

## Supporting Figure Legends

### **Figure 6. Example scatter plots showing binding of p65 and p50 to gene promoters in living cells.**

Microarray data from p65 ChIP in unstimulated cells and p50 ChIP following LPS stimulation. The x-axis represents the hybridization signal intensity for input whole cell extract (WCE) DNA ( $\log_{10}$  Cy3 background-subtracted signal). The y-axis shows the intensity for DNA enriched by ChIP using antibodies specific for p65 or p50 ( $\log_{10}$  Cy5 background-subtracted signal). Microarray elements that were flagged as not found by the Axon software were not analyzed. The red lines either side of the scatter plots represent the ratio of IP signal to WCE signal necessary to achieve a significance score of 0.002. The position of the promoters for the known NF- $\kappa$ B target genes *HLA-B*, *NFKB2*, *CCL1*, *PTX3*, *CSF2* and *RAC2*, the novel NF- $\kappa$ B target genes *NFKBIB* and *P4HA1* and  $\beta$ -actin, a negative control, are marked in both scatter plots. No genes were enriched above the p-value threshold of 0.002 by p65 ChIP in unstimulated cells. In contrast, *HLA-B*, *NFKB2*, *CCL1*, *PTX3*, *CSF2*, *RAC2*, *NFKBIB* and *P4HA1* were among the 193 genes enriched above this threshold by p50 ChIP in stimulated cells.  $\beta$ -actin was not enriched in either p65 or p50 ChIP.

### **Figure 7. Gene-specific PCRs of immunoprecipitated and whole cell extract DNA.**

A) Gene-specific PCRs of promoters at which binding was detected using the promoter arrays. All gene-specific PCRs were performed with DNA from LPS-

stimulated cells.  $1/30^{\text{th}}$  of the immunoprecipitated (IP) DNA (equivalent to  $1.5 \times 10^6$  cells) was amplified with the designated promoter specific primers and the results compared to the amplification of serial dilutions of whole cell extract DNA ( $1/18000^{\text{th}}$ ,  $1/6000^{\text{th}}$  and  $1/2000^{\text{th}}$ ). Thus, a similar intensity DNA band in the IP amplification and the highest WCE dilution amplification indicates that the DNA sequence is present in the IP fraction at an amount  $\sim 600\text{x}$  lower than in the WCE. We amplified the  $\beta$ -actin promoter from the IP and WCE DNA as a negative control ( $\beta$ -actin was not bound by NF- $\kappa$ B in the array results, see Supporting Information Figure 6). The intensities of the bands were quantified using ImageQuant (Amersham). The enrichment ratio is the average fold-enrichment of the promoter of interest in the IP fraction compared to WCE, normalized to the  $\beta$ -actin control (ie. a ratio greater than 1 indicates that the ratio of IP to WCE is greater for the NF- $\kappa$ B target gene than for  $\beta$ -actin).

B) Gene-specific PCRs of promoters at which binding was not detected using the promoter arrays.

C) Intersection between previously known directly bound NF- $\kappa$ B target genes and targets identified in this study. The complete list of previously known targets is compiled from all the cell types and conditions in the literature and is available through the Literature Targets link at [web.wi.mit.edu/young/nfkb](http://web.wi.mit.edu/young/nfkb) (based on Pahl, H.L. (1999). *Oncogene* 18, 6853-6866 and the Gilmore lab database at <http://people.bu.edu/gilmore/nf-kb/target/index.html>). Of the 224 literature targets, our array covered the promoters of 154 genes. Of these, 38 NF- $\kappa$ B targets were bound at a p-value of 0.002 (24%,  $p=4 \times 10^{-19}$ ) and 49 at a p-value of 0.01 (32%,

$p=2 \times 10^{-19}$ ). As a negative control, we compared the NF- $\kappa$ B literature targets to those identified for E2F4 using location analysis and found no significant overlap ( $p=0.96$ ). A list of the E2F4 bound genes can be found in Supporting Information, Table 4.

**Figure 8. Immunoblotting of NF- $\kappa$ B proteins in the cytoplasm and nucleus of U937 cells before and after LPS stimulation.**

U937 cells were treated with LPS (2.5  $\mu$ g/ml) and nuclear and cytosolic extracts were harvested at 0, 30, 60 and 180 minutes following stimulation. p50 and p52 were both present in the nuclei of unstimulated cells and increased in nuclei after LPS stimulation, consistent with the results of location analysis. The p52 antibody (Upstate, 06-413) detects an additional band in the nucleus corresponding to a protein of approximately 58 kD. This protein is unlikely to be an NF- $\kappa$ B subunit because it is not present in the cytoplasm and does not increase upon LPS stimulation. This unspecific band is also apparent in western blots performed using this antibody by other investigators (Senftleben et al. 2001. Science 293,1495-1499). We do not believe that this unspecific binding significantly affects our location analysis results because the vast majority of the p52 targets we identify were also detected by antibodies to other NF- $\kappa$ B subunits, either before or after LPS stimulation. Small amounts of RelB and c-Rel are apparent in the nuclei of unstimulated U937 cells after long exposures to film. We also detect a relatively small number of targets for these proteins in unstimulated cells (Figure 1). Location analysis can be more sensitive than western blotting for

small amounts of DNA binding proteins because the technique uses immunoprecipitation to concentrate protein from  $\sim 5 \times 10^7$  cells and then LM-PCR to amplify co-precipitating DNA.

Materials and methods: Extracts were prepared using a protocol modified from Dignam and colleagues (Dignam et al. (1983). *Nucleic Acids Res.* 11, 1475-1489). Cells were washed with PBS and lysed on ice in hypertonic buffer with NP-40 (10mM Hepes, pH 7.9, 1.5mM  $MgCl_2$ , 10mM KCl, 0.5mM DTT, 0.1% NP-40 with Complete protease inhibitor (Roche)) for 5 minutes. The cytosolic fraction was harvested by centrifuging the lysate at 4500g for 3 min and collecting the supernatant. The nuclear pellet was washed twice in hypertonic buffer without NP-40. Nuclear proteins were then extracted on ice in high-salt buffer (20mM Hepes, pH 7.9, 420mM NaCl, 1.5mM  $MgCl_2$ , 0.2mM EDTA, 0.5mM DTT, 10% glycerol with Complete protease inhibitor (Roche)) for 30 minutes. The nuclear extract was harvested by centrifuging the lysate at 7000g for 3 min and collecting the supernatant. Protein concentrations were determined using the BCA kit (Pierce) and 8 $\mu$ g of protein extracts were loaded per lane and separated by SDS-polyacrylamide gel electrophoresis. Proteins were transferred onto Hybond-P membrane (Amersham) and the membrane blocked with 5% milk in PBS containing 0.1% Tween-20 (Sigma). The primary antibodies used were identical to those used for location analysis. The blots were developed with ECL (Amersham).

**Figure 9. Recruitment of activatory NF- $\kappa$ B subunits to activated genes upon LPS stimulation.**

A. NF- $\kappa$ B binding at genes to which RNA polymerase II is recruited and to genes where it is lost (top 5% of genes for both categories). The change in RNA polymerase binding is represented as a ratio, with red indicating an increase and green a decrease, according to the scale on the left. Only genes bound by RNA polymerase II in either unstimulated or activated cells were used. NF- $\kappa$ B subunits that were already bound ( $p < 0.002$ ) before LPS stimulation are marked in blue and the recruitment of new subunits is marked in red.

B. NF- $\kappa$ B binding at genes that are upregulated or downregulated over time in response to LPS in U937 cells. Genes were either upregulated by 2-fold or downregulated by 2-fold at two consecutive time points. Red indicates an increase in expression and green a decrease according to the scale on the left.

**Figure 10. Model of the transcriptional regulatory network coordinated by NF- $\kappa$ B family members.**

Genes (boxes) bound by NF- $\kappa$ B subunits (circles) in U937 cells before and after stimulation with LPS. Among the NF- $\kappa$ B target genes identified here are many transcription factors with roles in regulating the immune response. Therefore we suggest a model in which NF- $\kappa$ B controls the response to pathogens, not only by binding to its direct targets, but also through the activation of other transcription factors.