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Binding Loci of RelA-containing Nuclear Factor-kappaB (NF-κB) Dimers in Promoter Regions of PHM1-31 Myometrial Smooth Muscle Cells

Running Title: Myometrial Gene Regulation by NF-κB

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Abstract

Human parturition is associated with many pro-inflammatory mediators which are regulated by the Nuclear Factor kappa B (NF-κB) family of transcription factors. In the present study, we employed a ChIP-on-chip approach to define genomic loci within chromatin of PHM1-31 myometrial cells that were occupied by RelA-containing NF-κB dimers in response to a TNF stimulation of one hour. In TNF-stimulated PHM1-31 cells, anti-RelA serum enriched 13,300 chromatin regions; importantly, 11,110 regions were also enriched by anti-RelA antibodies in the absence of TNF. DNA sequences in these regions, from both unstimulated or TNF-stimulated PHM1-31 cultures, were associated with genic regions including IκBα, COX-2, IL6RN, Jun and KCNMB3. TNF-induced binding events at a consensus κB site numbered 1,667; these were represented by 112 different instances of the consensus κB motif. Of the 1,667 consensus κB motif occurrences, 770 (46.2%) were identified within intronic regions. In unstimulated PHM1-31 cells, anti-RelA-serum-enriched regions were associated with sequences corresponding to open reading frames of ion channel subunit genes including CACNB3 and KCNB1. Moreover, in unstimulated cells, the consensus κB site was identified 2,116 times, being defined by 103 different sequence instances of this motif. Of these 2,116 consensus κB motifs, 1,089 (51.5%) were identified within intronic regions. Parallel expression array analyses in PHM1-31 cultures demonstrated that TNF stimulated a >2-fold induction in 51 genes and a fold repression of >1.5 in 18 others. We identified 14 anti-RelA-serum-enriched genomic regions that correlated with 17 TNF-inducible genes, such as COX2, Egr-1, Jun, IκBα and IL6, as well as five regions associated with TNF-mediated gene repression, including Col1A2.

Keywords: NF-kappaB / chromatin / labour / microarray / pre-term birth
Introduction

In the developed world, premature birth (that before 37 weeks completed gestation) complicates 6-12% of pregnancies (Khashan et al., 2010). Annually it is estimated that 1.1 million babies worldwide die from being born prematurely (Blencowe et al., 2012; Chang et al., 2013); surviving infants having an elevated risk of major long-term mental and physical handicap (Marlow et al. 2005; Costeloe et al. 2012). Moreover, such infants also have a disproportionate effect on health-care budgets worldwide: a recent U.K. estimate of the total cost of preterm birth to the public sector was £2.95 billion (Mangham et al., 2009). Tocolytic therapies (drugs which stop premature contractions of the womb) are few in number and are associated with complications for both infant and mother (Oei, 2006). This problem is compounded by the fact that, despite many years of research, we remain ignorant of the fundamental biological principles governing uterine function during pregnancy and labour.

NF-κB Biology and the Myometrium

Regulatory networks between transcription factors and DNA ensure cells function normally. The Nuclear Factor kappaB (NF-κB) family are one set of transcription factors which govern a wide variety of cellular activities (reviewed in Perkins, 2007; Perkins, 2012; Hayden and Ghosh, 2012; Cookson and Chapman, 2010). NF-κB, which is rapidly induced by over 400 different stimuli including TNF (Perkins, 2007; Hayden and Ghosh, 2012; Cookson and Chapman, 2010), is present in virtually every cell type within the body. NF-κB is composed of dimeric complexes formed from five distinct subunits: RelA (p65), RelB, c-Rel, NF-κB 1 (p105/p50) and NF-κB 2 (p100/p52) (Perkins, 2007; Perkins, 2012; Hayden and Ghosh, 2012; Cookson and Chapman, 2010). DNA binding by NF-κB dimers is mediated by a conserved N-terminal domain termed the Rel Homology Region (Chen and Ghosh, 1999). Combinations of
subunits determine the specificity of transcriptional activation (Perkins et al., 1992; Chen and
Ghosh, 1999); indeed NF-κB can modulate prolonged gene expression through the exchange
of NF-κB dimers at a given promoter (Saccani et al., 2003).

There are predicted to be in excess of 3,000 κB sites within the human genome with the
consensus NF-κB binding site generally viewed as 5′-G_{5-4}G_{3}R_{2}N_{1}Y_{0}Y_{+1}Y_{+2}C_{+3}C_{+4}-3′
(where R = A or G; N = A, C, T or G and Y = C or T; Natoli et al., 2005). Importantly, there
are a great many functional variants of this consensus κB motif and there is now a wealth of
studies describing how κB DNA motifs associate with various NF-κB dimers (Ghosh et al.,
1995; Müller et al., 1995; Cramer et al., 1997; Huang et al., 1997; Chen et al., 1998a; Chen et
al., 1998b; Chen and Ghosh, 1999; Phelps et al., 2000; Hoffman et al., 2003; Leung et al.,
2004; Huang et al., 2005; Moorothy et al., 2007; Trinh et al., 2008; Wan and Lenardo, 2009;
Wang et al., 2012).

At term, the smooth muscle of the uterus, the myometrium, is exposed to a complex milieu of
inflammatory signalling factors (Aguilar and Mitchell, 2010; Cookson and Chapman, 2010;
Golightly et al., 2011; Webster et al., 2013). Moreover, there is now a body of evidence that
NF-κB dimers containing the RelA NF-κB subunit play a pivotal role in regulating human
parturition (Belt et al., 1999; Allport et al., 2001; Elliot et al., 2001; Yan et al., 2002a; Yan et
al., 2002b; Lappas et al., 2003; Lee et al., 2003; Chapman et al., 2004; Lappas and Rice, 2004;
Lappas et al., 2004; Soloff, et al., 2004; Chapman et al., 2005; Lindström and Bennett, 2005,
Soloff et al., 2006; Terzidou et al., 2006; Mohan et al., 2007; Lindström et al., 2008).
Consequently, it would seem highly likely that, based on the evidence above, the myometrial
smooth muscle cell could have evolved suitable mechanisms to ensure those NF-κB-regulated
promoters are expressed only at the correct spatio-temporal juncture. Consistent with this notion, we have previously demonstrated that temporal changes in NF-κB subunit composition and associated DNA-binding activity occurs between non-pregnant (NP), pregnant (P) and spontaneously labouring (SL) myometrium (Chapman et al. 2004). At present, the importance of this change in NF-κB dimer composition within the uterine smooth muscle is unclear. Temporal changes in NF-κB subunit composition on NF-κB-regulated promoters, however, can permit fine-tuning of the transcriptional response ensuring the gene is expressed at the correct level for the appropriate length of time (Saccani et al., 2003).

The obvious corollary to those observations, therefore, is that it is highly likely that a similar temporal manner of regulation is being employed in the uterus ensuring parturition occurs at the correct juncture. The study described herein examines where NF-κB complexes bind to chromatin in myometrial cells and whether this binding influenced gene expression in such cells. Essentially this allows us to determine if NF-κB promoter occupancy is associated with transcriptional activation, transcriptional repression or homeostasis.

Materials and Methods

PHM1-31 Cell Passaging

PHM1-31 immortalised human myometrial myocytes were the kind gift of Prof. Barbara Sanborn, Colorado State University, USA (Monga et al. 1996). Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) FCS and 2 mM L-glutamine and 0.1 mg/ml Geneticin using published cell culture procedures (Chapman et al. 2005, Webster et al., 2013, Waite et al., 2014).
Transient Transfections, Plasmids and Luciferase Assays

Transient transfection of PHM1-31 myometrial cells was performed using the LT-1 reagent from Miras (Geneflow, Staffordshire UK) as described by Chapman et al., (2005) for primary myometrial cells. The 3x-κB-ConA-luciferase (3x-κB-ConA-Luc) and enh-ConA-luciferase (ΔκB-ConA-Luc) vectors were the generous gift of Prof. Ron Hay (University of Dundee, U.K.) and the construction of these has been reported in detail (Rodriguez et al., 1996). All transfection experiments were performed a minimum of three times and results are expressed as the mean ± SEM. All data analyses were conducted on GraphPad Prism Version 5.02 (GraphPad Software, San Diego, California). Comparison of data from two matched samples were compared using a paired, two-tailed t-test; p<0.05 was considered statistically significant.

RelA Immunocytochemistry in PHM1-31 Cells Following TNF Stimulation

PHM1-31 cells were cultured in a 24-well plate, washed in PBS and fixed in 1% (v/v) formaldehyde overnight at 4°C. Endogenous cellular peroxidase was quenched with 1% (v/v) hydrogen peroxide for 10 minutes. The Vectastain® Elite ABC kit (Vector Labs) was used for the following reactions. Endogenous biotin was blocked with PBS containing horse serum and avidin for one hour at room temperature, followed by incubation with primary antibody (anti-RelA, #sc-372, Santa Cruz Biotechnology Inc.) in antibody diluent and biotin at 4°C overnight. Secondary anti-mouse IgG (Dako) was added for 30 minutes at room temperature before the addition of the ABC reagent for 30 minutes at room temperature and finally DAB (3, 3′-diaminobenzidine). Cells were stored in PBS and photographed. Negative control experiments included the substitution of the primary antibody with an isotype control (Abcam, # ab46450).

Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assay (EMSA)
Nuclear extracts were prepared essentially as described in Dignam et al., (1983). In this study, the EMSA utilised an oligonucleotide consisting of the HIV-1 3’ long terminal repeat (LTR) κB site (in bold; 5’-GATCCGCTGGGACTTTCCAGGCG-3’). The EMSA was carried out as detailed in Chapman et al., (2002 and 2005).

**Western Immunodetection**

Expression of the RelA NF-κB subunit was examined using Western analysis with immunoblots probed with antibodies that recognize either the amino terminal or carboxy terminal of RelA (p65) (Santa Cruz Biotechnology Inc. Santa Cruz CA #sc-109 and #sc-372 respectively) and developed using EZ-ECL detection reagents (Geneflow, Staffs. U.K.) as detailed in Chapman et al., (2004).

**Chromatin Immunoprecipitation (ChIP) Assay**

The ChIP assay was performed on eight T-75 flasks of PHM1-31 cells (~2.6-2.8x10^6 cells/flask) grown to 100% confluence using the Magna-ChIP ChIP assay kit (#17-611, Millipore U.K. Ltd. Dundee) following the manufacturer’s guidelines and detailed in Webster et al., (2013). ChIP antibodies used were RelA and RNA Polymerase II (#sc-372 and #sc-899 respectively; Santa Cruz Biotechnology Inc. Santa Cruz CA). Briefly, four flasks were stimulated with 10ng/ml TNF for one hour while the remaining four were unstimulated controls. TNF has been demonstrated to be present in myometrium at term (Opsjln et al., 1993; Fitzgibbon et al. 2009; reviewed in Golightly et al., 2011) and has been used regularly by our group studying cytokine-induced myometrial NF-κB function (Chapman et al., 2005, Webster et al., 2013; Waite et al., 2014). The rationale for this time point was that it would represent an early response to TNF. We believe such early binding events play pivotal roles in the cell’s
choice of subsequent signalling pathway usage (reviewed in Perkins 2007). It was accepted
that TNF-induced gene regulation events occurring after one hour would not be investigated
(Campbell et al., 2001; Rocha et al., 2003). Three biological replicates of these ChIP assays
were completed. The work-flow utilised to generate the appropriate chromatin samples is
illustrated in Figure 1.

Quality Control PCR of Immunoprecipitated DNA

Prior to microarray analyses, ChIP efficacy was determined by enrichment of RelA on the
IκBα promoter. PCR was carried out on the immunoprecipitated DNA using primers flanking
the κB sites within the IκBα promoter as a positive control (Chapman et al. 2005). The Gαs
promoter, which is not regulated by RelA was chosen as a negative control (Webster et al.,
2013). Once it was determined that the chromatin was of sufficiently high quality, it was then
prepared ready to probe Affymetrix 1.0R Human promoter Arrays (Affymetrix, Santa Clara,
CA).

Affymetrix Microarrays

Full details of both arrays employed in this study can be found at the manufacturer’s web site:
GeneChip Human Promoter 1.0R Array:

http://www.affymetrix.com/estore/catalog/131461/AFFY/Human+Promoter+1.0R+Array#1

Human Genome U133 Plus 2.0 Array:

http://www.affymetrix.com/catalog/131455/AFFY/Human+Genome+U133+Plus+2.0+Array
ChIP DNA Amplification, Fragmentation and Labelling

ChIP DNA was amplified using the Whole Genome Amplification Kit (WGA; Sigma) as detailed in the manufacturer’s instructions with slight modifications. Briefly, 1 µl of ChIP DNA was diluted with 9 µl of ultrapure water. Then 2 µl of library preparation buffer together with 1 µl of library stabilisation solution was added to this and heated at 95ºC for 2 minutes before cooling on ice. After that, 1 µl of library preparation enzyme was added and the reaction was incubated in the thermal cycler for the following times: 20 minutes at 16ºC (pre-cooled to this temperature), 20 minutes at 20ºC, 20 minutes at 37ºC, 5 minutes at 75ºC, and on hold at 4ºC.

The amplified DNA was re-amplified using the WGA re-amplification kit to generate the 7.5 µg required. This method is described in the manufacturer’s guidelines (Sigma). The re-amplified DNA was then fragmented and labelled according to the Affymetrix ChIP Protocol using the GeneChip WT Double-Stranded DNA Terminal Labelling Kit.

Human Promoter 1.0R Array Procedures

Hybridisation of amplified DNA to Affymetrix Human Promoter 1.0R arrays was carried out using the GeneChip Hybridization, Wash and Stain kit (Affymetrix) according to the Affymetrix ChIP Protocol. The hybridisation cocktail (7.5 µg fragmented labelled DNA, 50pM control oligonucleotide B2, hybridisation mix, 7% (v/v) DMSO) was hybridised in the Affymetrix GeneChip Hybridisation Oven 640. Washing and staining was carried out using the GeneChip Fluidics Station 450 as described in the GeneChip Expression Wash, Stain and Scan User manual (Affymetrix). The GeneChip Scanner 3000 7G, operated by the GeneChip Operatating Software (GCOS, Affymetrix), was used to scan the Human Promoter 1.0R Arrays.
The raw data (.CEL) files, generated by the GCOS software, were imported into Partek Genomics Suite (PGS; Version 6.6; www.partek.com/pgs) and subjected to Robust Multi-array Average (RMA) background correction, quantile normalisation and Log (base 2) transformation utilising the software’s tiling workflow. Prior to invoking an ANOVA, the data were normalised to the baseline by subtraction of all values ascribed to IgG samples from both unstimulated controls and TNF-treated samples since these values would represent non-specific binding events (Fig.1; illustration of work-flow). A two-way ANOVA was then completed within PGS to undertake multiple comparisons and determine the difference between unstimulated controls and TNF-treated samples at probe-level. Differences between control and TNF-stimulated samples were considered significant if $p \leq 0.05$.

Upon completion of the ANOVA, the Model-based Analysis of Tiling (MAT) algorithm (T statistic) was employed to detect enriched regions of chromatin in un-stimulated and TNF-stimulated data sets (Johnson et al. 2006). The MAT algorithm allows a rapid method of detecting regions enriched by a given transcription factor (in this study RelA-containing NF-kB dimers). The MAT algorithm was then applied across a sliding window of 600bp, using a minimum of 10 probes per region with ChIP-enriched regions deemed to be statistically significant when $p \leq 0.01$: this output was the MAT score. Those regions with a positive MAT score and $p \leq 0.05$ from the ANOVA indicated significant enrichment by anti-RelA antiserum in those samples treated with TNF compared to untreated controls. Conversely, a negative MAT score and $p \leq 0.05$ from the ANOVA represented those samples where enrichment of genomic loci was greater in unstimulated controls compared to those exposed to TNF. Promoters of known genes within the significantly enriched regions were then identified.
using the RefSeq database, based on the hg18 build of the human genome. All of the array data has been archived with the NCBI Gene Expression Omnibus (NCBI GEO) with the accession number GSE65721 [www.ncbi.nlm.nih.gov/geo]; this number covers all associated experimental sub-series. All tables of original data sets can be accessed and downloaded from the folder entitled Cookson et al 2015 Public Access MHR Original Data Sets at the following hyperlink:

https://drive.google.com/folderview?id=0B4bwcdSzbmn8OXdLWEtKemxtb0k&usp=sharing

**RNA Extraction**

On reaching 90% confluence, PHM1-31 cells were stimulated with 10ng/ml TNF for one hour with non-stimulated flasks serving as controls. RNA was extracted using the EZ-RNA extraction system (Geneflow, Staffs. U.K.) and quantified using the nanophotometer (Implen; supplied by Geneflow, Staffs. U.K.). Prior to first strand synthesis, the quality of isolated RNA was verified using Agilent’s Eukaryote Total RNA Nano Chip (5067-1511) in conjunction with the Agilent 2100 bioanalyser following the manufacturer’s guidelines. Three biological replicates were performed.

**Affymetrix U133plus2 Human Expression Array Procedures**

RNA was reverse transcribed using Bio-Rad iScript cDNA synthesis Kit according to the manufacturer’s guidelines (Bio-Rad Laboratories Ltd. Hertfordshire U.K.). The preparation and hybridisation of cDNA to U133Plus2 expression arrays was performed according to published protocols (Kirby et al. 2011; Simpson et al., 2011; Brockington et al., 2013; Raman et al., 2015).

**Data Analysis in Partek Genomics Suite 6.6**
The raw data files (.CEL) were imported into PGS V6.6 and analysed following the software’s Gene Expression workflow. Differentially expressed genes were identified using ANOVA to generate p values. Linear contrast was used to calculate fold-change and mean ratio from the contrast between unstimulated and TNF stimulated samples. Genes with fold change ≥ 2 or fold change ≤ -1.5 and with p values ≤ 0.05 were identified using RefSeq.

**Data Analysis of Combined Human Promoter 1.0R and U133plus2 Human Expression Arrays**

Using PGS, the enriched region list from both Criteria-A and Criteria-C were merged with the respective gene list from the expression array analyses. This provided data defining NF-κB-enriched regions that were either expressed or repressed.

**Validation of Microarray Results by qRT-PCR**

Quantitative RT-PCR using SYBR Green Jumpstart Taq ReadyMix (Sigma, UK) was undertaken following MIQE guidelines (Bustin et al. 2009). GAPDH and β-Actin were selected as housekeeping genes. The primer sequences were as follows:

- **TNFAIP3** Forward: 5’-TGAGCCCTTGGCGTGGAACC-3’
- **TNFAIP3** Reverse: 5’-AAAGGGCTGGGTGCTGTCGG-3’
- **NFKBIA** Forward: 5’- CGCCCAAGCACCACCCGGATA-3’
- **NFKBIA** Reverse: 5’- GGGCAGCTCGTCCTCTGTGA-3’
- **GAPDH** Forward: 5’-TGTTGCAGTCAGCCGCATCT-3’
- **GAPDH** Reverse: 5’- CAGGCGCCCAATACGACCAAATC-3’
- β-Actin Forward: 5’-CGAGCACAGAGCCTCGCTT-3’
β-Actin Reverse: 5’- CGAGCAGAGGCCTCGCCTT -3’.

qPCR was performed in triplicate using a 7900HT fast qPCR Machine (Applied Biosystems) and gene expression data were analysed using ΔΔCT using SDS 2.0 Software (Applied Biosystems).

Results

TNF induces RelA Nuclear Localisation and Occupancy of the IκBα Promoter in PHM1-31 Cells

A number of pro-inflammatory cytokines, including TNF, are associated with the onset of both normal and preterm birth (Aguilar and Mitchell, 2010; Golightly et al., 2011). Prior to the ChIP-on-chip experiments, it was important to confirm that in PHM1-31 cells, NF-κB RelA was activated by stimulation with TNF. RelA immunocytochemistry of PHM1-31 cells showed diffuse staining in the control, unstimulated cells and those stained with control IgG, (Fig. 2A; Panels I and II) whilst translocation of the RelA subunit into the nucleus (black arrows) was apparent following 1 hour TNF stimulation (Fig. 2A; Panel III). Moreover, increased RelA binding to the consensus κB site in EMSA was also seen and that binding activity could be specifically super-shifted with anti-RelA antiserum (Fig. 2B). Furthermore, when PHM1-31 cells were transiently transfected with the RelA-responsive 3x-κB-luc reporter and subsequently exposed to TNF for one hour, increased reporter activity was seen in those cells harbouring the 3x-κB-luc vector, but not those with the ΔκB-luc control (Fig. 2C).

Immunoprecipitation using the RelA antiserum (sc-372) showed specific binding to the RelA protein, while the control IgG failed to precipitate any RelA complexes (Fig. 2D). To demonstrate the specificity of the ChIP, the RelA antiserum detected low level binding of RelA
complexes to the IκBα promoter region without TNF stimulation; as predicted, this increased upon TNF exposure. No binding was seen at the RelA-insensitive Gαs promoter. Supporting this, after exposure to TNF, RNA PolII binding was also observed at the IκBα promoter. These results illustrate that the immunoprecipitation and ChIP methods were specific for the RelA NF-κB subunit. Interestingly, while we see a low-level of NF-κB occupancy of the IκBα promoter under unstimulated conditions, we could not detect a similar pattern of immunostaining (Fig. 2A, Panels I and II).

**TNF Induces RelA NF-κB Promoter Occupancy at a Variety of Promoters**

In this study of PHM1-31 myometrial myocytes, statistically significant differences in promoter occupancy between unstimulated and TNF-treated cultures were observed in 24,410 genomic regions enriched by the anti-RelA antiserum (p≤0.05); these data were termed Criteria-B and represent all enriched regions from both unstimulated and TNF-treated cultures (Fig. 3A; [Original Data Set - Table 1](#)). Within the set Criteria-B, defining the MAT algorithm parameters to enriched values of >0 and p≤0.01 (i.e. a positive MAT score) generated 13,300 genomic loci that were significantly more enriched by the anti-RelA antiserum in TNF-treated cells compared to unstimulated controls; this subset was termed Criteria-A (Fig. 3A; [Original Data Set - Table 2](#)) and represents TNF-induced enrichment.

Similarly within the Criteria-B dataset, defining the MAT algorithm parameters to enriched values of <0 and p≤0.01 (i.e. a negative MAT score), we identified 11,110 genomic regions that were significantly more enriched by the anti-RelA antiserum in the unstimulated control set compared to the TNF-treated samples. This subset was termed Criteria-C and represents unstimulated enrichment. Therefore, in this study, the Criteria-C dataset identifies genomic
loci bound by NF-κB dimers containing the RelA subunit (homo- or heterodimers of RelA) when the cell population is not exposed to an exogenous stimulant such as TNF (Fig. 3A; Original Data Set - Table 3). While we cannot rule out the possibility that some RelA-enriched regions may be non-specific, the removal of the IgG-associated regions will minimise such interference.

RelA-enriched loci from both Criteria-A and Criteria-C datasets represent given regions of chromatin and thus may map to different aspects of a gene including exons and introns. Fig. 3B illustrates a schematic representation of chromosome 14 (the IκBα locus) illustrating such differentially enriched regions listed in either Criteria-A (TNF-induced; red vertical lines) or Criteria-C (unstimulated; blue vertical lines) datasets.

RelA-Enriched Regions Encoding or Juxtaposed to Genes Associated with Parturition

Enrichment of chromatin from TNF-treated cells by anti-RelA serum was seen to contain or be juxtaposed to regions encoding many genes believed to play a significant role in myometrial quiescence or labour itself including, but not limited to, PTGS2, Jun, IL6RN, IL6, CACNB3, KCNMB3, TRPC2 and VCAM-1 (Original Data Set - Table 4; Criteria-A and Original Data Set - Table 5; Criteria-C). Figure 4 illustrates examples of genes identified in RelA-enriched chromatin after cells were stimulated with TNF. Of these, regions harbouring PTGS2 and Jun were enriched in the presence of TNF (Figs. 4A-4B; Criteria-A regions are red bars) and those regions also encoded κB motif(s) that were in agreement with the published κB consensus site. For PTGS2 a region was also enriched in the absence of TNF but no consensus κB motif was identified therein. Interestingly, genes encoding IL6RN and KCNMB3 were also enriched in the presence of TNF but no discernible consensus κB motif could be identified in those
respective Criteria-A enriched regions, suggesting a non-consensus κB motif was being
utilised (Figs. 4C-4D; red bars). Moreover, we also observed that intronic regions of both
IL6RN and KCNMB3 were also enriched by RelA NF-κB in the absence of TNF stimulation
suggesting that RelA-containing dimers do have a function in governing expression of these
genes (Figs. 4C-4D; blue bars). Finally, regions that were only enriched by RelA-containing
dimers in the absence of TNF (i.e. unstimulated) were also examined. Examples of such regions
were those encoding regulatory subunits of calcium and potassium channels such as CACNB3
and KCNB1 (Figs. 4E-4F; blue bars). Significantly, regions encoding these ion channel
subunits also harboured κB motifs corresponding to the consensus κB sequence (Figs. 4E-4F).

To ensure our experimental system was functioning correctly, we also examined the NF-κB-
regulated the IκBα promoter region (LeBail et al., 1993). As expected, in TNF-treated cells,
aspects of the IκBα promoter were enriched by anti-RelA serum. Interestingly, however, no
enrichment of the IκBα promoter was observed in the absence of TNF (Fig. 5A) despite this
region being weakly amplified in the control ChIP assay (Fig. 2E). Increasing the resolution of
the schematic representation in Fig. 5A illustrates two regions within the IκBα gene enriched
in TNF-treated cells (Fig. 5B). The region upstream of the IκBα transcription start site was
also seen to encode the three κB motifs believed to be responsible for governing IκBα
expression (LeBail et al., 1993). A second intra-genic region within IκBα open reading frame
was seen to be enriched in TNF-treated cells although no consensus κB motifs were identified
therein (Fig. 5B). In contrast to genes in close proximity to the anti-RelA serum enriched loci,
other enriched regions were decidedly more remote from the nearest genes

Original Data Set
- Table 4, Criteria-A and Original Data Set - Table 5, Criteria-C; full annotated gene lists associated with these enriched regions can also be viewed therein).

**Frequency of κB site Motif Occurrence in Criteria-A and Criteria-C Datasets**

In the Criteria-A dataset (i.e. RelA-enriched regions in response to TNF), 1,667 occurrences of the κB consensus sequence were identified, defined by 112 different sequence representations. Of the 1,667 occurrences, 1,604 resided in non-repetitive genomic regions. To aid clarity, we focussed the study on those motifs from the non-repetitive regions. These motifs were defined by 65 different sequence instances (Table 1). The remaining 63 occurrences, whilst not studied further, were defined by 47 different sequence instances many of which were also observed in the non-repetitive dataset (data not shown). Of the 1,667 consensus κB motif occurrences in the Criteria-A dataset, 770 (46.2%) were identified within intronic regions. 

In the Criteria-C data set (i.e. RelA-enriched regions in absence of TNF), the consensus κB motif occurred 2,116 times defined by 103 different sequence representations. Of the 2,116 occurrences, 2,064 were seen to reside in non-repetitive elements of the genome. These were chosen for further study. In turn, these motifs were also represented by 65 different sequence instances (Table 1). The remaining 52 occurrences, whilst not studied further as they were from highly repetitive sequences, were defined by 38 different sequence instances many of which were also observed in the non-repetitive dataset (data not shown). Of the 2,116 consensus κB motif occurrences in the Criteria-C dataset, 1,089 (51.5%) were identified within intronic regions.
Overall, the consensus κB motif was observed a total of 3,783 times in anti-RelA serum-enriched genomic regions with 3,720 κB motifs identified in non-repetitive elements of the genome of PHM1-31 myometrial myocytes. Alignment of the 65 representations of the κB sequence was performed using WebLogo 3 open access software (Crooks et al. 2004; http://weblogo.threeplusone.com/). Consensus κB sequence variability was seen to be 5’GGG(A/G)(A/C/T/G)(C/T)(C/T)(C/T)CC-3’ but no difference was observed with this between Criteria-A (TNF-induced enrichment) and Criteria-C (un-stimulated enrichment) (Fig. 6).

Expression Array Analysis of PHM1-31 Gene Expression Induced by TNF

As illustrated thus far, promoter occupancy by RelA-containing NF-κB dimers per se, does not provide information on associated transcriptional levels (i.e. expressed, repressed or quiescent; akin to chromatin-bound RNA polymerase II and promoter-proximal stalling (Core and Lis, 2009)). Consequently, we undertook expression array analyses in PHM1-31 cells utilising total RNA extracted from cells exposed to TNF for one hour (an identical time course to those used to isolated chromatin for ChIP-on-chip studies).

A statistically significant change in expression of 2,963 genes was induced by TNF (Supplementary Information - Data Table 8 p-value region<0.05). Expression of six genes remained unchanged between unstimulated and TNF-treated cells (SLC35F5, DEFB106A, RFTN1, DTWD2, SLC34A3 and SEZ6L). Removal of duplicates from the original data gave a total of 2,223 genes that were differentially expressed when PHM1-31 cells were stimulated with TNF for one hour (Fig. 7; Criteria-B1, p<0.05; and Original Data Set - Table 8). Of this total, 51 genes were seen to have a fold-induction of >2. Of these, two were discounted as they
were not annotated, leaving a total of 49 genes; these were termed Criteria-A1. Physiologically relevant genes identified in this dataset were EGR 1-4, FOS, FOSB, JUN, JUNB, ATF3, NFKBIA, NFKBIZ, TNFAIP3, COX2, CXCL2, CXCL3, CCL20, LIF, IL-6, MAP3K8, THBS1 and TNF. Eighteen genes were seen to have a fold repression of >1.5. Of these, one was not annotated and was thus excluded leaving a total of 17 genes; these were termed Criteria-C1 (Fig. 7). Included in this dataset were HOXA11, COL1A2 and STAT2. To summarise, Table II lists those genes whose expression was induced by at least two-fold in response to TNF while Table III lists those genes subject to repression by at least 1.5 fold in the presence of TNF. Further details from the expression array analyses can be viewed in

Original Data Set - Tables 8-10.

Combined Analysis of PHM1-31 Promoter Occupancy and Gene Expression

Merging the Criteria-A TNF-enriched promoter dataset with Criteria-A1 (TNF-induced gene expression), 14 TNF-induced, RelA-enriched genomic regions from the promoter array screen also encoded TNF-inducible genes (Table II; genes annotated with * and Supplementary Information - Data Table 11). These included known NF-κB-regulated genes IL-6, Jun, NFKBIA, PTGS2, TNC and TNFAIP3. Genes not yet conclusively demonstrated to be under NF-κB control included DUSP-2, DUSP-5, ERRFI1, THBS1 and CTGF (Table II; genes annotated with * and Original Data Set - Table 11). Moreover, by combining the Criteria-A promoter array dataset with the expression array Criteria-C1 dataset (TNF-repressed genes), we also identified five RelA-enriched genomic regions that harboured TNF-repressed genes, including COL1A2 (Table III; genes annotated with * and Original Data Set - Table 12).

Gene Ontology and Validation of Selected RelA-enriched Regions Modulated by TNF
PGS Gene Ontology analysis identified two functional groups of interest in the Biological Processes category: Intra-cellular Signal Transduction and Cell Surface Receptor Protein Signalling. These included TNFAIP3 (Cytokine-Induced Signalling) and IκBα (NIK/NF-κB Signalling; Fig. 8A). These targets were subsequently used to validate the expression array data because both have important roles in governing TNF signalling and NF-κB function respectively (Perkins, 2007; Chen and Ghosh, 1999; Hayden and Ghosh, 2012). Real-time qRT-PCR on total RNA from PHM1-31 cells treated with TNF for one hour demonstrated that both IκBα and TNFAIP3 were expressed in response to TNF. Good agreement was observed for induction of gene expression for IκBα (Fig. 8B; 2.94 on array vs. 4.43 for qRT-PCR) while a slightly greater margin of difference was noted for TNFAIP3 expression (Fig. 6B; 7.96 on array vs. 3.85 for qRT-PCR).

Discussion

NF-κB Binding and Distribution in the Genome of TNF-stimulated and Unstimulated Cells

This report is the first to describe a promoter array-based approach to define chromatin regions of myometrial myocytes occupied by RelA-containing NF-κB dimers. Our data demonstrated that in PHM1-31 myometrial cells, NF-κB-mediated enrichment of 13,300 chromatin regions in the presence of TNF and 11,110 in unstimulated cells. Some of these regions were juxtaposed to genes known to function in human labour, for example PTGST2 (Chan et al., 2014) and KCNMB3. NF-κB-controlled regulation of PTGS2 in the myometrium and amnion is well documented (Allport et al., 2001; Soloff et al., 2004; Lindström and Bennett, 2005) while a putative role in governing gene activity of the potassium channel subunit KCNMB3 is less so. In contrast, other RelA-enriched regions were decidedly more remote from the nearest transcription start with distances being measured in numbers of kilobases. Many of these loci
were also noted to be within intronic regions as discussed below. We did not examine the influence of such remote binding events in this study but we cannot rule out that they represent enhancer sequences or non-coding RNA transcriptional units. Indeed, supporting this notion are the observations that many transcription factor binding sites are arranged many kilobases from the transcription start site of the genes they regulate (Deaton and Bird, 2011).

κB Binding Site Loci

Intriguingly, RelA appeared to exhibit stimulus dependent binding to different loci of the same gene; this was evidenced with PTGS2, IL6RN and KCNMB3. In contrast, however, a section of the promoter region for Jun was only enriched in the presence of TNF while for CACNB3 and KCNB1, sections of promoter regions for these respective genes were only enriched by RelA-containing dimers in the absence of TNF; the molecular mechanisms accounting for these observations remain to be established. We did, however, determine that in both datasets, the consensus κB motif was represented a total of 3,783 times, including 1,859 (49.14%) sites identified within intronic regions. A number of studies in various cell lines have reported such intronic binding by NF-κB (Martone et al., 2003; Schreiber et al., 2006; Lim et al., 2007; Wong et al., 2011; Satohn, 2013; Xing et al., 2013) and, given that the early work describing its function demonstrated it was bound to the first intron of the κ-light chain enhancer (Schjerven et al., 2001), our observations support such previous data. Interestingly there are reports of transcription of certain regulatory proteins initiating from within the 3′-intronic regions of the parental gene. The calcium channel associated transcriptional regulator (CCAT), for example, is generated through initiation of independent transcription of exons 46 and 47 at a 3′-intronic site of the parental Cav1.2 calcium channel gene (Gomez-Ospina et al., 2013). Clearly, our data does not illustrate if such NF-κB binding directly modifies gene activity in this manner but it
offers a likely rationale for such intronic binding, perhaps as a means of governing post-
transcriptional RNA splicing, and a further avenue for investigating the complexities of
myometrial gene activity as labour commences. Due to the apparent promiscuity of RelA
binding to multiple loci and limited sensitivity of the ChIP-on-chip methodology, ChIP-exo
(see below) would be a superior means by which to narrow down the precise binding location
of RelA-containing dimers to near single base pair resolution (Rhee and Pugh, 2012).

Selection of \( \kappa B \) Motifs

The consensus \( \kappa B \) binding motif is viewed as 5'-G_5G_4G_3R_2N_1Y_0Y_1Y_2C_3C_4+3'- with
many functional variants on this being reported (Perkins, 2007; Hayden and Ghosh, 2012; Chen
and Ghosh, 1999). This motif does offer a level of subunit selectivity and crystallographic
studies of various NF-\( \kappa B \) dimers bound to different \( \kappa B \) DNA sequences support this (Ghosh et
al., 1995; Müller et al., 1995; Cramer et al., 1997; Huang et al., 1997; Chen et al., 1998a; Chen
et al., 1998b; Phelps et al., 2000; Huang et al., 2005; Moorthy et al., 2007; Trinh et al., 2008).
The actual \( \kappa B \) DNA sequence clearly does impose binding constraints upon certain dimers; for
example, within the \( \kappa B \) motif, the 5'-G_5G_4G_3R_2N_1-3' half site is bound by p50; in contrast,
the 5'-Y_1Y_2C_3C_4+3' half site is necessary for RelA binding (Huang et al., 2005 and
references therein). Whether region-enrichment is dimer-specific and dependent on the nature
of the stimulus (in this case TNF or not) could not be determined herein. Since we focussed on
immunoprecipitated RelA, this would recover four possible groups of RelA-containing dimers;
RelA:RelA, RelA:c-Rel RelA:p50 and RelA:p52 (RelA:RelB heterodimers are not thought to
bind DNA; Marienfeld et al., 2003). Given the published physical constraints imposed upon
certain NF-\( \kappa B \) dimer: \( \kappa B \) motif interactions (Phelps et al., 2000; Huang et al., 2005) one could
propose that, at this time-point of one hour, it is likely the RelA:p50 or RelA:p52 heterodimers
are being physiologically favoured. Given that we focussed on RelA, the obvious confounder of our work is that those enriched regions would likely reflect only contributions from such RelA-containing dimers; other non-RelA dimers would be missed. As above, further studies employing ChIP-seq would be required to provide the higher resolution data defining whether dimer composition on individual promoters/loci changed over multiple time points.

**Limitations of ChIP-on-chip**

While ChIP-on-chip is a robust, well-documented method to analyse transcription factor-mediated chromatin enrichment on a whole genome scale, it is associated with limitations when compared with more recent sequencing technologies including ChIP-seq (reviewed in Hurd and Nelson, 2009; Park, 2009). The obvious corollary, therefore, is that we cannot rule out the possibility that bias occurred in our system and influenced the data presented herein. A comparison between both ChIP-on-chip and ChIP-seq methodologies has been highlighted in Ho et al. (2011) who address the limitation and benefits associated with both technologies. With whole genome sequencing now available in most institutions, and methods including ChIP-exo (essentially ChIP-seq but using lambda exonuclease to trim the immunoprecipitated DNA to within a few base pairs of the binding residues on a given transcription factor) being developed, the next step would be to conduct ChIP-seq with all NF-κB subunit antisera, at different time points to obtain an unbiased genome-wide signature of binding events taking place within myometrial cells.

In our study, we employed the GeneChip 1.0R Human Promoter array. This contains 25,500 human promoter regions but it lacks full genomic coverage of the corresponding tiling arrays. Consequently, we cannot rule out the possibility that the low correlation between region
enrichment and subsequent transcription expression/repression could arise because the Human 1.0R Promoter array was not fully representative of the complete human genome.

PHM1-31 cultures were not cell-cycle synchronised prior to the ChIP assay because the induction of synchronisation itself (serum starvation) has been documented to effect expression of key genes involved in cell function and ion transport including the MaxiK potassium channel as well both the L- and T-type calcium channels (Woodfork et al., 1995; Panner et al., 2005; Patel et al., 2005). Essentially, potassium channels are responsible for hyperpolarising the plasma membrane, an event necessary for the cells to move from G1 to S (Wonderlin and Strobl, 1996; Ouadid-Ahidouch and Ahidouch, 2013). Calcium channels are thought to provide transient signals at checkpoints within the cell cycle which are necessary for the cell to continue cycling (Whitaker, 2006). We believe it highly likely that these events would have influenced those TNF-induced effects reported herein (Perkins, 2012).

Promoter Occupancy and Transcriptional Activity

Occupancy of a given promoter by NF-κB does not necessarily mean transcriptional activity directed by that promoter will change and there is robust data to support this notion (Hoffman et al., 2003; Leung et al., 2004; Wan and Lenardo, 2009; Wang et al., 2012). Of the TNF-induced NF-κB-enriched regions identified in our work, 14 were correlated with an increase in gene expression while five targets were repressed. Therefore, one must ask why the disparity between the number of enriched regions and the number of genes with altered activity? Clearly under normal physiological conditions, the myometrium would be bathed in a milieu rich in cytokines including IL-1β, IL-6, IL-8 and TNF to name but a few (Aguilar and Mitchell, 2010; Golightly et al., 2011; Webster et al., 2013). The manner by which these other proinflammatory
stimulants influence NF-κB activity was not examined herein but it is reasonable, based on
published evidence, to assume these factors would also moderate myometrial gene activity.
Secondly, it is without doubt that we will have missed many key binding events at promoters
at immediate early time points, ranging from seconds to minutes, as well as more prolonged
stimuli after a number of hours. Indeed, this approach may underlie why we did not observe
enrichment of regions that encoded genes for IL-1β, oxytocin or oxytocin receptor, all of which
have previously been shown to require NF-κB for induction of expression (Belt et al., 1999;
Lee et al., 2003; Soloff et al., 2006; Terzidou et al., 2006).

Differential Gene Expression

In the context of premature birth research, many authors have published expression array
studies in attempts to define genes responsible for promoting myometrial quiescence and
myometrial contraction. Such studies have focussed on native tissues (amnion, decidua and
myometrium) as well as both immortalised and primary cell lines (Charpigny et al., 2003;
Bethin et al., 2003; Bailey and Europe-Finner, 2005; Bailey et al., 2005; Esplin et al., 2005;
Havelock et al., 2005; Han et al., 2008; Khanjani et al., 2011; Lim et al., 2012).

A recent study by Chan et al. (Chan et al., 2014), used a robust RNA-seq-based approached to
define 764 differentially expressed genes in human myometrium from pregnant, non-labouring
women and those women in active labour. Salient examples of up-regulated genes from that
list included IL6, IL8, IL13, MCP, enzymes governing prostaglandin biosynthesis (PTGS2),
THBS2, DUSP family members, members of the NF-κB family of proteins, and intermediates
in the TGF-β and TNF-signalling pathway (Chan et al., 2014). Significantly, our study of TNF-
stimulated differential gene expression identified 49 induced genes; of these expressed genes,
17 were also represented in the list of genes up-regulated in labouring human myometrium documented by Chan et al. (Chan et al., 2014). Furthermore, 14 of the TNF-induced genes were also seen to be in regions of chromatin enriched by RelA. This is a key observation because it indirectly validates our own expression array work. Moreover, it supports the notion that NF-κB plays a pivotal role in controlling expression of genes involved in human parturition since many of those targets identified by Chan et al. are documented to be regulated by NF-κB (www.bu.edu/nf-kb/gene-resources/target-genes/).

Interestingly, a recent meta-analysis of gestational tissue-based transcriptomic studies highlighted significant variation in expression of individual genetic regions; essentially only 23 common sites were identified out of 10,993 unique transcriptionally active units (Eidem et al., 2015). Focussing on studies of myometrial gene expression, Eidem et al. identified 15 genes present in four or more studies. In our study, four TNF-induced genes were also present in the myometrial group identified by Eidem et al., including FOSB, NR4A1, LIF and PTGS2. Moreover, those genes reported to act as biomarkers of pre-term birth, including IL6 and TNF, were also represented in our work giving further validation of the TNF-induced gene expression data presented herein (Table II and Eidem et al., 2015). The meta-analysis described by Eidem et al., does not, however, consider changes in promoter occupancy of those gene targets, essentially because, other than our work, there are no data in the reproductive field describing such investigations.

**TNF-Induced Gene Expression**

In terms of NF-κB function, the NFKBIA gene, which encodes the IκBα protein, was induced 2.9 fold by TNF. As our positive control, this observation also validates the data presented
here. A second IκB family member, NFKBIZ, which encodes the IκBζ protein, was induced 5.2-fold in response to TNF. Significantly, other groups (Eto et al., 2003) have observed that IκBζ induction is not TNF-mediated. The differences in these observations may be accounted for through cell-type specific effects (macrophage or kidney versus myometrium) but it is noteworthy that one effector, TNF, can exert diametrically opposed effects on the same gene in different cell types, suggesting other nuclear-based factors are influencing the NF-κB-mediated gene regulation process. Significantly, IκBζ is known to bind specifically to p50 homodimers forming a robust ternary complex on the IL6 promoter activating expression of this gene (Trinh et al., 2008). Indeed, in our study TNF stimulation also caused a three-fold induction of IL-6 expression and we speculate this is mediated by p50 homodimer:IκBζ complex although further experimental analyses would be required to confirm this. TNFAIP3 (also termed A20) was also up-regulated. This protein also plays a significant role in termination of the NF-κB signal by inhibiting NF-κB DNA binding (Perkins, 2012; Hayden and Ghosh 2012).

Our study also identified various transcription factors that were significantly up-regulated in response to TNF, including members of the early growth response transcription factor family, Egr-1, -2, -3 and -4, as well as those of the AP1 family, namely ATF3, Fos, FosB, Jun, JunB. The Egr family are well-described zinc-finger containing proteins recognising the consensus sequence of 5-GCGG/TGGGCG-3’ (Christy and Nathans, 1989). The function of the Egr family in human myometrium is not clear but they have been shown to co-operate with the RelA subunit of NF-κB in embryonic kidney cells through an interaction between the RelA Rel Homology Domain and the zinc-finger region of Egr-1 (Chapman and Perkins, 2000), as well as competing for Sp1 sites in pro-inflammatory promoters such as PDGF-B (Khachigian...
et al., 1996). Many of the promoters identified in our study were GC-rich and up-regulation of factors which readily bind to such regions offers a potential insight into the control of complex myometrial gene expression networks.

Members of the AP1 transcription factor family, including Fos, FosB and Jun, bind to the consensus AP1 motif 5’-TGAG/CTCA-3’ or 5’-TGACGTCA-3’ (Shaulian and Karin, 2002) and have been shown to be differentially expressed in pregnant versus labouring rat myometrium. Importantly, studies in rat myometrium have described the differential expression of members of this family, between pregnant and labouring states. The salient observation is that peak levels of Fos, FosB, Jun and JunB occur during active labour (Mitchell and Lye, 2002; Mohan et al., 2007); importantly, those observations are in keeping with our data from human cells. Moreover, since Fos/Jun are immediate early genes (Shaulian and Karin, 2002), they are likely regulators of more extensive transcriptional networks within the cell and it is therefore interesting to speculate that NF-κB dimers may orchestrate a hierarchy of transcriptional activity within the myometrial cell. Interestingly, NF-κB has also been shown to play a key regulatory role in JunB expression as part of the JunB-mediated induction of VEGF in response to hypoxia (Schmidt et al., 2007). It is well documented that uterine contractions during labour do induce local hypoxic regions (Bugg et al., 2006); whether such myometrial hypoxia initiates NF-κB-induced AP1 family expression remains unclear at present but induction of such factors may be how the uterus responds to such a hypoxic stress and utilises NF-κB to instigate this protective mechanism.

Conclusions
In conclusion, our data demonstrate that RelA-containing dimers of NF-κB bind to numerous loci throughout the genome of PHM1-31 myometrial myocytes. For some promoters, this binding occurred in the presence of TNF as well as in unstimulated cells; this was mediated by different regions of the same promoter. Occupancy of other promoters was seen either only in unstimulated conditions or only after cells were stimulated by TNF. The consensus κB motif was identified 3,783 times in this study with over 100 different sequence instances potentially mediating NF-κB DNA binding. Of these κB motifs, 41% were found within intronic regions of the PHM1-31 cell chromatin. Of the 49 TNF-induced genes, 17 were shown to have promoters enriched by NF-κB in response to TNF. Interestingly, five genes with promoters occupied by NF-κB were repressed by TNF. Together, our data illustrate that NF-κB influences a wide range of regulatory gene networks within myometrial cells; we must decipher how these interactions govern myometrial function during pregnancy and labour if we are to begin to understand the syndrome of premature birth.
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Authors' Roles

VJC and SLW performed the experimental work, undertook initial data analyses and read and helped edit the manuscript. PRH performed the array work (promoter and expression) and read the manuscript. PJH and SVG assisted with the data analysis and manuscript preparation. NRC conceived the study, obtained study funding, designed the experiments, undertook the data analyses and prepared the manuscript.

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Conflict of Interest
The authors declare they have no competing interests, financial or otherwise, that would affect the publication of this data.

**Figure Legends**

**Fig. 1:** Schematic Representation of the Work-flow used to Generate Chromatin for this Study.

**Fig 2:** TNF Induces RelA NF-κB Activity in PHM1-31 Myometrial Cells. Immunostaining was used to demonstrate TNF-mediated induction of RelA nuclear localisation in PHM1-31 myometrial cell lines (A; **Panel-I**, unstimulated; **Panel-II**, negative control; **Panel-III**, TNF-stimulated; scale bar = 100μm). Nuclear extracts were prepared from PHM1-31 cells and incubated with α-^{32}P-labelled oligonucleotide harbouring the 3′-HIV-LTR κB site. Three main complexes were seen to form and, using supershift analyses, these were demonstrated to be p50:RelA heterodimers, RelA homodimers and a lower non-specific complex. An increased shift in RelA in TNF-treated cells illustrates TNF was inducing RelA NF-κB translocation to the nucleus. Specificity of the experiment was confirmed by including an excess (100 ng) of cold, HIV κB DNA (B). PHM1-31 cells were transfected with 200 ng of either 3x-κB-ConA-luc (NF-κB-responsive; C; **Panel-I**) or ΔκB-ConA-luc (NF-κB unresponsive; C; **Panel-II**). After 24 hours cells were stimulated with TNF (10 ng/ml) for one hour. Promoter activity was quantified using a Berthold Sirius tube luminometer. All experiments were performed three times in triplicate. Data were analysed using an unpaired, two-tailed t-test and results are expressed as the mean ± S.E.M. (error bars); p<0.05 was considered statistically significant.
As expected, TNF induced NF-κB activity (C; Panel-I; p = 0.0001). No NF-κB activity was observed in a control reporter lacking the κB site (C; Panel-II). Nuclear extracts were prepared from PHM1-31 cells and subjected to immunoprecipitation with anti-RelA anti-serum. RelA was recovered from both control and TNF-stimulated samples, illustrating the effectiveness of the antiserum. Minimal non-specific binding was observed with IgG (D). RelA occupancy of the IκBα promoter was seen under basal conditions. In the presence of TNF, both RelA and RNA Pol II were seen to be associated with the IκBα promoter (E; Upper Panels). No RelA occupancy of the control Gαs promoter was observed, illustrating the specificity of the ChIP assay (E; Lower Panels).

**Fig. 3: Comparison of RelA-enriched Chromatin Regions.** The Criteria-B dataset represents all regions with a p-value <0.05. Within this, the Criteria-A dataset (red circle) represent those regions where p<0.05 and the MAT score is >0; essentially, these regions are enriched over the control in response to TNF. The Criteria-C dataset (blue circle) represents those regions where p<0.05 and the MAT score is <0; essentially, these regions are enriched in the absence of TNF stimulation (A). An illustrative heat map of chromosome 14 illustrating the loci of Criteria-A regions (red bars) and Criteria-C regions (blue bars). Known transcripts from each strand are represented by green blocks (B).

**Fig. 4: Genes within Criteria-A and Criteria-C Enriched Regions and Associated κB Motif loci.** Schematic representation of the loci of both Criteria-A (TNF-induced NF-κB enrichment) and Criteria-C (unstimulated NF-κB enrichment) regions around selected genes including COX-2 (A); Jun (B), IL6RN (C); KCNMB3 (D); CACNB3 (E) and KCNB1 (F). For COX-2 and Jun, κB motifs corresponding to the consensus were found in the TNF-induced RelA
enriched regions (3A; 3B). With IL6RN and KCNMB3, both TNF-induced RelA enriched regions and unstimulated RelA-enriched regions were observed. The former were not associated with a consensus κB motif while the latter, unstimulated RelA-enriched, both harboured consensus κB motifs (3C; 3D). Regions around CACNB3 and KCNB1 were not enriched by RelA in the presence of TNF but were enriched by RelA in unstimulated cells, possibly by the consensus κB motifs identified (3E; 3F). Arrows indicate the direction of transcription, not actual transcription start sites.

Fig. 5: Differential Enrichment of Regions around the NFKBIA (IkBα) Gene Locus on Chromosome 14. Low resolution schematic illustration of the IkBα locus and surrounding regions. In the absence of TNF, no enrichment around the IkBα promoter is observed (A; blue bars). In the presence of TNF, a number of regions around the promoter are enriched by the anti-RelA antiserum (A; red bars). High resolution schematic illustration of the IkBα promoter illustrating TNF-induced regions enriched by the anti-RelA antiserum. One region encompasses the IkBα promoter and harbours the three reported κB motifs in that region. A second intra-genic region, encompassing the 3’ portion of exon 2 and all of exons 3 and 4, is also enriched but no consensus κB motifs were identified therein (B).

Fig. 6: Sequence Logo to Illustrate the Variability of κB Consensus Motif in Chromatin from PHM1-31 Cells.

The consensus κB motifs presented in Table I were aligned in the open-access software WebLogo 3. The probability of a given bases occurring at the proscribed position is illustrated.
No difference in \( \kappa B \) motif variability was noted between Criteria-A (TNF-induced) and Criteria-C (unstimulated) datasets.

Fig. 7: Comparison of TNF-Induced Differential Gene Expression. The Criteria-B1 dataset represents 2,223 genes modified by TNF with a p-value <0.05. The Criteria-A1 and -B1 dataset (red circle) represent those genes where \( p<0.05 \) and expression was increased 2-fold or more; 49 genes populated this intersection. The Criteria-C1 and -B1 (blue circle) represents those genes regions where \( p<0.05 \) and expression was repressed by more than than 1.5 fold; 17 genes populated this intersection.

Fig. 8: GO Enrichment – Biological Process and Validation of Expression Array Analyses by qRT-PCR. Within the Biological Processes group, two functional groups of interest were Intra-cellular Signal Transduction and Cell Surface Receptor Protein Signalling. This included TNFAIP3 (Cytokine-mediated Signalling) and I\( \kappa B \alpha \) (NIK/NF-\( \kappa B \) Cascade) (A). qRT-PCR on total RNA from PHM1-31 cells treated with TNF for one hour demonstrated that both I\( \kappa B \alpha \) and TNFAIP3 were expressed in response to TNF (B). A close agreement was seen for induction of gene expression for I\( \kappa B \alpha \) (4.43 for qRT-PCR; stippled bars vs. 2.94 on array; diagonal stripes) while a slightly greater margin of difference was noted for TNFAIP3 expression (3.85 for qRT-PCR; stippled bars vs. 7.96 on array; diagonal stripes).
Figure 1

Work-flow for ChIP-on-chip Experiments

1) Initial ChIP assays. Four groups of chromatin: U/S IgG, U/S αRelA; TNF IgG, TNF αRelA:

<table>
<thead>
<tr>
<th>Un-stimulated (U/S)</th>
<th>+TNF</th>
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<tr>
<td>IgG</td>
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2) Chromatin processed and quality-control PCR undertaken

3) Chromatin labelled; used to probe four Human Promoter 1.0R arrays (repeated three times)

4) Arrays scanned and raw data (.CEL files) processed by Partek Genomics Suite.

5) Values ascribed to IgG from three individual experiments deemed as non-specific and subtracted giving a new control baseline

6) PGS analysis between αRelA-enriched U/S chromatin and TNF-induced, αRelA-enriched chromatin:

Un-stimulated +TNF

Criteria-C dataset

Criteria-B = Criteria-A + Criteria-C
Figure 2
TNF Induces RelA NF-κB Activity in PHM1-31 Myometrial Cells

A Anti-RelA Staining

Panel I
Un-stimulated

Panel II
Negative Control

Panel III
+TNF

B RelA EMSA

<table>
<thead>
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<th>+TNF</th>
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<tr>
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<tr>
<td>Cold κB oligo</td>
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<td>Non-specific oligo</td>
<td>αRelA</td>
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\[ ^3P-κB oligo \]

Control vs. TNF: p = 0.0001

C 3x-κB-luc

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<td>TNF</td>
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Control vs. TNF: p = 0.0001

D RelA Immunoprecipitation

<table>
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\[ ^3P-κB oligo \]

I=Input; IP = αRelA

E RelA ChIP Analysis

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</tr>
</thead>
<tbody>
<tr>
<td>IgG</td>
<td>αRelA</td>
</tr>
<tr>
<td>αRelA</td>
<td>αRelA</td>
</tr>
</tbody>
</table>

\[ ^3P-κB oligo \]

I=Input (IxBα primers); IG= Input (Gαs primers)
IP = αRelA or αPolII

IgG Heavy chain

IxBα promoter

Primers

Gαs promoter

Primers
Figure 3
Comparison of Enriched Regions

A Venn Diagram of Enriched Regions

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Condition</th>
<th>Number of Enriched Regions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Criteria A</td>
<td>(+TNF; MAT score &gt; 0)</td>
<td>13,300</td>
</tr>
<tr>
<td>Criteria B</td>
<td>(p-value region ≤ 0.05)</td>
<td>24,410</td>
</tr>
<tr>
<td>Criteria C</td>
<td>(Un-stim.; MAT score &lt; 0)</td>
<td>11,110</td>
</tr>
</tbody>
</table>

B Heat Map of Chromosome 14 Illustrating Enriched Regions with a Positive MAT score (TNF-stimulated; Criteria-A; Red) and Enriched Regions with a Negative MAT Score (Un-stimulated; Criteria-C; Blue)

RefSeq Transcripts (plus strand) 10/05/2013:

RefSeq Transcripts (minus strand) 10/05/2013:

Detected Regions
(+TNF; Criteria A)

Detected Regions
(Un-stim.; Criteria C)
Figure 4
Genes Associated with Criteria-A and Criteria-C Enriched Regions

A) COX2 gene (PTGS2; NM_000963; minus strand)

B) Jun (NM_002228; minus strand)

C) IL6RN gene (NM_000577; plus strand)

D) KCNMB3 (NM_001163677; minus strand)

E) CACNB3 gene (NM_000725; plus strand)

F) KCNB1 (NM_004975; minus strand)
Figure 5
Differential Enrichment of Regions around the NFKBIA (IκBα) Gene Locus on Chromosome 14

A Chromosome 14 Enriched Regions and the NFKBIA (IκBα) Promoter

RefSeq Transcripts (minus strand) 10/05/2013:
IκBα gene (NFKBIA; NM_020529)

Detected Regions (Criteria A)

Detected Regions (Criteria C)

Regions expanded in Fig. 3B

Chr 14

Distance (Kbps)

B NFKBIA (IκBα) Promoter and κB-binding Motifs

RefSeq Transcripts (minus strand) 10/05/2013:
IκBα gene (NFKBIA; NM_020529)

Detected Regions (+TNF; Criteria A)

κB-Binding Motifs:
Consensus κB motif (5'-GGGRNYYYCC-3'):
NFKBIA Promoter κB motif 1 (-63 to -53; 5'-GGAAATTCCCC-3'):
NFKBIA Promoter κB motif 2 (-319 to -310; 5'-GGGAACCCCC-3'):
NFKBIA Promoter κB motif 3 (-225 to -216; 5'-AGGACTTTCC-3'):

Chr 14

Distance (Kbps)
Figure 6

Summary of κB Sequence Motif Variability in PHM1-31 Cells

Nucleotide Position within the κB Motif
(5' → 3')
Figure 7
Comparison of TNF-Induced Differential Gene Expression

B1 Total: 2,223 genes

A1 Total: 51 genes

Criteria A1
(Fold Change >2)
2 genes
(unannotated)

Criteria B1
(p-value region ≤0.05)
2,154 genes

A1 AND B1
(Fold Change >2)
49 genes

C1 AND B1
(Fold Change <-1.5)
17 genes

Criteria C1
(Fold Change <-1.5)
1 gene
(unannotated)

C1 Total: 18 genes

B1 Total: 2,223 genes
A GO Enrichment Biological Processes - Signal Transduction Groups

Biological Processes

- Response to Stimulus
- Signal Transduction
  - Intra-cellular Signal Transduction
    - Small GTPase-mediated Signal Transduction (2.2)
    - Inositol lipid-mediated Signalling (2.8)
    - NIK/NF-κB Cascade (4.1)
    - Cytoplasmic Pattern Recognition Receptor Signalling (5.7)
    - Intra-cellular Protein Kinase Cascade (6.1)
    - Second Messenger-mediated Signalling (8.3)

- Cell Surface Receptor Signalling Pathway
  - GPCR Signalling (0.83)
  - Immune response-regulating Cell Surface Receptor Signalling (0.88)
  - Neurotrophin Signalling (1.7)
  - Extrinsic Apoptotic Signalling (2.9)
  - Integrin-mediated Signalling (4.0)
  - Cytokine-mediated Signalling (4.7)
  - LPS-mediated Signalling (5.9)
  - Enzyme-linked Receptor Protein Signalling (11.0)

B Validation of Expression Array Analyses by qRT-PCR

![Graph showing qRT-PCR and Expression Array levels for TNFAIP3 and NFKBIA3.](image)