Exploring the role of NF-κB in TNF mediated repression of gene expression

A Thesis submitted to

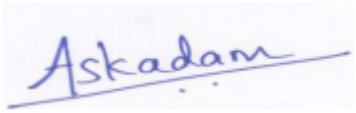
Indian Institute of Science Education and Research Pune
in partial fulfillment of the requirements for the BS-MS Dual Degree Programme
by

Aditee Kadam

April, 2018
Supervisor: Dr Soumen Basak

Certificate

This is to certify that this dissertation entitled “Exploring the role of NF-κB in TNF mediated repression of gene expression” towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents work carried out by Aditee Kadam at National Institute of Immunology under the supervision of Dr Soumen Basak, Staff Scientist-V, Department of Systems Immunology Department, during the academic year 2018-2019.



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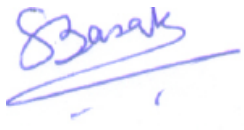

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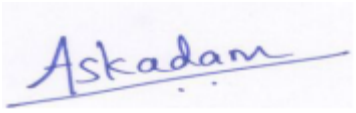
Declaration

I hereby declare that the matter embodied in the report entitled “Exploring the role of NF-κB in TNF mediated repression of gene expression” are the results of the work carried out by Aditee Kadam, at the Department of Systems Immunology in the National Institute of Immunology, under the supervision Dr Soumen Basak, and the same has not been submitted elsewhere for any other degree.



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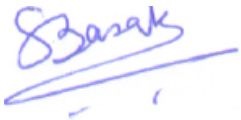

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Exploring the role of NF-κB in TNF mediated gene repression

Abstract:

Inflammation is one of the first symptomatic events in an immune response. A controlled inflammatory response is beneficial. However, it can have certain detrimental effects if unchecked (Takeuchi and Akira, 2010). Tumour Necrosis Factor (TNF) is an important mediator of inflammation, which induces NF-κB during this inflammatory response. NF-κB plays diverse roles including induction of genes involved in inflammation and producing pro-survival factors (Medzhitov and Horng, 2009). However, a plausible role of TNF-activated NF-κB factors in transcriptional repression has not been systematically investigated at the genome scale.

Here, I obtained transcriptomic data of TNF-stimulated mouse embryonic fibroblasts (MEFs) genetically devoid of one or more NF-κB monomers or monomer precursors. I then interrogated this data to dissect the relative contributions of different NF-κB heterodimers in the repression of gene expression. First, I included genes that exhibited NF-κB-dependent transcriptional downregulation in the analyses utilising appropriate controls in this data set. Using a clustering algorithm, I catalogued these genes into four groups, based on their repression in different genetic backgrounds. Next, in collaboration, I verified the repression of representative genes from each gene-groups by performing qRT-PCR experiments. Further, I carried out Gene Ontology analysis to explore the biological processes and pathways which are enriched in the NF-κB repressed gene sets. I evaluated binding of specific NF-κB factors at proximal locations of the downregulated genes by analysing the ChIP-Seq data that I received, generated using antibodies against specific NF-κB factors in TNF-stimulated MEFs. I finally examined the involvement of secondary transcription factors in repression via motif *de novo* enrichment and transcriptomic analysis.

Background:

The NF-κB family consists of five monomeric factors such as RelA, RelB, cRel, p50, p52 etc, which may form 15 possible combinatorial dimers (Basak et al., 2012). Amongst the NF-κB monomers, p52 and p50 get processed from p100 and p105 respectively, by partial proteolysis. The most prevalent dimers are RelA-p50 and RelB-p52 (Hayden and Ghosh, 2014).

NF-κB system has two distinct signalling arms – namely the canonical and the non-canonical arms. The major inhibitors of the canonical pathway are the inhibitors of NF-κBs (IκBs), which sequester the RelA-p50 heterodimers in resting cells (Hoffmann and Levencheko, 2002). Once the cell is stimulated by a canonical stimulus such as TNF, IκBs are phosphorylated through signal transduction and tagged for degradation via the proteasome. This results in the nuclear translocation of RelA-p50, which further transcribes close to hundreds of genes with pro-inflammatory, immune response and pro-survival functions (Hoffmann and Baltimore, 2006). RelA-p50 also synthesizes IκBs, thus constituting a negative feedback loop (Hayden, S. Ghosh, 2011).

On the other hand, Lymphotoxin-β (LT-β) is a non-canonical stimulus. Here, IkBsome, i.e., an oligomer of p100 acts as the inhibitor (Basak, 2007), and sequesters the RelB NF-κB subunit in the cytoplasm (Savinova 2009; Yilmaz 2014). The LTβ stimulation leads to processing of p100 intoto p52 and release of the RelB-p52 heterodimer into the nucleus (Xiao, 2004). The non-canonical pathway controls processes such as B-cell survival and maturation and lymphoid organogenesis (Weih et al.,2001). Of note, p100 is encoded by *Nfkb2*, which is a RelA-target gene (Banoth et al., 2015).

In *Nfkb2*-deficient cells, RelB binds to the canonical NF-kB subunit p50 forming the RelB:p50 heterodimer because of the lack of its primary binding

partners p52 and p100. Both RelA:p50 and RelB:p50 bind to the inhibitor IκBs and are regulated by canonical signaling. TNF stimulation degrades IκBs and releases both these heterodimers into the nucleus in *Nfkb2*-null cells (Roy et al., 2016).

This allowed us to analyze an interesting system where we could observe the activity of one or more NF-κB transcription factors in a panel of knockout mouse embryonic fibroblasts (MEFs) treated with TNF. More specifically, *Nfkb2*$^{-/-}$ cells exhibit the activity of both RelA and RelB dimers, *Nfkb2*$^{-/-}$*Relb*$^{-/-}$ double knockout elicited the RelA activity only, and analogously, *Nfkb2*$^{-/-}$*Rela*$^{-/-}$ activated solely the RelB dimers (Roy et al., 2016).

Introduction:

TNF is an important cytokine involved in regulating a wide array of processes such as inflammation, cell survival, proliferation, differentiation, and death. Although, TNF is known to predominantly induce genes via the NF-κB pathway, there were reports suggesting of its role in gene repression. Specifically, RelB was involved in downregulating the production of CXCL12 during inflammatory signaling (Madge et al., 2011). Thus, we hypothesized a general role of NF-κB in TNF-mediated gene repressions.

Repression of NF-κB-dependent genes requires the occurrence of three events (Mingming Zhao, 2018). Firstly, in the presence of NF-κB, abundance of mRNAs encoded by NF-κB-dependent genes should decrease with time. Secondly, abolishing NF-κB activity should result in no change or increase in the transcript levels of these genes. In my study, this was accomplished by analyzing the transcriptomic data of various cell-knockouts in which various NF-κB dimers were either present or absent. And thirdly, for direct NF-κB mediated gene repressions, an event of NF-κB factors binding to the genes should take place. This was examined by analyzing the ChIP-Seq data consisting of the binding locations of the NF-κB factors in the whole genome in the *Nfkb2*$^{-/-}$ cells.

Methods:

a) Microarray mRNA analyses

MEF cell knockouts of the genotypes-Wild-type, *Nfkb2*$^{-/-}$, *Nfkb2*$^{-/-}$ *Rela*$^{-/-}$, *Nfkb2*$^{-/-}$ *Relb*$^{-/-}$, *Rela*$^{-/-}$ *Relb*$^{-/-}$ cRel$^{-/-}$(NF-κB deficient) were used for microarray mRNA analyses as detailed in Roy et al., 2016. In two independent experiments, the knockouts were treated with TNFc (10ng/ml) for 6hrs following which total RNA was isolated. The experiments at each time points were performed in duplicates. For microarray analysis- labeling, hybridization to Illumina MouseRef-8 v2.0 Expression BeadChip, data processing and quantile normalization were performed by Sandor Pvt Ltd (Hyderabad, India).

Thus, I obtained the quartile normalized microarray data consisting of the transcriptome of ~18000 genes of the TNF treated and the untreated cells.

b) Data processing pipeline

I began my microarray analysis by selecting genes with consistent gene expression by using the Irreproducibility Discovery Rate (IDR) method (Li et al, 2011). In this method, replicate gene expressions are associated with an IDR value which is a representative of their reproducibility. Those genes which passed the criteria of IDR<=0.05 in each genotypes were chosen for analysis. IDR was performed by using the package “idr” in R platform with the default parameters. Then, the gene expression values of the replicates in each of the genotypes at each time points were averaged. To investigate for repression, fold change was calculated as 0hr expression/ 6hr expression. Among these genes, NF-κB dependent genes were filtered by using the condition that the gene expression in NF-κB deficient cells should be between 1/1.3 and 1.3-fold. Further, the repressed genes were detected by applying the condition of fold repression in *Nfkb2*$^{-/-}$ >= 1.3. In doing so, I arrived at a list of 492 repressed genes.

c) Clustering analysis:

I used the clustering algorithm 'Partition Around Medoids' (PAM) to categorize the genes into six clusters (Reynolds et al., 2006). This was achieved by using the "clusters" package in R and supplying the log2-transformed values of fold repression in each genotype to the function "pam". The PAM algorithm assigns dataset candidates to the closest medoid out of 'k' medoids, where 'k' is specified by the user (here, six). It then swaps a medoid with another data-point and checks if it incurs a lesser cost of swapping by producing clusters that are closer to the medoids. This process occurs recursively until the cost can no longer be reduced. Here, PAM was chosen over other algorithms because of the greater reproducibility of medoids over numerous runs and also, its lesser sensitivity to outliers. I plotted the resulting clusters in MATLAB as heat-maps using the "imagesc" function. Next, the six gene-clusters were clubbed to form four gene-groups, because of the similarity in patterns between them. Further, I generated violin plots from the four groups using the "violin" function in MATLAB. The violin plot is used to observe the distribution of the data sets and their mean and the median values.

d) Gene expression studies

Representative genes from each gene-groups based on high repression status and biological importance were chosen for quantitative Real Time Polymerase Chain (qRT-PCR) reactions. I received total RNA isolated from MEFs with the genotypes WT, *Nfkb2*$^{-/-}$ and NF-κB deficient cells, stimulated chronically with 10ng/ml of TNF using Trizol. TNFc treatment was carried out for the time courses of 0hr and 6hrs. Then, in collaborative effort, RNA was converted to cDNA using the Takara's PrimeScript 1st strand cDNA Synthesis Kit and amplified using Sybr Green PCR mix. The relative gene expressions were quantified using ΔΔCT method upon normalizing to β-actin mRNA level. (Banoth, 2015).

The equation to calculate ΔΔCT used was:

$$2^{(\text{target gene } \Delta CT \text{ value at a given time})} - 2^{(\text{target gene } \Delta CT \text{ value at 0hr})}$$

Then, the fold change calculated as 6hr expression/0hr expression, were subjected to a one-tailed t-test between genotype samples taken two at a time, assuming equal variance between those samples.

e) *Gene ontology analysis*

I subjected the gene-groups to gene ontology (GO) analysis (Fig 3). Functional enrichment of various Gene Ontology terms for biological process in the indicated gene-groups was determined by 'topGO' library in R (Alexa et al 2006). The p-value threshold for the GO terms in each group was set to 0.05. The resulting GO terms' p-values in a gene-group were compared with other groups. The list was further narrowed down by selecting a few terms and broadly categorizing them under physiological processes.

f) *ChIP-Seq analysis*:

I obtained the processed ChIP-Seq data containing peak intensities of RelA and RelB binding in the whole genome at 0hr and 6hr in duplicates. Three regions were annotated for a gene: Intergenic (50kb), Gene, Promoter (-3kb to +1kb, with respect to the transcription start site. I first chose regions in which peak intensity of RelA and RelB binding at 6hr in both the replicates was greater than zero. Next, the 6hr replicates were associated with a IDR value, and regions containing peaks with IDR<0.1 were selected for further analysis. The genes annotated in these regions were compared with genes in each group. In doing so, I obtained information about number of genes bound by NF-κB factors in each group. Enrichment score was calculated by comparing genes bound by NF-κB factors in a gene-group versus 1000 random genes (Fig 4).

g) Motif enrichment analysis

I used HOMER (Hypergeometric Optimization of Motif EnRichment) to find enrichment of motifs in the repressed gene-groups (Heinz et al., 2010). The HOMER program findMotifs.pl was used to find enrichment of the target genes compared to the background. For my analysis, I used the default background, which is the total gene list in Homer software minus the genes used in each gene-groups. I then searched for motifs of default length, i.e., 8,10,12 in the region from -2kb to +1kb relative to the Transcription Start Site (TSS). The transcript levels of selected factors binding to these motifs at 0hr and 6hr were examined in various genotypes. A heatmap demonstrating 6hr/0hr transcript levels was plotted in MATLAB.

Results:

1. Distribution of the gene clusters:

Clustering algorithm of gene expression data obtained using various NF-κB knockouts depicted that NF-κB dependent genes, in which fold downregulation was >=1.3 in *Nfkb2*$^{-/-}$, can be grouped into four groups (Gr-I-IV). Each group represents genes repressed by one or more transcription factors. It was inferred from our clustering analyses that the first gene-group was repressed by RelB; second group consisted genes repressed by either RelA or RelB, third by RelA, and the fourth by both RelA and RelB. Then, the distribution and probability density of fold-downregulation in each gene-groups was plotted using the Violin plot. The comparison of mean (black) and median (red) values in each gene-groups corroborated the partitioning of the gene-groups.

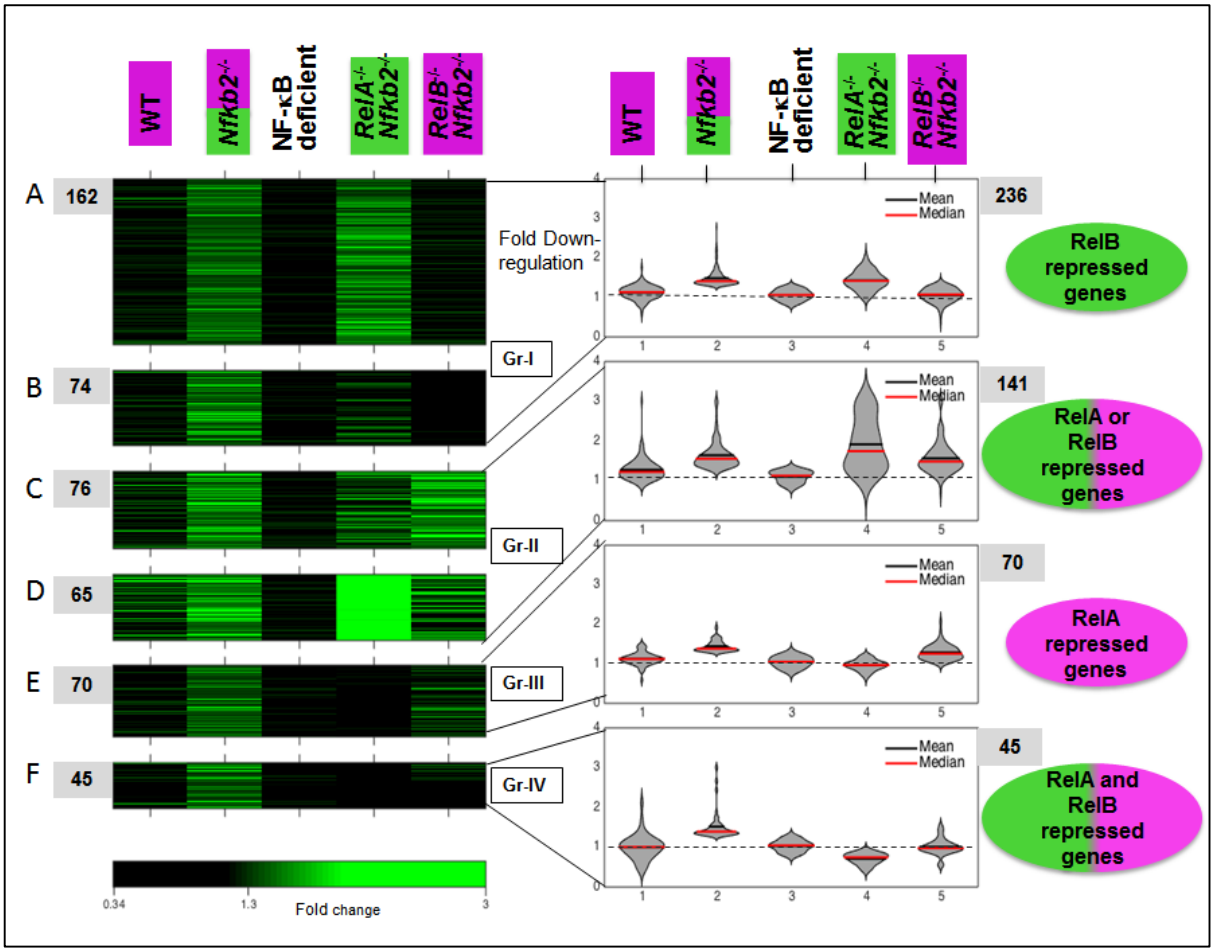


Fig 1: Heatmap and Violin plot for the four gene-groups
a) Heatmap demonstrates binary logarithmic transform of TNF-induced fold changes in the expressions of these genes in the indicated knockout cells clustered using the partition around medoids algorithm(left).
b) Violin plots show relative frequency distributions of fold change values and the corresponding mean and medians for various genotypes as well as the number of members in each gene-group (Right).

2. *Quantitative RT-PCR measuring time courses of gene expressions*

Selected candidates from each gene-groups were subjected to qRT-PCR analysis based on their high suppression status in the required genotypes. The time-course analyses demonstrated that TNFc treatment repressed accumulation of mRNAs of genes from each gene groups in *Nfkb2*$^{-/-}$ compared to the abcTKO(Rela$^{-/-}$Relb$^{-/-}$cRel$^{-/-}$), thus indicating the role of NF-κB in repression. The results are significant for the genes Pdcd4 and Pdgfc, while not for Foxc1 and Acin1.

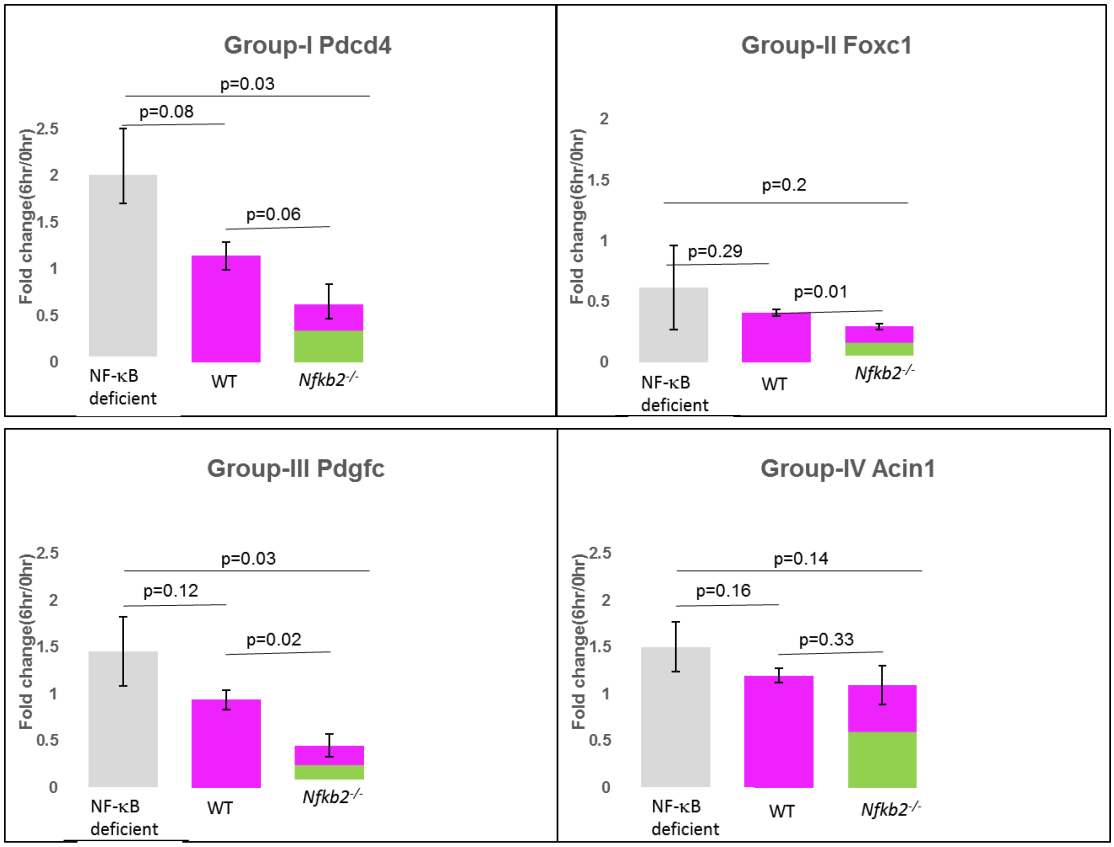


Fig 2: qRT-PCR experiments to validate the repression of genes
a)qRTPCR was carried out using RNA extracted from MEFs cells stimulated with TNF for 0hr and 6hrs. Data shown are the average of 3 independent experiments with qRTPCR carried out in duplicate; Error bars represent the standard error of the mean between the triplicate experiments.

3. *Distribution of the ontology terms:*

The GO terms associated with the genes in each groups were compared with GO terms of the background of ~18000 genes. In a group, GO terms that were significant upto p-value 0.05 were selected, and then compared with the terms in other gene-groups. Next, some of the resultant GO terms were broadly categorized under biological processes. Irrespective of the stringent cutoff for the GO terms, I observed that NF-κB repressed genes were involved in regulating numerous processes, such as immune regulation, metabolism, anatomical development etc., thus, hinting towards a wide impact of NF-kB mediated downregulation. I also observed that a few processes were exclusively governed by a single transcription factor; for instance, the immune system processes and pigmentation related processes were seemingly regulated by RelA. On the other hand, metabolic processes required the contribution of both RelA and RelB, alone

or together, to cause the repression. Thus, in our study, we have identified both specific and generic NF-κB regulators of biological processes likely varying the trajectory for therapeutic interventions.

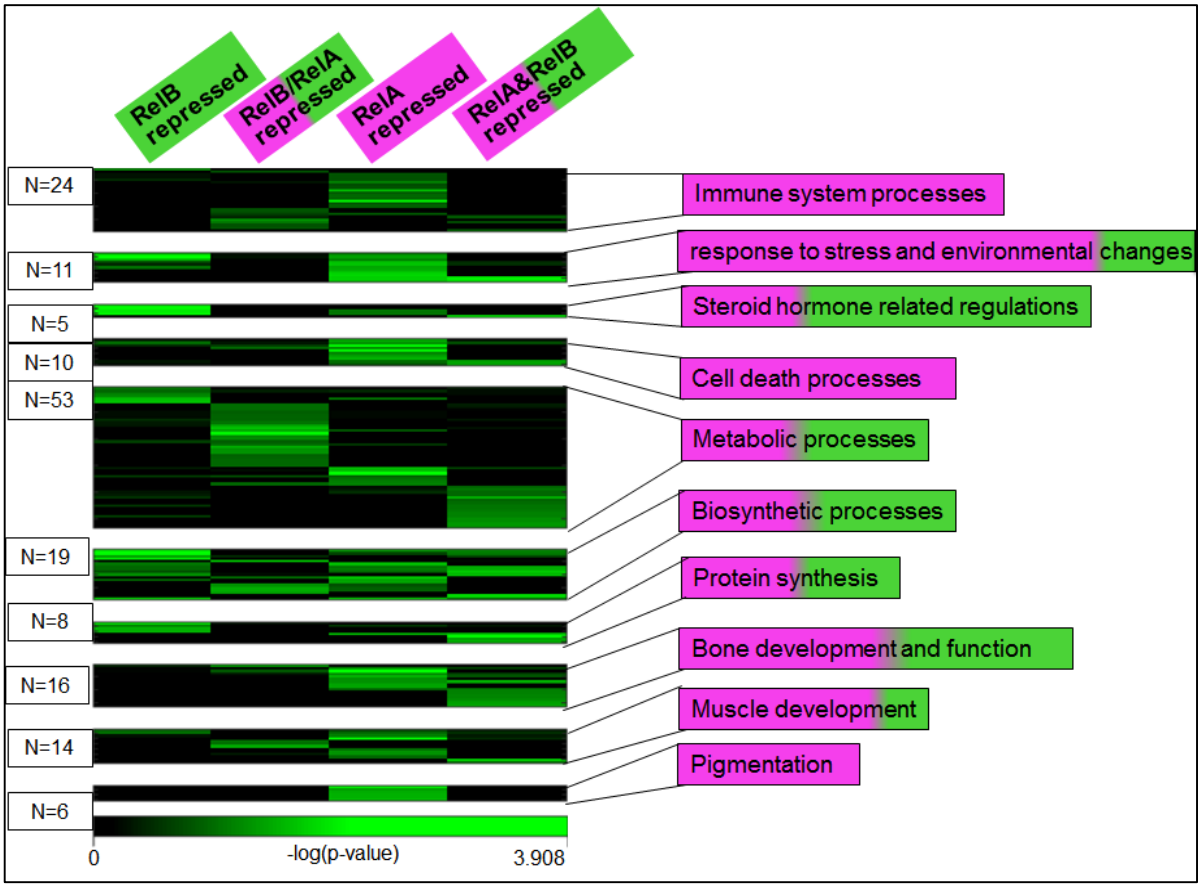


Fig 3: Heatmap for the GO terms:
Heatmap demonstrates negative of the logarithmic transform of the Fischer weight for each of the terms in four gene-groups.

4. *Profile of NF-κB binding in each cluster*

I observed that there was redundant binding of the transcription factors. This is because though the genes were inferred to be repressed by a single transcription factor (Group1 and Group3 genes), both RelA and RelB transcription factors were present at one or more locations in the annotated regions of a gene. Also, as the enrichment scores for each group were low, I surmised that there was a little or no binding of the NF-κB factors to the genes. In conclusion, NF-κB factors weren't directly involved in causing the repression.

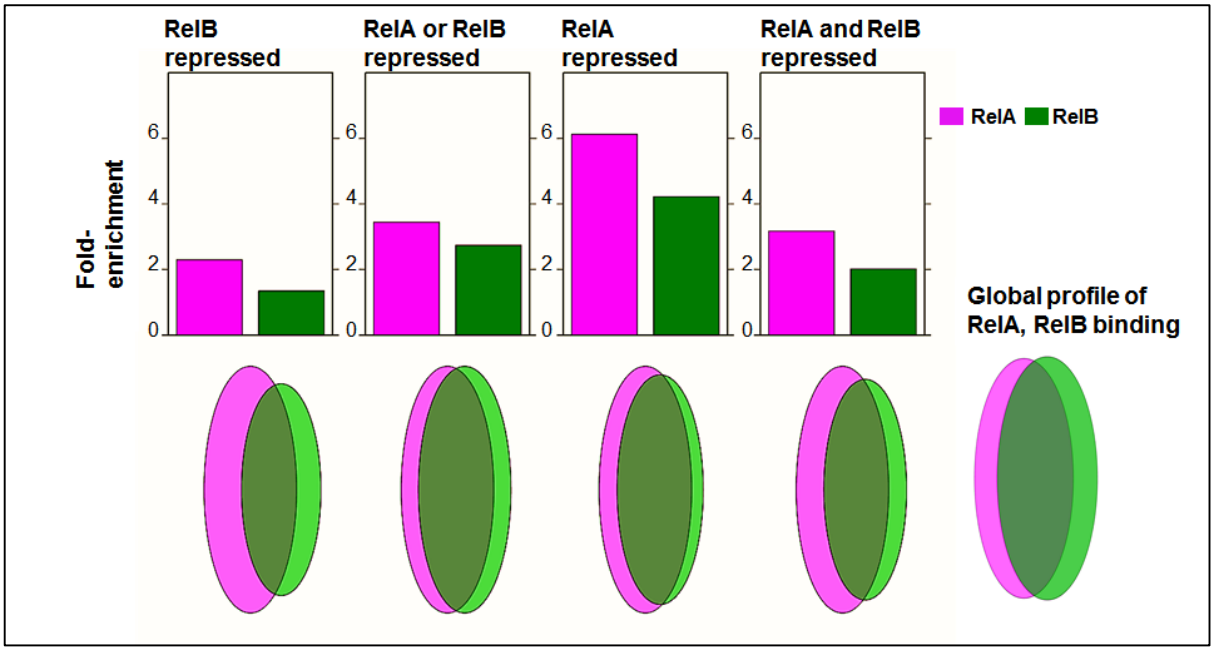


Fig 4: ChIP-seq analysis for the four gene-groups
a) The bar graph demonstrates fold enrichment score of binding of RelA or RelB to the genes in a group compared to 1000 random genes.
b) The Venn-diagram shows the number of genes in a group bound by RelA (magenta), bound by RelB (green), and bound by both(dark-green).

5. Homer de novo Motif analysis

From the low binding of NF-κB factors to the gene-groups, I next looked for indirect mechanisms of repression. Using Homer, I probed for de novo common motifs at the promoter regions of each groups and the likely transcription factor that could bind to those motifs (Fig 5b-e). Further, I checked the transcript levels of selected factors in the cell-knockouts harboring or lacking NF-κB activity. Thus, I arrived at a list of NF-κB dependent transcription factors that seemingly acted as secondary transcription factors (Fig 5a).

Transcript levels of factors involved in downregulation of genes

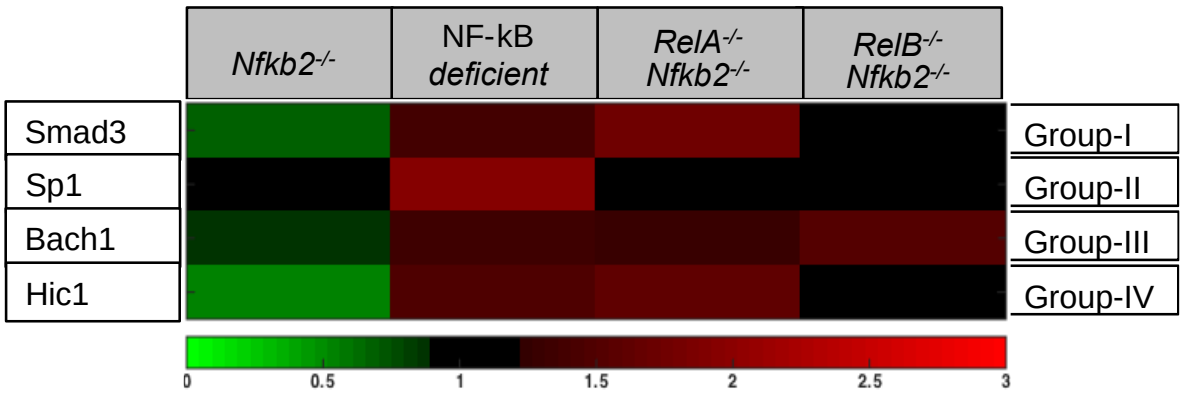


Fig 5a: Transcript levels of the putative secondary transcription factors
a) Heatmap showing 6hr/0hr transcript levels of transcription factors in various cell knockouts in response to TNFc treatment. Each transcription factor was inferred to regulate a set of genes from each group, thus indirectly aiding in repression.

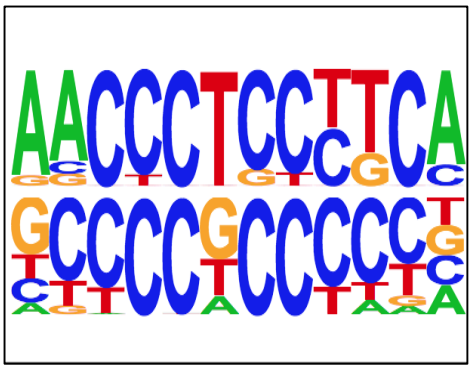
Group1:
Total target sequences = 198
Total background sequences = 29037
Motif name:Smad3

p-value:	1e-11
Number of Target Sequences with motif	40.0
Percentage of Target Sequences with motif	20.20%
Number of Background Sequences with motif	1707.4
Percentage of Background Sequences with motif	5.88%

Fig 5b: Alignment of de novo sequence to Smad3.

In Group1, motif for Smad3 was enriched (Fig 5b). The transcript analysis shows that mRNA levels of Smad3 increased in Rela$^{-/-}$*Nfkb2*$^{-/-}$ post TNFc treatment (Fig 5a), suggesting that Smad3 was induced by RelB. Thus, it invokes a plausible mechanism where Smad3 acts as an intermediate factor in repressing Group1, i.e., RelB repressed genes.

Group2:
Total target sequences = 115
Total background sequences = 26636
Motif name:Sp1

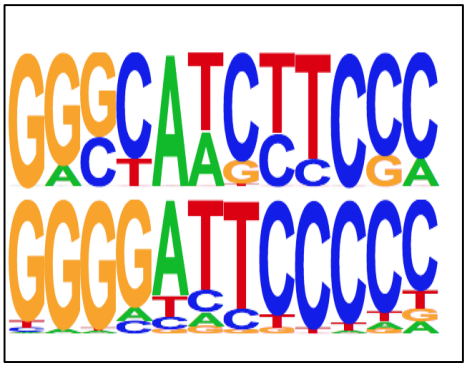


p-value:	1e-9
Number of Target Sequences with motif	19.0
Percentage of Target Sequences with motif	16.52%
Number of Background Sequences with motif	716.1
Percentage of Background Sequences with motif	2.69%

Fig 5c: Alignment of de novo sequence to Sp1

Sp1, an enriched motif in Group2 (Fig 5c), was induced in the absence of NF-κB, but relatively repressed in the presence of either NF-κB factors. In conclusion, RelA or RelB activity was sufficient for suppression of Sp1, which possibly was a factor regulating Group2 genes.

Group3:
Total target sequences = 56
Total background sequences = 26796
Motif name: Bach1

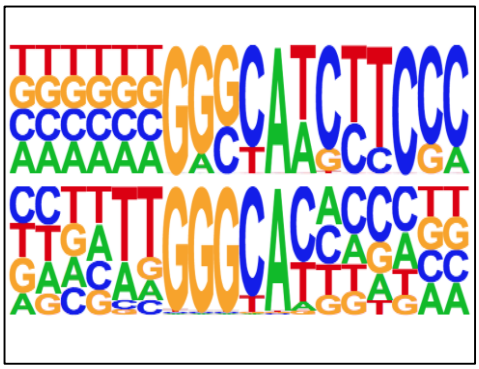


p-value:	1e-8
Number of Target Sequences with motif	9.0
Percentage of Target Sequences with motif	16.07%
Number of Background Sequences with motif	233.2
Percentage of Background Sequences with motif	0.87%

Fig 5d: Alignment of de novo sequence to Bach1

In Group3, i.e., RelA repressed genes, motif for Bach1 was enriched (Fig 5d). Bach1 transcript levels increase in the presence of RelA, and therefore, Bach1 could act as a secondary transcription factor in repressing Group3 genes.

Group-4
Total target sequences = 35
Total background sequences = 21116
Motif name:Hic1



p-value:	1e-10
Number of Target Sequences with motif	11.0
Percentage of Target Sequences with motif	31.43%
Number of Background Sequences with motif	399.5
Percentage of Background Sequences with motif	1.89%

Fig 5e: Alignment of de novo sequence to Hic1

Finally, Group4 genes were enriched for the motif Hic1 (Fig 5e). Hic1 is repressed only in *Nfkb2*$^{-/-}$, indicating the necessity of both the NF-κB factors for its suppression. As a result, Hic1 could act as a candidate factor in downregulating the Group4 genes.

Discussion:

First, I observed that NF-κB factors play a role in repression of gene expression upon pro-inflammatory stimulus TNF. I obtained a set of genes influenced by TNF-mediated repression by NF-κB factors. These genes could be categorized into four distinct groups, where each group was inferred to be repressed by either one or more transcription factors. The repressed genes were involved in regulating a variety of processes such as immune system regulation, metabolic processes, etc. Out of these processes, some were seemingly regulated by only a single NF-κB factor while others required more than one factors. Next, in the ChIP-Seq data analysis, I observed low enrichment scores due to poor direct binding of NF-κB factors.

Combining all of the above analyses, I concluded that NF-κB is extensively involved in repression, primarily through indirect measures. I then probed for common de novo motifs at the proximal promoter regions of gene-groups and arrived at a list of putative candidates acting as secondary transcription factors. Finally, I found that transcriptional repression via secondary transcription factors is an important mechanism leading to the repression of NF-κB dependent genes.

Future prospective:

Using the data processing pipeline, I obtained a set of repressed genes. I interpreted that most of these genes are repressed by indirect mechanisms. To probe the involvement of secondary transcription factors in this process, I carried out the promoter analysis (using HOMER) of the repressed genes. We look forward to make a mathematical model to know about the kinetics of gene repression mechanism. Also, we plan to verify the status of secondary transcription factors in various cell-knockouts by carrying out the qRT-PCR experiments. Further, we would like to check whether the secondary transcription factors bound to the repressed set of genes by performing a Ch-IP Seq analysis.

Physiological significance:

The NF-κB transcription factors are usually not good targets for therapeutic intervention. This is because they are involved in regulating a wide array of processes and thereby also affecting processes other that the intended one. However, the detection of indirect feedforward mechanism of gene repressions (or gene activation) may provide for an additional source for therapeutic targeting, with possibly diminished side effects. Thus, from our analysis, genes acting as secondary transcription factors having a role in NF-κB mediated repression provide an avenue as soft-targets for health interventions.

Our study was focused on p100-deficient system. Interestingly, inactivating mutations in *Nfkb2* have been frequently associated with the multiple-myeloma disease (Annunziata et al., 2007). Also, dendritic cells have reduced levels of p100. In conclusion, we claim that our study involving *Nfkb2*-deficient system has physiological and pathophysiological relevance.

Appendix:

1)List of genes in each group presented in Figure 1

Cluster1

"Fads1""2700094K13Rik""Aplp2""Ngfrap1""Gabarap""Scd1""Wbp5""Klf9""Arl6ip1""Uap1l1""Ddrgk1""Itgb5""Tpst1""Tulp4""Ldb1""Igf2bp2""4930403O06Rik""Fnip1""Impact""Rbbp9""Tmem43""Cdt1""Midn""Tcf4""AW549877""Lztr1""Zfyve21""Fnta""Mbnl1""Atp6v0d1""Arhgef18""Gpr177""Fam134a""Slc38a2""Neu1""Flcn""Rbms2""Tsc2""Ift81""Pold1""Mxra8""Fcho2""Slc44a2""Pias1""Zfp251""Angel2""BC039093""Fars2""LOC100045359""0610031J06Rik""Tmem106c""Zadh2""Obrgrp""Mic2l1""Itpr3""Abhd4""Glt8d1""Ints7""Sh3gl1""Man2c1""2410004L22Rik""LOC100047651""Grcc10""Echdc2""Tspan14""Hexa""Cul7""Kank1""Ttc3""9030624J02Rik""Cat""Bcs1l""E430025E21Rik""Chkb""Rev3l""Rbm4b""Tpp1""Ankmy2""N6amt1""Cdan1""Nacc2""Enpp5""D10Ertd610e""Snx21""Zcchc14""Ddah2""Ids""Rgl2""Paox""Pdcd4""Klhl17""Klhl22""Camk2n1""4933428G20Rik""Zbtb24""Ppp2r5d""Kif3a""Rab3a""Iqce""Ocrl""Smpd4""Sall2""Suv420h2""Sft2d2""Zfp512""Ccdc120""Zkscan17""Gps2""Ctdspl""Mpv17""Zfp30""1700030K09Rik""Atp5sl""1700034H14Rik""Dedd2""Aplf""Rreb1""Zfp579""Tmem62""Taz""Pick1""Cc2d1b""Mknk1""Krba1""Zkscan14""Fam171a2""Med12""Slc9a3r2""Tsc22d4""Spg20""Hist1h2bk""Igbp1""Rhobtb2""Epc1""Zscan21""Zfp354a""Nek3""Ezh2""Commd9""Casp9""Cfp""Zfp524""Dhx57""Tbc1d9b""Ethe1""Dcp1b""Ap4m1""Snx15""Ptpdc1""Dgka""Rshl2a""Psg23""Ap1gbp1""D930015E06Rik""Hrsp12""Pigo""Polg2""Athl1""Nphp4""Fam149b""Ttll5""ORF19"	Group1

Cluster2

"Ak3""Ckb""Ahcyl1""Lrig3""Pqlc1""Gtpbp2""Hdac3""LOC100045343""Klhl26""Slc6a8""Ctsz""Man2a1""Mib2""Agap1""Tpcn1""Clk4""Trp53inp1""Rxrb""1500012F01Rik""Col4a5""Mospd1""Plcb3""2410025L10Rik""Prkd3"B930041F14Rik""Mrpl24""Zfp187""Slc39a13""Ep300""Bdh2"Exoc3""Frs2""Fzd5""Luc7l2""Ifi30""Sdf4"Sh2b1""2410018M08Rik""Pold4""Cep164""Xpc""Eml3""Hspa12b""BC046404""Mtvr2""Snrk""2310066E14Rik""Zfpl1"BC031353""Rsad1""Plcd3""Nav1""Wrb""Ddx6"Map3k12""Tnrc6c""Dennd2a""Chchd5""Dalrd3""Hic2""Gsto2""Sntb2""Gnpda1""Mepce""Cetn4""Spnb2""Zfp661""Foxn3""Ccl27""Six5""Prkcbp1""Fbxo31""Kifc3""Ogfod2"	

Cluster3

"Scara3""Osbpl9""Scarf2""Olfml2b""D4Bwg0951e""Acadm""2310022B05Rik""Fhl1""Echs1""Nrp1""Adk""Vim""Rhobtb3""6330406I15Rik""Kctd10""Ccdc80""Snx24""Flot1""Sdc2""Rpl22""Slc35b2""Abhd5""LOC100046883""2310036D22Rik""Lrrk1""Sema3f""Il33""Cxcl12""Zfp637""Ugp2""Meis1""Ermp1""Mxra7""Npr2""Itfg3""1110032E23Rik""LOC100047012""Ube2e3""Tmem150""Letmd1""Gstz1""1110001A07Rik""Nagk""Zxdc""Kif21a""C87436""Zhx1""Ulk2""8430432M10Rik""2310003H01Rik""Cd99l2""Slc25a12""AW540478""Bre""Ndrg3""Tef""Ddit4l""Dtd1""Eps8""9630058J23Rik""Aasdh""Aldh16a1""Zfp467""Cobll1""Adamtsl4""BC029214""Ece1""Gm1673""Tdrd3""Abhd12""Evi5l""Smarcd3""AA536717""6720460F02Rik""Nit2""Ahnak2"	Group2

Cluster4

"Emb""St6gal1""Nrn1""Spon2""Bscl2""Il11ra1""Crip2""Id1""Zer1""Supt3h""Hsd3b7""Trib2""Cnnm2""Gab1""Ssbp3""Retsat""Wasf1""Aldh4a1""Eno3""Foxc1""1500010J02Rik""Tmem53""Rab3d""Pfkfb2""Prps2""Nrbp2""Inpp5e""Tlcd1""D6Wsu163e""Mical1""Dync2li1""Tspan17""Rfx2""Irs2""2300002D11Rik""Ctnnal1""Ntn2l""4930455F23Rik""Zfp101""Ctns""Peli2""Senp7""Zmym3""Fam13c""Mcoln1""Gamt""2510009E07Rik""Cyp4f13""Hectd3""Btbd6""Arhgef19""1110013L07Rik""5730419I09Rik""Mfsd11""Wrn""Gpr19""Nhsl1""Txnrd2""37499""Ganc""Pxmp4""Kat2b""Hist1h2bm""Rcbtb2""Spsb2"	

Cluster5

"Anxa3""Ctnna1""Emp1""Prnp""Grb10""Hnrpl""Cav1""Lhfp""Mum1""Tgfbr2""Pls3""Lasp1""Dap""1810063B05Rik""Rbm5""AW555464""2400001E08Rik""Sumo3""Wwc2""Itga3""Fbxo21""Snx8""Gpx8""2310016C16Rik""Trappc2l""BC025076""Hirip3""Ap3d1""Raly""Ccdc56""Mrpl30""B4galt1""Hdac7""Pacs2""Rell1""E330036I19Rik""Ak1""Zfml""Rsu1""Ranbp9""Nln""Pdgfc""Sh3rf1""Cryab""Tle1""Avpi1""Zfp317""Tmed3""Wiz""D5Ertd579e""Ahnak""Fam110a""Prpf38b""Flii""Bcl2""Cbara1""Pmm1""Zfp768""Nab2""Gpsm1""Slc12a9""Bmi1""B230317C12Rik""Stx3""Usp30""Pigt""1810015A11Rik""Ankrd25""Smg6""Tcea2"	Group3

Cluster6

"Timp3""Hnrpdl""Nudt4""Por""Lbh""Mta2""Morc2a""Klhl7""Akap12""Apbb2""Vat1""Phlda3""Evc2""Cdc2l5""Pitpnm2""Cln3""Tbl1x""Wwc1""Stard5""Qtrt1""Gas8""D430028G21Rik""Clk1""Fgfrl1""Grem1""Polh""Dtx4""Stau2""Terf2""C85492""4631427C17Rik""LOC100047427""Zmiz1""Tada2l""Zbtb2""Cnot6l""Gatad2b""Metapl1""Mthfsd""Git1""Kremen""Egln2""Chka""Rpusd1""Acin1"	Group4

2) List of the primers used in our quantitative real-time PCR in Figure 2

	Gene Name	Primer Sequence
1	Pdcd4	Fwd: ACTGACCCTGACAATTTAAGCG Rev: TTTTCCGCAGTCGTCTTTTGG
2	Foxc1	Fwd: TATGAGCGTGTACTCGCACCCT Rev: CGTACCGTTCTCCGTCTTGATGTC
3	Pdgfc	Fwd: GCCCGAAGTTTCCTCATACA Rev: ACACTTCCATCACTGGGCTC
4	Acin1	Fwd: ATGTGGGGACGGAAACGAC Rev: CTTCGGGCATCTTCGGTAATTT

3) List of GO terms and their enrichment scores presented in Figure 3

GO ID	GO Term	Group1	Group2	Group3	Group4	Processes
GO:2000973	regulation of pro-B cell differentiation	2.530	0.000	0.000	0.000	Immune system processes
GO:0002826	negative regulation of T-helper 1 type immune response	0.000	1.322	0.000	0.000	
GO:0033077	T cell differentiation in thymus	0.520	0.000	1.424	0.000	
GO:0071594	thymocyte aggregation	0.520	0.000	1.424	0.000	
GO:0033083	regulation of immature T cell proliferation	0.872	0.000	1.408	0.000	
GO:0030890	positive regulation of B cell proliferation	0.000	0.595	2.046	0.000	
GO:0032700	negative regulation of interleukin-17 production	0.000	0.000	1.330	0.000	
GO:0043374	CD8-positive, alpha-beta T cell differentiation	0.000	0.000	1.452	0.000	
GO:0032703	negative regulation of interleukin-2 production	0.000	0.000	2.690	0.000	
GO:0046007	negative regulation of activated T cell proliferation	0.000	0.000	1.503	0.000	
GO:0002326	B cell lineage commitment	0.000	0.000	1.704	0.000	
GO:0033091	positive regulation of immature T cell	0.000	0.000	1.704	0.000	

	proliferation					
GO:0051138	positive regulation of NK T cell differentiation	0.000	0.000	3.824	0.000	
GO:0002283	neutrophil activation involved in immune response	0.000	0.000	1.560	0.000	
GO:0002645	positive regulation of tolerance induction	0.000	0.000	1.560	0.000	
GO:0002448	mast cell mediated immunity	0.000	1.381	0.000	0.000	
GO:0008063	Toll signaling pathway	0.000	1.399	0.000	0.000	
GO:0034139	regulation of toll-like receptor 3 signaling pathway	0.000	1.151	1.452	0.000	
GO:0090025	regulation of monocyte chemotaxis	0.000	0.889	0.000	1.390	
GO:2000107	negative regulation of leukocyte apoptotic process	0.000	2.214	0.000	0.000	
GO:0002686	negative regulation of leukocyte migration	0.000	1.949	0.000	1.320	
GO:1903236	regulation of leukocyte tethering or rolling	0.000	1.399	0.000	0.000	
GO:1904994	regulation of leukocyte adhesion to vascular endothelial cell	0.000	1.399	0.000	0.000	
GO:0002689	negative regulation of leukocyte chemotaxis	0.000	0.000	0.000	1.712	
GO:0031669	cellular response to nutrient levels	1.664	0.225	1.141	0.000	Response to stress and environmental changes
GO:0009267	cellular response to starvation	1.986	0.277	1.285	0.000	
GO:0042149	cellular response to glucose starvation	1.373	0.000	1.057	0.000	
GO:0070482	response to oxygen levels	0.638	0.123	1.514	0.464	
GO:0001666	response to hypoxia	0.387	0.134	1.574	0.483	
GO:0042542	response to hydrogen peroxide	0.901	0.000	1.337	0.000	
GO:0071453	cellular response to oxygen levels	0.170	0.000	1.414	0.000	
GO:0055093	response to hyperoxia	0.000	0.000	1.626	0.000	

GO:0071455	cellular response to hyperoxia	0.000	0.000	1.704	0.000	
GO:0045852	pH elevation	0.000	0.000	1.626	1.836	
GO:0051454	intracellular pH elevation	0.000	0.000	1.626	1.836	
GO:0043401	steroid hormone mediated signaling pathway	1.372	0.000	0.000	0.000	Steroid hormone related regulations
GO:0030518	intracellular steroid hormone receptor signaling pathway	1.426	0.000	0.000	0.000	
GO:0031960	response to corticosteroid	1.336	0.000	0.698	0.000	
GO:0051384	response to glucocorticoid	1.376	0.000	0.712	0.000	
GO:0032352	positive regulation of hormone metabolic process	0.000	0.000	0.000	1.415	
GO:2001238	positive regulation of extrinsic apoptotic signaling pathway	0.266	0.000	1.710	0.000	Cell death processes
GO:0043154	negative regulation of cysteine-type endopeptidase activity involved in apoptotic process	1.033	0.340	1.445	0.774	
GO:0060561	apoptotic process involved in morphogenesis	0.478	0.691	2.259	0.000	
GO:1904748	regulation of apoptotic process involved in development	0.000	1.031	1.330	0.000	
GO:2001240	negative regulation of extrinsic apoptotic signaling pathway in absence of ligand	0.000	0.000	2.322	0.000	
GO:0060544	regulation of necroptotic process	0.000	0.000	1.330	0.000	
GO:0046666	retinal cell programmed cell death	0.000	0.000	1.560	0.000	
GO:0060057	apoptotic process involved in mammary gland involution	0.000	0.000	1.704	0.000	
GO:0010941	regulation of cell death	0.332	0.063	1.070	1.489	
GO:0042981	regulation of apoptotic process	0.283	0.097	0.899	1.663	
GO:0051171	regulation of nitrogen	1.687	0.010	0.000	0.523	Metabolic processes

	compound metabolic process					
GO:0046486	glycerolipid metabolic process	1.371	0.564	0.650	0.372	
GO:0034248	regulation of cellular amide metabolic process	1.688	0.000	0.000	0.355	
GO:0020027	hemoglobin metabolic process	1.843	0.000	0.000	0.000	
GO:0006013	mannose metabolic process	2.388	0.000	1.560	0.000	
GO:0006689	ganglioside catabolic process	2.388	0.000	0.000	0.000	
GO:0019637	organophosphate metabolic process	0.215	1.447	0.098	0.575	
GO:0032787	monocarboxylic acid metabolic process	0.043	1.439	0.070	0.498	
GO:0006631	fatty acid metabolic process	0.198	1.435	0.152	0.278	
GO:0009161	ribonucleoside monophosphate metabolic process	0.356	1.306	0.306	0.000	
GO:0009126	purine nucleoside monophosphate metabolic process	0.367	1.329	0.311	0.000	
GO:0009144	purine nucleoside triphosphate metabolic process	0.375	1.345	0.315	0.000	
GO:0006575	cellular modified amino acid metabolic process	0.687	1.361	0.000	0.481	
GO:0044242	cellular lipid catabolic process	0.512	1.529	0.000	0.000	
GO:0046128	purine ribonucleoside metabolic process	0.443	2.121	0.241	0.000	
GO:1901605	alpha-amino acid metabolic process	0.127	2.405	0.000	0.436	
GO:0010906	regulation of glucose metabolic process	0.120	2.084	1.236	0.000	
GO:0046395	carboxylic acid catabolic process	0.209	2.936	0.000	0.000	
GO:0044282	small molecule catabolic process	0.224	1.767	0.000	0.000	
GO:0009063	cellular amino acid catabolic process	0.207	1.768	0.000	0.000	
GO:0006749	glutathione metabolic process	0.883	1.306	0.000	0.000	
GO:0006111	regulation of gluconeogenesis	0.000	1.443	0.862	0.000	
GO:0050994	regulation of lipid catabolic process	0.000	2.188	0.000	0.000	
GO:0046461	neutral lipid catabolic process	0.000	1.869	0.000	0.000	
GO:0046464	acylglycerol catabolic process	0.000	1.869	0.000	0.000	

GO:0006558	L-phenylalanine metabolic process	0.000	1.322	0.000	0.000	
GO:0010815	bradykinin catabolic process	0.000	1.322	0.000	0.000	
GO:0006600	creatine metabolic process	0.000	1.399	0.000	0.000	
GO:0072366	regulation of cellular ketone metabolic process by positive regulation of transcription from RNA polymerase II promoter	0.000	1.399	0.000	0.000	
GO:1902222	erythrose 4-phosphate/phosphoenolpyruvate family amino acid catabolic process	0.000	1.399	0.000	0.000	
GO:0060255	regulation of macromolecule metabolic process	0.530	0.015	2.292	0.893	
GO:0019318	hexose metabolic process	0.162	0.344	2.297	0.000	
GO:2000378	negative regulation of reactive oxygen species metabolic process	0.000	0.000	3.125	0.000	
GO:0046033	AMP metabolic process	0.833	1.067	1.367	0.000	
GO:0006012	galactose metabolic process	0.000	0.000	1.503	0.000	
GO:0019673	GDP-mannose metabolic process	0.000	0.000	1.626	0.000	
GO:0044857	plasma membrane raft organization	0.000	1.399	1.704	0.000	
GO:0046483	heterocycle metabolic process	0.094	0.027	0.389	1.337	
GO:1901360	organic cyclic compound metabolic process	0.091	0.035	0.481	1.517	
GO:0032269	negative regulation of cellular protein metabolic process	0.477	0.019	0.697	1.393	
GO:0097164	ammonium ion metabolic process	0.563	0.190	0.000	1.416	
GO:0042439	ethanolamine-containing compound metabolic process	0.808	0.000	0.000	2.197	
GO:0042987	amyloid precursor protein catabolic process	0.000	0.000	0.000	1.365	
GO:0045540	regulation of cholesterol biosynthetic	0.000	0.000	0.000	1.443	

	process					
GO:0006684	sphingomyelin metabolic process	0.799	0.000	0.000	1.538	
GO:1901160	primary amino compound metabolic process	0.000	0.000	0.000	1.538	
GO:0006677	glycosylceramide metabolic process	0.000	0.000	0.000	1.575	
GO:0019471	4-hydroxyproline metabolic process	0.000	0.000	0.000	1.662	
GO:0046337	phosphatidylethanolamine metabolic process	0.914	0.000	0.000	1.662	
GO:0006678	glucosylceramide metabolic process	0.000	0.000	0.000	1.836	
GO:0046116	queuosine metabolic process	0.000	0.000	0.000	1.836	
GO:0019374	galactolipid metabolic process	0.000	0.000	0.000	1.915	
GO:0019695	choline metabolic process	0.000	0.000	0.000	1.915	
GO:1901576	organic substance biosynthetic process	2.079	0.115	1.444	1.313	Biosynthetic processes
GO:0044271	cellular nitrogen compound biosynthetic process	2.175	0.065	0.685	1.096	
GO:1901566	organonitrogen compound biosynthetic process	1.413	0.492	0.010	0.740	
GO:0043604	amide biosynthetic process	1.825	0.035	0.000	0.143	
GO:0006163	purine nucleotide metabolic process	0.052	1.589	0.118	0.232	
GO:0009133	nucleoside diphosphate biosynthetic process	1.081	0.000	1.626	0.000	
GO:0019438	aromatic compound biosynthetic process	0.966	0.100	1.066	1.488	
GO:0034654	nucleobase-containing compound biosynthetic process	0.953	0.082	1.136	1.557	
GO:1901362	organic cyclic compound biosynthetic process	0.786	0.158	0.967	1.763	
GO:0071897	DNA biosynthetic process	1.243	0.251	0.000	1.598	
GO:0016051	carbohydrate biosynthetic process	0.215	0.958	1.715	0.000	

GO:0009226	nucleotide-sugar biosynthetic process	0.914	0.000	1.452	0.000	
GO:0009312	oligosaccharide biosynthetic process	0.000	0.000	1.452	0.000	
GO:0033692	cellular polysaccharide biosynthetic process	0.000	1.324	0.802	0.000	
GO:0005978	glycogen biosynthetic process	0.000	1.511	0.896	0.000	
GO:0009250	glucan biosynthetic process	0.000	1.511	0.896	0.000	
GO:0006561	proline biosynthetic process	0.000	1.399	0.000	0.000	
GO:0008616	queuosine biosynthetic process	0.000	0.000	0.000	1.836	
GO:0016070	RNA metabolic process	1.853	0.005	1.749	1.226	
GO:0006518	peptide metabolic process	2.316	0.351	0.051	0.131	Protein synthesis
GO:0018205	peptidyl-lysine modification	1.398	0.077	0.658	0.376	
GO:0043038	amino acid activation	1.772	0.000	0.000	0.000	
GO:0043039	tRNA aminoacylation	1.772	0.000	0.000	0.000	
GO:1900084	regulation of peptidyl-tyrosine autophosphorylation	0.000	0.000	1.704	1.915	
GO:0006400	tRNA modification	0.000	0.000	0.000	2.509	
GO:0019511	peptidyl-proline hydroxylation	0.000	0.000	0.000	1.662	
GO:0031119	tRNA pseudouridine synthesis	0.000	0.000	0.000	1.915	
GO:0098751	bone cell development	0.533	1.796	0.000	0.000	Bone development and function
GO:0060349	bone morphogenesis	0.179	0.000	1.445	0.774	
GO:0060348	bone development	0.212	0.952	2.590	0.000	
GO:0030279	negative regulation of ossification	0.000	0.371	1.523	0.813	
GO:0060350	endochondral bone morphogenesis	0.000	0.000	1.878	0.990	
GO:0003413	chondrocyte differentiation involved in endochondral	0.000	0.000	1.503	0.000	

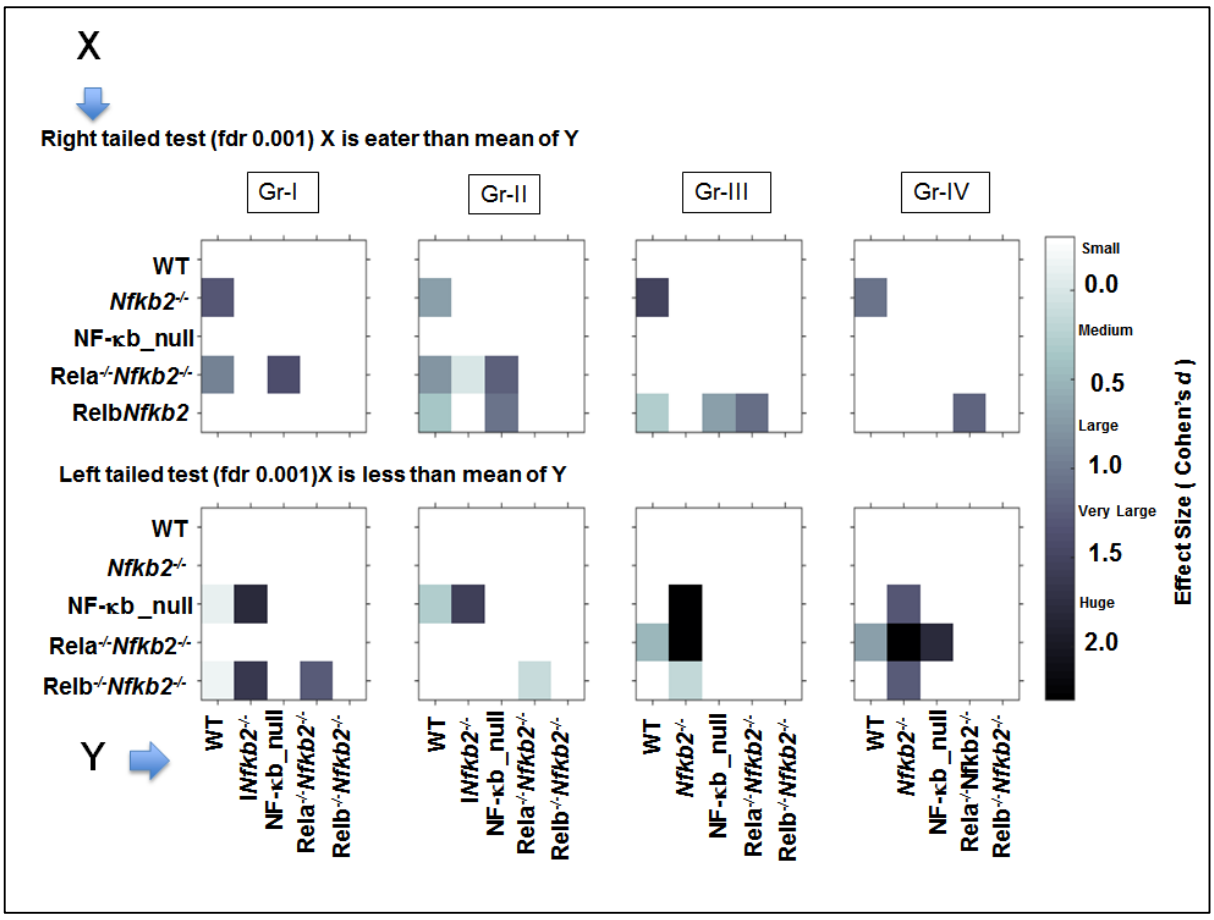
	bone morphogenesis					
GO:0033689	negative regulation of osteoblast proliferation	0.000	0.000	1.560	1.770	
GO:0003418	growth plate cartilage chondrocyte differentiation	0.000	0.000	1.704	0.000	
GO:0003433	chondrocyte development involved in endochondral bone morphogenesis	0.000	0.000	1.704	0.000	
GO:0003416	endochondral bone growth	0.000	0.000	1.115	1.320	
GO:0070977	bone maturation	0.000	0.000	0.000	1.415	
GO:0043931	ossification involved in bone maturation	0.000	0.000	0.000	1.443	
GO:0046851	negative regulation of bone remodeling	0.000	0.000	0.000	1.443	
GO:0061430	bone trabecula morphogenesis	0.000	0.000	0.000	1.504	
GO:0035630	bone mineralization involved in bone maturation	0.000	0.000	0.000	1.662	
GO:0002158	osteoclast proliferation	0.000	0.000	0.000	1.836	
GO:0051147	regulation of muscle cell differentiation	1.327	0.211	0.986	0.544	Muscle development
GO:0051153	regulation of striated muscle cell differentiation	1.426	0.000	1.319	0.711	
GO:0060538	skeletal muscle organ development	0.412	0.142	1.616	0.496	
GO:0016202	regulation of striated muscle tissue development	0.097	0.225	3.004	0.622	
GO:0014743	regulation of muscle hypertrophy	0.000	1.381	0.000	0.000	
GO:0010611	regulation of cardiac muscle hypertrophy	0.000	1.422	0.000	0.000	
GO:1901741	positive regulation of myoblast fusion	0.000	2.039	0.000	0.000	
GO:0014902	myotube differentiation	0.000	0.000	1.328	0.000	
GO:0010830	regulation of myotube	0.000	0.000	1.789	0.000	

	differentiation					
GO:0048641	regulation of skeletal muscle tissue development	0.000	0.497	1.824	0.000	
GO:0048742	regulation of skeletal muscle fiber development	0.000	0.000	1.330	0.000	
GO:0032330	regulation of chondrocyte differentiation	0.000	0.000	0.000	2.435	
GO:0060379	cardiac muscle cell myoblast differentiation	0.000	0.000	0.000	1.616	
GO:0033059	cellular pigmentation	0.000	0.000	2.094	0.000	pigmentation
GO:0048070	regulation of developmental pigmentation	0.000	0.000	1.367	0.000	
GO:0043476	pigment accumulation	0.000	0.000	1.452	0.000	
GO:0043485	endosome to pigment granule transport	0.000	0.000	1.503	0.000	
GO:0048757	pigment granule maturation	0.000	0.000	1.503	0.000	
GO:0045634	regulation of melanocyte differentiation	0.000	0.000	1.626	0.000	

4) Determination of the difference in gene-expression between various genotypes

I tested whether, for a group, gene-expression mean in various genotypes are significantly different, using a t-test. I subjected my data set to right and left tailed t-tests, using a false discovery rate(fdr) < 0.001 (Maitra and Melnykov, 2010). I also determined the effect size as a measure of the numerical significance of these differences. The data has been presented using q-value maps (Sawilowsky, 2009). Absence of a block in a q-value map indicates insignificant difference between a pair of distributions. In other words, it signifies that the expression of genes belonging to a given groups is not significantly different between the indicated pair of genotypes. The color bar captures effect sizes for statistically significant pairs. WT, *Nfkb2*$^{-/-}$, *Relb*$^{-/-}$*Nfkb2*$^{-/-}$, *Rela*$^{-/-}$*Nfkb2*$^{-/-}$, *Rela*$^{-/-}$*Relb*$^{-/-}$*Rel*$^{-/-}$. Right tailed test (fdr 0.001): if significant, mean of Y is greater than mean of X Left tailed test (fdr 0.001): if significant, mean of Y is less than mean of X. We implemented individually right

and left tailed t-tests for false discovery rate < 0.001, and examined if the mean of a distribution was significantly different than the mean of another distribution.



Supplementary Fig 1: q-value maps indicating the differences between various genotypes.

Thesis contributions:

Aditee Kadam carried out the microarray, Gene Ontology, ChIP-Seq and motif analyses with the assistance from Budhaditya Chatterjee. The data for microarray experiments was obtained from the Systems Immunology Lab that was performed as a part of Roy et al., 2016. The ChIP Seq data was kindly provided by Dr Ranjan Sen, a Principal Investigator at National Institute on Aging. qRT-PCR experiments were performed by Aditee Kadam with extensive assistance from Uday Sarkar. The thesis was carried out under the supervision of Dr Soumen Basak and Dr L S Shashidhara.

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