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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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1   **Abstract**

2   **Human parturition is associated with many pro-inflammatory mediators which are**

3   **regulated by the Nuclear Factor kappa B (NF-κB) family of transcription factors. In the**

4   **present study, we employed a ChIP-on-chip approach to define genomic loci within**

5   **chromatin of PHM1-31 myometrial cells that were occupied by RelA-containing NF-κB**

6   **dimers in response to a TNF stimulation of one hour. In TNF-stimulated PHM1-31 cells,**

7   **anti-RelA serum enriched 13,300 chromatin regions; importantly, 11,110 regions were**

8   **also enriched by anti-RelA antibodies in the absence of TNF. DNA sequences in these**

9   **regions, from both unstimulated or TNF-stimulated PHM1-31 cultures, were associated**

10   **with genic regions including IκBα, COX-2, IL6RN, Jun and KCNMB3. TNF-induced**

11   **binding events at a consensus κB site numbered 1,667; these were represented by 112**

12   **different instances of the consensus κB motif. Of the 1,667 consensus κB motif**

13   **occurrences, 770 (46.2%) were identified within intronic regions. In unstimulated PHM1-**

14   **31 cells, anti-RelA-serum-enriched regions were associated with sequences corresponding**

15   **to open reading frames of ion channel subunit genes including CACNB3 and KCNB1.**

16   **Moreover, in unstimulated cells, the consensus κB site was identified 2,116 times, being**

17   **defined by 103 different sequence instances of this motif. Of these 2,116 consensus κB**

18   **motifs, 1,089 (51.5%) were identified within intronic regions. Parallel expression array**

19   **analyses in PHM1-31 cultures demonstrated that TNF stimulated a >2-fold induction in**

20   **51 genes and a fold repression of >1.5 in 18 others. We identified 14 anti-RelA-serum-**

21   **enriched genomic regions that correlated with 17 TNF-inducible genes, such as COX2,**

22   **Egr-1, Jun, IκBα and IL6, as well as five regions associated with TNF-mediated gene**

23   **repression, including Col1A2.**

25   **Introduction**

26   In the developed world, premature birth (that before 37 weeks completed gestation)

27   complicates 6-12% of pregnancies (Khashan et al., 2010). Annually it is estimated that

28   1.1million babies worldwide die from being born prematurely (Blencowe et al., 2012; Chang

29   et al., 2013); surviving infants having an elevated risk of major long-term mental and physical

30   handicap (Marlow et al. 2005; Costeloe et al. 2012). Moreover, such infants also have a

31   disproportionate effect on health-care budgets worldwide: a recent U.K. estimate of the total

32   cost of preterm birth to the public sector was £2.95 billion (Mangham et al., 2009). Tocolytic

33   therapies (drugs which stop premature contractions of the womb) are few in number and are

34   associated with complications for both infant and mother (Oei, 2006). This problem is

35   compounded by the fact that, despite many years of research, we remain ignorant of the

36   fundamental biological principles governing uterine function during pregnancy and labour.

37

38   **NF-κB Biology and the Myometrium**

39   Regulatory networks between transcription factors and DNA ensure cells function normally.

40   The Nuclear Factor kappaB (NF-κB) family are one set of transcription factors which govern

41   a wide variety of cellular activities (reviewed in Perkins, 2007; Perkins, 2012; Hayden and

42   Ghosh, 2012; Cookson and Chapman, 2010). NF-κB, which is rapidly induced by over 400

43   different stimuli including TNF (Perkins, 2007; Hayden and Ghosh, 2012; Cookson and

44   Chapman, 2010), is present in virtually every cell type within the body. NF-κB is composed of

45   dimeric complexes formed from five distinct subunits: RelA (p65), RelB, c-Rel, NF-κB 1

46   (p105/p50) and NF-κB 2 (p100/p52) (Perkins, 2007; Perkins, 2012; Hayden and Ghosh, 2012;

47   Cookson and Chapman, 2010). DNA binding by NF-κB dimers is mediated by a conserved N-

48   terminal domain termed the Rel Homology Region (Chen and Ghosh, 1999). Combinations of

49   subunits determine the specificity of transcriptional activation (Perkins et al., 1992; Chen and

50   Ghosh, 1999); indeed NF-κB can modulate prolonged gene expression through the exchange

51   of NF-κB dimers at a given promoter (Saccani et al., 2003).

52

53   There are predicted to be in excess of 3,000 κB sites within the human genome with the

54   consensus NF-κB binding site generally viewed as 5'-$G_{-5}G_{-4}G_{-3}R_{-2}N_{-1}Y_0Y_{+1}Y_{+2}C_{+3}C_{+4}$-3'

55   (where R = A or G; N = A, C, T or G and Y = C or T; Natoli et al., 2005). Importantly, there

56   are a great many functional variants of this consensus κB motif and there is now a wealth of

57   studies describing how κB DNA motifs associate with various NF-κB dimers (Ghosh et al.,

58   1995; Müller et al., 1995; Cramer et al., 1997; Huang et al., 1997; Chen et al., 1998a; Chen et

59   al., 1998b; Chen and Ghosh, 1999; Phelps et al., 2000; Hoffman et al., 2003; Leung et al.,

60   2004; Huang et al., 2005; Moorthy et al., 2007; Trinh et al., 2008; Wan and Lenardo, 2009;

61   Wang et al., 2012).

62

63   At term, the smooth muscle of the uterus, the myometrium, is exposed to a complex milieu of

64   inflammatory signalling factors (Aguilar and Mitchell, 2010; Cookson and Chapman, 2010;

65   Golightly et al., 2011; Webster et al., 2013). Moreover, there is now a body of evidence that

66   NF-κB dimers containing the RelA NF-κB subunit play a pivotal role in regulating human

67   parturition (Belt et al., 1999; Allport et al., 2001; Elliot et al., 2001; Yan et al., 2002a; Yan et

68   al., 2002b; Lappas et al., 2003; Lee et al., 2003; Chapman et al., 2004; Lappas and Rice, 2004;

69   Lappas et al., 2004; Soloff, et al., 2004; Chapman et al., 2005; Lindström and Bennett, 2005,

70   Soloff et al., 2006; Terzidou et al., 2006; Mohan et al., 2007; Lindström et al., 2008).

71   Consequently, it would seem highly likely that, based on the evidence above, the myometrial

72   smooth muscle cell could have evolved suitable mechanisms to ensure those NF-κB-regulated

73 promoters are expressed only at the correct spatio-temporal juncture. Consistent with this

74 notion, we have previously demonstrated that temporal changes in NF-κB subunit composition

75 and associated DNA-binding activity occurs between non-pregnant (NP), pregnant (P) and

76 spontaneously labouring (SL) myometrium (Chapman et al. 2004). At present, the importance

77 of this change in NF-κB dimer composition within the uterine smooth muscle is unclear.

78 Temporal changes in NF-κB subunit composition on NF-κB-regulated promoters, however,

79 can permit fine-tuning of the transcriptional response ensuring the gene is expressed at the

80 correct level for the appropriate length of time (Saccani et al., 2003).

81

82 The obvious corollary to those observations, therefore, is that it is highly likely that a similar

83 temporal manner of regulation is being employed in the uterus ensuring parturition occurs at

84 the correct juncture. The study described herein examines where NF-κB complexes bind to

85 chromatin in myometrial cells and whether this binding influenced gene expression in such

86 cells. Essentially this allows us to determine if NF-κB promoter occupancy is associated with

87 transcriptional activation, transcriptional repression or homeostasis.

88

89 **Materials and Methods**

90 **PHM1-31 Cell Passaging**

91 PHM1-31 immortalised human myometrial myocytes were the kind gift of Prof. Barbara

92 Sanborn, Colorado State University, USA (Monga et al. 1996). Cells were cultured in

93 Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) FCS and 2 mM

94 ʟ-glutamine and 0.1 mg/ml Geneticin using published cell culture procedures (Chapman et al.

95 2005, Webster et al., 2013, Waite et al., 2014).

96

97   **Transient Transfections, Plasmids and Luciferase Assays**

98   Transient transfection of PHM1-31 myometrial cells was performed using the LT-1 reagent

99   from Miras (Geneflow, Staffordshire UK) as described by Chapman et al., (2005) for primary

100   myometrial cells. The 3x-κB-ConA-luciferase (3x-κB-ConA-Luc) and enh-ConA-luciferase

101   (ΔκB-ConA-Luc) vectors were the generous gift of Prof. Ron Hay (University of Dundee,

102   U.K.) and the construction of these has been reported in detail (Rodriguez et al., 1996). All

103   transfection experiments were performed a minimum of three times and results are expressed

104   as the mean ± SEM. All data analyses were conducted on GraphPad Prism Version 5.02

105   (GraphPad Software, San Diego, California). Comparison of data from two matched samples

106   were compared using a paired, two-tailed t-test; $p < 0.05$ was considered statistically significant.

107

108   **RelA Immunocytochemistry in PHM1-31 Cells Following TNF Stimulation**

109   PHM1-31 cells were cultured in a 24-well plate, washed in PBS and fixed in 1% (v/v)

110   formaldehyde overnight at 4°C. Endogenous cellular peroxidase was quenched with 1% (v/v)

111   hydrogen peroxide for 10 minutes. The Vectastain® Elite ABC kit (Vector Labs) was used for

112   the following reactions. Endogenous biotin was blocked with PBS containing horse serum and

113   avidin for one hour at room temperature, followed by incubation with primary antibody (anti-

114   RelA, #sc-372, Santa Cruz Biotechnology Inc.) in antibody diluent and biotin at 4°C overnight.

115   Secondary anti-mouse IgG (Dako) was added for 30 minutes at room temperature before the

116   addition of the ABC reagent for 30 minutes at room temperature and finally DAB (3, 3'-

117   diaminobenzidine). Cells were stored in PBS and photographed. Negative control experiments

118   included the substitution of the primary antibody with an isotype control (Abcam, # ab46450).

119

120   **Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assay (EMSA)**

121 Nuclear extracts were prepared essentially as described in Dignam et al., (1983). In this study,

122 the EMSA utilised an oligonucleotide consisting of the HIV-1 3'long terminal repeat (LTR)

123 κB site (in bold; 5'-GATCCGCTG**GGGACTTTCC**AGGCG-3'). The EMSA was carried out

124 as detailed in Chapman et al., (2002 and 2005).

125

126 **Western Immunodetection**

127 Expression of the RelA NF-κB subunit was examined using Western analysis with

128 immunoblots probed with antibodies that recognize either the amino terminal or carboxy

129 terminal of RelA (p65) (Santa Cruz Biotechnology Inc. Santa Cruz CA #sc-109 and #sc-372

130 respectively) and developed using EZ-ECL detection reagents (Geneflow, Staffs. U.K.) as

131 detailed in Chapman et al., (2004).

132

133 **Chromatin Immunoprecipitation (ChIP) Assay**

134 The ChIP assay was performed on eight T-75 flasks of PHM1-31 cells (~2.6-2.8x10^6

135 cells/flask) grown to 100% confluence using the Magna-ChIP ChIP assay kit (#17-611,

136 Millipore U.K. Ltd. Dundee) following the manufacturer's guidelines and detailed in Webster

137 et al., (2013). ChIP antibodies used were RelA and RNA Polymerase II (#sc-372 and #sc-899

138 respectively; Santa Cruz Biotechnology Inc. Santa Cruz CA). Briefly, four flasks were

139 stimulated with 10ng/ml TNF for one hour while the remaining four were unstimulated

140 controls. TNF has been demonstrated to be present in myometrium at term (Opsjln et al., 1993;

141 Fitzgibbon et al. 2009; reviewed in Golightly et al., 2011) and has been used regularly by our

142 group studying cytokine-induced myometrial NF-κB function (Chapman et al., 2005, Webster

143 et al., 2013; Waite et al., 2014). The rationale for this time point was that it would represent an

144 early response to TNF. We believe such early binding events play pivotal roles in the cell's

145   choice of subsequent signalling pathway usage (reviewed in Perkins 2007). It was accepted

146   that TNF-induced gene regulation events occurring after one hour would not be investigated

147   (Campbell et al., 2001; Rocha et al., 2003). Three biological replicates of these ChIP assays

148   were completed. The work-flow utilised to generate the appropriate chromatin samples is

149   illustrated in Figure 1.

150

151   **Quality Control PCR of Immunoprecipitated DNA**

152   Prior to microarray analyses, ChIP efficacy was determined by enrichment of RelA on the

153   IκBα promoter. PCR was carried out on the immunoprecipitated DNA using primers flanking

154   the κB sites within the IκBα promoter as a positive control (Chapman et al. 2005). The Gαs

155   promoter, which is not regulated by RelA was chosen as a negative control (Webster et al.,

156   2013). Once it was determined that the chromatin was of sufficiently high quality, it was then

157   prepared ready to probe Affymetrix 1.0R Human promoter Arrays (Affymetrix, Santa Clara,

158   CA).

159

160   **Affymetrix Microarrays**

161   Full details of both arrays employed in this study can be found at the manufacturer's web site:

162   GeneChip Human Promoter 1.0R Array:

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

164   1

165   Human Genome U133 Plus 2.0 Array:

166   http://www.affymetrix.com/catalog/131455/AFFY/Human+Genome+U133+Plus+2.0+Array

167   #1_1

168

169   **ChIP DNA Amplification, Fragmentation and Labelling**

170   ChIP DNA was amplified using the Whole Genome Amplification Kit (WGA; Sigma) as

171   detailed in the manufacturer's instructions with slight modifications. Briefly, 1 µl of ChIP DNA

172   was diluted with 9 µl of ultrapure water. Then 2 µl of library preparation buffer together with

173   1 µl of library stabilisation solution was added to this and heated at 95ºC for 2 minutes before

174   cooling on ice. After that, 1 µl of library preparation enzyme was added and the reaction was

175   incubated in the thermal cycler for the following times: 20 minutes at 16ºC (pre-cooled to this

176   temperature), 20 minutes at 20ºC, 20 minutes at 37ºC, 5 minutes at 75ºC, and on hold at 4ºC.

177   The amplified DNA was re-amplified using the WGA re-amplification kit to generate the 7.5

178   µg required. This method is described in the manufacturer's guidelines (Sigma). The re-

179   amplified DNA was then fragmented and labelled according to the Affymetrix ChIP Protocol

180   using the GeneChip WT Double-Stranded DNA Terminal Labelling Kit.

181

182   **Human Promoter 1.0R Array Procedures**

183   Hybridisation of amplified DNA to Affymetrix Human Promoter 1.0R arrays was carried out

184   using the GeneChip Hybridization, Wash and Stain kit (Affymetrix) according to the

185   Affymetrix ChIP Protocol. The hybridisation cocktail (7.5 µg fragmented labelled DNA, 50pM

186   control oligonucleotide B2, hybridisation mix, 7% (v/v) DMSO) was hybridised in the

187   Affymetrix GeneChip Hybridisation Oven 640. Washing and staining was carried out using

188   the GeneChip Fluidics Station 450 as described in the GeneChip Expression Wash, Stain and

189   Scan User manual (Affymetrix). The GeneChip Scanner 3000 7G, operated by the GeneChip

190   Opertating Software (GCOS, Affymetrix), was used to scan the Human Promoter 1.0R Arrays.

191

192 **Data Analysis in Partek Genomics Suite 6.6**

193 The raw data (.CEL) files, generated by the GCOS software, were imported into Partek

194 Genomics Suite (PGS; Version 6.6; www.partek.com/pgs) and subjected to Robust Multi-array

195 Average (RMA) background correction, quantile normalisation and Log (base$_2$) transformation

196 utilising the software's tiling workflow. Prior to invoking an ANOVA, the data were

197 normalised to the baseline by subtraction of all values ascribed to IgG samples from both

198 unstimulated controls and TNF-treated samples since these values would represent non-

199 specific binding events (Fig.1; illustration of work-flow). A two-way ANOVA was then

200 completed within PGS to undertake multiple comparisons and determine the difference

201 between unstimulated controls and TNF-treated samples at probe-level. Differences between

202 control and TNF-stimulated samples were considered significant if $p \leqslant 0.05$.

203

204 Upon completion of the ANOVA, the Model-based Analysis of Tiling (MAT) algorithm (T

205 statistic) was employed to detect enriched regions of chromatin in un-stimulated and TNF-

206 stimulated data sets (Johnson et al. 2006). The MAT algorithm allows a rapid method of

207 detecting regions enriched by a given transcription factor (in this study RelA-containing NF-

208 κB dimers). The MAT algorithm was then applied across a sliding window of 600bp, using a

209 minimum of 10 probes per region with ChIP-enriched regions deemed to be statistically

210 significant when $p \leqslant 0.01$: this output was the MAT score. Those regions with a positive MAT

211 score and p value $\leqslant 0.05$ from the ANOVA indicated significant enrichment by anti-RelA

212 antiserum in those samples treated with TNF compared to untreated controls. Conversely, a

213 negative MAT score and p value $\leqslant 0.05$ from the ANOVA represented those samples where

214 enrichment of genomic loci was greater in unstimulated controls compared to those exposed to

215 TNF. Promoters of known genes within the significantly enriched regions were then identified

216   using the RefSeq database, based on the hg18 build of the human genome. All of the array data

217   has been archived with the NCBI Gene Expression Omnibus (NCBI GEO) with the accession

218   number GSE65721 (www.ncbi.nlm.nih.gov/geo); this number covers all associated

219   experimental sub-series. All tables of original data sets can be accessed and down-loaded from

220   the folder entitled Cookson et al 2015 Public Access MHR Original Data Sets at the following

221   hyperlink:

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

223

RNA Extraction

225   On reaching 90% confluence, PHM1-31 cells were stimulated with 10ng/ml TNF for one hour

226   with non-stimulated flasks serving as controls. RNA was extracted using the EZ-RNA

227   extraction system (Geneflow, Staffs. U.K.) and quantified using the nanophotometer (Implen;

228   supplied by Geneflow, Staffs. U.K.). Prior to first strand synthesis, the quality of isolated RNA

229   was verified using Agilent's Eukaryote Total RNA Nano Chip (5067-1511) in conjunction with

230   the Agilent 2100 bioanalyser following the manufacturer's guidelines. Three biological

231   replicates were performed.

Affymetrix U133plus2 Human Expression Array Procedures

233   RNA was reverse transcribed using Bio-Rad iScript cDNA synthesis Kit according to the

234   manufacturer's guidelines (Bio-Rad Laboratories Ltd. Hertfordshire U.K.). The preparation

235   and hybridisation of cDNA to U133Plus2 expression arrays was performed according to

236   published protocols (Kirby et al. 2011; Simpson et al., 2011; Brockington et al., 2013; Raman

237   et al., 2015).

238

Data Analysis in Partek Genomics Suite 6.6

240   The raw data files (.CEL) were imported into PGS V6.6 and analysed following the software's

241   Gene Expression workflow. Differentially expressed genes were identified using ANOVA to

242   generate p values. Linear contrast was used to calculate fold-change and mean ratio from the

243   contrast between unstimulated and TNF stimulated samples. Genes with fold change ≥ 2 or

244   fold change ≤ -1.5 and with p values ≤ 0.05 were identified using RefSeq.

245

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

247   **Arrays**

248   Using PGS, the enriched region list from both Criteria-A and Criteria-C were merged with the

249   respective gene list from the expression array analyses. This provided data defining NF-κB-

250   enriched regions that were either expressed or repressed.

251

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

253   Quantitative RT-PCR using SYBR Green Jumpstart Taq ReadyMix (Sigma, UK) was

254   undertaken following MIQE guidelines (Bustin et al. 2009). GAPDH and β-Actin were

255   selected as housekeeping genes. The primer sequences were as follows:

256   TNFAIP3 Forward: 5'-TGAGCCCTTGGCGTGGAACC-3';

257   TNFAIP3 Reverse: 5'-AAAGGGCTGGGTGCTGTCGG-3';

258   NFKBIA   Forward: 5'- CGCCCAAGCACCCGGATACA-3'

259   NFKBIA Reverse: 5'- GGGCAGCTCGTCCTCTGTGA-3';

260   GAPDH Forward: 5'-TGTTCGACAGTCAGCCGCATCT-3';

261   GAPDH Reverse: 5'-CAGGCGCCCAATACGACCAAATC-3';

262   β-Actin Forward: 5'-CGAGCACAGAGCCTCGCCTT-3'

263   β-Actin Reverse: 5'- CGAGCACAGAGCCTCGCCTT -3'.

264   qPCR was performed in triplicate using a 7900HT fast qPCR Machine (Applied Biosystems)

265   and gene expression data were analysed using ΔΔCT using SDS 2.0 Software (Applied

266   Biosystems).

267

268   **Results**

269   **TNF induces RelA Nuclear Localisation and Occupancy of the IκBα Promoter in PHM1-**

270   **31 Cells**

271   A number of pro-inflammatory cytokines, including TNF, are associated with the onset of both

272   normal and preterm birth (Aguilar and Mitchell, 2010; Golightly et al., 2011). Prior to the

273   ChIP-on-chip experiments, it was important to confirm that in PHM1-31 cells, NF-κB RelA

274   was activated by stimulation with TNF. RelA immunocytochemistry of PHM1-31 cells showed

275   diffuse staining in the control, unstimulated cells and those stained with control IgG, (Fig. 2A;

276   Panels I and II) whilst translocation of the RelA subunit into the nucleus (black arrows) was

277   apparent following 1 hour TNF stimulation (Fig. 2A; Panel III). Moreover, increased RelA

278   binding to the consensus κB site in EMSA was also seen and that binding activity could be

279   specifically super-shifted with anti-RelA antiserum (Fig. 2B). Furthermore, when PHM1-31

280   cells were transiently transfected with the RelA-responsive 3x-κB-luc reporter and

281   subsequently exposed to TNF for one hour, increased reporter activity was seen in those cells

282   harbouring the 3x-κB-luc vector, but not those with the ΔκB-luc control (Fig. 2C).

283

284   Immunoprecipitation using the RelA antiserum (sc-372) showed specific binding to the RelA

285   protein, while the control IgG failed to precipitate any RelA complexes (Fig. 2D). To

286   demonstrate the specificity of the ChIP, the RelA antiserum detected low level binding of RelA

287   complexes to the IκBα promoter region without TNF stimulation; as predicted, this increased

288   upon TNF exposure. No binding was seen at the RelA-insensitive Gαs promoter. Supporting

289   this, after exposure to TNF, RNA PolII binding was also observed at the IκBα promoter. These

290   results illustrate that the immunoprecipitation and ChIP methods were specific for the RelA

291   NF-κB subunit. Interestingly, while we see a low-level of NF-κB occupancy of the IκBα

292   promoter under unstimulated conditions, we could not detect a similar pattern of

293   immunostaining (Fig. 2A, Panels I and II).

294

295   **TNF Induces RelA NF-*κ*B Promoter Occupancy at a Variety of Promoters**

296   In this study of PHM1-31 myometrial myocytes, statistically significant differences in

297   promoter occupancy between unstimulated and TNF-treated cultures were observed in 24,410

298   genomic regions enriched by the anti-RelA antiserum ($p \leq 0.05$); these data were termed

299   Criteria-B and represent all enriched regions from both unstimulated and TNF-treated cultures

300   (Fig. 3A; Original Data Set - Table 1). Within the set Criteria-B, defining the MAT algorithm

301   parameters to enriched values of >0 and $p \leq 0.01$ (i.e. a positive MAT score) generated 13,300

302   genomic loci that were significantly more enriched by the anti-RelA antiserum in TNF-treated

303   cells compared to unstimulated controls; this subset was termed Criteria-A (Fig. 3A; Original

304   Data Set - Table 2) and represents TNF-induced enrichment.

305

306   Similarly within the Criteria-B dataset, defining the MAT algorithm parameters to enriched

307   values of <0 and $p \leq 0.01$ (i.e. a negative MAT score), we identified 11,110 genomic regions

308   that were significantly more enriched by the anti-RelA antiserum in the unstimulated control

309   set compared to the TNF-treated samples. This subset was termed Criteria-C and represents

310   unstimulated enrichment. Therefore, in this study, the Criteria-C dataset identifies genomic

311 loci bound by NF-κB dimers containing the RelA subunit (homo- or heterodimers of RelA)

312 when the cell population is not exposed to an exogenous stimulant such as TNF (Fig. 3A;

313 Original Data Set - Table 3). While we cannot rule out the possibility that some RelA-enriched

314 regions may be non-specific, the removal of the IgG-associated regions will minimise such

315 interference.

316

317 RelA-enriched loci from both Criteria-A and Criteria-C datasets represent given regions of

318 chromatin and thus may map to different aspects of a gene including exons and introns. Fig.

319 3B illustrates a schematic representation of chromosome 14 (the IκBα locus) illustrating such

320 differentially enriched regions listed in either Criteria-A (TNF-induced; red vertical lines) or

321 Criteria-C (unstimulated; blue vertical lines) datasets.

322

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

324 Enrichment of chromatin from TNF-treated cells by anti-RelA serum was seen to contain or be

325 juxtaposed to regions encoding many genes believed to play a significant role in myometrial

326 quiescence or labour itself including, but not limited to, PTGS2, Jun, IL6RN, IL6, CACNB3,

327 KCNMB3, TRPC2 and VCAM-1 (Original Data Set - Table 4; Criteria-A and Original Data

328 Set - Table 5; Criteria-C). Figure 4 illustrates examples of genes identified in RelA-enriched

329 chromatin after cells were stimulated with TNF. Of these, regions harbouring PTGS2 and Jun

330 were enriched in the presence of TNF (Figs. 4A-4B; Criteria-A regions are red bars) and those

331 regions also encoded κB motif(s) that were in agreement with the published κB consensus site.

332 For PTGS2 a region was also enriched in the absence of TNF but no consensus κB motif was

333 identified therein. Interestingly, genes encoding IL6RN and KCNMB3 were also enriched in

334 the presence of TNF but no discernible consensus κB motif could be identified in those

335   respective Criteria-A enriched regions, suggesting a non-consensus κB motif was being

336   utilised (Figs. 4C-4D; red bars). Moreover, we also observed that intronic regions of both

337   IL6RN and KCNMB3 were also enriched by RelA NF-κB in the absence of TNF stimulation

338   suggesting that RelA-containing dimers do have a function in governing expression of these

339   genes (Figs. 4C-4D; blue bars). Finally, regions that were only enriched by RelA-containing

340   dimers in the absence of TNF (i.e. unstimulated) were also examined. Examples of such regions

341   were those encoding regulatory subunits of calcium and potassium channels such as CACNB3

342   and KCNB1 (Figs. 4E-4F; blue bars). Significantly, regions encoding these ion channel

343   subunits also harboured κB motifs corresponding to the consensus κB sequence (Figs. 4E-4F).

344

345   To ensure our experimental system was functioning correctly, we also examined the NF-κB-

346   regulated the IκBα promoter region (LeBail et al., 1993). As expected, in TNF-treated cells,

347   aspects of the IκBα promoter were enriched by anti-RelA serum. Interestingly, however, no

348   enrichment of the IκBα promoter was observed in the absence of TNF (Fig. 5A) despite this

349   region being weakly amplified in the control ChIP assay (Fig. 2E). Increasing the resolution of

350   the schematic representation in Fig. 5A illustrates two regions within the IκBα gene enriched

351   in TNF-treated cells (Fig. 5B). The region upstream of the IκBα transcription start site was

352   also seen to encode the three κB motifs believed to be responsible for governing IκBα

353   expression (LeBail et al., 1993). A second intra-genic region within IκBα open reading frame

354   was seen to be enriched in TNF-treated cells although no consensus κB motifs were identified

355   therein (Fig. 5B). In contrast to genes in close proximity to the anti-RelA serum enriched loci,

356   other enriched regions were decidedly more remote from the nearest genes (Original Data Set

357 - Table 4, Criteria-A and Original Data Set - Table 5, Criteria-C; full annotated gene lists

358 associated with these enriched regions can also be viewed therein).

359

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

361 In the Criteria-A dataset (i.e. RelA-enriched regions in response to TNF), 1,667 occurrences

362 of the κB consensus sequence were identified, defined by 112 different sequence

363 representations (Original Data Set - Table 6). Of the 1,667 occurrences, 1,604 resided in non-

364 repetitive genomic regions. To aid clarity, we focussed the study on those motifs from the non-

365 repetitive regions. These motifs were defined by 65 different sequence instances (Table 1). The

366 remaining 63 occurrences, whilst not studied further, were defined by 47 different sequence

367 instances many of which were also observed in the non-repetitive dataset (data not shown). Of

368 the 1,667 consensus κB motif occurrences in the Criteria-A dataset, 770 (46.2%) were

369 identified within intronic regions (Original Data Set - Table 6).

370

371 In the Criteria-C data set (i.e. RelA-enriched regions in absence of TNF), the consensus κB

372 motif occurred 2,116 times defined by 103 different sequence representations (Original Data

373 Set - Table 7). Of the 2,116 occurrences, 2,064 were seen to reside in non-repetitive elements

374 of the genome. These were chosen for further study. In turn, these motifs were also represented

375 by 65 different sequence instances (Table 1). The remaining 52 occurrences, whilst not studied

376 further as they were from highly repetitive sequences, were defined by 38 different sequence

377 instances many of which were also observed in the non-repetitive dataset (data not shown). Of

378 the 2,116 consensus κB motif occurrences in the Criteria-C dataset, 1,089 (51.5%) were

379 identified within intronic regions (Original Data Set - Table 7).

380

381　Overall, the consensus κB motif was observed a total of 3,783 times in anti-RelA serum-

382　enriched genomic regions with 3,720 κB motifs identified in non-repetitive elements of the

383　genome of PHM1-31 myometrial myocytes. Alignment of the 65 representations of the κB

384　sequence was performed using WebLogo 3 open access software (Crooks et al. 2004;

385　http://weblogo.threeplusone.com/). Consensus κB sequence variability was seen to be

386　5'GGG(A/G)(A/C/T/G)(C/T)(C/T)(C/T)CC-3' but no difference was observed with this

387　between Criteria-A (TNF-induced enrichment) and Criteria-C (un-stimulated enrichment)

388　(Fig. 6).

389

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

391　As illustrated thus far, promoter occupancy by RelA-containing NF-κB dimers per se, does not

392　provide information on associated transcriptional levels (i.e. expressed, repressed or quiescent;

393　akin to chromatin-bound RNA polymerase II and promoter-proximal stalling (Core and Lis,

394　2009)). Consequently, we undertook expression array analyses in PHM1-31 cells utilising total

395　RNA extracted from cells exposed to TNF for one hour (an identical time course to those used

396　to isolated chromatin for ChIP-on-chip studies).

397

398　A statistically significant change in expression of 2,963 genes was induced by TNF

399　(Supplementary Information - Data Table 8 p-value region<0.05). Expression of six genes

400　remained unchanged between unstimulated and TNF-treated cells (SLC35F5, DEFB106A,

401　RFTN1, DTWD2, SLC34A3 and SEZ6L). Removal of duplicates from the original data gave

402　a total of 2,223 genes that were differentially expressed when PHM1-31 cells were stimulated

403　with TNF for one hour (Fig. 7; Criteria-B1, p<0.05; and Original Data Set - Table 8). Of this

404　total, 51 genes were seen to have a fold-induction of >2. Of these, two were discounted as they

405   were not annotated, leaving a total of 49 genes; these were termed Criteria-A1. Physiologically

406   relevant genes identified in this dataset were EGR 1-4, FOS, FOSB, JUN, JUNB, ATF3,

407   NFKIBIA, NFKBIZ, TNFAIP3, COX2, CXCL2, CXCL3, CCL20, LIF, IL-6, MAP3K8,

408   THBS1 and TNF. Eighteen genes were seen to have a fold repression of >1.5. Of these, one

409   was not annotated and was thus excluded leaving a total of 17 genes; these were termed

410   Criteria-C1 (Fig. 7). Included in this dataset were HOXA11, COL1A2 and STAT2. To

411   summarise, Table II lists those genes whose expression was induced by at least two-fold in

412   response to TNF while Table III lists those genes subject to repression by at least 1.5 fold in

413   the presence of TNF. Further details from the expression array analyses can be viewed in

414   (Original Data Set - Tables 8-10).

415

416   **Combined Analysis of PHM1-31 Promoter Occupancy and Gene Expression**

417   Merging the Criteria-A TNF-enriched promoter dataset with Criteria-A1 (TNF-induced gene

418   expression), 14 TNF-induced, RelA-enriched genomic regions from the promoter array screen

419   also encoded TNF-inducible genes (Table II; genes annotated with * and Supplementary

420   Information - Data Table 11). These included known NF-κB-regulated genes IL-6, Jun,

421   NFKBIA, PTGS2, TNC and TNFAIP3. Genes not yet conclusively demonstrated to be under

422   NF-κB control included DUSP-2, DUSP-5, ERRFI1, THBS1 and CTGF (Table II; genes

423   annotated with * and Original Data Set - Table 11). Moreover, by combining the Criteria-A

424   promoter array dataset with the expression array Criteria-C1 dataset (TNF-repressed genes),

425   we also identified five RelA-enriched genomic regions that harboured TNF-repressed genes,

426   including COL1A2 (Table III; genes annotated with * and Original Data Set - Table 12).

427

428   **Gene Ontology and Validation of Selected RelA-enriched Regions Modulated by TNF**

429   PGS Gene Ontology analysis identified two functional groups of interest in the Biological

430   Processes category: Intra-cellular Signal Transduction and Cell Surface Receptor Protein

431   Signalling. These included TNFAIP3 (Cytokine-Induced Signalling) and IκBα (NIK/NF-κB

432   Signalling; Fig. 8A). These targets were subsequently used to validate the expression array data

433   because both have important roles in governing TNF signalling and NF-κB function

434   respectively (Perkins, 2007; Chen and Ghosh, 1999; Hayden and Ghosh, 2012). Real-time

435   qRT-PCR on total RNA from PHM1-31 cells treated with TNF for one hour demonstrated that

436   both IκBα and TNFAIP3 were expressed in response to TNF. Good agreement was observed

437   for induction of gene expression for IκBα (Fig. 8B; 2.94 on array vs. 4.43 for qRT-PCR) while

438   a slightly greater margin of difference was noted for TNFAIP3 expression (Fig. 6B; 7.96 on

439   array vs. 3.85 for qRT-PCR).

440

441   **Discussion**

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

443   This report is the first to describe a promoter array-based approach to define chromatin regions

444   of myometrial myocytes occupied by RelA-containing NF-κB dimers. Our data demonstrated

445   that in PHM1-31 myometrial cells, NF-κB-mediated enrichment of 13,300 chromatin regions

446   in the presence of TNF and 11,110 in unstimulated cells. Some of these regions were

447   juxtaposed to genes known to function in human labour, for example PTGST2 (Chan et al.,

448   2014) and KCNMB3. NF-κB-controlled regulation of PTGS2 in the myometrium and amnion

449   is well documented (Allport et al., 2001; Soloff et al., 2004; Lindström and Bennett, 2005)

450   while a putative role in governing gene activity of the potassium channel subunit KCNMB3 is

451   less so. In contrast, other RelA-enriched regions were decidedly more remote from the nearest

452   transcription start with distances being measured in numbers of kilobases. Many of these loci

453   were also noted to be within intronic regions as discussed below. We did not examine the

454   influence of such remote binding events in this study but we cannot rule out that they represent

455   enhancer sequences or non-coding RNA transcriptional units. Indeed, supporting this notion

456   are the observations that many transcription factor binding sites are arranged many kilobases

457   from the transcription start site of the genes they regulate (Deaton and Bird, 2011).

458

459   **κB Binding Site Loci**

460   Intriguingly, RelA appeared to exhibit stimulus dependent binding to different loci of the same

461   gene; this was evidenced with PTGS2, IL6RN and KCNMB3. In contrast, however, a section

462   of the promoter region for Jun was only enriched in the presence of TNF while for CACNB3

463   and KCNB1, sections of promoter regions for these respective genes were only enriched by

464   RelA-containing dimers in the absence of TNF; the molecular mechanisms accounting for these

465   observations remain to be established. We did, however, determine that in both datasets, the

466   consensus κB motif was represented a total of 3,783 times, including 1,859 (49.14%) sites

467   identified within intronic regions. A number of studies in various cell lines have reported such

468   intronic binding by NF-κB (Martone et al., 2003; Schreiber et al., 2006; Lim et al., 2007; Wong

469   et al., 2011; Satohn, 2013; Xing et al., 2013) and, given that the early work describing its

470   function demonstrated it was bound to the first intron of the κ-light chain enhancer (Schjerven

471   et al., 2001), our observations support such previous data. Interestingly there are reports of

472   transcription of certain regulatory proteins initiating from within the 3'-intronic regions of the

473   parental gene. The calcium channel associated transcriptional regulator (CCAT), for example,

474   is generated through initiation of independent transcription of exons 46 and 47 at a 3'-intronic

475   site of the parental $C_{av}1.2$ calcium channel gene (Gomez-Ospina et al., 2013). Clearly, our data

476   does not illustrate if such NF-κB binding directly modifies gene activity in this manner but it

477   offers a likely rationale for such intronic binding, perhaps as a means of governing post-

478   transcriptional RNA splicing, and a further avenue for investigating the complexities of

479   myometrial gene activity as labour commences. Due to the apparent promiscuity of RelA

480   binding to multiple loci and limited sensitivity of the ChIP-on-chip methodology, ChIP-exo

481   (see below) would be a superior means by which to narrow down the precise binding location

482   of RelA-containing dimers to near single base pair resolution (Rhee and Pugh, 2012).

483

484   **Selection of κB Motifs**

485   The consensus κB binding motif is viewed as 5'-$G_{-5}G_{-4}G_{-3}R_{-2}N_{-1}Y_{0}Y_{+1}Y_{+2}C_{+3}C_{+4}$-3' with

486   many functional variants on this being reported (Perkins, 2007; Hayden and Ghosh, 2012; Chen

487   and Ghosh, 1999). This motif does offer a level of subunit selectivity and crystallographic

488   studies of various NF-κB dimers bound to different κB DNA sequences support this (Ghosh et

489   al., 1995; Müller et al., 1995; Cramer et al., 1997; Huang et al., 1997; Chen et al., 1998a; Chen

490   et al., 1998b; Phelps et al., 2000; Huang et al., 2005; Moorthy et al., 2007; Trinh et al., 2008).

491   The actual κB DNA sequence clearly does impose binding constraints upon certain dimers; for

492   example, within the κB motif, the 5'-$G_{-5}G_{-4}G_{-3}R_{-2}N_{-1}$-3' half site is bound by p50; in contrast,

493   the 5'-$Y_{+1}Y_{+2}C_{+3}C_{+4}$-3' half site is necessary for RelA binding (Huang et al., 2005 and

494   references therein). Whether region-enrichment is dimer-specific and dependent on the nature

495   of the stimulus (in this case TNF or not) could not be determined herein. Since we focussed on

496   immunoprecipitated RelA, this would recover four possible groups of RelA-containing dimers;

497   RelA:RelA, RelA:c-Rel RelA:p50 and RelA:p52 (RelA:RelB heterodimers are not thought to

498   bind DNA; Marienfeld et al., 2003). Given the published physical constraints imposed upon

499   certain NF-κB dimer:κB motif interactions (Phelps et al., 2000; Huang et al., 2005) one could

500   propose that, at this time-point of one hour, it is likely the RelA:p50 or RelA:p52 heterodimers

501   are being physiologically favoured. Given that we focussed on RelA, the obvious confounder

502   of our work is that those enriched regions would likely reflect only contributions from such

503   RelA-containing dimers; other non-RelA dimers would be missed. As above, further studies

504   employing ChIP-seq would be required to provide the higher resolution data defining whether

505   dimer composition on individual promoters/loci changed over multiple time points.

506

507   **Limitations of ChIP-on-chip**

508   While ChIP-on-chip is a robust, well-documented method to analyse transcription factor-

509   mediated chromatin enrichment on a whole genome scale, it is associated with limitations when

510   compared with more recent sequencing technologies including ChIP-seq (reviewed in Hurd

511   and Nelson, 2009; Park, 2009). The obvious corollary, therefore, is that we cannot rule out the

512   possibility that bias occurred in our system and influenced the data presented herein. A

513   comparison between both ChIP-on-chip and ChIP-seq methodologies has been highlighted in

514   Ho et al. (2011) who address the limitation and benefits associated with both technologies.

515   With whole genome sequencing now available in most institutions, and methods including

516   ChIP-exo (essentially ChIP-seq but using lambda exonuclease to trim the immunoprecipitated

517   DNA to within a few base pairs of the binding residues on a given transcription factor) being

518   developed, the next step would be to conduct ChIP-seq with all NF-κB subunit antisera, at

519   different time points to obtain an unbiased genome-wide signature of binding events taking

520   place within myometrial cells.

521

522   In our study, we employed the GeneChip 1.0R Human Promoter array. This contains 25,500

523   human promoter regions but it lacks full genomic coverage of the corresponding tiling arrays.

524   Consequently, we cannot rule out the possibility that the low correlation between region

525   enrichment and subsequent transcription expression/repression could arise because the Human

526   1.0R Promoter array was not fully representative of the complete human genome.

527

528   PHM1-31 cultures were not cell-cycle synchronised prior to the ChIP assay because the

529   induction of synchronisation itself (serum starvation) has been documented to effect expression

530   of key genes involved in cell function and ion transport including the MaxiK potassium channel

531   as well both the L- and T-type calcium channels (Woodfork et al., 1995; Panner et al., 2005;

532   Patel et al., 2005). Essentially, potassium channels are responsible for hyperpolarising the

533   plasma membrane, an event necessary for the cells to move from G1 to S (Wonderlin and

534   Strobl, 1996; Ouadid-Ahidouch and Ahidouch, 2013). Calcium channels are thought to provide

535   transient signals at checkpoints within the cell cycle which are necessary for the cell to continue

536   cycling (Whitaker, 2006). We believe it highly likely that these events would have influenced

537   those TNF-induced effects reported herein (Perkins, 2012).

538

539   **Promoter Occupancy and Transcriptional Activity**

540   Occupancy of a given promoter by NF-κB does not necessarily mean transcriptional activity

541   directed by that promoter will change and there is robust data to support this notion (Hoffman

542   et al., 2003; Leung et al., 2004; Wan and Lenardo, 2009; Wang et al., 2012). Of the TNF-

543   induced NF-κB-enriched regions identified in our work, 14 were correlated with an increase in

544   gene expression while five targets were repressed. Therefore, one must ask why the disparity

545   between the number of enriched regions and the number of genes with altered activity? Clearly

546   under normal physiological conditions, the myometrium would be bathed in a milieu rich in

547   cytokines including IL-1β, IL-6, IL-8 and TNF to name but a few (Aguilar and Mitchell, 2010;

548   Golightly et al., 2011; Webster et al., 2013). The manner by which these other proinflammatory

549   stimulants influence NF-κB activity was not examined herein but it is reasonable, based on

550   published evidence, to assume these factors would also moderate myometrial gene activity.

551   Secondly, it is without doubt that we will have missed many key binding events at promoters

552   at immediate early time points, ranging from seconds to minutes, as well as more prolonged

553   stimuli after a number of hours. Indeed, this approach may underlie why we did not observe

554   enrichment of regions that encoded genes for IL-1β, oxytocin or oxytocin receptor, all of which

555   have previously been shown to require NF-κB for induction of expression (Belt et al., 1999;

556   Lee et al., 2003; Soloff et al., 2006; Terzidou et al., 2006).

557

558   **Differential Gene Expression**

559   In the context of premature birth research, many authors have published expression array

560   studies in attempts to define genes responsible for promoting myometrial quiescence and

561   myometrial contraction. Such studies have focussed on native tissues (amnion, decidua and

562   myometrium) as well as both immortalised and primary cell lines (Charpigny et al., 2003;

563   Bethin et al., 2003; Bailey and Europe-Finner, 2005; Bailey et al., 2005; Esplin et al., 2005;

564   Havelock et al., 2005; Han et al., 2008; Khanjani et al., 2011; Lim et al., 2012).

565

566   A recent study by Chan et al. (Chan et al., 2014), used a robust RNA-seq-based approached to

567   define 764 differentially expressed genes in human myometrium from pregnant, non-labouring

568   women and those women in active labour. Salient examples of up-regulated genes from that

569   list included IL6, IL8, IL13, MCP, enzymes governing prostaglandin biosynthesis (PTGS2),

570   THBS2, DUSP family members, members of the NF-κB family of proteins, and intermediates

571   in the TGF-β and TNF-signalling pathway (Chan et al., 2014). Significantly, our study of TNF-

572   stimulated differential gene expression identified 49 induced genes; of these expressed genes,

573   17 were also represented in the list of genes up-regulated in labouring human myometrium

574   documented by Chan et al. (Chan et al., 2014). Furthermore, 14 of the TNF-induced genes

575   were also seen to be in regions of chromatin enriched by RelA. This is a key observation

576   because it indirectly validates our own expression array work. Moreover, it supports the notion

577   that NF-κB plays a pivotal role in controlling expression of genes involved in human

578   parturition since many of those targets identified by Chan et al. are documented to be regulated

579   by NF-κB (www.bu.edu/nf-kb/gene-resources/target-genes/).

580

581   Interestingly, a recent meta-analysis of gestational tissue-based transcriptomic studies

582   highlighted significant variation in expression of individual genetic regions; essentially only

583   23 common sites were identified out of 10,993 unique transcriptionally active units (Eidem et

584   al., 2015). Focussing on studies of myometrial gene expression, Eidem et al. identified 15

585   genes present in four or more studies. In our study, four TNF-induced genes were also present

586   in the myometrial group identified by Eidem et al., including FOSB, NR4A1, LIF and PTGS2.

587   Moreover, those genes reported to act as biomarkers of pre-term birth, including IL6 and TNF,

588   were also represented in our work giving further validation of the TNF-induced gene

589   expression data presented herein (Table II and Eidem et al., 2015). The meta-analysis described

590   by Eidem et al., does not, however, consider changes in promoter occupancy of those gene

591   targets, essentially because, other than our work, there are no data in the reproductive field

592   describing such investigations.

593

594   **TNF-Induced Gene Expression**

595   In terms of NF-κB function, the NFKBIA gene, which encodes the IκBα protein, was induced

596   2.9 fold by TNF. As our positive control, this observation also validates the data presented

597   here. A second IκB family member, NFKBIZ, which encodes the IκBζ protein, was induced

598   5.2-fold in response to TNF. Significantly, other groups (Eto et al., 2003) have observed that

599   IκBζ induction is not TNF-mediated. The differences in these observations may be accounted

600   for through cell-type specific effects (macrophage or kidney versus myometrium) but it is

601   noteworthy that one effector, TNF, can exert diametrically opposed effects on the same gene

602   in different cell types, suggesting other nuclear-based factors are influencing the NF-κB-

603   mediated gene regulation process. Significantly, IκBζ is known to bind specifically to p50

604   homodimers forming a robust ternary complex on the IL6 promoter activating expression of

605   this gene (Trinh et al., 2008). Indeed, in our study TNF stimulation also caused a three-fold

606   induction of IL-6 expression and we speculate this is mediated by p50 homodimer:IκBζ

607   complex although further experimental analyses would be required to confirm this. TNFAIP3

608   (also termed A20) was also up-regulated. This protein also plays a significant role in

609   termination of the NF-κB signal by inhibiting NF-κB DNA binding (Perkins, 2012; Hayden

610   and Ghosh 2012).

611

612   Our study also identified various transcription factors that were significantly up-regulated in

613   response to TNF, including members of the early growth response transcription factor family,

614   Egr-1, -2, -3 and -4, as well as those of the AP1 family, namely ATF3, Fos, FosB, Jun, JunB.

615   The Egr family are well-described zinc-finger containing proteins recognising the consensus

616   sequence of 5-GCGG/TGGGCG-3' (Christy and Nathans, 1989). The function of the Egr

617   family in human myometrium is not clear but they have been shown to co-operate with the

618   RelA subunit of NF-κB in embryonic kidney cells through an interaction between the RelA

619   Rel Homology Domain and the zinc-finger region of Egr-1 (Chapman and Perkins, 2000), as

620   well as competing for Sp1 sites in pro-inflammatory promoters such as PDGF-B (Khachigian

621   et al., 1996). Many of the promoters identified in our study were GC-rich and up-regulation of

622   factors which readily bind to such regions offers a potential insight into the control of complex

623   myometrial gene expression networks.

624

625   Members of the AP1 transcription factor family, including Fos, FosB and Jun, bind to the

626   consensus AP1 motif 5'-TGAG/CTCA-3' or 5'-TGACGTCA-3' (Shaulian and Karin, 2002)

627   and have been shown to be differentially expressed in pregnant versus labouring rat

628   myometrium.. Importantly, studies in rat myometrium have described the differential

629   expression of members of this family, between pregnant and labouring states. The salient

630   observation is that peak levels of Fos, FosB, Jun and JunB occur during active labour (Mitchell

631   and Lye, 2002; Mohan et al., 2007); importantly, those observations are in keeping with our

632   data from human cells. Moreover, since Fos/Jun are immediate early genes (Shaulian and

633   Karin, 2002), they are likely regulators of more extensive transcriptional networks within the

634   cell and it is therefore interesting to speculate that NF-κB dimers may orchestrate a hierarchy

635   of transcriptional activity within the myometrial cell. Interestingly, NF-κB has also been shown

636   to play a key regulatory role in JunB expression as part of the JunB-mediated induction of

637   VEGF in response to hypoxia (Schmidt et al., 2007). It is well documented that uterine

638   contractions during labour do induce local hypoxic regions (Bugg et al., 2006); whether such

639   myometrial hypoxia initiates NF-κB-induced AP1 family expression remains unclear at present

640   but induction of such factors may be how the uterus responds to such a hypoxic stress and

641   utilises NF-κB to instigate this protective mechanism.

642

643   **Conclusions**

644   In conclusion, our data demonstrate that RelA-containing dimers of NF-κB bind to numerous

645   loci throughout the genome of PHM1-31 myometrial myocytes. For some promoters, this

646   binding occurred in the presence of TNF as well as in unstimulated cells; this was mediated by

647   different regions of the same promoter. Occupancy of other promoters was seen either only in

648   unstimulated conditions or only after cells were stimulated by TNF. The consensus κB motif

649   was identified 3,783 times in this study with over 100 different sequence instances potentially

650   mediating NF-κB DNA binding. Of these κB motifs, 41%were found within intronic regions

651   of the PHM1-31 cell chromatin. Of the 49 TNF-induced genes, 17 were shown to have

652   promoters enriched by NF-κB in response to TNF. Interestingly, five genes with promoters

653   occupied by NF-κB were repressed by TNF. Together, our data illustrate that NF-κB influences

654   a wide range of regulatory gene networks within myometrial cells; we must decipher how these

655   interactions govern myometrial function during pregnancy and labour if we are to begin to

656   understand the syndrome of premature birth.

657

658

659

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1063

Authors' Roles

1064

1065 VJC and SLW performed the experimental work, undertook initial data analyses and read and

1066 helped edit the manuscript. PRH performed the array work (promoter and expression) and read

1067 the manuscript. PJH and SVG assisted with the data analysis and manuscript preparation. NRC

1068 conceived the study, obtained study funding, designed the experiments, undertook the data

1069 analyses and prepared the manuscript.

1070

1076

1077 **Conflict of Interest**

1078   The authors declare they have no competing interests, financial or otherwise, that would affect

1079   the publication of this data.

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1081

1082

1083   **Figure Legends**

1084   **Fig. 1: Schematic Representation of the Work-flow used to Generate Chromatin for this**

1085   **Study.**

1086

1087   **Fig 2: TNF Induces RelA NF-κB Activity in PHM1-31 Myometrial Cells.** Immunostaining

1088   was used to demonstrate TNF-mediated induction of RelA nuclear localisation in PHM1-31

1089   myometrial cell lines (**A**; **Panel-I,** unstimulated; **Panel-II**, negative control; **Panel-III**, TNF-

1090   stimulated; scale bar = 100μm). Nuclear extracts were prepared from PHM1-31 cells and

1091   incubated with α-^{32}P-labelled oligonucleotide harbouring the 3'-HIV-LTR κB site. Three main

1092   complexes were seen to form and, using supershift analyses, these were demonstrated to be

1093   p50:RelA heterodimers, RelA homodimers and a lower non-specific complex. An increased

1094   shift in RelA in TNF-treated cells illustrates TNF was inducing RelA NF-κB translocation to

1095   the nucleus. Specificity of the experiment was confirmed by including an excess (100 ng) of

1096   cold, HIV κB DNA (**B**). PHM1-31 cells were transfected with 200 ng of either 3x-κB-ConA-

1097   luc (NF-κB-responsive; **C; Panel-I**) or ΔκB-ConA-luc (NF-κB unresponsive; **C; Panel-II**).

1098   After 24 hours cells were stimulated with TNF (10 ng/ml) for one hour. Promoter activity was

1099   quantified using a Berthold Sirius tube luminometer. All experiments were performed three

1100   times in triplicate. Data were analysed using an unpaired, two-tailed t-test and results are

1101   expressed as the mean ± S.E.M. (error bars); $p<0.05$ was considered statistically significant.

1102   As expected, TNF induced NF-κB activity (**C; Panel-I**; p = 0.0001). No NF-κB activity was

1103   observed in a control reporter lacking the κB site (**C; Panel-II**). Nuclear extracts were prepared

1104   from PHM1-31 cells and subjected to immunoprecipitation with anti-RelA anti-serum. RelA

1105   was recovered from both control and TNF-stimulated samples, illustrating the effectiveness of

1106   the antiserum. Minimal non-specific binding was observed with IgG (**D**). RelA occupancy of

1107   the IκBα promoter was seen under basal conditions. In the presence of TNF, both RelA and

1108   RNA Pol II were seen to be associated with the IκBα promoter (**E; Upper Panels**). No RelA

1109   occupancy of the control Gαs promoter was observed, illustrating the specificity of the ChIP

1110   assay (**E; Lower Panels**).

1111

1112   **Fig. 3: Comparison of RelA-enriched Chromatin Regions**. The Criteria-B dataset represents

1113   all regions with a p-value <0.05. Within this, the Criteria-A dataset (red circle) represent those

1114   regions where p<0.05 and the MAT score is >0; essentially, these regions are enriched over the

1115   control in response to TNF. The Criteria-C dataset (blue circle) represents those regions where

1116   p<0.05 and the MAT score is <0; essentially, these regions are enriched in the absence of TNF

1117   stimulation (**A**). An illustrative heat map of chromosome 14 illustrating the loci of Criteria-A

1118   regions (red bars) and Criteria-C regions (blue bars). Known transcripts from each strand are

1119   represented by green blocks (**B**).

1120

1121   **Fig. 4: Genes within Criteria-A and Criteria-C Enriched Regions and Associated κB Motif**

1122   **loci.** Schematic representation of the loci of both Criteria-A (TNF-induced NF-κB enrichment)

1123   and Criteria-C (unstimulated NF-κB enrichment) regions around selected genes including

1124   COX-2 (**A**); Jun (**B**), IL6RN (**C**); KCNMB3 (**D**); CACNB3 (**E**) and KCNB1 (**F**). For COX-2

1125   and Jun, κB motifs corresponding to the consensus were found in the TNF-induced RelA

1126   enriched regions (**3A; 3B**). With IL6RN and KCNMB3, both TNF-induced RelA enriched

1127   regions and unstimulated RelA-enriched regions were observed. The former were not

1128   associated with a consensus κB motif while the latter, unstimulated RelA-enriched, both

1129   harboured consensus κB motifs (**3C; 3D**). Regions around CACNB3 and KCNB1 were not

1130   enriched by RelA in the presence of TNF but were enriched by RelA in unstimulated cells,

1131   possibly by the consensus κB motifs identified (**3E; 3F**). Arrows indicate the direction of

1132   transcription, not actual transcription start sites.

1133

1134   **Fig. 5: Differential Enrichment of Regions around the NFKBIA (IκBα) Gene Locus on**

1135   **Chromosome 14.** Low resolution schematic illustration of the IκBα locus and surrounding

1136   regions. In the absence of TNF, no enrichment around the IκBα promoter is observed (**A; blue**

1137   **bars**). In the presence of TNF, a number of regions around the promoter are enriched by the

1138   anti-RelA antiserum (**A; red bars**). High resolution schematic illustration of the IκBα

1139   promoter illustrating TNF-induced regions enriched by the anti-RelA antiserum. One region

1140   encompasses the IκBα promoter and harbours the three reported κB motifs in that region. A

1141   second intra-genic region, encompassing the 3' portion of exon 2 and all of exons 3 and 4, is

1142   also enriched but no consensus κB motifs were identified therein (**B**).

1143

1144   **Fig. 6: Sequence Logo to Illustrate the Variability of κB Consensus Motif in Chromatin**

1145   **from PHM1-31 Cells.**

1146   The consensus κB motifs presented in Table I were aligned in the open-access software

1147   WebLogo 3. The probability of a given bases occurring at the proscribed position is illustrated.

1148   No difference in κB motif variability was noted between Criteria-A (TNF-induced) and

1149   Criteria-C (unstimulated) datasets.

1150

1151   **Fig. 7: Comparison of TNF-Induced Differential Gene Expression.** The Criteria-B1 dataset

1152   represents 2,223 genes modified by TNF with a p-value <0.05. The Criteria-A1 and -B1 dataset

1153   (red circle) represent those genes where p<0.05 and expression was increased 2-fold or more;

1154   49 genes populated this intersection. The Criteria-C1 and -B1 (blue circle) represents those

1155   genes regions where p<0.05 and expression was repressed by more than than 1.5 fold; 17 genes

1156   populated this intersection.

1157

1158   **Fig. 8: GO Enrichment – Biological Process and Validation of Expression Array Analyses**

1159   **by qRT-PCR.** Within the Biological Processes group, two functional groups of interest were

1160   Intra-cellular Signal Transduction and Cell Surface Receptor Protein Signalling. This included

1161   TNFAIP3 (Cytokine-mediated Signalling) and IκBα (NIK/NF-κB Cascade) (**A**). qRT-PCR on

1162   total RNA from PHM1-31 cells treated with TNF for one hour demonstrated that both IκBα

1163   and TNFAIP3 were expressed in response to TNF (**B**). A close agreement was seen for

1164   induction of gene expression for IκBα (4.43 for qRT-PCR; stippled bars vs. 2.94 on array;

1165   diagonal stripes) while a slightly greater margin of difference was noted for TNFAIP3

1166   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:
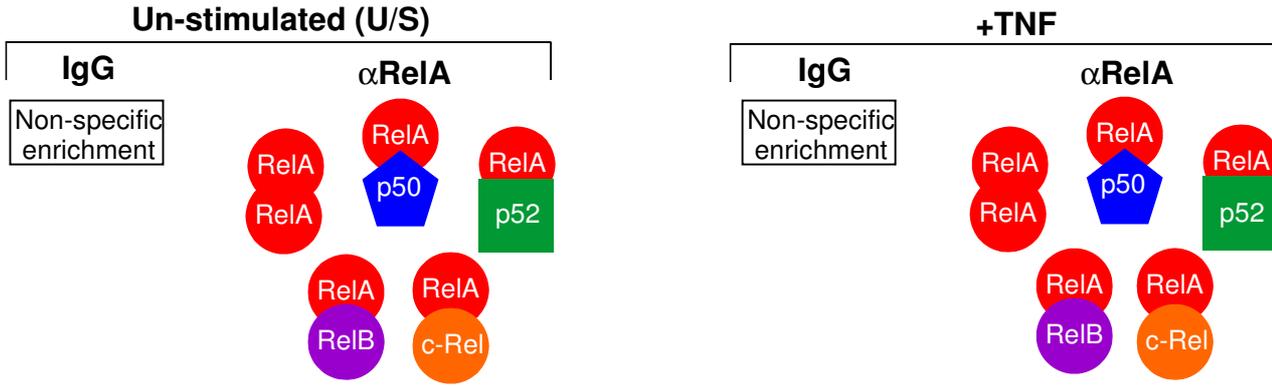
2) Chromatin processed and quality-control PCR undertaken

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.

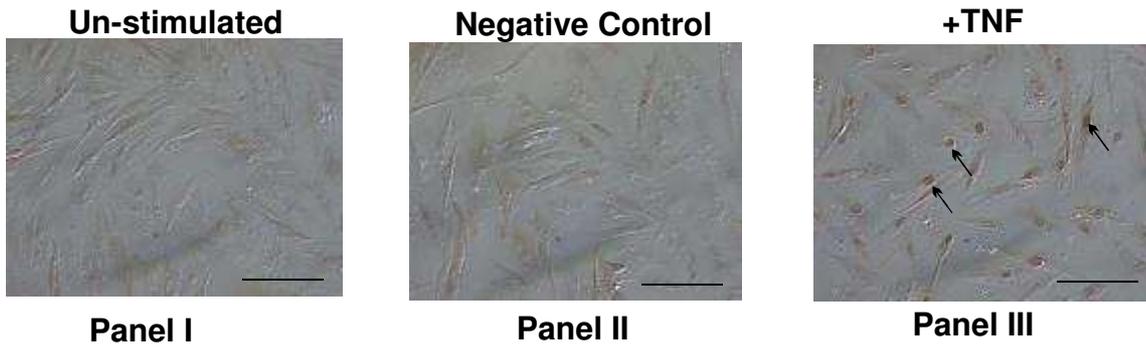5) Values ascribed to IgG from three individual experiments deemed as non-specific and subtracted giving a new control baseline

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

Criteria-C dataset   *Criteria-A* dataset

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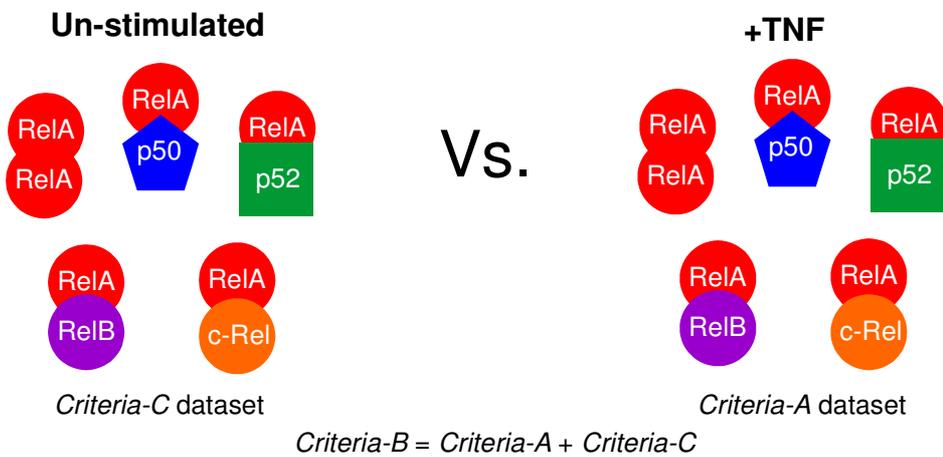
Figure 2
TNF Induces RelA NF-κB Activity in PHM1-31 Myometrial Cells

A Anti-RelA Staining

Un-stimulated   Negative Control   +TNF

Panel I   Panel II   Panel III

B RelA EMSA

RelA supershift
RelA homodimers
RelA:p50 heterodimers

^{32}P-κB oligo + + + + +   + + + + +

I=Input; supershift = αRelA

C 3x-κB-luc   ΔκB-luc

*Control vs. TNF: p = 0.0001

Control vs. TNF: p = 0.072

D RelA Immunoprecipitation

RelA
IgG Heavy chain

I=Input; IP = αRelA

E RelA ChIP Analysis

IκBα promoter
Primers

Gαs promoter
Primers

I=Input (IκBα primers); IG= Input (Gαs primers)
IP = αRelA or αPolII
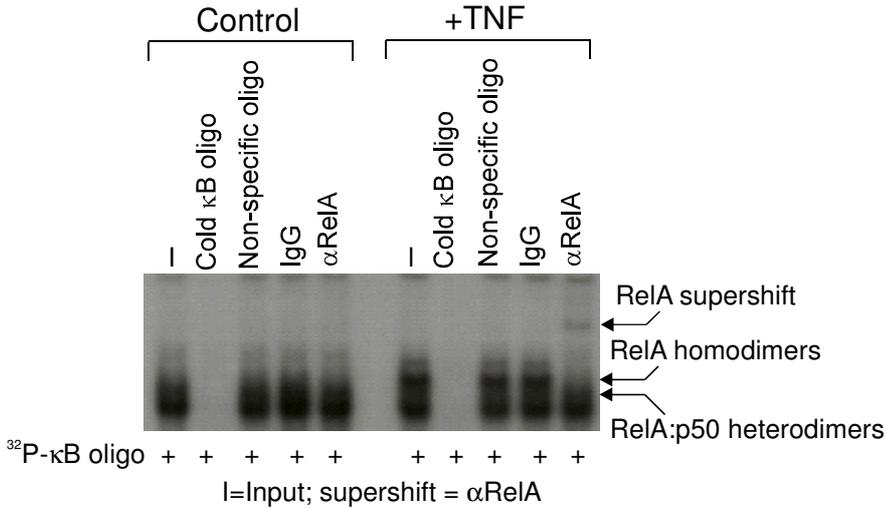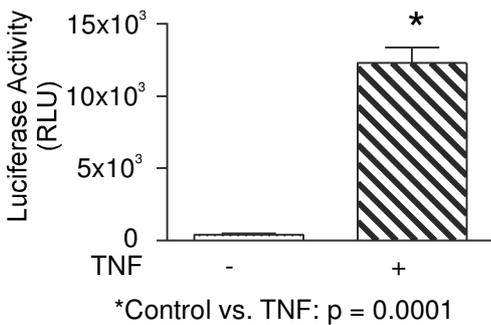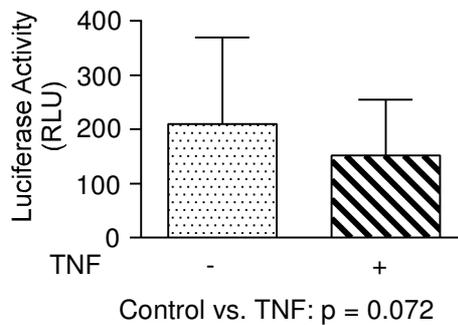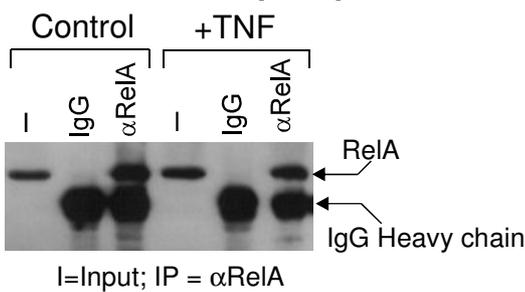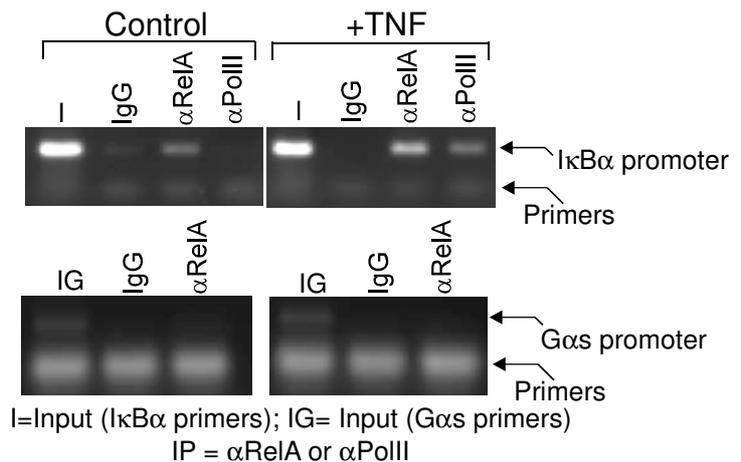
Figure 3
Comparison of Enriched Regions

A Venn Diagram of Enriched Regions

**All enriched regions
(Criteria B)**
(p-value region ≤0.05)
24,410 enriched regions

Criteria A
(+TNF; MAT score >0)
13,300 enriched regions

Criteria C
(Un-stim.; MAT score <0)
11,110 enriched regions
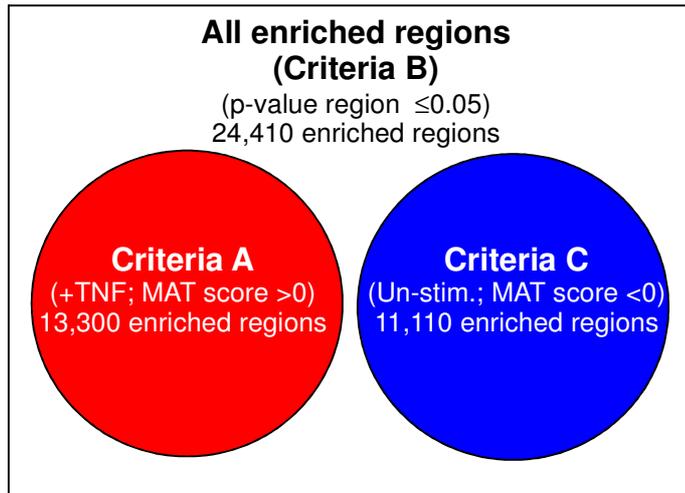
B Heat Map of Chromosome 14 Illustrating Enriched Regions with a Positive MAT score (TNF-stimulated; *Criteria-A*; Red) and Enriched Regions with a Negative MAT Score (Un-stimulated;*Criteria-C*; Blue)

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*)

0   26.6   53.2   79.8   106.4

Distance (Mbps)
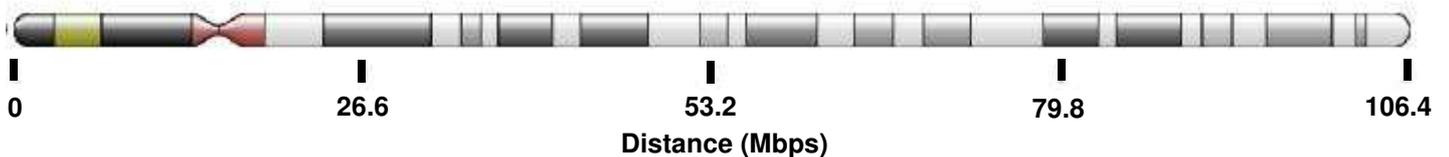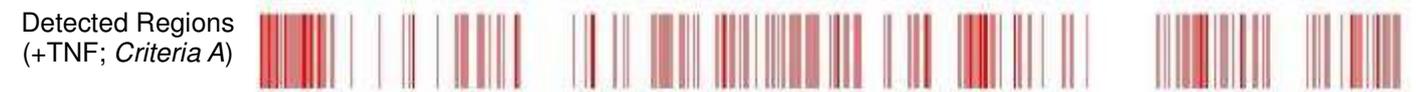
Figure 4

Genes Associated with *Criteria-A* and *Criteria-C* Enriched Regions
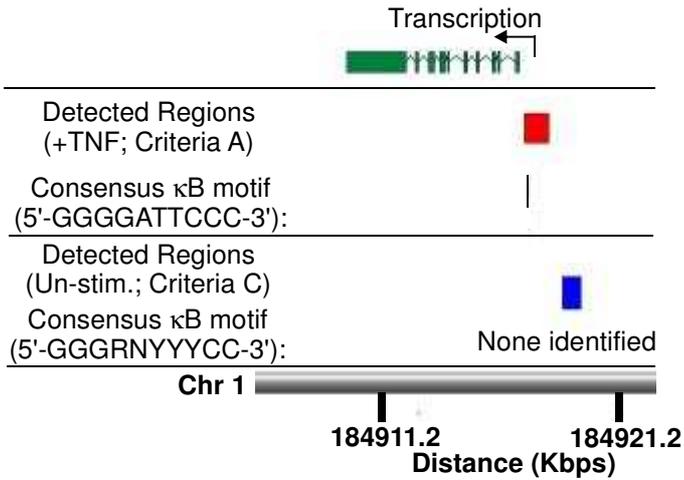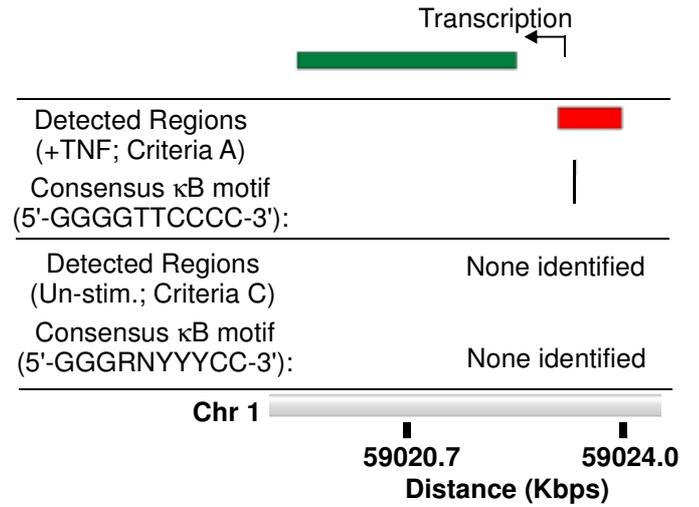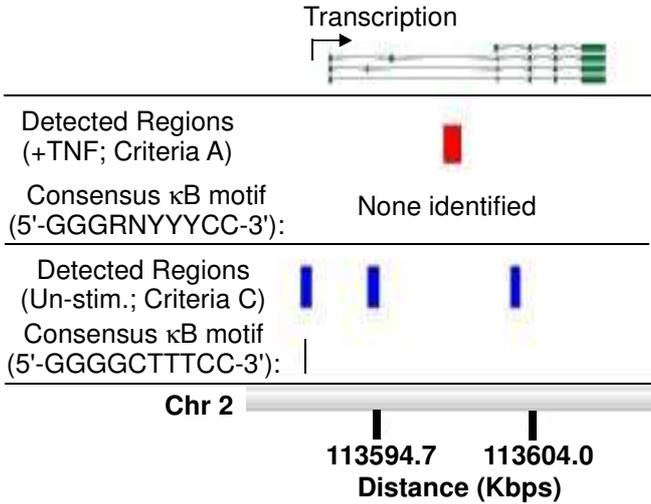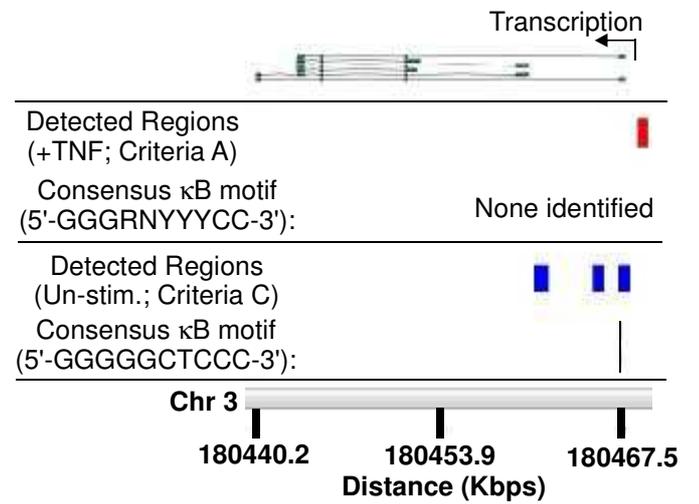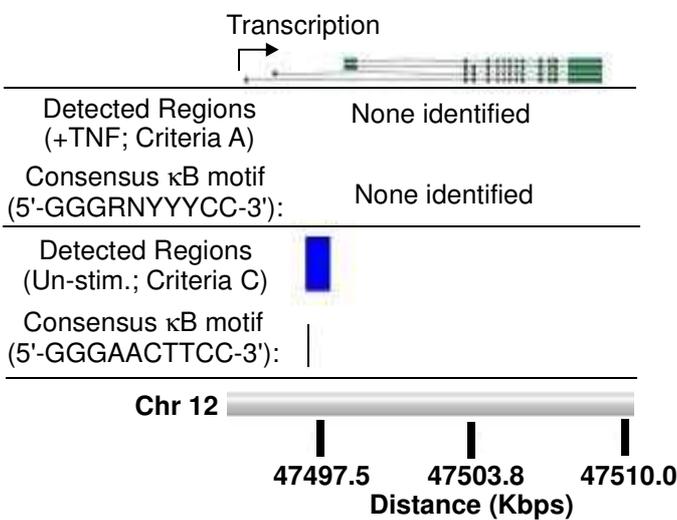
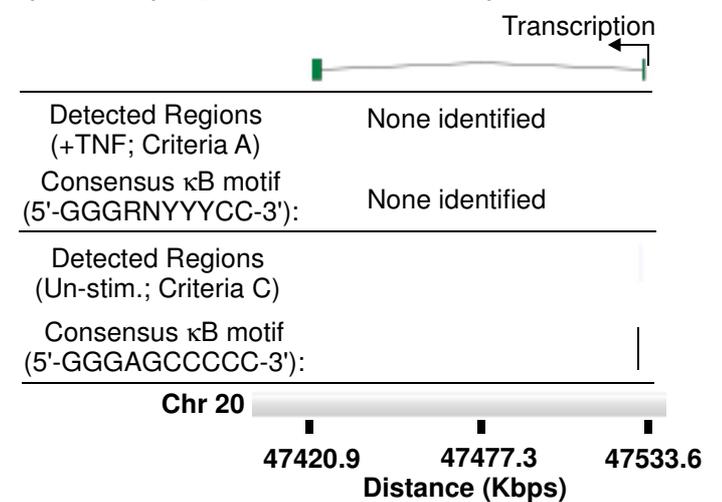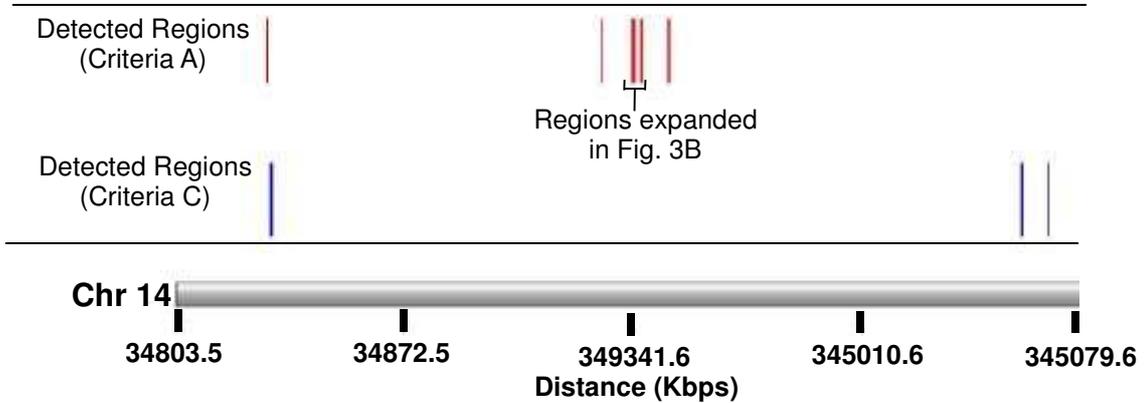**Figure 5**

Differential Enrichment of Regions around the NFKBIA (IκBα) Gene Locus on Chromsome 14

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

B **NFKBIA (IκBα) Promoter and κB-binding Motifs**

Figure 6

Summary of κB Sequence Motif Variability in PHM1-31 Cells
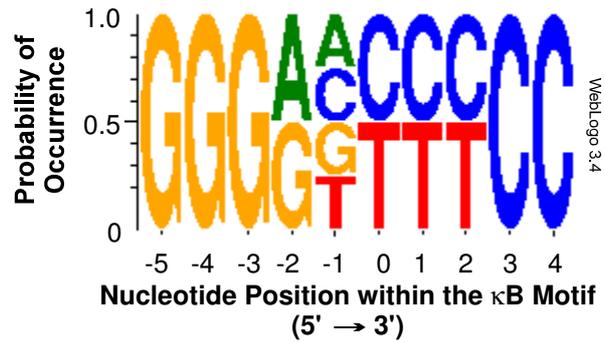
Figure 7
Comparison of TNF-Induced Differential Gene Expression

B1 Total: 2,223 genes

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

A1 Total: 51 genes

C1 Total: 18 genes

A1 AND B1
(p-value region ≤0.05;
Fold Change >2)
49 genes

C1 AND B1
(p-value region ≤0.05
Fold Change <-1.5)
17 genes

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

Criteria C1
(Fold Change <-1.5)
1 gene
(unannotated)
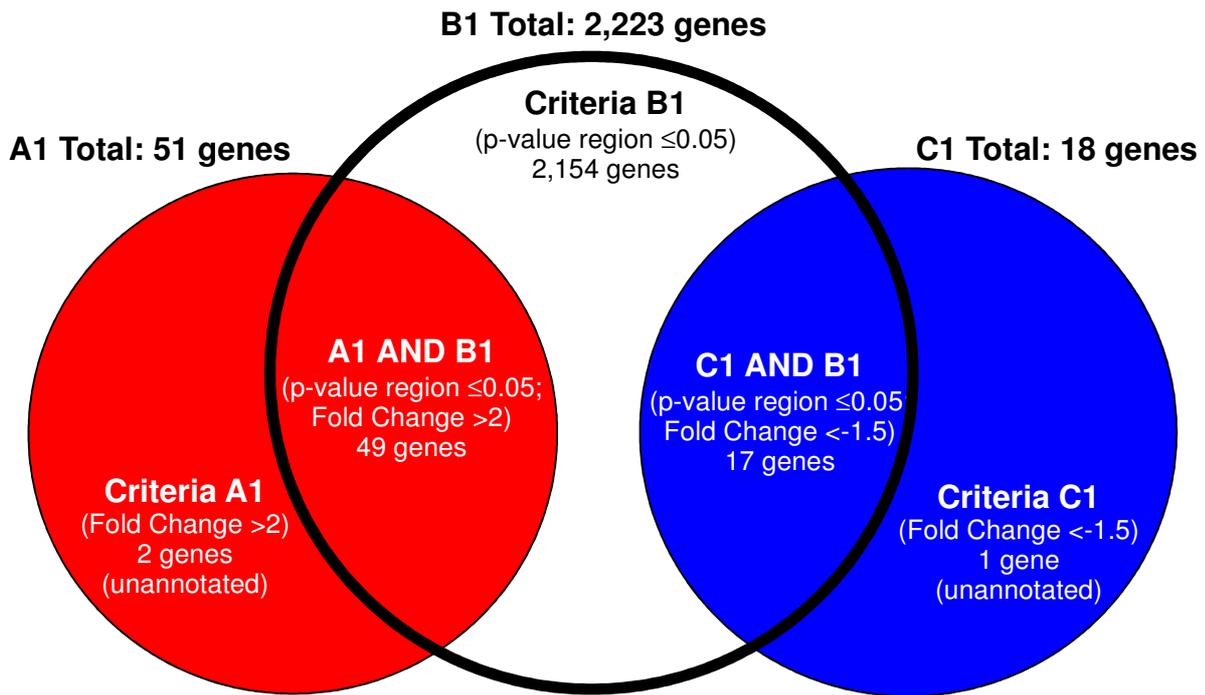
Figure 8

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**