $\kappa$ B gene targets, qPCR analysis was performed on cDNA generated from these reconstituted cell lines. Initially, known NF- $\kappa$ B target genes; *Bcl2/1* (Bcl-xl); *Cxcl1* (KC); *Cxcl2* (MIP2) & *Tnfaip3* (A20) [32-35] were investigated in unstimulated cells, to allow analysis of Thr-435 mutation on basal level expression. In agreement with previous studies, expression of the chemokines *Cxcl1* and *Cxcl2* was severely diminished in the absence of RelA and was found to be enhanced following reconstitution with all forms of RelA (Fig. 3A&B) [21, 33, 35]. However, some differences between the Thr-435 mutant forms of RelA were observed. *Cxcl2* expression was dramatically increased in the phospho-mimicking T435D cell line, indicating that phosphorylation at this site may play a role in the induction of this gene (Fig. 3A). In agreement with this, a *Cxcl2* promoter luciferase reporter plasmid was also found to have higher expression levels with the T435D mutant (Fig. 3E). Similar enhancement of mRNA expression by the T435D mutation was not observed for other members of the CXC chemokine superfamily whose expression was detectable in these cell lines (Supp. Fig. 1). Expression levels of the CXC chemokine *Cxcl1* was significantly reduced in the unphosphorylatable T435A cell line, suggesting that phosphorylation at this site is important for basal expression of this gene (Fig 3B). Levels of the ubiquitin editing enzyme *Tnfaip3* also became elevated following the introduction of RelA, but no significant differences were detected between the

wild type and Thr-435 mutant cell lines (Fig. 3C). Finally, expression of the anti-apoptotic gene *Bcl2l1* was reduced following reconstitution with RelA (Fig. 3D).

Taken together, these results indicate that the Thr-435 residue regulates the transcriptional activity of full length RelA in a gene specific manner. To begin a more mechanistic evaluation of these effects, the role played by the T435D mutant in the upregulation of *Cxcl2* expression levels was investigated using chromatin immunoprecipitation (ChIP) analysis.

### **Protein occupancy at the *Cxcl2* promoter in reconstituted cell lines.**

Increased expression of the murine *Cxcl2* gene is known to occur in a RelA dependent manner, following exposure to a variety of NF- $\kappa$ B inducing stimuli, including TNF $\alpha$ , LPS and hypoxia [21, 33, 36, 37]. The *Cxcl2* promoter contains not only an NF- $\kappa$ B binding motif, but also SP1 and AP1 binding motifs, all of which are required for optimal promoter activation [36, 38]. ChIP analysis revealed that, in unstimulated cells, consistent with enhanced levels of gene expression, elevated levels of RelA, RNA Pol II and SP1 were found at the *Cxcl2* promoter in the T435D cell line (Fig. 4A-C). In contrast, enhanced binding of the T435D mutant form of RelA was not observed at either the *Cxcl1* or *Tnfaip3* promoters (Sup. Fig. 2A&B). This indicated that *Cxcl2* promoter specific recruitment is regulated by the T435D mutation and that this is not an intrinsic effect on DNA-binding, consistent with the earlier EMSA analysis (Fig. 2C).

We next examined effects on histone modification at the *Cxcl2* promoter and enhanced acetylation of Histones H3 (Lysine 9 & 14) and H4 (Lysine 12), both markers for active regions of transcription, were observed following the introduction of wild type RelA or the T435D mutant (Fig. 4D&E). In the T435D cells, acetylated Histone H3 levels were higher than those found in the phospho-null T435A cells, but not significantly higher than in the wild type RelA cells (Fig. 4D). However, acetylated Histone H4 levels were significantly higher in the T435D cells, compared to both wild type RelA and T435A mutant cells, consistent with increased expression of this gene in the T435D cell line (Fig. 4E). To determine if the reduced acetylation detected in the T435A cell line results from enhanced binding of a deacetylase enzyme, levels of HDAC1 binding to the *Cxcl2* promoter were determined and enhanced HDAC1 binding was detected at this promoter (Fig. 4F). These results illustrate that mutating Thr-435, in RelA's TAD, influences the ability of RelA and other transcription factors, such as SP1 and Pol II, to bind specifically to the *Cxcl2* promoter. Additionally, differences in the acetylation status of histone tails at this promoter were also detected and can be accounted for, at least in part, by elevated levels of HDAC1 binding to the promoter in the T435A cell line.

### **HDAC1 interaction with RelA.**

To investigate potential differences in HDAC1 interaction with RelA, co-immunoprecipitation analyses were performed using PK-Tagged wild type and

Thr-435 mutant forms of RelA, along with HA-Tagged HDAC1. Significantly, the phosphomimicking T435D mutation decreased HDAC1 interaction with RelA, corroborating data obtained through ChIP analysis of the *Cxcl2* promoter (Fig. 4G). As  $\text{TNF}\alpha$  stimulation is known to result in decreased HDAC1 interaction with RelA [39], this data is consistent with Thr-435 phosphorylation contributing to this process.

### Regulation of NF- $\kappa$ B gene targets following $\text{TNF}\alpha$ stimulation

Taken together, this data suggested that the effect of the T435D mutation was to mimic the effect of  $\text{TNF}\alpha$  stimulation on the RelA dependent regulation of *Cxcl2* expression. Therefore, we next investigated the effects of  $\text{TNF}\alpha$  stimulation on the RelA reconstituted MEFs. Significantly, a RelA-dependent,  $\text{TNF}\alpha$  induced upregulation of both *Cxcl1* and *Cxcl2* was observed (Fig. 5A & B). In the case of *Cxcl1* this induction resulted in similar levels of mRNA in the reconstituted cells, bypassing the reduced basal expression seen in the T435A cell line. However, in the case of *Cxcl2* there were differences in the induction of this gene between the Thr-435 mutants. Cells containing wild type RelA showed a robust induction in *Cxcl2* mRNA levels, reaching levels similar to that found basally in the T435D cell line, suggesting that this mutation did indeed mimic the effects of  $\text{TNF}\alpha$  stimulation for this NF- $\kappa$ B gene target. This increase was much reduced in cells containing the T435A mutant. The already enhanced levels of expression seen with the T435D mutant were somewhat further stimulated by  $\text{TNF}\alpha$  treatment, as might be expected from the enhanced translocation of RelA to the nucleus under these conditions. These results are consistent with RelA Thr-435 phosphorylation playing an important role in the  $\text{TNF}$  dependent induction of *Cxcl2* expression.

Effects on other NF- $\kappa$ B target genes were also examined. RelA also enhanced *Tnfaip3* expression following  $\text{TNF}\alpha$  treatment, but significant differences were not detected between wild type and Thr-435 mutants (Fig. 5C). However, little effect of  $\text{TNF}\alpha$  was seen on *Bcl2l1* mRNA levels (Fig. 5D). This result is consistent with ChIP analysis, where we could not observe RelA binding to the *Bcl2l1* promoter in the reconstituted MEF cell lines (Data not shown), suggesting that in this context this gene is not a RelA target and the effects we see on its expression are indirect. We also examined the expression levels of the other members of the CXC chemokine superfamily expressed in these cells, before and after  $\text{TNF}\alpha$  stimulation (Supp. Fig. 1). Interestingly, although some effects with Thr-435 mutation are seen, these are associated with reductions in the levels of gene expression (see *Cxcl3* and *Cxcl7* for example). However, in this analysis, no other genes were found with the enhanced levels of expression, seen with *Cxcl2*, associated with Thr-435 phosphorylation. This confirmed that in this context, the effect of Thr-435 phosphorylation on RelA dependent gene expression is highly specific.

### Protein occupancy at the *Cxcl2* promoter in wild type MEFs.

We next investigated endogenous RelA binding to the *Cxcl2* promoter

in wild type MEFs. As expected, ChIP analysis revealed enhanced RelA binding following  $\text{TNF}\alpha$  stimulation (Fig. 6A). Importantly, and consistent with its predicted role as a regulator of *Cxcl2* expression, we also detected enhanced phospho-Thr-435 RelA bound to the *Cxcl2* promoter following  $\text{TNF}\alpha$  stimulation (Fig. 6B). Levels of RelA binding to the *Cxcl2* promoter, both before and after  $\text{TNF}\alpha$  stimulation, were comparable between the reconstituted MEFs and wild type MEFs (Supp. Fig. 3). Furthermore, consistent with the earlier data from the reconstituted MEFs, specific Thr-435 dependent effects at the *Cxcl2* promoter were also observed in the wild type MEFs after  $\text{TNF}\alpha$  stimulation, with acetylated Histone H4 levels being significantly enhanced while levels of HDAC1 present were reduced. However, some differences were observed between these experimental systems with acetylated Histone H3 levels not being seen to increase in the wild type MEFs (Fig. 6C-E).

Since this ChIP analysis indicated that Thr-435 modified RelA is bound to the *Cxcl2* promoter, we investigated what proportion of total promoter-associated RelA this represented. To determine this, the ChIP samples were immuno depleted with the phospho-Thr-435 antibody prior to RelA immunoprecipitation and PCR analysis. Significantly, preclearing Thr-435 modified RelA negated the  $\text{TNF}\alpha$  induced increase in RelA binding to the *Cxcl2* promoter but had little effect on RelA binding to the *Cxcl1* promoter, indicating that a very specific pool of activated RelA binds to and regulates the expression of *Cxcl2* (Fig. 6F).

Taken together, these results support the data obtained from the reconstituted MEF cell lines and demonstrate that phosphorylation of Thr-435 plays an important role in the regulation of *Cxcl2* mRNA expression in wild type MEF cells following  $\text{TNF}\alpha$  stimulation.

## Discussion

Although Thr-435 had been identified as a putative phosphorylation site in RelA's transactivation domain, subject to dephosphorylation by PP4, this had relied upon *in vitro* phosphorylation assays and no analysis of modification in cells was performed [27]. Therefore, we generated a phospho specific antibody against Thr-435 which allowed the detection of endogenous RelA phosphorylation at this site following calyculin A treatment of U-2 OS cells (Fig. 1A). Phosphorylation was also detected following TNF $\alpha$  treatment in MEF cells and at the *Cxcl1*, *Cxcl2* and *Tnfaip3* promoters following ChIP analysis (Fig. 1C, 6B and Supp. Fig. 2). Although this analysis establishes Thr-435 phosphorylation as a regulator of RelA transactivation, the identity of the kinase responsible is currently unknown. Phospho-site prediction algorithms identify Thr-435 as a potential CK2 or polo like kinase 1 (PLK1) target. Interestingly, CK2 was previously shown to phosphorylate RelA at Ser-529 in response to TNF $\alpha$  treatment [20]. However, phosphorylation of Thr-435 by CK2 could not be verified empirically during the course of this study (data not shown). Identification of the Thr-435 kinase is an important question that should be investigated in future studies. Furthermore, Thr-435 phosphorylation in response to other NF- $\kappa$ B inducers is also currently unknown and will require further analysis. That this site is only weakly phosphorylated after TNF $\alpha$  stimulation in U-2 OS cells implies a possible cell type specific role *in vivo*.

In contrast with the dramatic transcriptional effects seen with Gal4 fusion proteins (Fig. 1D), full-length RelA versions of Thr-435 phospho-mutants did not reproduce significant effects on transcription, when used with a selection of standard NF- $\kappa$ B reporter plasmids (Fig. 1E and data not shown). Consistent with this, analysis of endogenous gene expression revealed that, at least in this context, the effects of Thr-435 phosphorylation are highly promoter specific, suggesting that this modification can help provide the specificity and selectivity required to produce a context dependent NF- $\kappa$ B transcriptional response to a stimulus. Interestingly, the effects seen with Thr-435 mutation on the endogenous *Cxcl2* gene were also mirrored with a *Cxcl2* promoter luciferase reporter (Fig. 3E). By contrast, other members of the CXC superfamily of genes did not display *Cxcl2* like sensitivity to RelA Thr-435 phosphorylation (Supp. Fig. 1) and gene expression array analysis will be required to identify other target genes similarly regulated by modification at this site.

These observations suggest that it is the specific organisation of the *Cxcl2* promoter that confers Thr-435 regulation. Importantly, immuno depletion, prior to ChIP analysis revealed that the majority of RelA bound to the *Cxcl2* promoter was Thr-435 phosphorylated, in contrast to the *Cxcl1* promoter where most RelA was not modified at this site (Fig. 6F). Moreover, the T435D mutant shows elevated levels of *Cxcl2* promoter binding, not seen with *Cxcl1* or *Tnfaip3* (Fig. 3A and Supp Fig 2A & B), suggesting that this modification facilitates RelA recruitment to the *Cxcl2* promoter. Given the location of this motif in the transactivation domain, we consider it unlikely that

this effect is due to an alteration in site specific RelA DNA binding. Rather, it is more likely to arise through effects on transcriptional coregulators required to appropriately modify *Cxcl2* promoter chromatin structure. Such effects may be mediated by the corepressor HDAC1, whose binding to RelA is inhibited by the T435D mutation (Fig. 4G). However, we cannot rule out effects on other interactions and it is entirely possible that Thr-435 phosphorylation not only decreases HDAC1 binding but also enhances binding to an, as yet unidentified, histone acetyltransferase (HAT). We also investigated binding of the CBP and TIP60 HATs to the Thr-435 mutants, as a potential explanation for the variations in histone acetylation observed, but no differences in their occupation of the *Cxcl2* promoter were detected in the RelA T435D reconstituted cells and no differences in binding to Thr-435 mutant forms of RelA were observed (data not shown). However other TNF $\alpha$  induced interactions, with additional co-transcriptional proteins such as SIRT6, Pin1, which is required for optimal *Cxcl2* expression, and RPS3 [40-42] were not investigated during the course of this study and may also emerge as being regulated by Thr-435 phosphorylation.

Our investigation of the consequences of RelA Thr-435 phosphorylation shows similarities and differences to previous analyses of point mutations to known RelA post-translational modification sites. Phosphorylation at S276 is known to play a role in activating RelA and regulating its interaction with either the co-activators p300/CBP or HDAC co-repressors [43]. Reconstituting *Rela*<sup>-/-</sup> MEFs, with a non-phosphorylatable S276A mutant form of human RelA, similarly resulted in distinctive expression profiles of NF- $\kappa$ B target genes, potentially due to decreased p300 recruitment [44-47]. Furthermore, the S276A mutation was found to severely impair RelA's ability to rescue *Rela*<sup>-/-</sup> MEFs from TNF $\alpha$ -mediated cell death [48], an effect not found with Thr-435 mutation. Phosphorylation at S536 is known to affect RelA's activity and reconstituting *Rela*<sup>-/-</sup> MEFs with a non-phosphorylatable S536A mutant form of human RelA was shown to decrease LPS induced NF- $\kappa$ B activation and also to reduce p300 recruitment, subsequently decreasing TNF $\alpha$  activation [45, 49]. Reconstituting *Rela*<sup>-/-</sup> MEFs with a phospho mimicking S536D mutant produced enhanced binding to TAFII31 and enhanced transcriptional activity, reciprocating effects of IL1 induced NF- $\kappa$ B activation [9]. Finally, phosphorylation at S468 has been reported to have both inhibitory and activatory effects on transactivation and reconstituting *Rela*<sup>-/-</sup> MEFs with a non-phosphorylatable S468A mutant form of human RelA has been shown to have a negative effect on both IL1- and TNF $\alpha$ -induced NF- $\kappa$ B transactivation [19]. TNF $\alpha$ -induced Ser-468 phosphorylation was also found to regulate ubiquitin mediated, proteasome-dependent removal of chromatin-bound RelA on certain NF- $\kappa$ B gene targets [13]. However, this effect was not observed for *Cxcl2*, suggesting other modifications, such as the Thr-435 phosphorylation, identified in this study, will have a primary regulatory role.

Taking these results together with the conclusions of previous studies, it is becoming clearer that phosphorylation of RelA helps to programme its

transcriptional functions by determining its activity at select target genes through modulating interactions with co-activators and co-repressors. Here we have identified a role for Thr-435 phosphorylation, which, following TNF $\alpha$  stimulation, modulates the interactions with HDAC1 and selectively regulates NF- $\kappa$ B dependent gene expression. This adds to the number of such modulatory phosphorylation events controlling NF- $\kappa$ B activity. Given the possible side effects associated with total NF- $\kappa$ B inhibition as a clinical therapy, drugs that selectively regulate RelA activity through affecting its post-translational modifications, have the potential to act as effective therapies for the wide range of diseases associated with aberrant NF- $\kappa$ B activity.

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## Figure Legends

### Figure 1: RelA Thr-435 is a phospho site that affects Transactivation Domain activity.

(A) U-2 OS cells were stimulated for the indicated times with calyculin A (50 nM). 40  $\mu$ g of whole cell extract was analysed by western blot using the P-Thr-435 or RelA antibodies (upper panel). 350  $\mu$ g whole cell extract was immunoprecipitated with RelA antibody and samples were western blotted with P-Thr-435 antibody (lower panel). Membranes were then stripped and reprobed with total RelA antibody.

(B) U-2 OS cells were stimulated with calyculin A (50 nM) for 15 minutes or  $\text{TNF}\alpha$  (20 ng/mL) as indicated. 40  $\mu$ g whole cell extract was western blotted with P-Thr-435 antibody. Membrane was then stripped and reprobed with total RelA antibody. Densitometry analysis indicates increase in intensity of the phospho band in relation to untreated sample, normalized to RelA input levels.

(C) MEF cells were stimulated with  $\text{TNF}\alpha$  (20 ng/mL) as indicated. 100  $\mu$ g whole cell extract was immunoprecipitated with P-Thr-435 antibody and samples were western blotted with total RelA antibody.

(D) U-2 OS cells were transfected with 1.5  $\mu$ g Gal4 E1B luciferase reporter plasmid and 750 pg of expression plasmids encoding the Gal4 DBD, the Gal4 DBD fused to amino acids 428–551 of RelA [RelA TAD], amino acids 428–551 of RelA with Thr-435 mutated to alanine [T435A] and amino acids 428–551 of RelA with Thr-435 mutated to aspartic acid [T435D]. A western blot demonstrating expression levels of Gal4 fusion proteins is shown. Luciferase assay results are expressed as fold activation over Gal4 DBD control, relative to wild type RelA TAD. Presented here are mean  $\pm$  SEM,  $n=8$ , ANOVA was performed followed by a Tukey-Kramer multiple comparisons test using Prism 4 software (GraphPad). \*  $P<0.05$  and \*\*\*  $P<0.001$  compared to wild type TAD.

(E) U-2 OS cells were transfected with 1  $\mu$ g 3x  $\kappa$ B ConA luciferase reporter plasmid and 0.6  $\mu$ g of expression plasmids encoding empty RSV vector, full length wild type RelA [RelA], RelA with Thr-435 mutated to alanine [T435A] and RelA with Thr-435 mutated to aspartic acid [T435D]. A western blot demonstrating expression levels of RelA proteins is shown. Luciferase assay results are expressed as fold activation over empty RSV plasmid.

Data presented here are the mean  $\pm$  SEM,  $n=3$ . ANOVA was performed followed by a Tukey-Kramer multiple comparisons test using Prism 4 software (GraphPad).

### Figure 2: Creation of stable cell lines constitutively expressing wild type and mutant forms of RelA.

(A) Western blot analysis of RelA protein levels in stably reconstituted *Rela*<sup>-/-</sup> mouse embryonic fibroblasts (MEF).

(B) Introduction of RelA protects cells from  $\text{TNF}\alpha$ -induced cell death. Whole cell extracts were prepared from reconstituted MEF cells stably expressing wild type and mutant forms of RelA, either unstimulated or stimulated with

TNF $\alpha$  (40 ng/mL) for 4 hours. Western blot analysis was performed for PARP cleavage.

(C) TNF $\alpha$ -induced DNA binding was not affected by mutating Thr-435. Nuclear protein extracts were prepared from reconstituted MEF cells stimulated with TNF $\alpha$  (40 ng/mL) for the indicated times and 5  $\mu$ g nuclear extract was subjected to EMSA analysis using  $^{32}$ P labeled IL8 and OCT1 oligonucleotides.

### Figure 3: Regulation of endogenous NF- $\kappa$ B target genes.

(A) Introduction of RelA into *Rela*<sup>-/-</sup> MEFs increases expression levels of *Cxcl2* (MIP2). Inserting a T435D mutation further enhances this effect.

(B) Introduction of RelA into *Rela*<sup>-/-</sup> MEFs increases expression levels of *Cxcl1* (KC).

(C) Introduction of RelA increases expression of *Tnfaip3* (A20).

(D) Introduction of RelA reduces expression of *Bcl2l1* (Bcl-xl).

RNA was extracted from reconstituted MEF cells, total cDNA was prepared and quantitative PCR analysis was performed using primers to mouse *Cxcl2*, *Cxcl1*, *Tnfaip3*, *Bcl2l1* and *Gapdh* (GAPDH) control. All results are normalized to *Gapdh* levels and expressed as fold induction relative to the level of gene expression in control cell line.

(E) U-2 OS cells were transfected with 0.5  $\mu$ g *Cxcl2* luciferase reporter plasmid and 0.5  $\mu$ g of expression plasmids encoding empty RSV vector, full length wild type RelA [RelA], RelA with Thr-435 mutated to alanine [T435A] and RelA with Thr-435 mutated to aspartic acid [T435D]. Results are expressed as fold activation over empty RSV plasmid.

Data presented here are the mean  $\pm$  SEM, (A-D) n=4, (E) n=3. ANOVA was performed followed by a Tukey-Kramer multiple comparisons test using Prism 4 software (GraphPad). \* P<0.05, \*\* P<0.01 and \*\*\* P<0.001. Asterisk in grey indicates P-value relative to Control cell line.

### Figure 4: ChIP analysis of the murine *Cxcl2* promoter in reconstituted cell lines.

(A-C) Elevated levels of RelA (A), RNA Polymerase II (Pol II) (B) and SP1 (C) were detected at the *Cxcl2* initiation site in the T435D cell line by qPCR.

(D & E) Introducing wild type RelA and T435D increases acetylation of both Histone H3 (K9/14) and Histone H4 (K12). Creating T435A mutation does not significantly increase the acetylation at this promoter.

(F) Enhanced binding of HDAC1 to the *Cxcl2* promoter in the T435A cell line. qPCR analysis was performed using primers to the mouse *Cxcl2* initiation site. All results were normalized to Input levels and control antibodies. Presented here are the mean enrichment levels relative to the Control cell line  $\pm$  SEM, (A) n=5, (B) n=3, (C) n=6, (D) n=3, (E) n=4, (F) n=7. ANOVA was performed followed by a Tukey-Kramer multiple comparisons test using Prism 4 software (GraphPad). \* P<0.05, \*\* P<0.01 and \*\*\* P<0.001. Asterisk in grey indicates P-value relative to Control cell line.

(G) HDAC1 interaction with RelA is reduced by mimicking phosphorylation at

T435. Co-immunoprecipitation analysis of the HDAC1 interaction with wild type and Thr-435 mutant forms of RelA was performed using the antibodies indicated and whole cell extracts (300  $\mu$ g) from HEK-293 cells transfected with expression plasmids encoding HA-HDAC1 and PK, PK-RelA, PK-T435A or PK-T435D. 20  $\mu$ g of whole cell extracts was included as Input

**Figure 5: Expression levels of NF- $\kappa$ B target genes following TNF $\alpha$  treatment.**

**(A-C)** TNF $\alpha$  stimulation differentially increases mRNA levels of *Cxcl2*, *Cxcl1* and *Tnfaip3* in the reconstituted cells.

**(D)** TNF $\alpha$  stimulation does not significantly alter the expression of *Bcl2l1* in the reconstituted cells. RNA was extracted from reconstituted MEFs cells, either unstimulated or stimulated with TNF $\alpha$  (40 ng/mL) for 30 minutes. Total cDNA was prepared and qPCR analysis was performed using primers to mouse **(A)** *Cxcl2*, **(B)** *Cxcl1*, **(C)** *Tnfaip3*, **(D)** *Bcl2l1* and *Gapdh* control.

All results were normalized to *Gapdh* levels and expressed as fold induction relative to the level of gene expression in control cell line. Data presented here are the mean  $\pm$  SEM, n=3. Two-way ANOVA was performed followed by a Bonferroni post-test to compare replicate untreated and TNF $\alpha$  means, using Prism 4 software (GraphPad). \* P<0.05 and \*\* P<0.01.

**Figure 6: ChIP analysis of the murine *Cxcl2* promoter in wild type MEFs.**

**(A&B)** TNF $\alpha$  stimulation increases binding of RelA and RelA phosphorylated at Thr-435 to the *Cxcl2* promoter region.

**(C)** TNF $\alpha$  stimulation does not significantly increase levels of acetylated Histone H3 (K9/14) at the *Cxcl2* promoter.

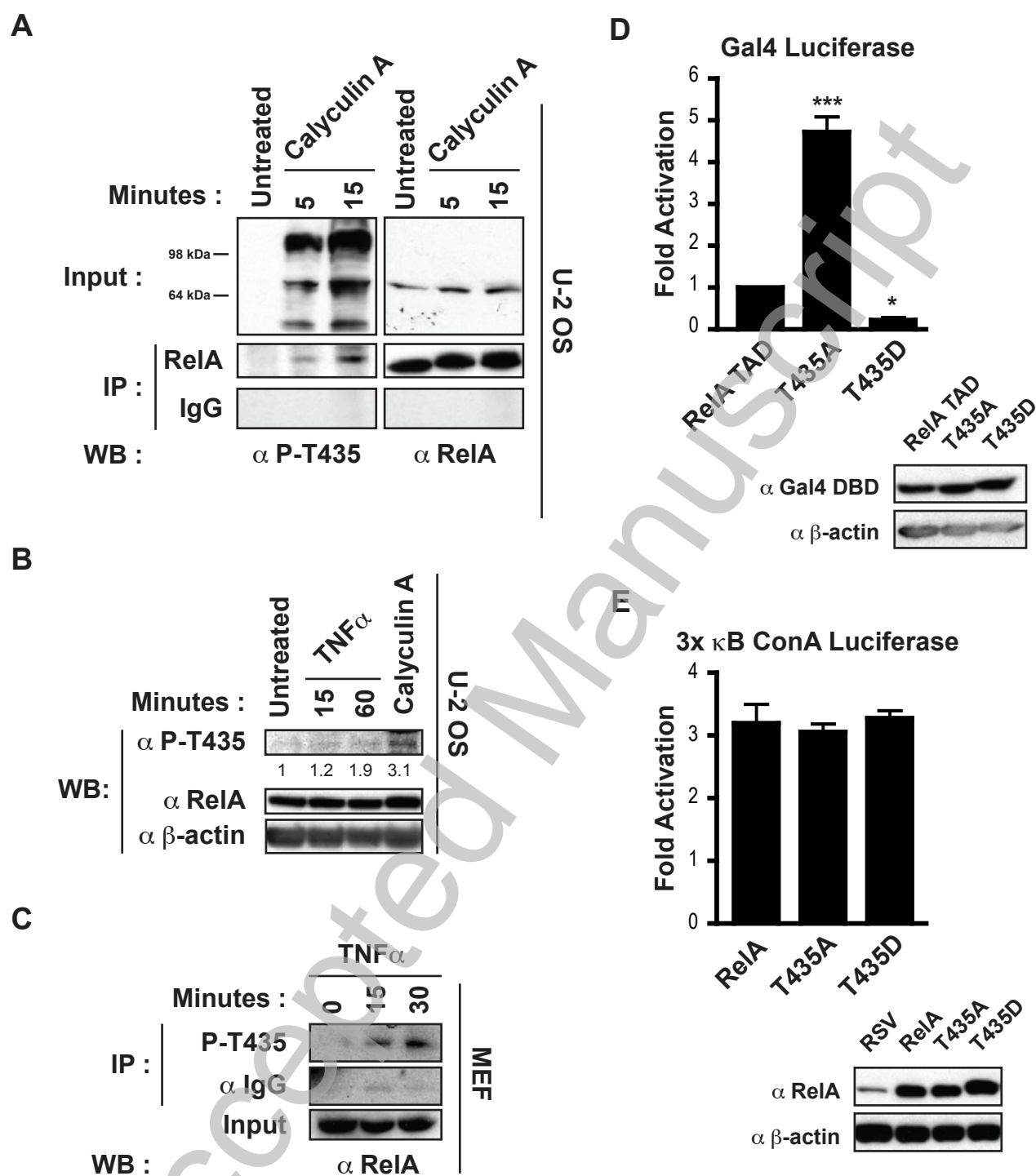
**(D)** Elevated levels of acetylated Histone H4 (K12) were found at the *Cxcl2* promoter following TNF $\alpha$  treatment.

**(E)** HDAC1 binding to the *Cxcl2* promoter was reduced after TNF $\alpha$  treatment. qPCR analysis was performed using primers to the mouse *Cxcl2* initiation site.

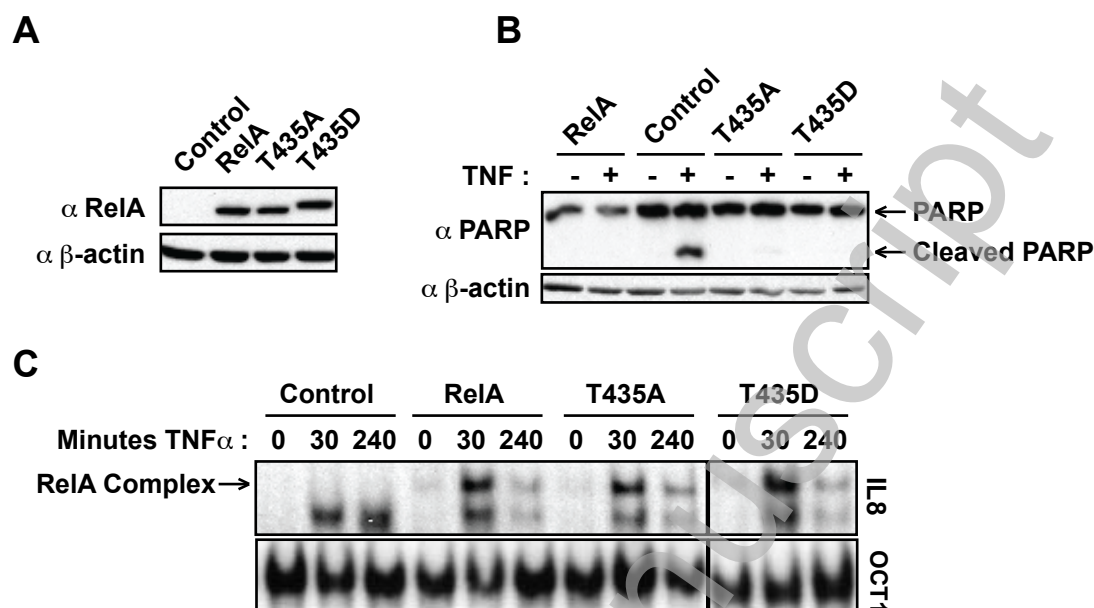
**(F)** Depleting samples of RelA phosphorylated at Thr-435 with phospho-specific antibody prior to performing ChIP analysis negates RelA binding to the *Cxcl2* promoter, but not the *Cxcl1* promoter following TNF $\alpha$  treatment.

All results were normalized to Input levels and control antibodies. Data presented here are the mean enrichment levels relative to Untreated cells  $\pm$  SEM, **(A-C)** n=7, **(D&E)** n=4, **(F)** n=3. Students t test was performed using Prism 4 software (GraphPad). \* P<0.05, \*\* P<0.01 and \*\*\*P<0.001.

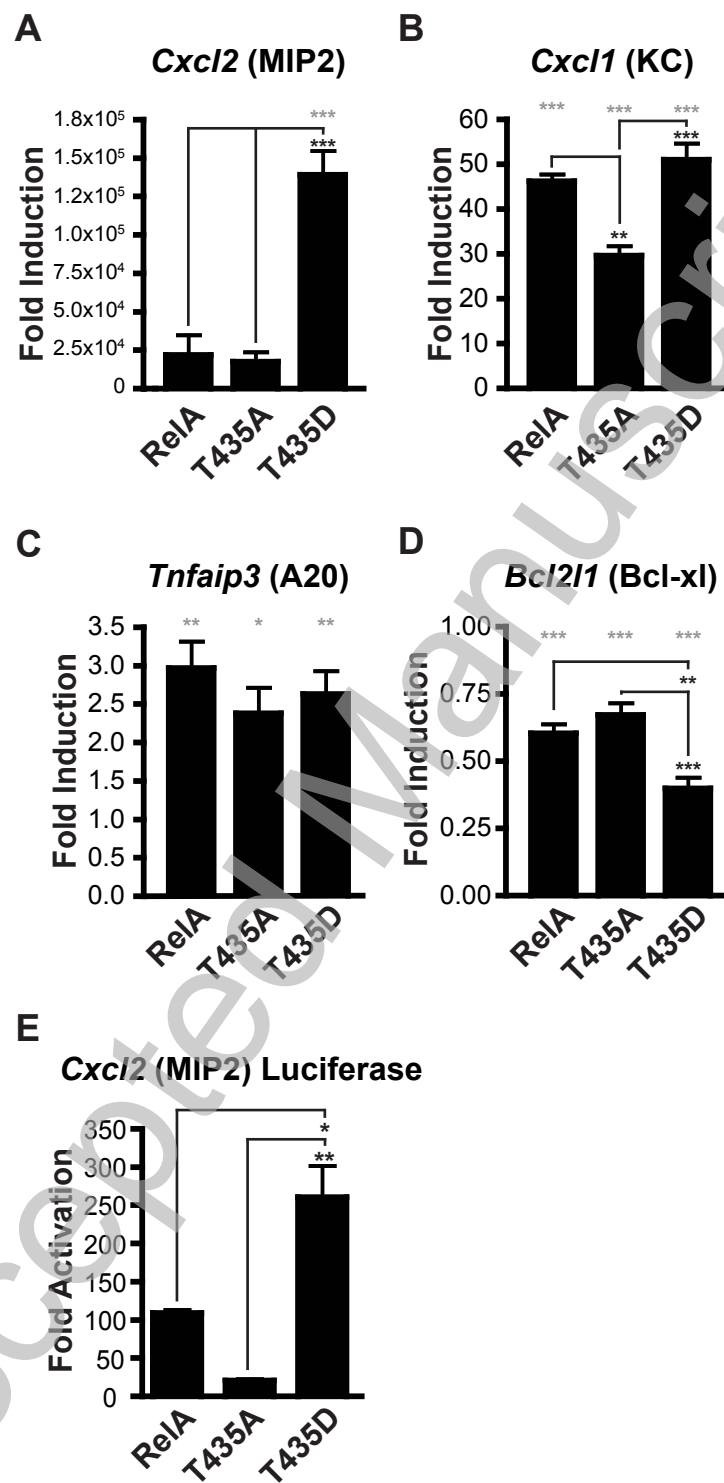
O'Shea & Perkins Figure 1



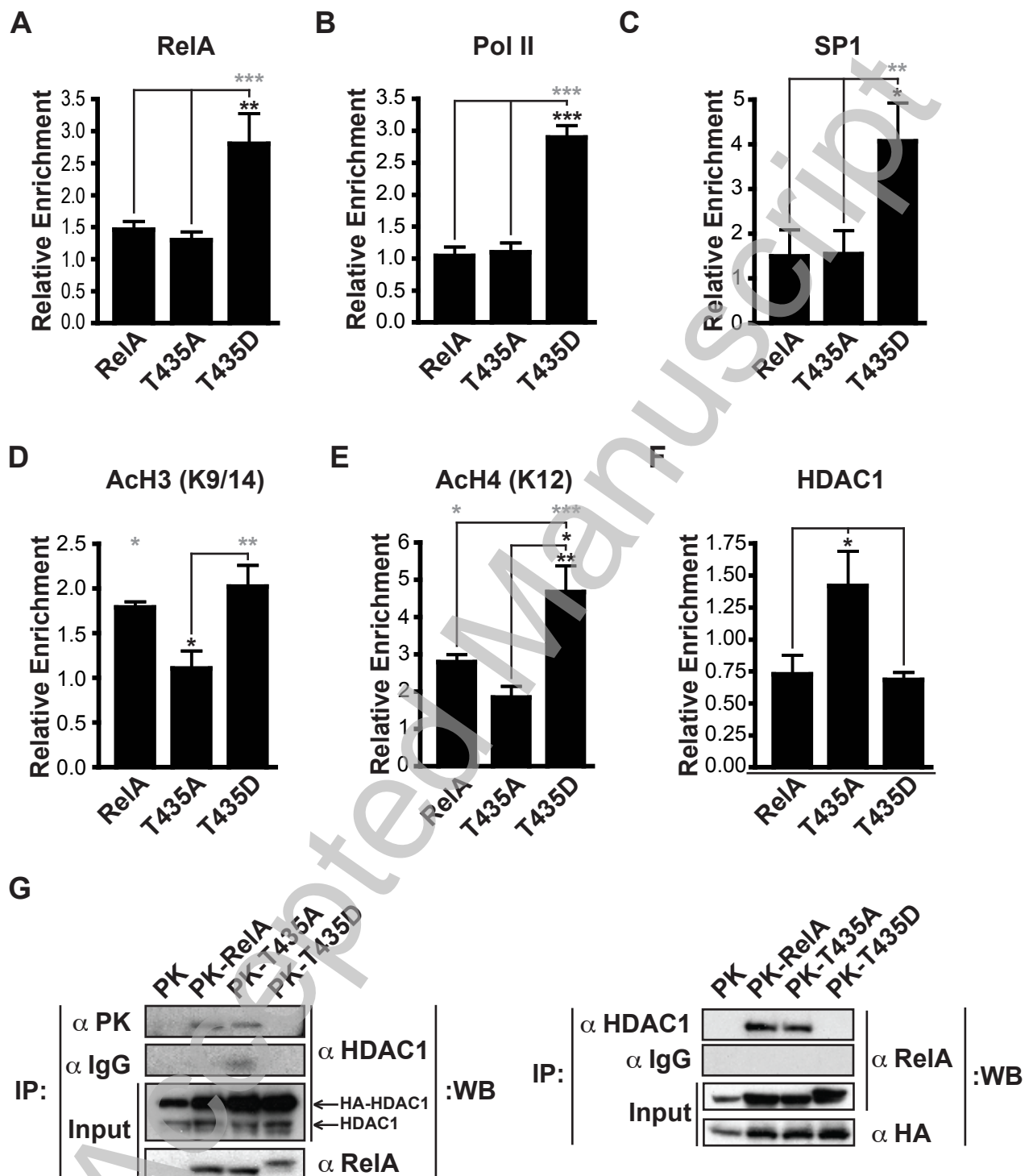
**O'Shea & Perkins Figure 2**



O'Shea & Perkins Figure 3

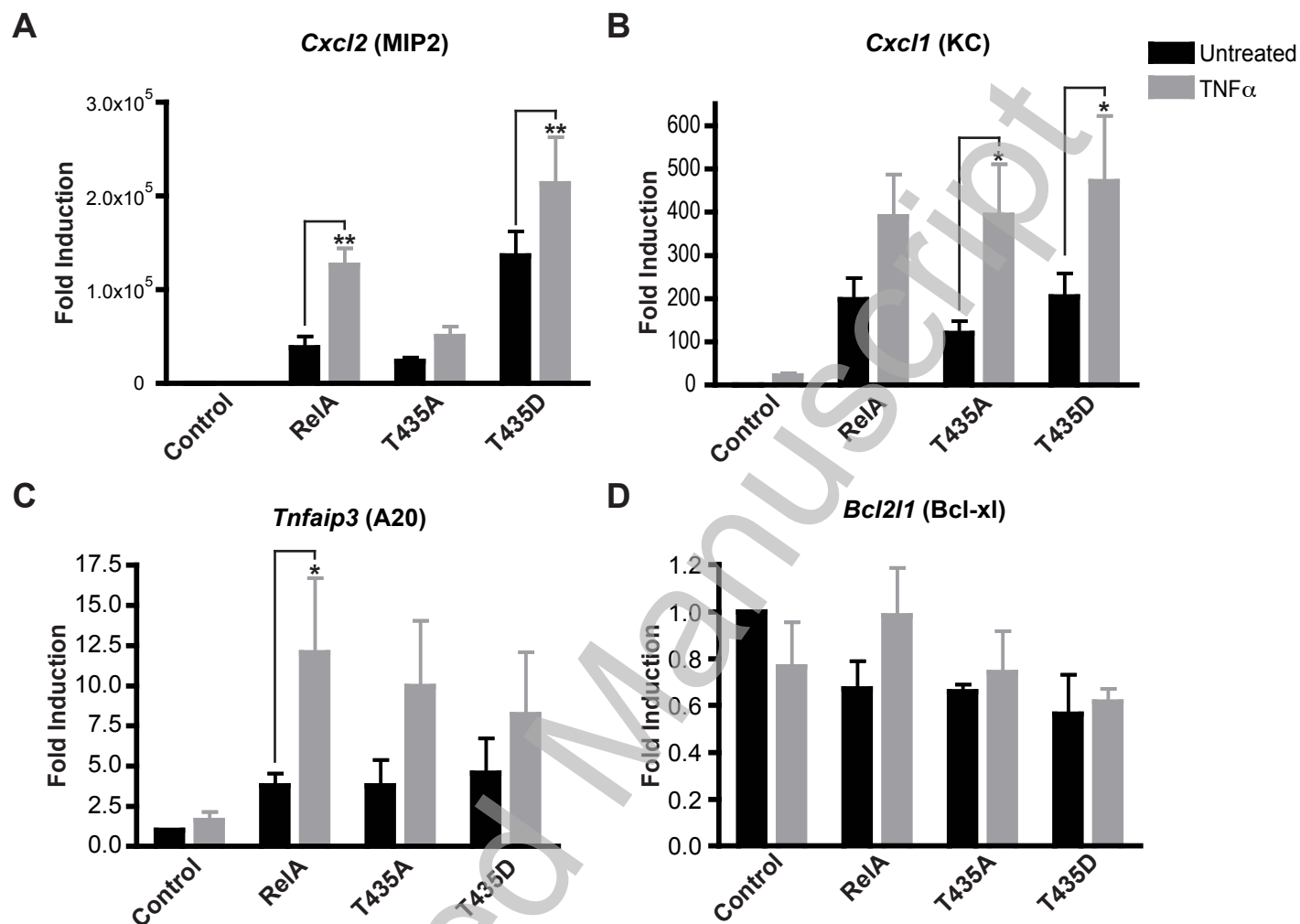


O'Shea & Perkins Figure 4



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O'Shea & Perkins Figure 5



O'Shea & Perkins Figure 6

