# The identification of transcriptional targets of Interferon Regulatory Factors (IRF) 1 and 8 in the context of pathogen challenge by ChIP on Chip and genome wide transcription profiling

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**Abstract:** Interferon regulatory factors (IRFs) is a family of transcriptional regulators that are essential for cell differentiation and growth, oncogenesis and the regulation of immunity. IRF1 and IRF8 play a crucial role in immunity by regulating the differentiation of the cells of myeloid lineage, particularly the development of immune cells including macrophages, NK and dendritic cells. Both transcription factors are essential for the resistance to viral and bacterial infections. We optimized the chromatin immunoprecipitation (ChIP) technique to be used with IRF8 and IRF1 antibodies in a J774 line stimulated with IFNγ/CpG. Using ChIP coupled to an exon promoter 244K microarray (ChIP on Chip) we identified 201 and 303 binding sites of high confidence for IRF1 and IRF8 respectively. Previously known IRF1 and IRF8 transcription targets (OAS1b for IRF1 and IFNb for IRF8) as well as novel transcriptional control loci were identified. One of the major gene ontology (GO) functional categories in the two gene lists was "immune response". The overlap of IRF1 and IRF8 transcription target genes from ChIP on Chip revealed an overlap of 19 genes most of which belonged to the "immune regulation" GO pathway and included targets of high confidence: Gbp6, Mx2, Tnfsf13b, H2-T24, and Ifit1. A parallel microarray transcription profiling study on mouse bone marrow macrophages (BMDMs) from an F2 generation of Balb/C IRF8-wildtype and BXH2 IRF8-mutant cross was performed. The BMDMs possessing the wildtype and the mutant IRF8 alleles were infected with *Legionella pneumophila* or stimulated with IFNγ/CpG to identify genes regulated by IRF8 in the context of infection. 1171 and 852 differentially regulated genes were differentially regulated in the infection and stimulation experiments respectively. Genes that displayed interaction between IRF8 allele and the infection conditions were isolated to yield high confidence gene lists of 31 and 129 genes for the *Legionella* and IFNγ/CpG experiments respectively. The overlap of the two gene lists revealed 7 genes in common, two of which belong to the JAK-STAT signalling pathway (IL10 and Ccd1). The comparison of the IFNγ/CpG microarray experiment gene list with the gene list from IRF8 ChIP on Chip revealed 15 genes in common. 2 of the 15 genes (Gbp6 and H2-T24) were also identified as potential targets common to IRF1 and IRF8 by ChIP on Chip. Gbp2 and H2-T24 represent interesting target genes for study and validation as high confidence targets of IRF8 and possibly, IRF1. Taken together, our data provides a set of novel IRF8 and IRF1 targets that may be studied to further our understanding of the regulatory networks controlled by IRF1 and IRF8 in the context of immunity.

**Résumé:** Les facteurs régulateurs interférons (IRFs) sont une famille de régulateurs transcriptionnels essentiels à la différenciation et la croissance cellulaire, à l'oncogénèse et au bon maintien du système immunitaire. IRF1 et IRF8 jouent un role clé dans l'immunité de l'organisme par la régulation de la différenciation des lignées cellulaires myéloïdes, nottament le développement de cellules immunitaires comme les macrophages, les cellules tueuses naturelles (NK) et les cellules dendritiques. Ces deux facteurs de transcription sont indispensables pour la résistance aux infections virales et bactériennes. Nous avons optimisé une technique d'immunoprécipitation de la chromatine (ChIP) afin d'y utiliser des anticorps ciblant IRF1 et IRF8, et ce à partir d'une lignée cellulaire J774 suivant un stimulation par interféron gamma et par oligonucléotides CpG (IFNγ/CpG). En combinant cette technique ChIP à un microarray 244K comprenant exons et promoteurs (ChIP on Chip), nous avons identifiés respectivement 201 et 303 sites d'ancrages pour chacun de IRF1 et IRF8. Certaines cibles transcriptionnelles déjà connues pour IRF1 (OAS1b) et IRF8 (IFNb) ainsi que de nouveaux loci de control transcriptionel ont été identifiés. Parmi les catégories par fonction relevées par Ontologie de gène (GO), une des catégories majeures des deux listes de gènes générées fut pour les gènes reliés à la ''réponse immunitaire''. La comparaison entre les gènes cibles de transcription pour IRF1 et IRF8 par ''ChIP on Chip'' a révélé 19 gènes représentés dans les deux cas. La majeure partie de ces gènes sont reliés à la ''réponse immunitaire'' par GO et ils incluent les cibles suivantes avec détection très significative: Gbp6, Mx2, Tnfsf13b, H2-T24, et Ifit1. En parallèle, des macrophages dérivés de la moëlle osseuse de souris (BMDMs) furent utilisés pour l'étude du profile transcriptionel par microarray d'une génération F2 générée avec des souris Balb/C (IRF8-wildtype) et des souris BXH2 (IRF8-mutant). Ces BMDMs comprenant les allèles normale et mutante d'IRF8 ont été infectés avec *Legionella pneumophila* ou stimulés avec IFNγ/CpG pour identifer les gènes dépendant d'IRF8 dans le cadre d'une infection. Pour chaque traitement, l'expression de respectivement 1171 et 852 gènes furent observés comme étant modifiés selon la situation relative à IRF8 dans les expériences d'infections et de stimulations. Selon leur intéraction avec les allèles d'IRF8 et pour chaque traitement des BMDBs, une liste de gènes fut établie avec haut niveau de confiance comprenant 31 et 129 gènes pour chacun des types de stimulation par Legionelle et par IFNγ/CpG, respectivement. Ces deux listes de gènes comprennent 7 gènes en commun, deux appartenant à la voie de signalement JAK-STAT (IL10 et Ccd1). La comparaison en les listes de gènes générées dans les expériences de stimulation IFNγ/CpG par microarray ainsi que celles générées par les expériences ''ChIP on Chip'' avec IRF8 ont révélés 15 gènes en commun. Deux de ces 15 gènes (Gbp6 et H2-T24) ont aussi été identifiés comme cibles potentielles pour IRF1 en plus de IRF8 par ''ChIP on Chip''. Gpb2 et H2-T24 représentent des gènes cibles intéressants pour d'autres séries d'études et de validations comme étant des cibles à haut niveau de confiance pour IRF8 et possiblement, IRF1. En somme, ces résultats fournissent un nouveau répertoire de cibles pour IRF1 et IRF8 qui pourront être étudiées pour approfondir nos connaissances sur les réseaux de régulation controlés par IRF1 et IRF8 dans le maintien de l'immunité de l'organisme.

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# Chapter I
# Introduction

# 1. INTRODUCTION

## 1.1. Rationale

The Interferon regulatory factor (IRF) family members are transcriptional regulators implicated in many cellular events including cell differentiation and growth, oncogenesis and as innate immunity. Mice with mutations in IRFs exhibit severe phenotypes including the inability to elicit a sufficient immune response to many types of infections. IRF1 and IRF8 are both important for the development of Th1 response, NK cell maturation, haematopoietic cell development, and resistance to viral and bacterial infections [1]. Both factors up-regulate a number of important immunogenic cytokines and chemokines including Interleukins 12 (IL12) and 1B (IL-1B), iNOS, IFNγ and TNFα [1, 2].

The transcriptome of IRF1 has been extensively studied in the context of cancer [3, 4], while the regulation of genes by IRF8 has been primarily studied in the context of myeloid progenitor cell maturation, and differentiation or during myeloproliferative disorders [5, 6]. Dror and colleagues studied the genes differentially expressed in peritoneal macrophages following stimulation with lipopolysaccharide (LPS) and IFNγ [7]. These latter studies have identified genes, the expression of which is likely dependent on IRF8 and/or IRF1; however, these experiments did not define the genes that are their direct transcriptional targets. The identification of direct IRF8 and IRF1 targets during infection can be better accomplished using a chromatin immunoprecipitation technique known as ChIP on Chip in primary macrophages activated with immunogenic ligands. In this project, we used both, ChIP on Chip and genome-wide transcript profiling to identify the transcriptomes of both IRF8/IRF1. Overlapping the results obtained by these two techniques provides a comprehensive list of genes which are directly cis-regulated by these two factors. The gene lists obtained from these studies may also include new candidate genes for genes which could be implicated in atypical susceptibility to infectious diseases in humans.

## 1.2. Specific aims of the project

The overlap of two techniques, ChIP on Chip and transcript profiling can allow for identification of genes that are directly regulated by IRF1 and/or IRF8 after pathogen challenge. The first essential step of the project was the optimization of the ChIP on Chip protocol, identification of the appropriate antibody conditions for immunoprecipitation of chromatin/protein complexes and optimal stimulation of the macrophages. The second goal of this project was to hybridize the IRF8- or IRF1-specific chromatin onto Agilent 244K microarray slides which would identify the precise regions of the genome where IRF8 and IRF1 bind in response to the immune stimuli. A parallel aim of the project was to generate a list of genes which are differentially regulated by IRF8 in response to *Legionella pneumophila* infection or IFNγ/CpG-oligodeoxynucleotide (CpG) stimulation which was done using Illumina whole mouse genome microarrays. In the final stages of the project the resulting gene lists from whole genome scanning will be merged with the list of promoter binding sites discovered using ChIP on Chip to create a comprehensive short list of genes directly regulated by IRF8 and IRF1 in the context of infection.

## 1.3. Interferons

### *1.3.1. Interferons as a family*

Interferons (IFNs) were the first of a long series of cytokines to be characterized more than fifty years ago. Their first description dates back to 1957 when Isaacs and Lindenmann noticed that the growth of virulent influenza was "interfered with" if it was grown in the presence of chick chorioallantoic egg membranes which had previously been incubated with heat inactivated influenza particles [8]. The active molecules studied by Isaacs and Lindenmann were type I Interferons that retained their descriptive name [9, 10]. Since their discovery, the role of IFNs in host defense against viral and other types of infections has been extensively described.

All IFNs bind to extracellular receptors and activate downstream signalling cascades that can result in a plethora of biological responses. In humans, IFNs are

separated into three subfamilies (IFN I, II and III) based on their amino acid sequence similarity [9]. Type I IFNs (IFNα/β/ω/ ε/κ) comprise the viral immune response and are produced by many types of cells and organs [9, 10]. They are up-regulated by many infection-related stimuli including double stranded RNA, LPS, umethylated CpG DNA and other pathogen related molecules [11-13]. Together with toll-like receptors (TLRs), type I IFNs are important for innate and adaptive immunity. In mammals, they function to enhance antibody response, up-regulate molecules necessary for the maturation of antigen presenting cells (CD40 and CD86), and stimulate antibody isotype switching [11]. Type II IFN (IFNγ) is exclusively secreted by activated immune cells such as T lymphocytes (Th1 subset and CD8+ cytotoxic T cells), NK cells and certain macrophages, especially the plasmocytoid dendritic cells [14, 15]. IFNγ is up-regulated by mitogens as well as antigenic and cytokine stimulation of sensitized cells [10, 14, 16]. This leads to increased pathogen resistance and clearance in the host organism, and induces macrophage activation. More specifically, IFNγ increases antigen processing and expression of MHC molecules, promotes immunoglobulin class switching to IgG2a antibody secretion, and controls the proliferation of B and NK cells. IFNγ secretion is the hallmark of Th1 cell response, which is necessary for both, cell-mediated immunity and intracellular pathogen resistance [17]. The significance of IFNγ in pathogen clearance and resistance was demonstrated by mouse knock-out studies where mice with a targeted disruption of the IFNγ receptor (IFNγR) gene showed heightened mortality in response to a non-lethal infection with BCG [18] Many other studies using either IFNγ or IFNγR knock-out mice have confirmed the central role of this cytokine in host defense in a variety of infection models including tuberculosis and malaria [19-23].

### *1.3.2. Interferon (IFN) receptors and interferon signalling*

The mechanism of IFN signalling has been thoroughly characterized. The primary and best known network is the JAK–STAT IFN signalling pathway, which begins when a ligand (IFN) binds to its cognate receptor causing the receptor to oligomerize. The Janus family protein kinases (JAK1, JAK2 or TYK2) associated with the IFN

receptors are activated and trans-phosphorylated by each other; this also results in the IFN receptor phosphorylation. The resulting activated protein kinase-receptor complex serves as a docking site for STAT (signal transducers and activators of transcription) proteins. STAT proteins are recruited to the active IFN receptor where they become phosphorylated by the associated JAK (or TYK2) kinases. Phospho-STAT proteins are able to homo- or hetero- dimerize, which allows them to translocate into the nucleus. Upon nuclear entry, STATs bind to DNA motifs termed gamma interferon activation sites (GAS) or IFN-stimulated response elements (ISREs) and modulate the transcription of IFN regulated genes [15, 24, 25].

The type I IFN receptor is a multi-chain structure consisting of two transmembrane proteins, IFNAR1 and IFNAR2, which heterodimerize upon ligand binding [26-28]. The interaction of the two receptor subunits triggers the activation of TYK-2 and JAK-1 kinases that are constitutively associated with the receptor complex in the membrane. Next, the kinases phosphorylate tyrosine residues on STAT-1, 2, 3 or 5 causing their homo- or hetero- dimerization [28] (Fig.1).

The IFNγ receptor also has two subunits - the constitutively expressed IFNGR1 and an accessory IFNGR2, which is recruited by IFNGR1 upon ligand binding [26, 24]. When IFNγ binds to IFNGR1, JAK-1 and JAK-2 kinases are activated and phosphorylate IFNGR2 and STAT-1. Phosphorylated STAT-1 proteins homodimerize and translocate into the nucleus and regulate the transcription of genes containing GAS and ISRE in their promoter sequences [24, 26]. The consensus sequences of these elements have been resolved as TTNCNNNAA and GGAAANNGAAACT respectively [2, 24, 29]. Along with STAT-1, two other STAT proteins, STAT-3 and STAT-5, have been identified in the DNA binding complex involved in IFNγ-activated JAK-STAT signalling pathway; however, their overall contribution to IFNγ signalling is considered minor [26].

Evidence suggests that IFNγ signalling has pleiotropic effects that can be manifested by pathways other than the canonical Jak-STAT cascade. For example, microarray data collected from STAT-1 deficient mononuclear phagocytes by Gil and colleagues showed a number of genes regulated in an IFN-dependent but STAT-1 independent manner [30]. Some of the other well-defined pathways activated by IFNγ

include the MAP kinase pathway, as well as the PI3-K, Akt and PKC pathways [15].

### *1.3.3. IFNγ-IL12 signalling axis*

Because IFNγ is central to the production of many inflammatory cytokines, there are mechanisms in place to ascertain sufficient production of IFNγ to prime the appropriate immune response. One positive feedback loop (Fig. 2), involving secreted IFNγ, IRF8, and IL12, has been described in which IFNγ signalling up-regulates the expression of IRF8 via a JAK-STAT pathway that increases the transcription of the IL12p40 gene. IL12 is a heterodimeric cytokine that, in its biologically active form, is composed of the constitutively expressed p35 subunit and the inducible p40 subunit. IL12 is an important regulator of macrophage proliferation as well as Th1 cells-mediated immunity and is secreted only by the cells of the monocyte/macrophage lineage [31, 32]. Expression of the IL12 receptor (IL12R) is restricted to the cells of haematopoietic origin, in particular, cells involved in innate immunity (NK cells, activated T cells, dendritic cells, and B cells). [33, 34, 35] Upon detecting IFNγ, immune cells up-regulate the expression of IRF8, which binds to an ISRE in the IL12p40 promoter, increasing its transcription. In addition, various studies have shown the ability of IL12 to potently induce IFNγ production in mouse and human macrophages, Th1 cells and NK cells. By activating the transcription of IL12p40 via IRF8, IFNγ is thus able to positively regulate its own production and induce a state of inflammation and infection resistance in the host [32, 36]. Interestingly, there is also a positive synergistic activation observed when macrophages are stimulated with IL12 potentiators LPS (lipopolysaccharide) or CpG DNA along with IFNγ.

LPS and CpG DNA are pathogen associated molecular patterns (PAMPs) that signal through the Toll-like family of receptors (TLRs). TLRs are a family of evolutionary conserved surface and intracellular receptors crucial for the development of early host immune response and Th1 cell mediated immunity [37]. Although they play a general pro-inflammatory role, each TLR can only respond to a specific set of ligands. Activation of TLRs triggers the transcription of a variety of genes via the nuclear factor κB (NFκB) and MAP kinases. For example, TLR2, 3, 4 and 9 can induce IRF8 expression via NFκB, ERK and JNK kinase signalling in response to

PGN (peptidoglycan found in bacterial cell walls), PolyI:C, LPS and CpG DNA respectively. [37-39]. Similar to other TLR-inducible genes, IL12p40 transcription can be triggered by either LPS or CpG DNA, via activating NFkB and binding to an NFκB half-site located upstream from the transcriptional activation site of the IL12p40 gene [32]. Coupled with IFNγ signalling, this mechanism results in the potent induction of IFNγ and IL12 secretion, which drives the differentiation and maturation of macrophages, the development of Th1 cells and the secretion of other pro-inflammatory cytokines including IFNα/β, iNOS, TNF, and IL1β [1, 2, 40]

## 1.2. Interferon Regulatory Factors

### *1.2.1. IRFs as a family of structurally related transcription factors*

Interferon regulatory factors (IRFs) are an important family of transcription factors that were first identified as the regulators of transcription of IFN-regulated genes. Now, this family is recognized as essential for many cellular processes other than immunity, including cell differentiation, cell cycle regulation and oncogenesis [41]. There are ten known members of IRFs, however, the mammals possess only nine of them (IRF1-9). All IRFs have an evolutionary conserved 120 amino acid-long DNA binding domain (DBD) that consists of a unique helix-turn-helix motif. This motif's signature five tryptophans repeat bears similarity to the DBD of Myb transcription factors [41-43]. The IRF DBD binds to specific DNA sequences found in the proximal promoters of genes regulated by type II IFNs. These DNA sequences consist of tandem repeats of the elementary IRF-binding DNA motif "GAAA". The ISRE is one of the best characterized IRF-binding motifs with the sequence GAAA(A)NNGAAA (tandem repeat underlined), which is found in a variety of interferon-regulated genes [42, 43].

In addition to the DBD, all IRFs except for IRF1 and IRF2 have a PEST-type interferon association domain (IAD) that binds other transcription factors or IRF members [43, 44]. The interaction of IRFs with other transcription factors or themselves through this domain is a necessary event for their association with DNA because all IRFs must hetero- or homo-dimerize with a partner to efficiently bind their

target DNA sequence. Moreover, the partner with which an IRF interacts determines the resulting transcription activation pattern. This was explained by the fact that the conformation that IRF and its binding partner acquire upon interaction determines their DNA affinity and the target binding selectivity [43, 45].

IRF8 and IRF4 are two unique IRF proteins because of their ability to bind to another DNA consensus sequence, the Ets/IRF composite element, or EICE. This element is composed of an IRF binding sequence GAAA and an Ets protein family binding sequence GGAA juxtaposed with each other (GGAA(A)-NNGAAA). [43] Interestingly, IRF8 and IRF4 can also heterodimerize with PU.1, an Ets family transcription factor. Similarly to ISRE, the EICE is found in the promoters of immune genes such as Igk, IL-1B, and TLR4. In addition, Kanno and colleagues describe a composite IRF/GAS element where the GAS and IRF binding motifs are found next to each other (TTTCNNGGAA). This element is important for IRF8-mediated IFN-gamma induced gene transcription and it provides IFNγ with a way to indirectly affect immune cell functions, particularly those of macrophages and dendritic cells [43].

### *1.2.1. IRF8 (ICSBP)*

IRF8 or ICSBP (Interferon Consensus Binding Protein) is an important IFN-inducible transcriptional regulator, whose unique expression pattern is restricted to cells of myeloid lineage and B cells. IRF8 plays a significant role in many homeostatic functions including myeloproliferation, cell differentiation, immune defenses, development of immune cells, apoptosis and oncogenesis [5, 44]. The physiological outcome of loss-of-function mutations in the IRF8 gene in mice and humans manifests in a severe impairment of immune responses to viral and bacterial pathogens that are under the control of IFN type II resistance, as well as in myeloproliferative disorders [40, 46, 47]. Like other IRFs (with the exception of IRF1 and IRF2), IRF8 elicits its transcriptional control by interacting with other transcription factors including IRF1, 2 or 4, PU.1 and E47 [40, 48]. In addition, IRF8 can cooperate with other factors such as Miz1 and Myc to regulate expression of certain gene targets.

### *1.2.2. IRF8 induction*

IRF8 expression is induced primarily by IFNγ via a phosphorylated STAT-1 homodimer binding to a conserved palyndromic GAS sequence in the IRF8 promoter. However, detectable induction can also occur in cells stimulated with various TLR ligands [42, 43, 45, 49]. Out of the ten members of the TLR family, TLR2, 4 and 9 have been implicated in IRF8-dependent induction of such pro-inflammatory molecules as IL-1, IL-6, IL18 NO, IL12, and TNFα [5]. It has been shown that IRF8 transcription is induced by TLRs via NFκB, which is the downstream signalling effector of many TLR-activated pathways. Consistently with these reports, an NFκB binding site was found in the promoter of IRF8 that spans the region at the position -531 to -540.

IFNγ and TLR can trigger the IRF8-mediated production of pro-inflammatory cytokines on their own, however, the highest levels of cytokine production are achieved when the IFNγ and TLR pathways are activated synergistically. This indicates that IRF8 is the central protein that mediates the cross talk between IFNγ and TLR signaling. The evidence also suggests that this mechanism involves IRF8 interactions with TRAF6 and the strengthening JAK and MAP kinase signalling [37]

### *1.2.3. Transcription targets of IRF8*

The transcriptome of IRF8 can be divided into two diverse sets of genes - those involved in the regulation of the cell cycle and differentiation in the myeloid lineage and those intrinsic to macrophage and denrtitic cell defenses against infections. The first set of genes drives the differentiation of myeloid progenitor cells into macrophages while inducing apoptosis in cells of the granulocytic lineage. These genes include positively regulated cyclin kinase inhibitors and pro-apoptotic genes such as Cdkn2b (Cyclin-dependent kinase 4 inhibitor B) and NF1 *(***neurofibromin 1***)* or genes that aid in the formation of the germinal centre, such as activation-induced cytidine deaminase (AICDA) and BCL6. The genes down-regulated by IRF8 primarily belong to the apoptotic and cell cycle regulation pathways, such as the anti-apoptotic Bcl2, Bclxl and Dab2 genes [6, 50, 51].

### *1.2.4. IRF8 in immune response*

The immune responses regulated by IRF8 are diverse but all require its interaction with at least one of the three following binding partners: PU.1/SpiB, IRF4, and IRF1 [40, 43, 52]. For example, IRF8/IRF1 together can induce the production of the respiratory burst oxidase components in macrophages, including NO, gp67 and gp91 [53].

The formation of the IRF8/PU.1 DNA-binding complex and the up-regulation of immune genes via ISRE or EICE is a pathway unique to the immune system [43]. The transcriptional activity of IRF8 within the IRF8/PU.1 complex favours the development of Th1 cellular response, magnifies macrophage anti-microbial and cytotoxic functions, stimulates the expression of MHC class II and enhances the antigen presentation capacity of T cells. Several of these responses are initiated, amplified and maintained by IRF8 via the establishment of a positive feedback loop between IFNγ and IL12p40 [43] (Fig.3). The feedback loop is triggered by IFNγ, which induces IRF8 and IL12 expression via GAS elements in their promoters. In addition, IRF8 up-regulates IL12 expression and the expression of other pro-inflammatory cytokines by binding to ISRE or EICE in their promoters. The up-regulation of these factors by IRF8 and IL12 culminates in the activation of T cells, macrophages and NK cells that secrete additional IFNγ.

The clinical manifestations of IRF-8 deficiency in mice and humans consist of defects of innate and adaptive immunity [37]. BXH2 mice that carry a spontaneous loss of function R294C mutation in a well-conserved portion of the IAD of IRF8 and IRF8 $^{-/-}$ mice are susceptible to infection with several pathogens, such as *Listeria monocytogenes, L. Pneumophila*, Bacillus Calmette-Guérin (BCG), *M. Tuberculosis*, and malaria (*Plasmodium chabaudi* ) [37, 43, 47].

### *1.2.5. IRF8 in myelopoiesis*

In myelopoiesis, IRF8 plays a dual inhibition/activation role depending on the cellular context and the interacting protein partners [5]. It is of particular importance in the development of most immune cell lineages including macrophages, monocytes,

dendritic cells, NK cells, CD4+ and CD8+ T cells. Absolutely all IRF8 knock-out mice display inadequate myeloid cell differentiation and most ultimately to develop chronic or acute myelogenous leukaemia (CML or AML) - like symptoms by 6 months of age [5, 40, 46, 47, 54]. These mice show numerous defects in the development of macrophages, monocytes, and dendritic cells, they lack $CD11c^+$ cells and show alterations in the T cell lineage. Additionally, most cells from IRF8-deficient mice are resistant to apoptosis and are hyper-responsive to cytokines. BXH2 mice develop splenomegaly and show abnormalities in lymph node tissues and bone marrow with infiltration of $Mac1^+/Gr1^+$ granulocyte precursors [46, 47]. These numerous defects point to the importance of IRF8 as the central regulator of myelopoiesis including cell development and differentiation. IRF8-deficient mice also lack $CD11\alpha^+$ dendritic cells suggesting a key role of IRF8 in the development of this specialized cell lineage. An example of IRF8-mediated cell regulation is the repression of transcription of the 3',5'-oligoadenylate synthase (*OAS*) in macrophages, which results in the down-regulation of their phagocytic functions during differentiation [5].

IRF8 also plays a complex role in the differentiation of T helper cells by promoting the Th1 cell development via an IFNγ-induced feedback mechanism [43, 42]. In this mechanism, IFNγ-induced IRF8 triggers the expression of IFNγ-inducing IL12 and IL23, while also cooperating with PU.1 to induce Th1-favouring IL18 expression [42].

### *1.2.6. IRF8 in cancer*

Studies have shown that IRF8 behaves as a tumour suppressor gene in certain malignancies [46, 54]. Lack of IRF8 leads to myeloid lineage aberrations that arise from the lack of IRF8-mediated induction of Fas-induced apoptotis. In addition to apoptosis, IRF8 governs a series of anti-tumour immune responses. It was shown that only IRF8-sufficient and not IRF8-/- mice are able to establish an immune response and prevent cell proliferation of passively transferred tumour cells [46]. Conversely, expressing IRF8 in such malignancies as soft tissue sarcoma and nasopharyngeal or

esophageal carcinoma sensitizes cells to apoptosis and increases the success of chemotherapy [40, 45, 54, 55] Microarray studies also revealed IRF8 as a potent leukaemia suppressor, identifying NF1 and PTPN13 as some of its repression target genes. NF1 and PTPN13 are responsible for cytokine sensitivity and apoptotic resistance, respectively [5]. These data suggest that loss of IRF8 may be one of the causative agents in the appearance and proliferation of cancers. In line with this, Yang et al showed that over 90% of high grade tumour cells from soft tissue sarcomas do not express nuclear IRF8 (active IRF8) compared to normal tonsilar tissue [45]. Some argue that the anti-tumour activities of IRF8 are due to the activation of the Caspase-8 and Bid in the mitochondrion-dependent apoptosis pathway; however, another mechanism involving caspase-9, caspase-3 and cytochrome c release may also play a role [45].

## 1.3. IRF1

Similarly to IRF8, IRF1 is a transcription factor with multiple functions in the regulation of host defense and cellular responses, including governing the transcription of IFN-dependent genes, the development and differentiation of thymocytes, cancer susceptibility, induction of DNA-damage associated growth arrest and cell death, as well as cell cycle regulation. The function of IRF1 is specified by its expression pattern, the cellular context and the availability of other transcription factors that can modulate its transcriptional effect. It is constitutively expressed in most cell types and its basal level of expression has a protective role against oncogenic transformation via cell growth arrest and apoptosis [4]. The role of IRF1 in immunity depends on the induction of highly expressed IRF1 transcripts that, in association with IRF8, up-regulate the transcription of IL12p40 and other pro-inflammatory genes.

### *1.3.1. IRF1 expression and induction*

High levels of IRF1 expression can be achieved in many cell types by stimulation with IFNα/β/γ, TNFa, IL-1, IL-6 and LIF [2, 56]. Synergistic induction of

IRF1 is observed in cells stimulated with TNFα and IFNγ, and in fact, it was shown that IRF1 is up-regulated by both pathways via a dual GAS/NFkB site at position -49/-40 in its promoter [57].

In addition to TNFα and IFNγ, IRF1 is directly and indirectly involved in the IL12 signalling pathway, a major pathway that links innate and adaptive immunity. IRF1 can be indirectly up-regulated by IL12 via IL12-induced IFNγ secretion by APCs (antigen presenting cells) but there is also a direct link between these two factors. Activation assays in T-cells and NK cells showed that IL12 can induce IRF1 expression independently of IFNγ production [58, 59]. Trans-activation luciferase reporter assays in fibrobalsts that were transiently transfected with STAT-4 showed that a STAT binding element in position -123/-113 is required for IL12-mediated IRF1 induction and that cells that do not express STAT-4 are not able to express IRF1 in response to IL12 [59]. The direct and indirect mechanism of IRF1 involvement in the IL12 signalling pathway also explains the existence of distinct stages of IRF1 induction by IL12, at 3-4 hours after stimulation in response to direct up-regulation by IL12 and a latent phase that can be seen as long as 24 hours after stimulation and which is a response to IL12-mediated IFNγ production [59, 60]. Similarly to IRF8, IRF1 is also induced by IFNγ via JAK/STAT signalling and a STAT-1 dimer binding to a GAS element in its promoter [24, 61, 62]. Although IRF1 is not induced by LPS on its own, there is great synergy in response to IFNγ/LPS. TLR ligands (LPS or CpG DNA) amplify the induction of IRF1 by activating p38 MAP and ERK1/2 kinases that phosphorylate the serine residues on STAT-1, thus aiding its activation and the binding of the GAS site [25, 63]. Generally, IRF1 down-regulates the anti-inflammatory and Th2-inducing genes and up-regulates the genes responsible for induction and amplification of immunity and Th1 mediated inflammation. For example, IRF1 down-regulates IL4, a signature Th2 cytokine, and IL6, which is an anti-inflammatory cytokine. In contrast, IRF1 positively regulates RANTES, IFNα/β, iNOS, IL12, IL15, CIITA and IL15 that are all pro-inflammatory or Th1-driving molecules. Some other intrinsic macrophage targets of IRF1 include gp130, GBP, Cox2, TAP1, β2 microglubulin, $gp91^{phox}$, $p21^{WAF1/CIP1}$, Caspase 1 and 7, and

Lysil oxidase [2].

### *1.3.2. IRF1 in immunity*

Although IRF1 was initially recognized for its regulation of type I IFNs, it has since then been shown to have remarkable functional diversity in the regulation of innate and adaptive immunity. Its effects include the stimulation of IL12 production by immune cells, stimulation of expression of IFN-inducible genes, and regulation of response to infection of NK cells, Th1 cells and macrophages [2, 56].

IRF1 is necessary for the formation of functional IL12p70 in immune cells via the regulation of its two subunits, IL12p35 and IL12p40. Although both subunits are crucial for the formation of IL12p70, the p40 subunit is normally expressed in excess of the constitutively expressed p35, making p35 the limiting component in the production of IL12. IRF1 controls IL12 formation by up-regulating the p35 subunit gene during infection via a small IRF-E region in the p35 exon 2 promoter [62].

Interestingly, the induction of IL12p40 in IRF1$^{-/-}$ macrophages is also diminished suggesting the role of IRF1 in the regulation of the second inducible subunit of this cytokine. Not surprisingly, transactivation assays with reporter genes and deletion analysis have shown that IRF1 binds to a site at the positions -72 to -58 in the IL12p40 gene promoter, where it forms part of a larger protein complex that includes the a c-Rel protein [64, 65]. Thus, similarly to IRF8, IRF1 is a major factor in regulating the production of IL12p40 and IFNγ [2, 66]. The importance of IRF1 in the IFNγ-IL12 signalling axis and its involvement in innate immunity is highlighted by the observation that IRF1 $^{-/-}$ mice are severely susceptible to infection with *Mycobacterium bovis, Listeria monocytogenes* and *Leishmania major* but are resistant to other infections (such as *Nippostrongylus brasiliensis*), where protection relies on a Th2 response [2, 66].

IRF1 is particularly important for innate and adaptive NK cell-mediated immunity. NK cells development is highly dependent on their interaction with their progenitor cells and the bone-marrow microenvironment. IRF1 modulates these interactions by altering the transcription of the IL15 gene. Consequently, NK cell-

mediated cytolytic activity is abrogated in spleen and liver of IRF1$^{-/-}$ mice, which indicates impairment in NK cell development [2].

Similarly to IRF8, the absence of IRF1 results in an enhanced production of Th2-related cytokines, which is manifested by the lack of IL12-secreting naïve CD4+ cells and an abundance of memory and effector cells. The skewed Th1:Th2 ratio in IRF1 deficient mice indicates that it plays an important role in the maintenance of the CD4+ cell subset [2, 67]. In addition, IRF1-/- mice show decreased numbers as well as a number of intrinsic deficiencies in CD8+ T cells, which suggests that IRF1 is required for lineage commitment and differentiation of CD8+ thymocytes as well. The inadequacy of the CD8+ thymocyte pool is thought to be due to the lack of regulation of the TAP1 and LMP2 genes by IRF1 [4, 68].

### *1.3.3. IRF1 in oncogenesis*

IRF1 is a potent tumour suppressor gene with multiple effects on malignant cell transformation. Its functions in oncogenesis include the regulation of cell growth inhibition, DNA-damage induced apoptosis and T-cell mediated cancer-directed immunity. Not surprisingly, IRF1 expression is tightly regulated throughout the cell cycle [2, 69]. The deletion of the chromosomal region that contains IRF1 is often observed in patients suffering from leukaemia or preleukaemic myelodysplastic syndrome (MDS) and its loss is also frequent in oesophageal and gastric cancers [2] [70]. Interestingly, the deletion of IRF1 alone does not initiate cell transformation or spontaneous tumour growth, but rather represents a secondary factor that exacerbates a pre-existing condition [2, 70].

The major cancer protective role of IRF1 is manifested by the induction of apoptosis and cell cycle arrest in response to DNA damage. The apoptotic activity of IRF1 is thought to be p-53 independent. The mechanism of IRF1-induced apoptosis is thought to involve the caspases 1, 7 and 8 [4, 71]. The secondary mechanism of IRF1 tumour suppressor activity is the induction of G1 cell cycle arrest. In line with this, over-expression of IRF1 leads to complete growth inhibition in many cell lines. Furthermore, ectopic or forced IRF1 expression inhibits cancer cell proliferation and

reverts the transformed cancer cell phenotype on agar plates and in nude mice [70]. The mechanism of IRF1 cell growth inhibition has been shown to be independent of Myc/Ras, but rather to rely on the down-regulation of Cyclin D1 [69, 70].

# Chapter 2

# Results

# 2. RESULTS

## 2.1. Materials and Methods

### Animals

C57BL/6J (B6) and Balb/C inbred mouse strains and a breeding stock of BXH-2/TyJ (BXH2) recombinant inbred strain were purchased from The Jackson Laboratory, Bar Harbor, ME. A breeding stock of BXH-2/TyJ (BXH2) recombinant inbred strain was obtained in the early 2000s from N. Jenkins and N. Copeland (National Cancer Institute, Frederick, MD) and subsequently expanded and maintained as a breeding colony at McGill University. F2 mice were bred by standard brother-sister mating of BXH2/TyJ and C57BL/6J mice. The mice were genotyped by PCR using $P^{32}$ (PerkinElmer, Waltham MA). Since the *IRF8* locus (*Myls*) maps within an 18-cM interval bounded by *D8Mit200* and *D8Mit13* on mouse chromosome 8, the *D8Mit13* microsattelite marker that is most proximal to the locus was used to identify the F2 individuals bearing wildtype and mutant *IRF8* alleles. The oligonutleotide primer pairs used were CCTCTCTCCAGCCCTGTAAG and AACGTTTGTGCTAAGTGGCC. $IRF1^{-/-}$ mice were purchased from the Jackson Laboratory and a breeding stock was maintained at McGill University. Maintenance and experimental manipulations of animals were performed according to the guidelines and regulations of the Canadian Council on Animal Care.

### Cell Culture and cell activation

J774 and RAW264.7 macrophage cell lines were maintained in DMEM (Sigma) supplemented with 10% heat-inactivated FBS (HI-FBS, Gibco), 100 U/ml penicillin, and 50 μg/ml streptomycin (Invitrogen) at 37°C, in 5% $CO_2$-containing humidified air. L929 cells were maintained in culture in our laboratory. To produce LCCM (L-cell conditioned media), L929 cells were plated in T75 flasks in 50ml DMEM with 10% HI-FBS, 100 U/ml penicillin and 50 μg/ml streptomycin and

cultured for 2-3 weeks until the cell monolayer was confluent. LCCM was collected and kept at -20$^{o}$C for future use as a macrophage growth and differentiation supplement.

In order to mimic infection conditions in macrophages, recombinant mouse IFNγ (Cell Sciences, Canton, MA), and CpG DNA oligonucleotides (5'-TCCATGACGTTCCTGACGTT-3', ordered from Invitrogen) were used at 400 U/ml and 1.5 μg/ml respectively. Mouse macrophages were incubated with IFNγ and CpG DNA for 3 hrs to study the effects of IRF1 or IRF8 expression in activated macrophages for microarray experiments. Prior to incubation, the mouse macrophages were primed with 50U/ml of IFNγ for 18 hrs. For ChIP application, J774 macrophages were stimulated with the same concentrations of IFNγ and CpG for 3 hrs with no priming.

**Bone Marrow Derived Macrophage (BMDM) Cultures**

Mice were sacrificed at ~10-14 weeks of age and tibia and femurs were removed and kept on ice and bone marrow cells were extracted by gently flushing tibia and femurs with 2.5-5ml of ice-cold DMEM. The bone marrow cells were pelleted by centrifugation at 2500 rpm for 4 min and resuspended and plated in 150 mm bacteriological grade culture dishes (Fischer) in 25 ml of warm DMEM supplemented with 10% FBS, 100 U/ml penicillin, 50 μg/ml streptomycin and 20% LCCM (L-cell conditioned media) to stimulate the differentiation of cells into bone marrow macrophages. The cells were allowed to differentiate for 5 days at 37°C, in 5% $CO_2$-containing humidified air. On the 5$^{th}$ day 2.5 ml of LCCM was added to the cell culture dishes and the cells were incubated for another 2 days. After 7 days in cell culture the cells were washed twice with warm PBS, after which they were incubated for 5 min in warm PBS-citrate. Cells were collected in PBS citrate by pipetting up and down and making sure all the cells became dislodged from the plate surface. Cells were washed twice more with warm PBS and the washes were added to the collected cell suspension in PBS-citrate. Cells were pelleted by centrifugation at 2500 RPM for

1.5 min and resuspended in warm DMEM with 10% HI-FBS (Gibco), 10% LCCM, 100 ug/ml of Thimidine (Sigma) and without antibiotics for infection with *L. pneumophila*. For the infection, the cells were seeded in either 24-well plates at the concentration of 2.5E5 cells/100 µl for CFU monitoring or in 150 mm tissue culture dishes at the concentration of 1.8E7 cells/25 ml for RNA collection. Alternatively, macrophages were plated at 1.8E7 cells/20 ml in DMEM, 10% HI-FBS, 10% LCCM with 100 U/ml penicillin and 50 µg/ml streptomycin for stimulation with IFN-γ/CpG DNA in 150mm tissue culture plates.

***Legionella pneumophila* Infection**

*L. pneumophila* strain Lp02, a thymidine auxotroph, was used to infect mouse BMDMs. Lp02 was grown at 37°C on buffered charcoal yeast extract (BCYE, from OXOID) agar plates supplemented with 100 µg $ml^{-1}$ of thymidine (Sigma) for 72 hrs, then the mature colonies were transferred into AYE media with the same amount of thymidine and grown at 37°C for 24 hrs with constant agitation. The culture was diluted with supplemented AYE to obtain 1:2, 1:4, 1:8 and 1:16 dilutions and the mixtures were incubated for another 24hrs. Bacterial growth curve was estimated by measuring optical density of each sample at 595 nm and the mature bacterial stock with the absorbency closest to 0.4 was chosen (Bacterial stock with OD of 0.4 is mature enough for infection). This bacterial stock was used to infect macrophages at the multiplicity of infection (MOI) 25:1 (bacteria : macrophages). Macrophages were incubated with the bacterial suspension for 1 h at 37°C, followed by three washes with warm DMEM to remove non-phagocytosed bacteria, and by subsequent incubation in DMEM supplemented with 10% HI-FBS, 10% LCCM, and 100 µg/ml of thymidine for 24, 48, and 72 hrs. After each time point the cells were washed and macrophage-internalized bacterial load was measured as CFUs following the lysis of macrophages with distilled water, and plating cell lysates onto thymidine-supplemented BCYE agar plates.

**RNA collection and transcriptional profiling**

Total cell RNA was prepared from ~1.8E7 cells per sample by Trizol extraction according to specifications (TriReagent, Sigma) from non-stimulated controls, Lp02 infected and IFNγ/CpG DNA stimulated BMDMs (8 groups per sample for infected, stimulated and control F2 mice with wt (*IRF8*$^{294C}$) and *IRF8* mutant (*IRF8*$^{294R}$) alleles). Shortly, the cells were suspended in 5ml of TriReagent. The samples were incubated for 5 min at RT, followed by chloroform extraction. The aqueous phase was removed and nucleic acids were precipitated with isopropanol. Pellets were washed with 75% ethanol, dissolved in RNAase-free water treated with 0.1% diethylpyrocarbonate (DEPC). Prior to application on microarrays, RNA was purified according to specifications on Qiaquick RNA purification columns and its quality was evaluated by electrophoresis on a 1.5% formaldehyde-agarose gel in a formaldehyde buffer and by RNA Bioanalyzer.

RNA was reverse transcribed into cDNA (see below) and hybridized on Mouse-6 v2 Expression BeadChip Illumina microarray at the McGill University and Génome Québec Innovation Centre functional genomics facility. The data was read and extracted with the Illumina GenomeStudio Software. The GeneSifter microarray data analysis system (VizX Labs; www.genesifter.net) was used to analyze data generated from comparisons between control and *L. pneumophila*-infected or between control and IFNγ/CpG DNA treated groups for the F2 generation bearing the wt and mutated IRF8 alleles. Data were normalized (all median) by the robust multiarray average algorithm included in the GeneSifter software and the fold changes were log transformed. Differential expression of genes between the groups was tested by using two-way ANOVA analysis with Benjamini Hochberg correction and p-value less or equal to 0.05 and a fold change threshold cut off of 1.5 on the logarithmic scale. Gene lists from the stimulation (IFNγ/CpG) and Lp02 infection transcription profiles were exported with the interaction filter applied (only genes that pass the interaction between strain and treatment were exported). Next, to identify genes involved in the

IRF8-dependent transcription during infection and stimulation conditions were found by comparing the two gene lists with the GeneSifter Intersector software (http://public.genesifter.net/intersector). The significant genes were annotated according to Gene Ontology (GO) terms in the GeneSifter program as well as by the DAVID functional annotation tool (http://david.abcc.ncifcrf.gov/).

**RT-PCR**

RT-PCR was carried out following a standard protocol. Briefly, 2 μ of RNA per sample was converted to cDNA in a 20μL reaction with 1 unit of MMLV reverse transcriptase (Invitrogen), dNTPs (500μM each) and oligo d(T) primers (5μM), First Strand buffer™, 0.1M DTT, and 1μl of RNA Guard (Invitrogen). The reaction was incubated for 50 min at 37°C and was inactivated by placing it at 70°C for 15 min. Expression of individual genes was tested by standard PCR amplification of RT-PCR products using a reaction mixture that contained 200μM dNTPs, 2mM $MgCl_2$, 3μL of cDNA (1:10 dilution), Taq polymerase (Invitrogen), and 10pmol of gene-specific primers. The standard amplification conditions were used: 25-28 cycles of (95°C for 1 min, 59°C for 45s, 72°C for 45s) following a 7.5 min extension step. PCR products were resolved by agarose gel electrophoresis stained with 0.1% ethidium bromide and visualized under UV. Oligonucleotide pairs used are the following: IL1β (5'CAGGATGAGGACATGAGCACC3' and 5'CTCTGCAGACTCAAACTCCAC), CXCL1 (5'GCTGGGATTCACCTCAAGAA3' and 5'TTGACACTTAGTGGTCTCCCAAT), β-actin (5'AGTGTGACGTTGACATCCGT3' and 5'TACTTGCGCTCAGGAGGAG), IL12p40 (5'GCTTCATCATCTGCAAGTTCTTGGG3' and 5'GTGAAGCACCAAATTACTCCGG3'), and IRF1 (5'TGTCGTCAGCAGTCTCT3') and (5'ACTCACTCAGGAGGGCAAGA3').

**Western blots**

Cells were incubated in ice-cold lysis buffer (1% Triton, 10 mM EDTA, 10 mM Tris (pH 7), 150 mM NaCl, and 30% glycerol) and complemented with protease inhibitors (PI: 2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 μg/ml pepstatin, and 100 μg/ml

PMSF) at 4°C for 5 min. Whole-cell lysates were heated for 5 min at 90°C, resolved on 10% SDS-polyacrylamide gels, and transferred by electroblotting on 0.45 µm nitrocellulose membranes (Whatman, Maidstone, England). Similar loading of protein lysates (50 µg/well) and equal amounts of protein transfer to membranes were verified by staining with Ponceau S red (Sigma-Aldrich). Non-specific reactions were avoided by incubating membranes in blocking solution containing 5% skim milk (w/v) in TBST (10 mM Tris-HCl (pH 8), 150 mM NaCl, 0.05% Tween 20) for 1 h at room temperature with gentle agitation. The blots were then incubated with 1:250 dilution of goat anti-IRF8 C-19 Ab, rabbit anti-IRF1-C20 Ab (Santa Cruz biotechnology inc., Santa Cruz, CA ) or domestic rabbit anti-IRF8 pAb in blocking solution overnight at 4°C, followed by six consecutive 5 min washes in TBST, and a final 1 h incubation with a 1:20,000 dilution of goat anti-mouse or rabbit anti-mouse secondary Ab conjugated to HRP (Jackson ImmunoResearch Laboratories, West Grove, PA) in blocking solution at room temperature. Immune complexes were revealed by chemiluminescence (SuperSignal West Pico; Biolynx, Brockville, ON) on film.

**Immunoprecipitation**

Cells were metabolically labelled with $^{35}$S-methionine (35 µCi/ml; PerkinElmer, Waltham, MA) by overnight incubation in methionine-free DMEM medium (Invitrogen) containing 10% heat-inactivated dialyzed fetal calf serum (Invitrogen). Prior to immunoprecipitation, labelled cells were washed in ice-cold PBS, pelleted, and lysed with 200 µl Buffer 1 (1% SDS, 50 mM Tris (pH 7.5)) in the presence of protease inhibitors at 4°C. Samples were then briefly placed in a sonicating water bath. Lysate volume was then brought to 1 ml by the addition of 800 µl of Buffer 2 (1.25% Triton-X, 200 mM NaCl, 50 mM Tris (pH 7.5)), thus giving a final concentration of 0.2% SDS, 1% Triton X-100, 160 mM NaCl, and 50 mM Tris (pH 7.5). $^{35}$S-methionine labelled clear cell extracts (~1 x $10^6$ TCA-precipitable cpm/µl) recovered via centrifugation were then incubated overnight at 4°C (~5 x $10^7$ TCA-precipitable cpm/sample) with either 1:10 or 1:100 dilutions of domestic rabbit

pAbs against Irf-8, or a commercial goat anti-IRF8 C-19 Ab (5 μg/sample; Santa Cruz Biotechnology inc., Santa Cruz, CA). Immune complexes were isolated after a 2-hr incubation at 4°C with a 1:1 mixture of protein A- and protein G-agarose beads (Millipore) that was followed by five consecutive washes in Buffer 3 (0.1% Triton X-100, 0.03% SDS, 150 mM NaCl, 50 mM Tris (pH 7.5), and 5 mg/ml BSA) and two washes in a solution containing 150 mM NaCl and 50 mM Tris (pH 7.5). The final pellet was incubated at room temperature in Laemmli sample buffer for 10 min. Supernatant fractions were recovered, while bead pellets were discarded. Immune complexes in the collected Laemmli eluate were heated at 90°C for 5 min and then analyzed by SDS-PAGE on 10% polyacrylamide gels. Fluorography of the gels were performed using a commercially available amplifier (Enhance; DuPont, Wilmington, DE) as recommended by the manufacturer. Gels were subsequently dried and exposed to film for 5 d to 2 wk at -80°C.

**Chromatin Immunoprecipitation (ChIP)**

ChIP assays were performed on J774 macrophages stimulated with IFNγ/CpG for 3 hrs. The cells for ChIP application were grown to 80% confluency and ~1.8 million (1/3 of a 150 mm plate) cells were used for regular chromatin IP or ~18 million cells (~3 X 150 mm plates) were used for ChIP on Chip. Before cross-linking, plates with stimulated cells were placed at room temperature for 2 min and then fresh 37% formaldehyde (540μL for 20ml of media) was added to the plates to the final concentration of 1% after which the plates were incubated at RT on a shaker at low speed for 10 min. Next, formaldehyde media was discarded and the cells were washed twice with ~25ml of ice-cold PBS (phosphate-buffered saline), the cross-linked cells were scraped from the plates and pelleted by centrifugation at 4°C for 10 min. The cell pellet was resuspended in cell lysis buffer (1% SDS, 10mM EDTA, 50mM Tris-HCl pH8) supplemented with protease inhibitor pellets (Roche) (300ul of buffer was used for each plate of J774 cells). The cell pellet was sonicated on ice with VirSonic 100 (Virtis) at power level 10 for 30 s pulses with a 30 sec pause to prevent sample over-

heating. The sonicated material was centrifuged at 13000rpm for 7 min at 4°C. The chromatin was diluted in a 2.5 ChIP dilution buffer (0.5% Triton X-100, 2mM EDTA, 100 mM NaCl, 20 mM Tris-HCl pH8.1) supplemented with protease inhibitor pellets and pre-cleared with 50% slurry of salmon sperm DNA/protein G agarose beads (Upstate/Millipore) for 2.5 hrs at 4°C (60 µL was used per IP for regular ChIP and 600 µL was used for ChIP on Chip application). 10% of pre-cleared chromatin was retained for standard curve generation by real time PCR. The rest of the pre-cleared chromatin was separated into equal amounts equivalent to approximately 1.8 million cells for ChIP or 18 million for ChIP on Chip and were either left with no antibody (control) or immunoprecipitated with an anti-IRF1H70 or anti-IRF8C19 antibody from Santa Cruz (sc-13041x and sc-6058x). 25µg of anti-IRF8 and 15µg of anti-IRF1 antibodies were used per IP for ChIP and 250µg of anti-IRF8 and 150 µg of anti-IRF1 antibodies were used for ChIP on Chip. The antibody and no antibody samples were left rotating at 4°C overnight after which 60µL (ChIP) or 600µL (ChIP on Chip) of 50% slurry of salmon sperm DNA/protein G beads was added to the samples with a subsequent 3 hr incubation on a rotor at 4°C. Immunoprecipitated and no antibody samples were washed for 10 min sequentially with each of the following buffers: low salt Buffer I (1% Triton X-100, 0.1% SDS, 150mM NaCl, 2 mM EDTA pH8.0, 20 mM Tris-HCl pH 8.1), high salt Buffer II ( 1% Triton X-100, 0.1% SDS, 500mM NaCl, 2 mM EDTA pH8.0, 20 mM Tris-HCl pH 8.1) and Buffer III (1% Igepal CA-630 from Sigma Aldrich, 0.25 mM LiCl, 1% Na-deoxycholate, 1mM EDTA pH8.0, 10mM Tris-HCl pH 8.1). In-between each wash the beads were spun for 1 min at 2500 rpm at 4°C and the supernatant was discarded. After the washes, the beads were washed briefly with TE buffer (10mM Tris-HCl pH 7.5, 1mM EDTA pH 8). Finally, the samples were de-crosslinked by incubating them at 65°C overnight in a decrosslinking buffer (1%SDS, 0.1M $NaHCO_3$). The next day, the samples were spun at 4000 rpm for 1 min, the supernatant containing DNA was collected and purified using the QIAquick PCR purifaction kit (Qiagen) according to product specifications.

**Sample preparation for hybridization to mouse extended promoter arrays (ChIP on Chip)**

Triplicate experiments were performed for ChIP on Chip and the samples were pooled together. Ligation-mediated PCR (LM-PCR) was performed on each of the pooled samples (IRF8, IRF1 and no Ab). Briefly,55 µL of each purified non-diluted sample was added to a mixture containing 11 µL 10X NEB buffer, 0.5 µL BSA (10mg/ml) (New England Biolabs, NEB), 1 µL 10mM dNTP mix (Invitrogen), 0.2 µL T4 DNA polymerase (3U/µL, NEB), and 42.3 µL of $H_2O$. The samples were incubated at 12°C for 20 min followed by the addition of a solution containing 11.5µL of 3M NaOAC pH5.2 and 0.5µL of glycogen (20mg/ml). The samples were vortexed and 120µl of phenol/chloroform/isoamyl (25:24:1) was added. After vortexing, the samples were centrifuged for 5 min at 13000 rpm. The upper phase was transferred to a new tube and precipitated with 2 volumes of ethanol kept at -20°C. After another vortex, the samples were left to precipitate at -80°C for 1hr. Next, the material was centrifuged at 13000rpm at 4°C for 30 min and the pellets were air-dried and resuspended in 20 µL of a mixture containing 2 µL 10X T4 DNA ligase buffer (NEB), 6.5 µL annealed linkers (15 µM), 0.5 µL 0.1 M ATP (generously provided by Dr. Giguere's lab), 1 µL T4 DNA ligase (400U/µL, NEB), and 10 µL of water. The re-suspended samples were allowed to ligate at 16°C overnight. The next day, LM-PCR was performed by adding to each sample 20 µL of solution containing 13.5 µL of H2O, 4 µL 10X Thermopol buffer (NEB), 1.25 µL of 10mM dNTP mix and 1.25 µL of 40 µM oligo oJW102 [72]. The LM-PCR program consisted of 4 min at 55°C, 3 min at 72°C, 2 min at 95°C, then 15 cycles of (30 sec at 95°C, 30 sec at 60°C, 1 min at 72°C), and finally, 5 min at 72°C. The PCR program was paused in step one after the temperature of 55°C had been held for 2 min and 10µL of solution containing 8µL of H2O, 1 µL of 10X Thermopol buffer, and 1µL of Taq polymerase (5U/µL, Invitrogen), after which the PCR run was continued. The LM-PCR samples were purified on QIAquick purification columns according to product specifications and eluted twice with 30µL and 40µL of elution buffer.

The LM-PCR samples underwent sixteen rounds of additional LM-PCRs with aminoallyl-dUTPs (Sigma) in order to generate enough DNA incorporated with aminoallyl-dUTPs for microarray application. Shortly, for each amplification 35 µL of solution containing 26.75 µL of water, 4 µL 10X Thermopol buffer, 3 µL 5mM dNTP mix containing aminoallyl-dUTPs (5mM dATP, 5mM dCTP, 5mM dGTP, 1.5 mM dTTP and 3.5 mM aminoallyl-dUTPs) and 1.25 µL of 40 µM oligo oJW102 [72] was added to 5 µL of a 5 ng/ µL dilution of the samples from the first LM-PCR amplification. The second LM-PCR program was identical to the first except for 18 cycles instead of 15 were used. The LM-PCR samples containing aminoallyl-dUTPs were purified on QIAquick PCR purification columns with a few modifications to the product procedure. A phosphate wash buffer was used to wash the columns (5mM $KPO_4$ pH 8.0 and 80% EtOH) and instead of the provided elution buffer, a phosphate elution buffer (4mM $KPO_4$ pH 8.0) was used to elute the DNA from columns twice with 30 µL and 40 µL of the buffer.

Next, the purified aminoallyl-dUTP conjugated samples were labelled with Cy3 and Cy5 dies. Briefly, three tubes per sample (3 tubes for IRF1, 3 for IRF8 and 6 for no Ab) were vacuum dried. The samples were resuspended in 4.5 µL of 0.1M $Na_2CO_3$ pH 9.0. Next, 4.5 µL of Cy5 (GE Healthcare) resuspended in 73 µL of DMSO or 4.5 µL of Cy3 (GE Healthcare) resuspended in 73 µL of DMSO were added to the enriched (IRF8 or IRF1) and control (no Ab) samples respectively. The samples were incubated in the dark at RT with occasional mixing for 1.5 hours. Subsequently, 35 µL of 0.1M NaOAc pH 5.2 was added to the samples that were then purified on QIAQuick PCR purification columns according to manufacturer's protocol. The samples were eluted with 52 µL of elution buffer and the eluates from similar samples were pooled together (3 tubes of IRF8 pooled into 1, 3 tubes of IRF1 pooled into 1 and 6 tubes of no Ab pooled into 1). Dye incorporation was quantified by performing a wavelength scan of the samples using the wavelengths: 260, 320, 550,650, and 750. The DNA amount was calculated by using the OD at 260 and 320, and the Cy3 and Cy5 incorporation was determined by using the following respective formulas: Cy3

dye/100bp = (100x(A550x6600))/(A260x150000), Cy5 dye/100bp = (100x(A650x6600))/(A260x250000). The samples were used for hybridization only if the incorporation was > 2 dye/100bp.

**Agilent ChIP on Chip hybridization and analysis**

Samples were hybridized to Agilent 244K mouse extended exon arrays containing ~17000 of the best-defined mouse transcripts as defined by RefSeq spanning the regions from -5.5kb upstream and +2.5kb downstream of the transcription start site. The procedure was done according to the Agilent mammalian ChIP on Chip protocol version 9.2. Following the hybridization at 65°C for 40 hours, the arrays were washed and scanned using a GenePix 4000B scanner and data was extracted from the images using Agilent Feature Extraction software as described in the mammalian ChIP on Chip protocol (Agilent, v.10) Data from ChIP on Chips were normalized and averaged using ChIP Analytics 1.3 software. Data was processed in ChIP Analytics using the intra-array Lowess normalization, Whitehead Error Model v1.0 and Whitehead Per-Array Neighbourhood Model v1.0 for peak detection and evaluation. The default parameters were used to identify significant binding events (1000 bp as the maximum distance for 2 probes to be considered neighbours in a probe set, probe set p-value <0.001 for a "bound" probe.

**Real time RT-PCR**

ChIP samples generated with anti-IRF8, anti-IRF1 or no antibody were used for real-time RT-PCR analysis to assess enrichment of IRF1 and IRF8 at known transcriptional targets binding sites. PCR amplification was performed using SYBR Green I PCR kit (Qiagen). Each reaction contained 1μL of the ChIP sample, 5 pmoles of the gene-specific primer pairs, 4.75 μL of PCR-grade water (Qiagen) and 6.25 μL of the SYBR Green master mix (includes SYBR Green, $MgCl_2$, dNTPs, Taq polymerase and buffer). Real time RT-PCR was performed on a Roche LightCycler with a program consisting of 15 min at 95°C followed by 45 cycles of (15 s at 95°C,

30 s at 60°C, and 30 s at 72°C). Oligonucleotides used for *Bcl6, iNOS, CathC, CysC*, and *IFNb* have been previously described for *CathC, CysC* and *iNOS* [6] [52], for *IFNb* and *HPRT* [52], and *Bcl6* [63]. Standard curves for PCR amplification efficiency were created for each set of oligonucleotide primer pairs using 10% of the pre-cleared non-immunoprecipitated chromatin used for ChIP. The chromatin was serially diluted 1:2, 1:4, 1:8, and 1:16. The standard curve allowed for the calculation of the efficiency of the PCR reaction ($Efficiency = 10^{(-1/slope)}$) [73]. Promoter occupancy of IRF1 and IRF8 was quantified using the following formula: $Occupancy = Efficiency^{(rtPCR\ cycle\ of\ no-Ab\ DNA)-(rtPCR\ cycle\ of\ Ab-enric\,hed\ DNA)}$ Further, enrichment of DNA fragments was normalized against an amplified control region that has no binding activity with IRF1 or IRF8 (a sequence 4kb upstream from HPRT)

### *2.2.1. Overview*

In the course of this project, we sought to identify genes that were transcriptionally regulated by either IRF1 or IRF8, the two important mediators of immune responses and oncogenesis. We first used a chromatin immunoprecipitation (chromatin IP or ChIP) technique to isolate DNA fragments that were bound by IRF1 or IRF1. Next, we used a ChIP on Chip method where the obtained IRF1/8-associated DNA was hybridized to Agilent 244k exon microarrays in order to identify which promoter regions or genes in the mouse genome were enriched for the IRF8 and IRF1 binding sites. In parallel, we used a microarray approach to generate a list of genes that were differentially expressed in response to infection with an intracellular pathogen (*L. pneumophila*) or to stimulation with TLR and IFNGR ligands in mouse BMDMs (IFNγ/CpG). This was done by using an F2 generation of mice that were homozygous for either wildtype or a mutant version of IRF8, but in which the rest of the genome was segregating. The gene lists obtained by the ChIP on Chip and microarray techniques underwent preliminary analysis and will further be used for target gene validation.

## 2.3. Optimization of the ChIP technique

### *2.3.1. Detection of IRF1 and IRF8 expression in J774 and RAW264.7 macrophages*

The ChIP on Chip method was used to identify promoter targets of IRF1 and IRF8. Prior to performing ChIP on Chip, we ascertained that the ChIP technique works in our specific cell lines and conditions of stimulation. First, we studied the activation times required for IRF8 and IRF1 to be induced in macrophages upon stimulation with IFNγ and CpG and established which conditions are most appropriate for the ChIP application. Next, we tested whether the anti-IRF8 antibodies available from our laboratory or commercially performed well in the ChIP protocol. Once the ChIP technique was validated for the anti-IRF8 antibodies, similar conditions were used with anti-IRF1 antibodies.

To determine whether murine macrophages express detectable amounts of IRF1 and IRF8 in their resting state and in the context of immune challenge, we first evaluated IRF8 and IRF1 expression in two cell lines, RAW 264.7 (mouse leukemic monocyte macrophages) and J774 macrophages. Previously, IFNγ treatment has been shown to up-regulate the transcription of immune modulatory genes (including IRF1 and IRF8) through the IFNGR and JAK-STAT signalling pathway [2, 37-39, 76]. Treated together with IFNγ and with TLR agonists such as LPS or CpG DNA, monocyte-macrophages display a stronger state of activation, enhanced transcription of immune genes, and the heightened secretion of chemokines and cytokines (IL12, TNFα, iNOS, IFNα/β/γ) [2, 38, 39, 76]. In accordance with these reports, we tested the induction of IRF8 and IRF1 in the two cell lines in response to recombinant murine IFNγ and CpG stimulation for 30 min, 1hr, 2hrs and 4 hrs. Protein expression was qualitatively assessed by Western blots of whole cell lysates using commercially available anti-IRF1 and anti-IRF8 antibodies. We observed an amplification of IRF8 and IRF1 expression in response to IFNγ/CpG challenge concomitant with an increase in stimulation time in J774 and RAW macrophage cell lines (Fig.4 A). Both, J774 and

RAW macrophages display a significant level of basal IRF8 expression with a noticeable increase after 2hrs of stimulation. Contrary to IRF8 expression, IRF1 was undetectable in resting macrophages and was induced only after 1hr and 2 hrs of stimulation with IFNγ/CpG in J774 and RAW cells respectively. The disparity between the strength of the IRF1 and IRF8 expression signal between RAW and J774 macrophages can be attributed to the distinct stages of differentiation of the two cell lines. RAW macrophages are arrested at an earlier state of maturation. J774 is a more mature cell line than RAW macrophages. It has been shown that J774 cells grow in a state of "priming" and that their immune responses are more easily triggered by pathogenic stimuli and physical shock [75]. In addition, others observed that RAW264.7 cells are inefficient in their induction of some immune genes, which may be an inherent characteristic of this cell line. For example, induction of IL12p40 protein following IFNγ and LPS stimulation is observed to be significantly lower in RAW cells compared to J774. Furthermore, according to the data deposited in the ATCC Global Bioresource centre (ATCC numbers TIB-71 for RAW264.7 and TIB-67 for J774), J774 macrophages constitutively produce IL-1β and possess receptors that are indicative of a mature macrophage cell line (C3, Fc receptor, IgG, high affinity I (Fcgr1)) whereas the RAW cells are negative for surface immunoglobulins and only express the C3 receptors. This data and our observations indicated that J774 cell line is a more suitable model for the dissection of the biological host response to infection. The J774 line was used for the subsequent ChIP on Chip experiments.

### *2.3.1. Detection of IRF8 by immunoprecipitation / immunoblotting*

To estimate whether the domestic and the commercial antibodies can precipitate IRF8 protein-DNA complexes we also attempted to detect IRF8 in the precipitates from chromatin IP by Western. In Figure 4 B, the presence of IRF8 at 49-50kD in the samples from RAW and J774 cell lysates immunoprecipitated with the domestic IRF8 (IRF8, lanes 3 and 5) and the commercial IRF8 (IRF8C19, lanes 4 and 6) is obscured by the appearance of the heavy IgG band. The characteristic heavy IgG

shape makes it difficult to judge whether the IRF8 protein is present in the samples. The appearance of the IgG band in samples immunoprecipitated with the domestic polyclonal rabbit antibody is attributed to the antigen primary Ab-secondary Ab interaction caused by the crosslinking between the IgGs of the rabbit antibody used to precipitate the samples and the rabbit IgG in the secondary HRP-coupled antibody used to visualized the proteins on the membrane. Although to a lesser degree, the cross-linking reactivity is also seen in samples immunoprecipitated with a goat anti-IRF8 antibody.

### *2.3.2. Optimization of anti-IRF8 antibody use for ChIP.*

Although we could not unambiguously show the presence of IRF8 protein in the post-IP samples (2.2.1), we proceeded to optimize the ChIP procedure for the specific antibodies by relying on the enrichment of DNA sequences of known IRF8 and IRF1 targets in the ChIP samples. *Cathepsin C (CathC), Cystatin C (CysC)* and *Bcl6 (B-Cell Lymphoma 6)* are some of the genes whose promoters have been previously reported to bind to and be regulated by IRF8 in the myeloid progenitor cell line Tot2 [6]. We used specific primes for the promoter regions of these genes to identify if their DNA is precipitated in the IRF-DNA complexes after ChIP. ChIP was performed on J774 cells stimulated with IFNγ/CpG for 30 min using antibodies to IRF1 (LifeSciences), the domestic antibody to IRF8, and a commercial antibody to IRF8-C19 (Santa Cruz). The relative DNA-binding of an antibody-precipitated sample at an IRF8-specific promoter site was calculated by amplifying the DNA at a) the specific promoter and b) a region 4kb upstream of the *HPRT* promoter (used as a negative control) and this for samples treated with an antibody and samples with no antibody. Further, the signal was normalized by calculating a comparative fold change between samples with or without antibody (log2 scale)

The results in Fig. 5A demonstrate subtle differences between the antibodies` pull-down potential with the commercial IRF8 being slightly more efficient at precipitating IRF8-DNA complexes. A combination of two antibodies (domestic IRF8

and IRF8 C19 Santa Cruz) did not result in a significant improvement of the ChIP signal. Interestingly, a small IRF1 binding signal at the *CathC* promoter was also observed. Although it has not been directly validated by chromatin IP, Storm van's Gravesande and colleagues reported IFNγ-induced IRF1-dependent CathC up-regulation in alveolar epithelial cells [76]. The positive ChIP signal for both, IRF1 and IRF8 antibodies, indicated that the chromatin IP was successful in precipitating the DNA of specific targets of IRF1 and IRF8 and thus, it could be further used for the systematic identification of novel IRF8 and IRF1 targets by ChIP on Chip.

### *2.3.3. ChIP analysis for IRF1 and IRF8 in macrophages*

The success of hybridization of ChIP-prepared DNA on a microarray depends on the quality of the DNA fragments isolated by ChIP. Higher ChIP enrichment values indicate the successful binding of protein of interest to DNA and the abundance of target-specific DNA segments in the sample. A low ChIP signal may be caused by either the failure of the protein-specific antibody to bind to its epitope, low affinity of the transcription factor for its target site, or by the low expression of the protein in the cell type tested. We attempted to obtain a higher ChIP DNA enrichment signal by increasing the time of IFNγ/CpG stimulation of J774 cells from 30 min to 3hrs. To verify that the difference in the resulting ChIP signal is due to the stimulation time, a parallel ChIP assay on non-stimulated J774 cells was performed. The rationale for increasing the stimulation time of the cells stems from our observation that IRF8 and IRF1 are more abundant in cell lysates after 2-4 hrs of stimulation with IFNγ/CpG (Fig. 4A), compared to 30 minutes. Furthermore, others have reported that both, IRF8 and IRF1 need other binding factors (IRF2, IRF4 or PU.1) to be present in the nucleus to facilitate IRF1/IRF8 DNA binding. Although IRF8 and some IRF1 are located in the nucleus, the stimulation time of 30 min may be insufficient for their binding partners to achieve maximal activation and to translocate into the nucleus [38, 59, 61, 70, 77]. As expected, the increase in stimulation time from 30 minutes to 3 hours elicited a much stronger ChIP signal for IRF8 targets (Fig. 5B). Moreover, the ChIP

signal for non-stimulated J774 cells was significantly weaker than that in the stimulated cells, confirming that the IRF1 and IRF8 binding activity is significantly improved with longer stimulation times. Further, the stimulation time of 3 hours was used for the ChIP on chip experiments.

Similarly to our previous findings for *CathC*, we noticed that some of the other IRF8 targets, such as *CysC* and *Bcl6* also display strong IRF1 binding, although direct regulation of these genes by IRF1 has not been previously reported. *Bcl*6 is an IFNγ-induced transcription factor involved in the formation of memory T-cells and *CysC* is an inhibitor of cysteine proteases [78, 79]. Both, *Bcl6* and *CysC* have an EICE element in their promoters, which suggests their responsiveness to IRFs [43]. Our results indicate that in addition to the reported IRF8 regulation, these genes may also be transcriptionally regulated by IRF1. Additionally, the IRF1 ChIP assay was validated by calculating the enrichment of iNOS (inducible nitric oxide synthase) binding. *iNOS* is a well-documented IRF1 regulation target that has been detected in ChIP studies by others [2].

## 2.4. ChIP on Chip for IRF8 and IRF1

To identify some transcription targets directly regulated by IRF1 or IRF8 we hybridized IRF1 and IRF8 – immunoprecipitated chromatin (ChIP) to Agilent 244K mouse promoter microarray. We used the 244K mouse array because it is specifically designed for the analysis of mouse DNA-binding proteins with probes that were optimized and validated for ChIP on Chip. This microarray optimizes the coverage of the genes and the promoter site by covering -5.5kb upstream to +2.5kb downstream of the transcriptional start sites for 17,000 best annotated RefSeq genes. The non-antibody treated ChIP was used as background for both, IRF1 and IRF8 samples. Using a stringent cut-off p-value <0.001 and based on their location within 1000 kb of transcription units, we assigned the gene segments present in the ChIP to 201 and 302 genomic loci for IRF1 and IRF8 respectively (Done with ChIP Analytics Software 1.3.1). Tables 1 shows 20 highest confidence target genes for IRF1 and IRF8

respectively. Full lists of IRF1 and IRF8 targets obtained by us are available in supplementary tables 1 and 2.

### *2.4.1. Functional annotation of IRF1 and IRF8 transcription targets*

In order to assign roles to IRF1 and IRF8 and their identified transcriptional targets to specific biological processes, we functionally annotated the genes obtained with ChIP on Chip into Gene Ontology (GO) categories by using the Babelomics FatiGO functional enrichment tool (http://www.babelomics.org/) [80]. FatiGO analyzes functional enrichment of biological processes according to GO terms within a gene list comparing it to a reference gene list (we used whole mouse genome) and looks for an over-representation of certain GO terms in the gene list of interest. Fisher´s exact test was used to generate a statistical significance rating for the comparisons [81]. FatiGO revealed several GO biological processes that were significantly over-represented in our list of target genes compared to the whole mouse genome. Among the enriched processes with p-value < 0.05 we observed a) immune response pathway for IRF1 and b) immune response, cell activation, defense response and cell proliferation for IRF8 (Fig. 6). The target gene list of IRF1 is highly enriched in genes responsible for immune processes (~79%) and IRF8 target gene list showed a large over-representation in the genes involved in immune response (~79%) and defence response ~73%. Our target gene lists may therefore contain novel targets that are regulated by IRF1 and IRF8 in the context of pathogen stimulation. These novel targets warrant further validation by ChIP assays, RP-PCR and/or microarray.

### *2.4.2. Presence of known transcription binding sites in genes identified by ChIP on Chip*

In addition to GO terms we simultaneously tested whether known IRF8 or IRF1 transcriptional targets were indeed identified in our ChIP on Chip-generated target lists. A number of genes identified by us had either a validated generic IRF,

IRF7, or IRF8 binding site (Fig. 7A and 8A). Surprisingly, one of the most highly enriched binding site in the IRF1 gene list is *Oct-1* (octamer-binding transcription factor 1), which regulates several house-keeping and tissue-specific genes [82]. *Oct-1* is also reported to be activated in response to DNA damage [83], which is also one of the previously described functions of IRF1 [2, 69]. The two most highly enriched known transcription factor binding sites in the IRF8 target gene list are the HNF-3alpha (hepatocyte nuclear factor-3alpha) and the FOXD3 binding sites. HNF-3alpha generally activates genes during cell development and FOXD3 is a stem cell regulatory gene [82]. The presence of previously reported IRF-transcription binding sites in the identified target gene lists of IRF1 and IRF8 serves as an indirect validation of the ChIP on Chip technique, as one would expect to identify genes that are known to be regulated by IRFs (Fig.7B and 8B). The presence of other transcription binding site on the target genes may indicate co-regulation of these genes by IRF1, IRF8 and other transcription factors and may help us implicate IRF1 and IRF8 in novel pathways.

### *2.4.3. Overlap between ChIP on Chip IRF1 and IRF8 target genes*

Comparison of the IRF1 and IRF8 target gene lists is helpful in identifying the genes that are co-regulated by two transcription factors as well as the pathways that they may share in common. The gene lists for IRF1 and IRF8 were overlapped with GeneSifter Intersector (http://public.genesifter.net/intersector) and the overlap yielded 19 genes that were found in both gene lists (Table 2). 17 out of 19 genes were identified as immune response regulation genes. The genes with highest confidence (p-value) identified were: *Gbp6, Mx2, Tnfsf13b, H2-T24, and Ifit1*.

## 2.5. Transcription profiling of IRF8 targets in macrophages in response to pathogen challenge.

In parallel with ChIP on Chip, we aimed to identify genes regulated by IRF8 by a separate method: transcription profiling with microarrays. By using this

technique we sought to obtain a list of genes that are differentially regulated in macrophages from wildtype (Balb/C) and IRF8 mutant (BXH2) mice. This list could be used to complement and possibly validate the gene list obtained by ChIP on Chip with the IRF8 antibody. The BXH2 recombinant inbred mouse strain has a loss of function mutation in the IRF8 allele, consisting of an arginine (R) to cysteine (C) substitution at position 294 that maps within the IAD of IRF8 [47]. Similarly to *IRF8*$^{-/-}$ mice, BXH2 mice display increased susceptibility to infections that require IFNγ-mediated immunity. A comparison of genes up-regulated in response to infection/stimulation in wildtype (wt) and BXH2 mice provided us with a list of genes that are regulated by IRF8 in response to infection.

### *2.5.1. Methods of macrophage activation used for the IRF8 transcriptional profiling study.*

We used two methods to imitate infection conditions in mouse BMDM. First, we used IFNγ and CpG DNA to stimulate the IFNGR and TLR9 receptors and trigger the activation of JAK-STAT and NFκB signalling. Secondly, we used infection with *L. pneumophila* (Lp02 strain), an intracellular bacterium that preferentially infects macrophages. *Legionella* is a good organism for the modeling of intracellular infection because it triggers diverse immune responses through the production of TLR agonists (LPS, lipopeptides, flagellin, unmethylated CpG DNA and peptidoglycan) [83]. IFNγ/CpG and *Legionella* infection therefore mimic different aspects of host pathogen interactions in macrophages. We used both methods in order to obtain gene lists with fuller representation of IRF8-dependent genes induced in macrophages by pathogens.

### *2.5.2. Generation of F2 mice aimed at reducing genetic background effects between BXH2 and Balb/C mice.*

Performing a transcription profiling study where gene expression in response

to a stimulus in two different mouse strain is compared, may be complicated by the presence of endogenous, strain specific, and genetically determined differences in gene expression (Expression quantitative trait loci; eQTLs). In our case, eQTLs distinguishing the genomes of Balb/C and BXH2 mice could potentially complicate detection of IRF1/IRF8-dependent differences in gene expression in response to infection or stimulation. In order to minimize such potentially interfering eQTLs, we generated an F2 cross between BXH2 and Balb/C mice, which allowed randomization of the different genetic background contributions intrinsic to either BXH2 or Balb/C parent and distinct from IRF1/IRF8-dependent effects (Fig. 9). Progeny that were homozygous for the mutant or the wildtype *IRF8* alleles were identified by genotyping, using the microsatellite marker *D8mit13* that is located approximately 3.1 Mb from the *IRF8* locus, as previously described [47]. This marker was used because of its proximal location to the *IRF8* locus on chromosome 8 (*Myls)*.

### *2.5.3. Validation of macrophage stimulation for the IRF8 transcription profiling study.*

BMDMs were extracted from F2 mice homozygous for either the $IRF8^{R294}$ or $IRF8^{C294}$ mutant alleles. Eight samples per group were either left untreated, infected with *L. pneumophila* (MOI 25:1, bacteria:macrophage) for 4 hours, or stimulated with IFNγ/CpG for 3hrs. The success of *L. pneumophila* infection was confirmed by RT-PCR of the *CXCL1* and *IL1β* genes (from RNA of infected macrophages) that are known to be up-regulated in macrophages following *L. pneumophila* infection (Fig. 10A). According to expectations, the infected mice (wildtype and mutant) showed up-regulated levels of *IL1β* and *CXCL1*. To verify activation of BMDM in response to IFNγ/CPG stimulation, RT-PCR for *IL12p40* and *IRF1* was performed (Fig. 10B). *IRF1* has been shown to be induced in response to IFNγ/TLR ligand stimulation [2, 56], which we observed in both, IRF8 wildtype and mutant macrophages (two lower panels of Fig. 10B). Not surprisingly, *IL12p40* was only induced in stimulated

macrophages from wild type F2s but not in F2s homozygote for the IRF8 mutant allele, in agreement with the previously reported absence of *IL12p40* expression in IRF8 mutant macrophages [2, 6, 43].

### *2.5.4. Transcription profiling results from infected or stimulated macrophages bearing wildtype or mutant IRF8 alleles*

After hybridizing cDNA from non-treated, infected and stimulated BMDM on Illumina mouse V.2 bead arrays, the transcript profiles associated with differential gene regulation in response to immune stimulus were investigated by a 2-way ANOVA analysis. Although n=8 samples were collected per group, only three samples per group were used for the initial analysis. Gene lists were generated by comparing *Legionella* (or IFNγ/CpG) -induced changes in F2 mice bearing either the WT or the $IRF8^{C294}$ allele. Initial analysis performed separately for each data set (Lp02 infected vs. non-infected and IFNγ/CpG stimulated vs. non-treated), with Benjamini and Hochberg corrected p-value less or equal to 0.05, revealed 1171 and 852 differentially regulated genes for the Lp02 and the IFNγ/CpG sets respectively (compared to controls). The GO (gene ontology) report showed a number of genes that are involved in cellular processes (16.8 and 18.6%). Moreover, a significant number of genes were found that regulated response to stimulus (6.3% and 7.6%) and immune system processes (2.8 and 4.6%) (Table 3).

From the complete differentially expressed gene lists generated from BMDMs infected with *L. pneumophila,* stimulated with IFNγ/CpG or left untreated, we identified 31 and 129 genes (for Lp02 and IFNγ/CpG lists, respectively) that were differentially expressed between strains as well as between different infection/stimulation conditions (supplementary tables 3 and 4). The manageable size of the gene lists we obtained demonstrate that the parental strain differences have been successfully diluted out by the application of the F2 breeding scheme.

### *2.5.4. Transcription profiling reveals genes regulated by IRF8 in response to L. pneumophila infection*

The list of genes that are differentially expressed as a result of the interaction of the infection with *L. pneumophila* and that show a dependence on IRF8 allelic combination contains 31 unique genes (Supplementary table 3) (Fig. 11A). The DAVID functional annotation clustering tool (http://david.abcc.ncifcrf.gov/) revealed that the two highly enriched functions in this gene list are the "regulators of GTPase and enzymatic activity" and the "immune processes". 9.4 % of the genes are directly involved in immune system development. Some of the high-scoring genes that are involved in immunity include: *H2-T24, IL7Rα*, haptoglobins, triggering receptor on myeloid cells-like *4 (Trem4)*, and *HMGA1*.

### *2.5.5. Transcription profiling reveals genes regulated by IRF8 in response to IFNγ/CpG stimulation.*

The IRF8-dependent IFNγ/CpG- stimulation specific list contains 129 genes, from which 7.55% genes regulate response to stimulus and 4.58% genes regulate immune system processes (Supplementary table 4) (Fig. 11B). The genes with the highest fold change that were more significantly up-regulated in wildtype BMDMs than in mutant BMDMs include transcripts coding for *Cd74*, serine peptidase inhibitor clade A *(Spi2/eb.1, Spi2A* or *Serpina3*), *Cxcl9, 10* and *16, Il1β*, formyl peptide receptor 2 (*Fpr2*), and MHC class II transactivator *CIITA*. The genes that were more significantly down-regulated in the wildtype macrophages include the regulator of G-protein signalling, platelet factor 4 (*Pf4*), myeloperoxidase (*Mpo*), *Bcl2, Bcl2-like* (*Bcl2l1*), and *interleukin receptor antagonist* (*Il1rn*). Some of the genes identified by us to be IRF8-dependent in the transcription profiles of IFNγ/CpG stimulated macrophages have been previously described in different cell lines. The previously reported IRF8 targets that were also identified by us include: *CXCL9* and *CXCL10* that were shown to be up-regulated by IRF8 in macrophage/microglia cells [86],

CXCL16 that has been reported to be regulated by IRF8 in peritoneal macrophages [7], *Mpo* that is down-regulated by IRF8 in Tot2 cells, and *Bcl2* that is repressed by IRF8 in the context of CML pathogenesis [87, 88].

### *2.5.6. Overlap between genes activated by L. pneumophila infection and by IFNγ/CpG stimulation*

GeneSifter Intersector (GeoSpiza) analysis of these two gene lists revealed an overlap of 7 genes between the Lp02 and IFNγ/CPG interaction lists. (Fig. 11C) (Table 4). Interestingly, 2 of the 7 genes (*Cyclin D1/Ccd1* and *IL10*) belong to the JAK-STAT signalling pathway, suggesting its involvement in both, Lp02 infection and IFNγ/CpG stimulation responses [89].

### *2.5.7. Overlap between genes regulated by IRF8 in the context of IFNγ/CpG stimulation and the IRF8 target genes isolated by ChIP on Chip.*

To find out whether some of the IRF8 target genes identified in the ChIP on Chip were present in the IFNγ/CpG-stimulated gene lists, we compared the gene lists by using GeneSifter Intersector software. The comparison of IRF8 ChIP on Chip gene list (302 genes) with the full differentially regulated IFNγ/CpG-stimulated gene list (852 genes) showed an overlap of 15 genes in common (Table 5). The 15 overlapping genes were classified according to GO terms by PhatiGO into the three pathways: "antigen processing and presentation", "defence response", and "immune response". The genes with the highest confidence value include *Ly86, Cd74, Gbp2*, and *H2-DMb1*. Interestingly, *Gbp6* and *H2-T24* were also identified as the genes common to IRF1 and IRF8 ChIP on Chip gene lists, suggesting the possible involvement of IRF1 in their regulation under in conditions of infection. Moreover, the *H2-T24* gene was also identified in the microarray on *Legionella*-infected samples. *Gbp2* and *H2-T24*

represent interesting target genes for study and validation as high confidence targets of IRF8.

# Chapter 3

# Conclusions

## 3. CONCLUSIONS:

IRF1 and IRF8 are two transcription factors playing a critical role in the initiation and amplification of innate and adaptive immune responses; they also regulate apoptosis and cellular transformation in myeloid cells. The susceptibility of IRF1/IRF8 deficient mice to viral and bacterial infections indicates that these 2 factors regulate many pathways important for eliciting immune responses to a wide range of microbial pathogens. To identify those genes and to better understand the role of IRF1/8 in immunity we performed a ChIP on Chip study for IRF8 and IRF1 to find novel transcriptional targets for these factors in the mouse genome. Additionally, we utilized a microarray approach to create a list of genes regulated by IRF8 in response to pathogen challenge. This gene list has the potential to be used as a separate gene-mining tool or as a validation tool for ChIP on Chip for IRF8.

### 3.1. ChIP on Chip identified novel targets for IRF1 and IRF8

Using the ChIP on Chip approach, we identified 201 and 303 novel loci that are enriched for IRF1 and IRF8 binding respectively after IFNγ/CpG stimulation. Functional Gene Ontology classification uncovered that most of the genes in the IRF1 and IRF8 lists (~79%) control immune responses in mammalian systems. These findings correspond with the literature reports that IRF1 and IRF8 are both important mediators of immunity. Importantly, we observed some of known IRF1 and IRF8 binding targets validated by others in ChIP on Chip experiments in our results: *OAS1b* for IRF1 or *IFNβ* for IRF8 [4, 6, 66].

We further analyzed the top 20 genes with the lowest p-value in both, IRF1 and IRF8 ChIP on Chip gene lists. We observed a number of genes that have been previously described as IRF targets but that were not validated by ChIP. Three of the genes on our list (*Irgm, tnfsf13b* and *Aif1*) have been described by Aly et al. as IRF1-dependent factors in a microarray study of IRF-1 KO mouse lungs infected with *Mycobacterium Avum* [88]. *Mx2* gene promoter site has been examined and an ISRE

site was identified within the promoter, although no specific IRF that is associated with that site has been described up to date [91]. Interestingly, our results identify *Mx2* as a transcriptional target of both, IRF1 and IRF8. Many of the other genes found to bind either IRF1, IRF8 or both in our experiment, have not been previously described to be associated with IRF1 or IRF8 and thus they represent novel targets that may be validated in subsequent experiments.

We also questioned whether there may be genes in the ChIP on Chip-generated gene lists that are able to be bound by IRF1 as well as IRF8. By performing comparative analysis between the gene lists we identified 19 such genes. These genes represent interesting targets for further validation. It is possible that they may be regulated by the immunity and hematopoesis-specific IRF8/IRF1/PU.1 transcription complex that has been previously described as essential for the induction of activation state in Tot2 myeloid precursor cells activated with IFNγ and LPS [7, 77]. Alternatively, IRF1 and IRF8 may have either separate binding sites or bind competitively to the promoters of the genes identified in both lists.

## 3.2. Transcription profiling of IRF8-dependent genes by microarray as a tool to validate ChIP on Chip results.

Validation by ChIP of all novel targets of IRF8/1 defined by ChIP on Chip is necessary in order to ascertain that the gene loci found were not simply a microarray aberration created during hybridization or array scanning. Genes from ChIP on Chip target lists may be further tested by comparing their protein or DNA expression in IRF8 mutant mice vs. wildtype mice (or alternatively, IRF1 wt vs. mutant). This would be the next logical step of this project.

In the scope of this project, we created a list of IRF8-dependent genes in the context of pathogen challenge by using microarrays. The microarray approach allowed us to compare gene expression in IRF8 wt and IRF8 mutant mouse bone marrow macrophages in response to infection. We identified 1171 and 852

differentially regulated genes. Only the genes that are differentially regulated by the IRF8 allele as well as by the stimulation conditions (infection with *Legionella* or stimulation with IFNγ/CpG) were filtered out yielding 31 and 129 unique genes for the *Legionella* and the IFNγ/CpG experiments. Only 7 genes were identified in the overlap between the *Legionella* and the IFNγ/CpG gene lists. The apparent small overlap may be explained by different signalling events that occur in macrophages in response to *Legionella* infection and IFNγ/CpG stimulation. *Legionella* is an intracellular microbe that induces an activation state in macrophages via signalling through several TLRs including TLR2, 4, 5, and 9. TLR signalling results in the induction of IFNγ and IL12 that up-regulate other immunity-related genes. The stimulation of macrophages with IFNγ and CpG activates TLR9 and the JAK-STAT signalling pathway via IFNGR signalling. Although the TLR signalling pathways have many genes in common the different models of mimicking infection will have inherent differences reflected in the gene lists.

The gene list generated by the IFNγ/CpG microarray provided us with a tool to validate some of the genes identified by ChIP on Chip for IRF8. After comparing the IRF8 ChIP on Chip and IRF8-dependent IFNγ/CpG stimulation gene lists we identified 15 genes in common between the two lists. The two main GO functions of these genes were identified by DAVID as “response to stimulus” and “inflammatory response”. Interestingly, the list of common genes contains a cluster of genes related to antigen processing and presentation: *CD74, Tapbpl* and *H2-DMb1* [92-95]. None of these genes have been previously reported to bind IRF1 or IRF8 and may therefore represent novel targets and possibly implicate IRF1/8 in the antigen presentation pathway.

### 3.3. Transcription profiling of IRF8-dependent genes revealed common pathways regulated by IRF8 in L. pneumophila and IFNγ/CpG stimulation.

We also used the microarray experiments as an independent tool for data-mining to determine which genes are regulated by IRF8 during infection. The GO pathways enriched in both gene lists (IFNγ/CpG and *Legionella*) include responses to stimulus and immune responses, among others. Some of the identified IRF8 targets, such as *CXCL9, 10* and *16*, *Mpo*, and *Bcl2* for the IFN IFNγ/CpG experiment [88, 96-99] and *IL7Rα* for the *Legionella* experiment [100]. We also observed an overlap of 7 genes between the target genes of IRF8 in infection and stimulation conditions. The overlap of the two gene lists shows the involvement of similar signalling pathways in the two models of infection. The genes that appear on both gene lists are of particular interest as they are most likely to be involved in essential immune signalling pathways regulated by IRF8. Interestingly, *IL10* and *Ccd1* are two of the 7 genes common to both, *Legionella* and IFNγ/CpG experiments, that belong in the JAK-STAT pathway. The involvement of IFNγ-triggered JAK-STAT signalling has been previously shown in *Legionella* infection as well as during IFNγ stimulation [37, 49, 85, 101]. However, IRF8 had not been implicated in the regulation of these two genes. This data may indicate a yet unidentified level of regulation of JAK-STAT pathway by IRF8.

### 3.4. Overlap between ChIP on Chip and microarray studies

The small overlap between the ChIP on Chip and microarray techniques observed in the IRF8-dependent gene lists could be explained by several factors. First, the samples that we used to hybridize to microarray slides for the ChIP on Chip and the transcription profiling studies will have inherent differences due to the techniques used to obtain the DNA (infection/stimulation followed by RNA extraction and RT-PCR vs. chromatin IP). The disparity between the lists could also be caused by the genetic variability between cell lines (primary macrophages and a cultured cell line

J774). Next, two different microarray techniques were used (Illimina Bead Chips for transcription profiling and Agilent promoter arrays for ChIP on Chip). Each technical approach used has the potential to identify targets not identified by another. Moreover, it has been observed that even though a binding event may be detected in a gene promoter, it may not necessarily indicate transcriptional regulation of the nearest gene. Since the ChIP on Chip gene loci are annotated according to the next proximal gene, some of the genes regulated by IRF1 or IRF8 and located further away from the binding locus would not be identified [102]. The gene lists obtained with ChIP on Chip and microarray transcription profiling should be viewed as two complementary approaches to identify genes regulated by a transcription factor. Merging the two techniques will result in a list of genes of "high confidence" that are regulated by the transcription factor of interest, however, this may also result in the loss of some possible transcriptional targets.

### 3.5. Other considerations

Although we have not performed the experiment, the IRF1 target gene list generated by IRF1 ChIP on Chip would benefit from being complemented by microarray transcription profiling similar to what has been done by us for IRF8. This and other validation techniques (comparison of specific target gene and protein expression in wildtype and knock-out mice for IRF1 and IRF8) are the next steps in this project. Furthermore, the validated genes of interest may be sequenced in some of the conditions thought to be at least partially caused by aberrant IRF signalling, such as the atypical Mendelian susceptibility to Mycobacterial disease (MSDS) [103]. Moreover, the transcription binding targets of IRF1 and IRF8 generated by us could potentially be compared with the published lists of PU.1-binding genes. PU.1 is a major IRF8/IRF1 partner in transcription of many genes involved in immunity and haematopoiesis. The generation of a list of genes regulated by PU.1 and IRF1, PU.1 and IRF8 or PU.1/IRF1/IRF8 would present us with an opportunity to study the involvement of IRF1 and IRF8 with other transcription binding factors in the context

of immunity.

We have successfully identified new IRF1 and IRF8 bound genes that may play a critical role in immune regulatory functions. We have been able to use the ChIP on Chip and microarray techniques to obtain novel transcriptional targets of these two factors. These data will provide us with novel IRF8 and IRF1-regulated genes that will undergo further functional characterization and validation. Ultimately, this study will further our knowledge of the roles of IRF1 and IRF8 in immunity.

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# Figures and legends

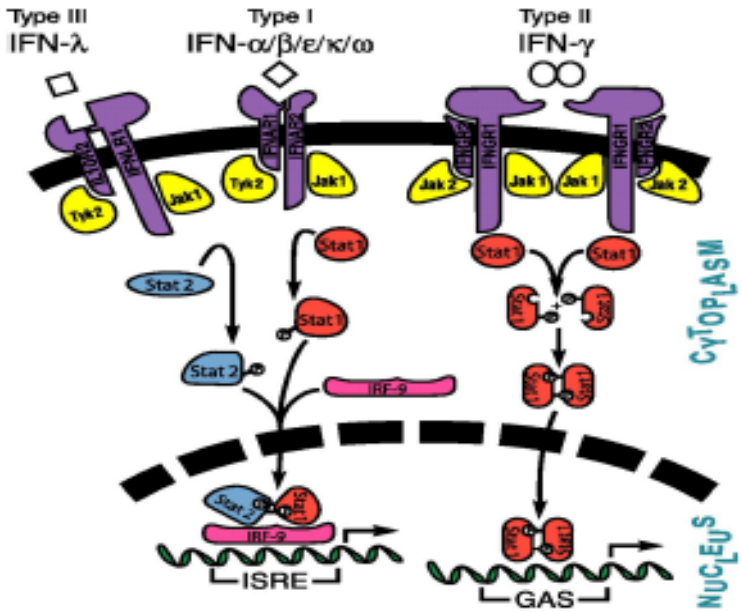



**Figure 1: Interferons and their signalling.** Type I IFNs bind to IFNAR1 and cause its dimerization with IFNAR2. The dimerization triggers the activation of the associated protein kinases Tyk2 and Jak1 as well as the docking and phosphorylation of of STATs (1,2,3 or 5). Phosphorylated STATs homo- or hetero-dimerize and translocate into the nucleus where they modulate transcription of GAS and/or ISRE-containing genes. STAT1snd STAT2 are often found in a trimeric protein complex with IRF9, termed ISGF3. Type II interferons signal in a manner similar to Type I and III via the activation of Jak1 and Jak2 protein kinases. STAT1 dimerization is primarily associated with IFN type II signalling, although STATs 3 and 5 have also been described. STAT1 homodimers bind to well defined GAS elements in IFN-regulated genes, including IRF8 and IRF1

*Adapted from Samuel et al., 2007, J. Biol. Chem. 282(28), 20045-46*

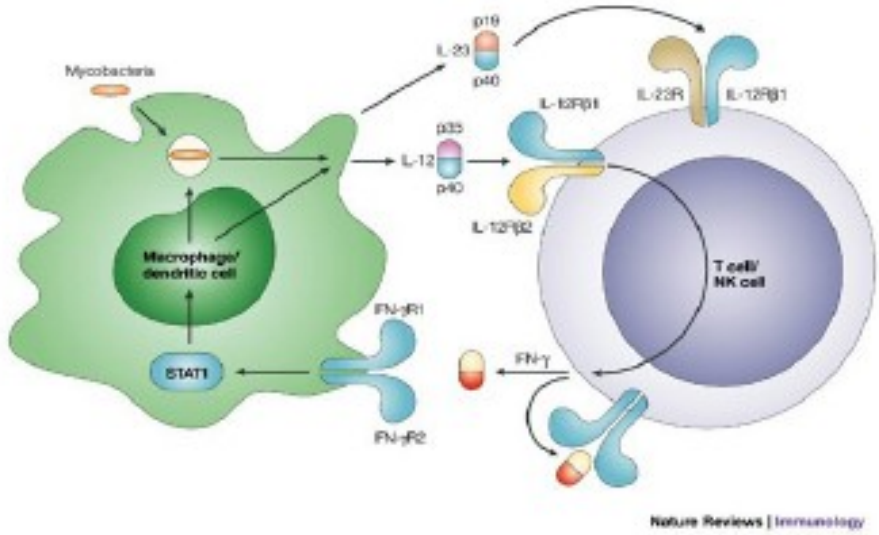



**Figure 2: IFNγ-IL12 signalling** IFNg-IL12 signalling cascade is initiated by IFNγ binding IFNγR. STAT1 is phosphorylated by Jak protein kinases and translocates into the nucleus where it binds to GAS elements on multiple IFNγ-regulated genes including. STAT1-induced IRF8 triggers IL12p40 transcription and the formation of a functional IL12 heterodimer. IL12 secreted into cell milieu induces differentiation and maturation of immune cells, which is accompanied by the secretion of IFNγ by mature NK cells, T cells, dendritic cells and macrophages.
*Adapted from Casanova et al,, 2004, Nature Reviews Immunology 4, 55-66*

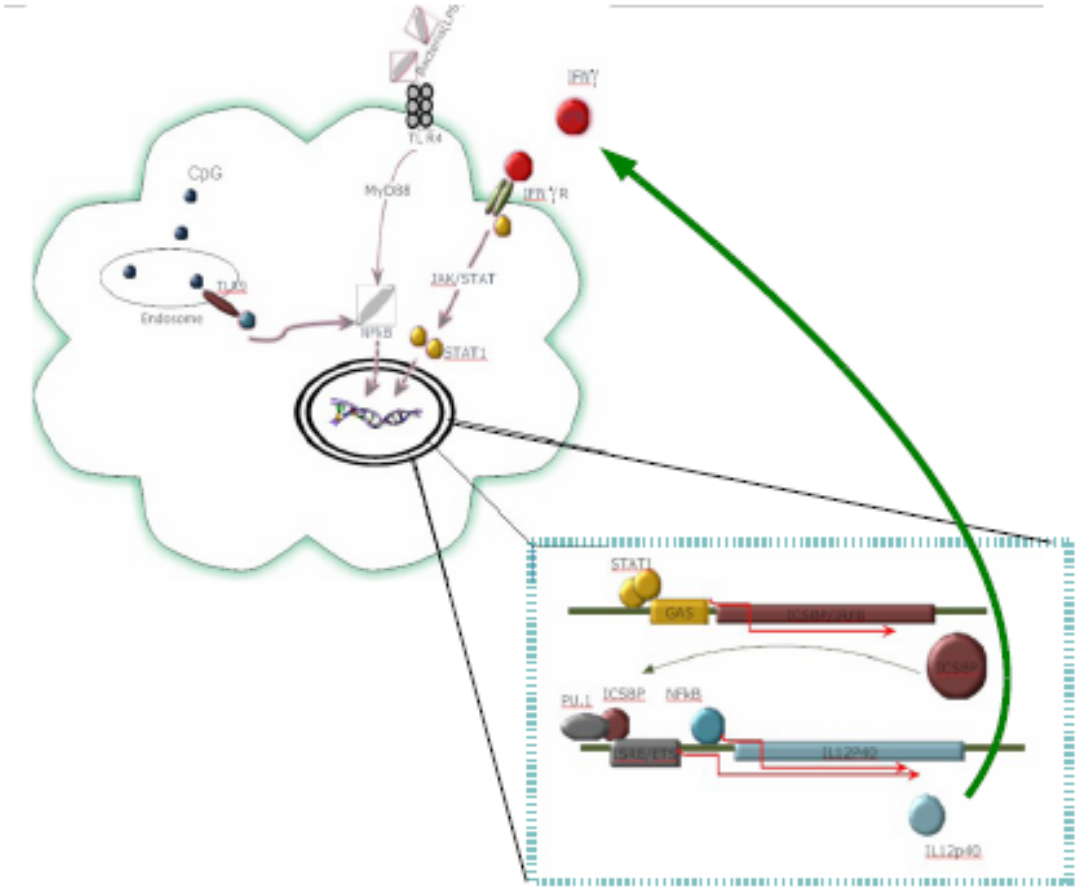



**Figure 3: IFNγ – IRF8 – IL12 positive feedback loop in monocytes/macrophages.** IFNγ signals through the Jak-STAT pathway. Resulting STAT1 dimer translocates into the nucleus and activates IRF8 (ICSBP) transcription by binding to a GAS in its upstream promoter. IRF8 forms a protein complex with PU.1 and attaches to an ISRE/Ets element in the proximal IL12p40 promoter. IRF8/PU.1 association triggers the transcription of IL12p40 subunit that forms a functional IL12. IL12 stimulates macrophages, dendritic cells, T cells and NK cells and promotes the secretion of more IFNγ consequently amplifying its own transcription. Transcription of IL12p40 may also be aided by TLR-activated NFκB during infection.

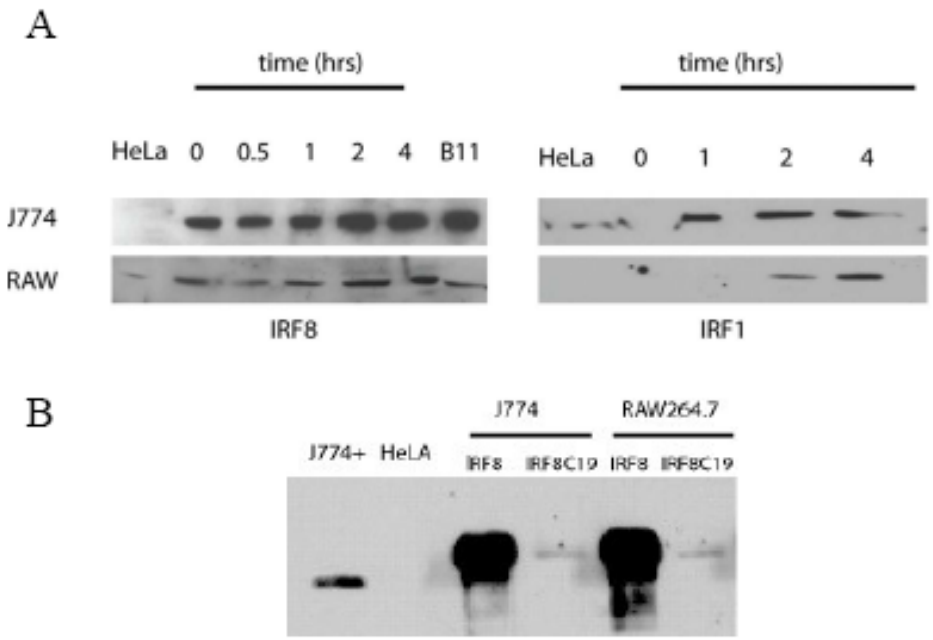



**Figure 4: Expression of IRF8 (ICSBP) and IRF1 in J774 and RAW264.7 macrophages and the detection of ICSBP in post-IP protein-DNA complexes. A)** RAW and J774 macrophages were stimulated with 400 U/ml of IFNγ and 1.5 ug/ml of CpG for up to 4 hrs. Shown are Western blots of whole cell lysates where IRF1 and IRF8 expression was detected by commercial anti-IRF1 and anti-IRF8 antibodies. **B)** Shown are whole cell lysates from J774 and RAW macrophages immunoprecipitated with the commerical IRF8 antibody (IRF8C19) or with a domestic antibody (IRF8). The presence of IRF8 in the precipitates was tested by commercial anti-IRF8 antibody. J774 macrophages stimulated with IFNγ/CpG are a positive control for IRF8 expression and non-IRF8 expressing HeLa cells are a negative control. The characteristic large heavy IgG band is seen at 50-55kD, whereas IRF8 is usually detected at 49kD (lanes 3 and 5). The commercial anti-IRF8 antibody appears to have much less IgG crosslinking due to the different species of antibodies used for immunoprecipitation and Western blotting (lanes 4 and 6)

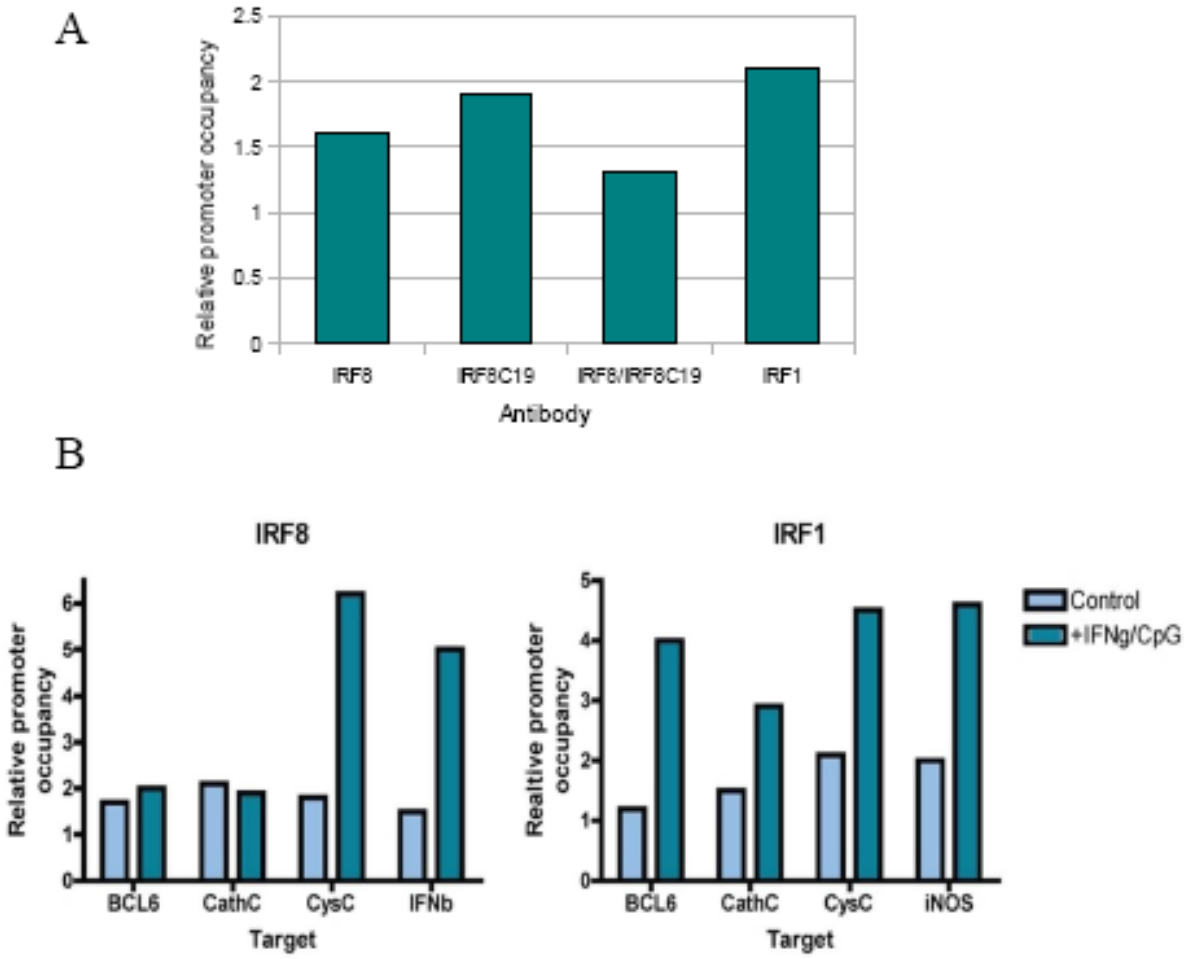
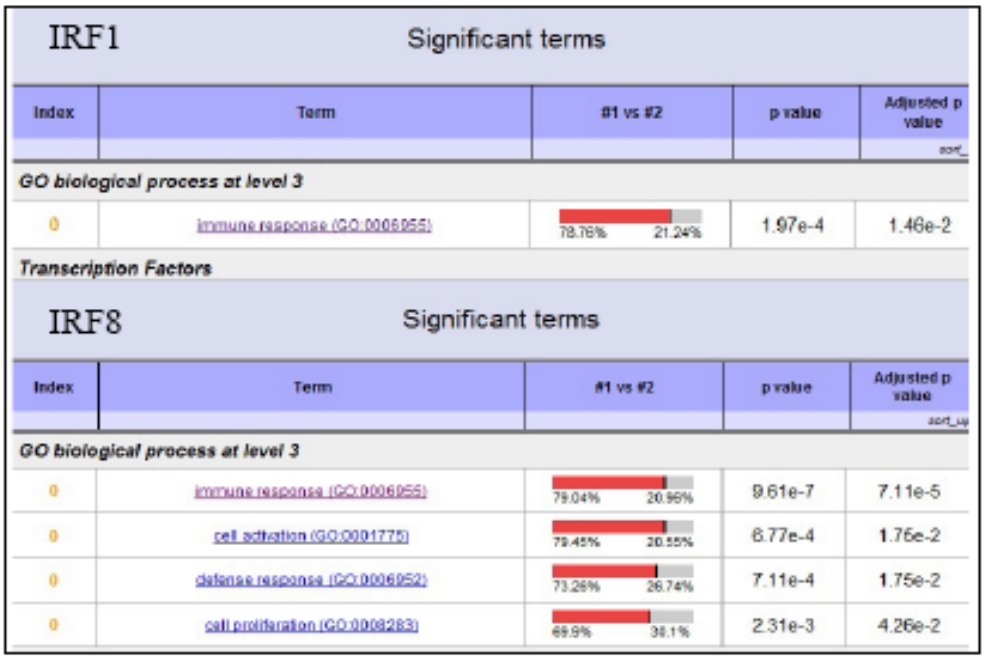



**Figure 5: ChIP assays show promoter binding enrichment for IRF8 and IRF1 transcription targets. A)** ChIP assays were performed on J774 macrophages stimulated with IFNγ/CpG for 30 minutes. Abundance of CathC DNA promoter elements (promoter occupancy) is shown for ChIP assays performed with commercial (IRF8C19) and domestic (IRF8) antibodies. The individual antibodies or their combination do not show a significant difference in ChIP signal. **B)** ChIP assays done on unstimulated J774 macrophages (control) or on cells stimulated with IFNγ/CpG for 3 hrs. Shown is the enrichment in promoter binding in IRF1 and IRF8 ChIP samples for documented IRF8 targets BCL6, CathC, CysC and IFNb. IRF1 ChIP shows enrichment for IRF8 targets described in literature as well as for iNOS, a documented IRF1 target.



IRF1 Significant terms

| Index | Term | #1 vs #2 | p value | Adjusted p value |
|---|---|---|---|---|
| *GO biological process at level 3* | | | | |
| 0 | immune response (GO:0006955) | 78.76% 21.24% | 1.97e-4 | 1.46e-2 |
| *Transcription Factors* | | | | |

IRF8 Significant terms

| Index | Term | #1 vs #2 | p value | Adjusted p value |
|---|---|---|---|---|
| *GO biological process at level 3* | | | | |
| 0 | immune response (GO:0006955) | 79.04% 20.96% | 9.61e-7 | 7.11e-5 |
| 0 | cell activation (GO:0001775) | 79.45% 20.55% | 6.77e-4 | 1.76e-2 |
| 0 | defense response (GO:0006952) | 73.26% 26.74% | 7.11e-4 | 1.75e-2 |
| 0 | cell proliferation (GO:0008283) | 69.9% 30.1% | 2.31e-3 | 4.26e-2 |

**Figure 6: Functional classification of IRF1 and IRF8 targets identified with ChIP on Chip according to GO terms.** IRF1 and IRF8 target gene lists were tested for over-representation of GO terms with FatiGO software (http://www.babelomics.org/). Percent over-representation in the gene list (#1, red) is shown over the representation of a GO term in a reference mouse genome (#2, gray).

**A)**

Transcription Factors

| Term | Genes | Percentage with term | #1 vs #2 | p value | Adjusted p value |
|---|---|---|---|---|---|
| IRF | #1: Ifit1 Mx2 Tn... #2: ENSMUSG00000... | 10.09% 1.81% | 84.8% 15.2% | 5.59e-6 | 1.5e-3 |
| IRF-7 | #1: Sfmbt1 Olfr8... #2: ENSMUSG00000... | 43.12% 24.68% | 63.6% 36.4% | 2.03e-5 | 2.72e-3 |
| Cdc5 | #1: Bst2 Mrpl50 ... #2: ENSMUSG00000... | 94.5% 85.59% | 52.47% 47.53% | 2.94e-3 | 2.63e-1 |
| ICSBP | #1: Ifit1 Bst2 R... #2: ENSMUSG00000... | 5.5% 1.45% | 79.18% 20.82% | 5.41e-3 | 3.63e-1 |
| Oct-1 | #1: Psmb10 E130... #2: ENSMUSG00000... | 88.99% 80.57% | 52.48% 47.52% | 1.37e-2 | 7.14e-1 |
| TCF-1P | #1: Fbxo30 Aggf1... #2: ENSMUSG00000... | 8.42% 2.38% | 72.97% 27.03% | 1.6e-2 | 7.14e-1 |
| AFP1 | #1: Dusp14 2610... #2: ENSMUSG00000... | 8.20% 4.06% | 67.09% 32.91% | 3.37e-2 | 9.04e-1 |
| AR | #1: Olfr770 Tor1... #2: ENSMUSG00000... | 8.26% 4.04% | 67.13% 32.87% | 3.34e-2 | 9.04e-1 |
| ISRE | #1: Rtp4 #2: ENSMUSG00000... | 0.92% 0.02% | 97.69% 2.31% | 2.71e-2 | 9.04e-1 |
| MEF-2 | #1: Olfr19 Kif5a... #2: ENSMUSG00000... | 57.8% 48.29% | 54.48% 45.52% | 2.96e-2 | 9.04e-1 |

**B)**

| Binding site | Gene names |
|---|---|
| IRF7 | Sfmbt1 Olfr836 Olfr934 Trappc2 Psmb9 Kif5a 2610029G23Rik Akt3 Cdc37l1 H2-T24 Taar7e Ifit1 Psma5 pdcd10 Bst2 Nmu Phex Asf1a Olfr874 BC057552 Rtp4 Cybb Ica1l Olfr166 Hoxd10 Gbp6 Olfr1023 Irgm Slc9a10 Tmem39a Hk3 Pxk Tor1aip1 Mrps21 Tas2r102 Oas1b Ifih1 Gm382 Hspa9 Socs1 Snx10 Amigo2 Psmb10 Mppe1 Trove2 Ssbp1 Nap1l1 |
| IRF | Ifit1 Mx2 Tnfsf13b Pxk Aurkaip1 Olfr1023 Tas2r102 Psmb10 Gbp6 Bst2 H2-T24 |
| ICSBP (IRF8) | Ifit1 Bst2 Rtp4 Aurkaip1 Gbp6 Olfr1480 |

**Figure 7: Known transcription factor binding sites identified in IRF1 ChIP on Chip target list. A)** FatiGO transcription binding site comparison of ChIP on Chip-generated IRF1 target list with the known transcription factor binding sites. Shown are the most represented transcription factor binding segments found in the IRF1 gene list. **B)** Genes in the IRF1 target gene list that contain a known IRF binding site.

**A)**

Transcription Factors

| Term | Genes | Percentage with term | #1 vs #2 | p value | Adjusted p value |
|---|---|---|---|---|---|
| IRF-7 | | 37.64% 24.68% | 60.4% 39.6% | 8.98e-5 | 2.41e-2 |
| IRF | | 5.82% 1.81% | 76.66% 24.35% | 1.72e-3 | 2.31e-1 |
| E2F-1 | | 29.78% 21.56% | 57.53% 42.47% | 9.6e-3 | 2.86e-1 |
| E2F-1:DP-1 | | 10.11% 5.56% | 65.26% 34.74% | 8.09e-3 | 2.86e-1 |
| E2F-1:DP-2 | | 9.55% 4.86% | 67.19% 32.81% | 4.42e-3 | 2.86e-1 |
| E2F-4:DP-2 | | 9.55% 4.86% | 67.19% 32.81% | 4.42e-3 | 2.86e-1 |
| FOXD3 | | 71.35% 62.38% | 53.35% 46.65% | 7.71e-3 | 2.86e-1 |
| MAZR | | 39.33% 30.72% | 56.14% 43.86% | 9.2e-3 | 2.86e-1 |
| STATx | | 4.49% 1.69% | 73.89% 26.11% | 8.35e-3 | 2.86e-1 |
| Nkx2-5 | | 84.61% 66.18% | 53.49% 46.51% | 1.39e-2 | 3.74e-1 |

**B)**

| Binding site | Gene names |
|---|---|
| IRF7 | Lamp1 Olfr934 Lpxn Itga2 Cd47 BC017643 Ftl1 Pfkp Pigl Impad1 Etv3 Capza2 H2-T24 Ifit1 Entpd1 Gpr68 Spop Il6ra Tspan8 Rbpsuh Carhsp1 Rsad2 Olfr488 Hmga2 Hoxc10 Rtp4 BC019943 Dtx3l Slc15a3 Stambpl1 Dnalc1 Hoxd10 Gbp6 Btla Asb11 Npal2 Phgdh Xdh BC027057 Plek Irgm Slc9a10 Hk3 Zfx Tapbpl Tas2r138 Bcdo2 Rxfp4 Edg5 Wipf1 Clec9a Eif4enif1 Pabpc1 Kcna3 Ccdc109b Zbtb32 Tyki Rgs2 Xcr1 Vdp Ccdc106 Gbp2 Trove2 Vps26a Arih1 Gpr171 Ube2w |
| IRF | Lamp1 Olfr934 Lpxn Itga2 Cd47 BC017643 Ftl1 Pfkp Pigl Impad1 Etv3 Capza2 H2-T24 Ifit1 Entpd1 Gpr68 Spop Il6ra Tspan8 Rbpsuh Carhsp1 Rsad2 Olfr488 Hmga2 Hoxc10 Rtp4 BC019943 Dtx3l Slc15a3 Stambpl1 Dnalc1 Hoxd10 Gbp6 Btla Asb11 Npal2 Phgdh Xdh BC027057 Plek Irgm Slc9a10 Hk3 Zfx Tapbpl Tas2r138 Bcdo2 Rxfp4 Edg5 Wipf1 Clec9a Eif4enif1 Pabpc1 Kcna3 Ccdc109b Zbtb32 Tyki Rgs2 Xcr1 Vdp Ccdc106 Gbp2 Trove2 Vps26a Arih1 Gpr171 Ube2w |
| ICSBP (IRF8) | Sla Ifit1 Entpd1 Lbr Rtp4 Gbp6 Edg5 |

**Figure 8: Known transcription factor binding sites identified in IRF8 ChIP on Chip target list. A)** FatiGO transcription binding site comparison of ChIP on Chip-generated IRF8 target list with the known transcription factor binding sites. Shown are the most represented transcription factor binding segments found in the IRF1 gene list. **B)** Genes in the IRF8 target gene list that contain a known IRF binding site.

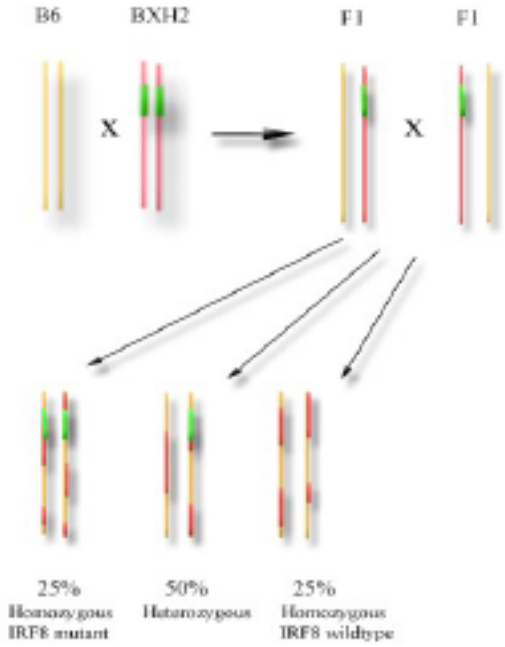



**Figure 9: F2 Balb/CxBXH2 cross for transcription profiling.** A) An F2 cross created by crossing Balb/C mice with a wildtype IRF8 allele with BXH2 (IRF8 mutant C294R). F1 progeny with 50% genetic contribution from each parent were crossed to each other to generate litters with a mixed genetic background (25% homozygous for the wildtype IRF8 allele and 25% homozygous for the mutant IRF8 allele).

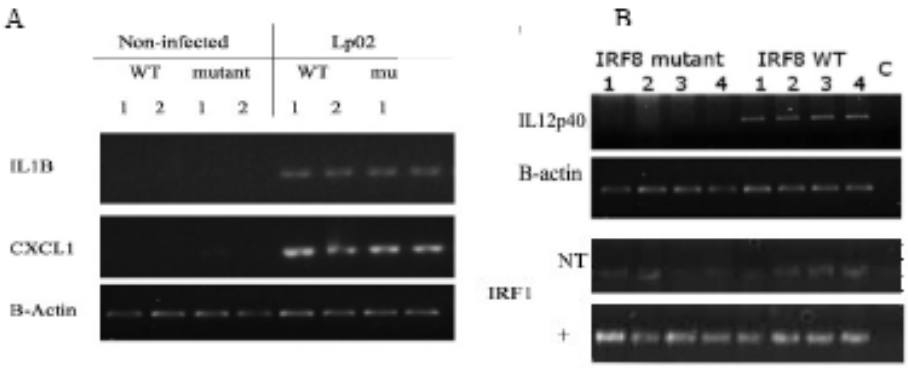
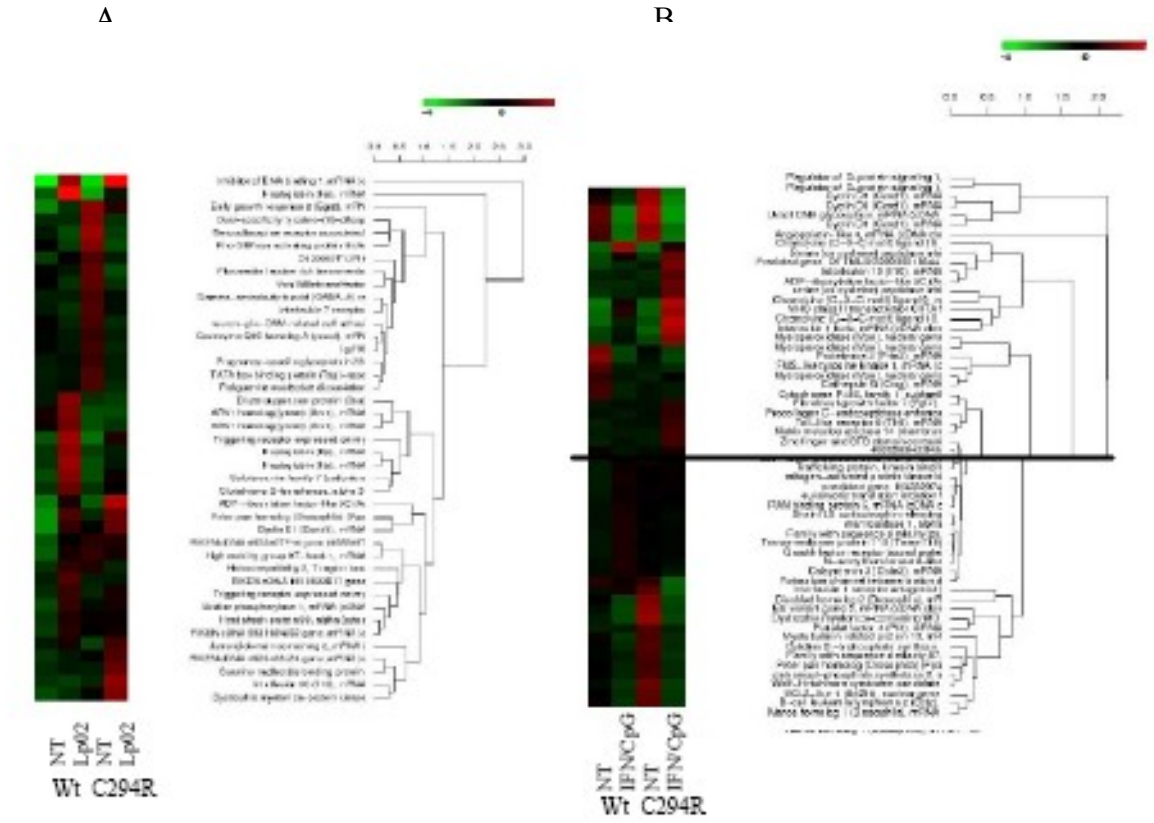



**Figure 10: F2 cross infection and stimulation for transcription profiling of IRF8 targets: A)** Bone marrow macrophages from mice homozygous for either wildtype or mutant IRF8 allele were infected with Lp02 and the expression of known IRF8 targets activated in response to infection was determined by RT-PCR. **B)** The induction of activation state in F2 bone marrow macrophages homozygous for either wildtype or mutant IRF8 allele in response to IFNγ/CpG stimulation was verified by RT-PCR for the IL12p40 subunit. IRF8 mutant macrophages are not able to trigger significant IL12p40 expression. Additionally, IRF1 expression as an indicator of macrophage stimulation was assessed to ascertain that the IRF8 mutant macrophages were activated. NT – non-treated macrophages, + - macrophages stimulated with IFNγ/CpG for 3 hrs.

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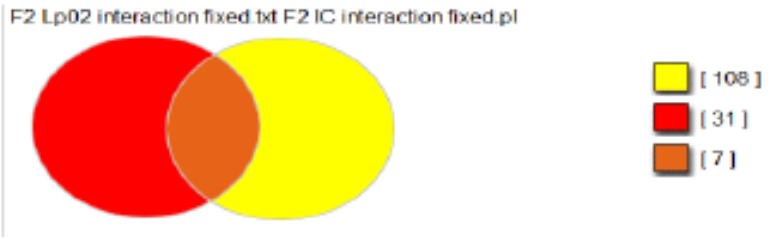


**Figure 11: Transcription profiles of genes regulated by IRF8 in infected or stimulated F2 BMDMs.** BMDMs from F2 mice homozygous for either the wildtype or mutant IRF8 allele were subjected to microarray analysis where untreated samples were compared to samples stimulated with IFNγ/CpG for 3 hours or infected with Lp02 for 4 hours. 3 samples per group were used for 2-way ANOVA analysis with Benjamini and Hochberg corrected p-val less or equal to 0.05 and a fold change cut off of 1.5 on a logarithmic scale. A) Transcription profile of Lp02 infected macrophages in infected and non-infected wt and mutant samples show differential gene regulation by 2-way ANOVA analysis. The total number of high confidence genes yielded is 41, although a much larger gene list is available with a lower fold change cut-off . B) Transcription profile of macrophages stimulated with IFNγ/CpG for infected and non-infected wt and mutant samples show differential gene regulation by 2-way ANOVA analysis. The total number of high confidence genes is 121. C) The overlap between high confidence genes regulated by IRF8 in the context of Lp02 infection and IFNγ/CpG stimulation yields 7 genes in common.

| *IRF8 targets by ChIP on Chip* | | *IRF1 targets by ChIP on Chip* | |
|---|---|---|---|
| *Gene ID* | *P[Xbar]* | *Gene ID* | *P[Xbar]* |
| Lpxn | 2.33E-15 | Olfr1271 | 0.000351 |
| BC017643 | 1.79E-14 | Olfr770 | 0.000176 |
| Prcp | 4.78E-14 | Olfr836 | 0.000269 |
| Zfp296 | 2.36E-13 | 1600029D21Rik | 0.000194 |
| Mnt | 5.53E-13 | 2610029G23Rik | 3.52E-11 |
| Gripap1 | 9.74E-13 | 4930431L04Rik | 0.000224 |
| Nckap1 | 1.15E-12 | Cox7b2 | 0.000805 |
| Tmem168 | 2.4E-12 | Atrip | 0.000141 |
| Clspn | 8.25E-12 | Alf1 | 1.41E-05 |
| Tas2r138 | 1.03E-11 | Arpp19 | 7.62E-06 |
| Gbp6 | 1.41E-11 | Asf1a | 1.77E-05 |
| H2-gs10 | 4.57E-11 | Cybb | 0.000773 |
| Ftl1 | 7.4E-11 | Dbpht2 | 7.13E-05 |
| Usf1 | 1.96E-10 | Dusp14 | 0.000493 |
| Pdlim2 | 7.48E-10 | E130308A19Rik | 0.000548 |
| Rhpn2 | 9.45E-10 | H2-M3 | 0.000525 |
| Rchy1 | 1.04E-09 | Hddc2 | 0.000495 |
| Dnalc | 1.3E-09 | Hspa9 | 0.000648 |
| D1Ertd622e | 3.14E-09 | Irgm | 1.23E-07 |
| Irgm | 3.67E-09 | Isg20l1 | 0.000326 |

**Table 1:Transcriptional targets of IRF1 and IRF8 identified by ChIP on Chip.** ChIP on Chip performed with anti-IRF1 and anti-IRF8 antibodies identified 201 and 302 novel transcriptional target sites for IRF1 and IRF8 respectively (p-value <0.001).

transcription target sites for IRF1 and IRF8 respectively (p-value <0.001).

| Genes in common between IRF1 and IRF8 ChIP on Chip | | |
|---|---|---|
| Gene ID | Gene Identifier | Gene Title |
| Aif1 | NM_019467 | allograft inflammatory factor 1 (Aif1), mRNA. |
| Arpp19 | NM_019467.2 | cAMP-regulated phosphoprotein 19 |
| Gbp6 | NM_145545.2 | guanylate binding protein 6 (Gbp6), mRNA. |
| H2-T24 | NM_008207 | histocompatibility 2, T region locus 24 |
| Hk3 | NM_001033245 | hexokinase 3 (Hk3), mRNA. |
| Hoxd10 | NM_013554 | homeo box D10 (Hoxd10), mRNA. |
| Ifit1 | NM_008331 | interferon-induced protein with tetratricopeptide repeats 1 |
| Igtm | NM_008207.2 | immunity-related GTPase family, M (Igtm), mRNA. |
| Mrpl50 | NM_178803 | mitochondrial ribosomal protein L50 (Mrpl50), mRNA. |
| Mx2 | NM_013606 | myxovirus (influenza virus) resistance 2 (mouse) |
| Olfr934 | NM_146442 | olfactory receptor 934 (Olfr934), mRNA. |
| Psma2 | NM_008944 | proteasome (prosome, macropain) subunit, alpha type 2 (Psma2), mRNA. |
| Rtp4 | NM_023398 | receptor transporter protein 4 (Rtp4), mRNA. |
| Slc9a10 | NM_198106 | solute carrier family 9, isoform 10 (Slc9a10), mRNA. |
| Tnfsf13b | NM_033622 | tumor necrosis factor (ligand) superfamily, member 13b |
| Trove2 | NM_013895 | TROVE domain family, member 2 (Trove2), mRNA. |
| Ube1l | NM_023738.2 | ubiquitin-activating enzyme E1-like (Ube1l), mRNA. |
| Usf1 | NM_009480 | Upstream transcription factor 1 |
| Zfp313 | NM_030749 | zinc finger protein 313 |

**Table 2: Overlap between target genes of IRF1 and IRF8.** Target genes from ChIP on Chip performed with IRF1 or IRF8 antibody are compared by GeneSifter Intersector software. The overlap of 201 and 303 genes for IRF1 and IRF8 respectively yields 19 genes in common.

| | Lp02 infection set | | | | IFNγ/CpGo stimulation set | | | |
|---|---|---|---|---|---|---|---|---|
| | List | Gene set | % genes | z-score | List | Gene set | % genes | z-score |
| cellular process | 24 | 10837 | 16.78 | 0.34 | 69 | 10837 | 18.6 | -0.01 |
| biological regulation | 18 | 6313 | 12.59 | 1.65 | 37 | 6313 | 9.97 | -0.7 |
| regulation of biological process | 16 | 5995 | 11.19 | 1.17 | 35 | 5995 | 9.43 | -0.69 |
| metabolic process | 15 | 6709 | 10.49 | 0.22 | 42 | 6709 | 11.32 | -0.16 |
| developmental process | 11 | 2910 | 7.69 | 2.15 | 27 | 2910 | 7.29 | 2.23 |
| multicellular organismal process | 11 | 3927 | 7.69 | 1.05 | 23 | 3927 | 6.2 | -0.48 |
| response to stimulus | 9 | 2013 | 6.29 | 2.44 | 28 | 2013 | 7.55 | 4.61 |
| localization | 5 | 2612 | 3.5 | -0.29 | 14 | 2612 | 3.77 | -0.72 |
| negative regulation of biological process | 5 | 1115 | 3.5 | 1.76 | 16 | 1115 | 4.31 | 3.5 |
| immune system process | 4 | 736 | 2.8 | 1.96 | 17 | 736 | 4.58 | 5.87 |
| positive regulation of biological process | 4 | 1147 | 2.8 | 1.02 | 10 | 1147 | 2.7 | 1.05 |
| anatomical structure formation | 3 | 680 | 2.1 | 1.31 | 10 | 680 | 2.7 | 2.8 |
| biological adhesion | 3 | 563 | 2.1 | 1.67 | 4 | 563 | 1.08 | 0.22 |
| establishment of localization | 3 | 2261 | 2.1 | -0.92 | 9 | 2261 | 2.43 | -1.56 |
| growth | 3 | 310 | 2.1 | 2.9 | 5 | 310 | 1.35 | 2.19 |
| multi-organism process | 2 | 270 | 1.4 | 1.89 | 5 | 270 | 1.35 | 2.53 |
| reproduction | 2 | 475 | 1.4 | 0.99 | 3 | 475 | 0.81 | -0.01 |
| reproductive process | 2 | 472 | 1.4 | 1 | 3 | 472 | 0.81 | 0 |
| cell killing | 1 | 34 | 0.7 | 3.44 | 0 | 34 | -- | -0.47 |
| pigmentation | 1 | 53 | 0.7 | 2.63 | 1 | 53 | 0.27 | 1.15 |
| rhythmic process | 1 | 76 | 0.7 | 2.08 | 3 | 76 | 0.81 | 3.64 |
| locomotion | 0 | 351 | -- | -0.88 | 9 | 351 | 2.43 | 4.6 |
| viral reproduction | 0 | 7 | -- | -0.12 | 1 | 7 | 0.27 | 454 |

**Table 3:** 3 samples per group from non-treated, stimulated, and infected cDNA from BMDM of F2 mice homozygous for either wildtype or BXH2 allele were applied to Illumina Mouse 6v2 microarray. The resulting gene lists were analyzed by two-way ANOVA with Benjamini-Hochberg correction and p-val less or equal to 0.05. The generated gene lists for stimulated and infected samples show a variety of genes involved in the regulation of biological processes, response to stimulus, and immune system processes.

| Genes common to the Lp02 and IFNγ/CpGo sets | | | | |
|---|---|---|---|---|
| NM_010548 | Interleukin 10 | IL10 | immune response, defense response to bacterium, negative regulation of B-cell proliferation, negative regulation of IL-12 | cytokine-cytokine receptor interaction, T cell signalling pathway, JAK STAT signalling pathway |
| NM_007631 | Cyclin D1 | Ccd1 | Cell cycle, cell division, cell differentiation | Melanoma, glioma, JAK STAT signalling, Chronic myeloid leukaemia, thyroid cancer, focal adhesion |
| NM_010118 | Early growth response 2 | Egr2 | regulation of transcription (DNA dependent), myelination, brain segmentation | |
| NM_145610 | Peter pan homolog (drosophila) | Ppan | regulation of cell growth by extracellular stimulus | |
| NM_172449 | Benzodiazapine receptor | Bzrap1 | receptor activity | |
| NM_207231 | ADP-rybosylation factor-like 5C | Arl5c | small GTPase mediated signal transduction | |

**Table 4: Genes regulated by IRF8 in response to *L. Pneumophila* infection and IFNγ/CpG stimulation.** IRF8-dependent gene lists from *L. Pneumophila* infection and IFNγ/CpG stimulation microarrays overlapped with GeneSifter Intersector. Fold change cut-off: 1.5, *P*-value <0.05, Benjamini Hochberg correction.

| Genes in common between IFR8 ChIP on Chip and microarray (IFNγ/CpG stimulation) | | |
|---|---|---|
| Gene ID | Gene Identifier | Gene title |
| Aif1 | NM_01946 | Allograft inflammatory factor 1, mRNA (cDNA clone MGC:35939 IMAGE:5346103) |
| Cd74 | NM_010545 | CD74 antigen (invariant polypeptide of major histocompatibility complex, class II antigen-associated), mRNA (cDNA clone MGC:6 |
| Cdca7 | NM_02586 | Cell division cycle associated 7 (Cdca7), mRNA |
| Chi3l4 | NM_14512 | Chitinase 3-like 4 (Chi3l4), mRNA |
| Clspn | NM_17555 | Claspin |
| Ercc6l | NM_146235 | Excision repair cross-complementing rodent repair deficiency complementation group 6 - like, mRNA (cDNA clone MGC:47180 IMAGE |
| Gbp2 | NM_01026 | Guanylate binding protein 2, mRNA (cDNA clone MGC:41173 IMAGE:1230883) |
| Gpr18 | NM_18280 | G protein-coupled receptor 18, mRNA (cDNA clone MGC:41272 IMAGE:1397739) |
| Gpr68 | NM_17549 | G protein-coupled receptor 68, mRNA (cDNA clone MGC:144863 IMAGE:40106886) |
| H2-DMb1 | NM_010387 | Histocompatibility 2, class II, locus Mb1, mRNA (cDNA clone MGC:5741 IMAGE:3486844) |
| Ly86 | NM_01074 | Lymphocyte antigen 86 (Ly86), mRNA |
| Ndufab1 | AK010307 | NADH dehydrogenase (ubiquinone) 1, alpha/beta subcomplex, 1, mRNA (cDNA clone MGC:106626 IMAGE:6815043) |
| Phgdh | NM_016966 | 3-phosphoglycerate dehydrogenase, mRNA (cDNA clone MGC:117966 IMAGE:6311873) |
| Rpa2 | NM_01128 | Replication protein A2, mRNA (cDNA clone MGC:6146 IMAGE:3586727) |
| Tapbpl | NM_145391 | TAP binding protein-like (Tapbpl), mRNA |

**Table 5: Genes in common between IRF8 ChIP on Chip and microarray:.** Target genes from ChIP on Chip performed with an IRF8 antibody are compared the gene list generated by microarray from IFNγ/CpG stimulated mouse BMDMs by GeneSifter Intersector software. The overlap of 303 and 845 genes yields 15 genes in common.

# Supplementary Tables

**Supplementary Table 1: IRF1 binding targets identified by ChIP on Chip**

| *IRF1 targets by ChIP on Chip* | | | | | |
|---|---|---|---|---|---|
| ***Refseq DNA*** | ***Chromosome*** | ***Start*** | ***End*** | ***Gene ID*** | ***P[Xbar]*** |
| NM_146793.1 | chr2 | 90065078 | 90066318 | Olfr1271 | 3.51E-004 |
| NM_146793 | chr10 | 128535077 | 128536246 | Olfr770 | 1.76E-004 |
| NM_146863.1 | chr9 | 18872871 | 18874081 | Olfr836 | 2.69E-004 |
| NM_146863 | chr9 | 50246902 | 50247941 | 1600029D21Rik | 1.94E-004 |
| NM_146564.1 | chrX | 101283080 | 101284273 | 2610029G23Rik | 3.52E-011 |
| NM_146564 | chr8 | 61801964 | 61803017 | 4930431L04Rik | 2.24E-004 |
| NM_029639 | chr5 | 71825710 | 71826882 | Cox7b2 | 8.05E-004 |
| NM_029639.2 | chr9 | 108921302 | 108922248 | Atrip | 1.41E-004 |
| NM_026312 | chr17 | 34781167 | 34782220 | Aif1 | 1.41E-005 |
| NM_026312.4 | chr9 | 74839210 | 74840390 | Arpp19 | 7.82E-006 |
| NM_175032 | chr10 | 53286661 | 53287843 | Asf1a | 1.77E-005 |
| NM_175032.1 | chrX | 8624301 | 8625654 | Cybb | 7.73E-004 |
| NM_030052 | chr12 | 75216690 | 75217901 | Dbpht2 | 7.13E-005 |
| NM_030052.3 | chr11 | 83882319 | 83883579 | Dusp14 | 4.93E-004 |
| N/A | chr4 | 59721127 | 59722278 | E130308A19Rik | 5.48E-004 |
| NM_019467.2 | chr17 | 36878406 | 36879569 | H2-M3 | 5.25E-004 |
| NM_019467 | chr10 | 31003129 | 31004349 | Hddc2 | 4.95E-004 |
| N/A | chr18 | 35077651 | 35078456 | Hspa9 | 6.48E-004 |
| NM_021548 | chr11 | 48714157 | 48715497 | Irgm | 1.23E-007 |
| NM_001142655 | chr7 | 78769731 | 78770918 | Isg20l1 | 3.26E-004 |
| NM_021548.4 | chr18 | 67369079 | 67369939 | Mppe1 | 1.14E-004 |
| NM_025541 | chr4 | 49536631 | 49537934 | Mrpl50 | 2.40E-004 |
| NM_025541.3 | chrX | 33332178 | 33333705 | Nkrf | 2.84E-004 |
| NM_007807 | chr5 | 121073051 | 121073882 | Oas1b | 4.23E-004 |
| NM_007807.4 | chrX | 152757717 | 152759066 | Phex | 1.55E-004 |
| NM_198866 | chr18 | 53541605 | 53542839 | Ppic | 6.29E-004 |
| NM_198866.2 | chr13 | 14405108 | 14406484 | Psma2 | 5.37E-006 |
| NM_019819 | chr3 | 108385526 | 108386851 | Psma5 | 1.11E-005 |
| NM_019819.3 | chr17 | 33796525 | 33797768 | Psmb9 | 1.97E-004 |
| NM_001015681 | chr5 | 74810858 | 74812077 | Scfd2 | 2.59E-004 |
| NM_153158 | chr11 | 109288221 | 109289649 | Slc16a6 | 3.17E-004 |
| NM_153158.4 | chr6 | 40401600 | 40402833 | Ssbp1 | 4.24E-004 |
| N/A | chr3 | 88666774 | 88667710 | Ssr2 | 2.58E-004 |
| NM_013819 | chr16 | 38481984 | 38483354 | Tmem39a | 3.41E-004 |
| NM_013819.2 | chr2 | 160338160 | 160339531 | Top1 | 2.83E-004 |
| NM_027168 | chrX | 161787680 | 161789000 | Trappc2 | 8.06E-004 |
| NM_027168.2 | chr1 | 145534302 | 145535779 | Trove2 | 1.19E-006 |
| NM_010481 | chr1 | 173248244 | 173249912 | Usf1 | 1.29E-006 |
| NM_010481.2 | chr2 | 167196048 | 167197311 | Zfp313 | 7.07E-006 |
| N/A | chr3 | 75638961 | 75639923 | pdcd10 | 5.14E-004 |
| N/A | chr3 | 75638961 | 75639923 | Serpini1 | 5.14E-004 |

| | | | | | |
|---|---|---|---|---|---|
| NM_172630.2 | chr6 | 38462990 | 38463970 | AK030470 | 1.12E-004 |
| NM_172630 | chr6 | 41244856 | 41245858 | Tcb2 | 1.34E-005 |
| NM_178603 | chrX | 91648529 | 91649698 | 2810002O09Rik | 1.07E-004 |
| NM_178603.4 | chr14 | 7463102 | 7464275 | 4930452B06Rik | 6.14E-004 |
| NM_029891.2 | chr4 | 111931350 | 111932707 | Skint9 | 9.04E-004 |
| NM_029891 | chr18 | 61304612 | 61305941 | Hmgxb3 | 6.91E-004 |
| NM_001083925 | chr7 | 79145812 | 79147047 | Abhd2 | 9.14E-004 |
| NR_003507.1 | chr13 | 96475909 | 96476826 | Aggf1 | 3.83E-004 |
| NR_003507 | chr1 | 179085211 | 179086362 | Akt3 | 3.09E-004 |
| N/A | chr11 | 68940408 | 68941739 | Aloxe3 | 1.87E-004 |
| NM_011077.2 | chr15 | 97076001 | 97077107 | Amigo2 | 2.42E-005 |
| NM_011077 | chr13 | 76568222 | 76569333 | Arsk | 5.68E-004 |
| NM_008908.4 | chr4 | 154671017 | 154672403 | Aurkaip1 | 8.03E-004 |
| NM_008908 | chr1 | 182686365 | 182687647 | BC031781 | 1.13E-004 |
| NM_008944 | chr7 | 119480166 | 119481470 | BC048390 | 1.65E-004 |
| NM_008944.2 | chr8 | 86994591 | 86995924 | BC057552 | 3.38E-004 |
| NM_011967.3 | chr19 | 29053332 | 29054454 | Cdc37l1 | 1.45E-004 |
| NM_011967 | chr11 | 72117202 | 72118438 | Fbxo39 | 8.47E-005 |
| NM_013585.2 | chrX | 69076502 | 69077745 | Gabrq | 3.21E-005 |
| NM_013585 | chr3 | 142467053 | 142468479 | Gbp6 | 9.95E-006 |
| NM_001114660 | chrX | 122505006 | 122506233 | Gm382 | 3.22E-005 |
| NM_178672.6 | chr17 | 35628266 | 35629479 | H2-T24 | 9.75E-007 |
| NM_178672 | chr13 | 55031651 | 55033031 | Hk3 | 2.78E-004 |
| N/A | chr2 | 74491063 | 74492576 | Hoxd10 | 2.14E-004 |
| N/A | chr2 | 62446613 | 62447831 | Ifih1 | 5.80E-004 |
| NM_001029842 | chrX | 149037237 | 149038222 | Kctd12b | 1.21E-004 |
| NM_134038.2 | chr3 | 144146210 | 144147613 | Lmo4 | 9.23E-004 |
| NM_134038 | chr3 | 95957773 | 95958632 | Mrps21 | 1.55E-004 |
| NM_028358.2 | chr16 | 97689344 | 97690659 | Mx2 | 2.72E-004 |
| NM_028358 | chr10 | 110881919 | 110883250 | Nap1l1 | 1.18E-004 |
| NM_212468 | chr10 | 79651584 | 79653110 | Ndufs7 | 4.54E-006 |
| NM_212468.3 | chr5 | 77442160 | 77443227 | Nmu | 3.73E-004 |
| NM_025448 | chr2 | 85686691 | 85688086 | Olfr1023 | 2.35E-006 |
| NM_025448.3 | chr2 | 85802979 | 85803953 | Olfr1032 | 1.16E-004 |
| NM_026407 | chr17 | 37219640 | 37221114 | Olfr115 | 1.91E-006 |
| NM_026407.2 | chr19 | 13592686 | 13593345 | Olfr1480 | 6.06E-004 |
| NM_009408 | chr16 | 19395490 | 19396543 | Olfr166 | 5.29E-004 |
| NM_009408.2 | chr16 | 58704759 | 58706001 | Olfr172 | 8.70E-004 |
| NM_025432.3 | chr16 | 16589787 | 16590945 | Olfr19 | 4.67E-006 |
| NM_025432 | chr7 | 86149777 | 86150682 | Olfr310 | 2.26E-004 |
| NM_013835 | chr6 | 42836654 | 42837899 | Olfr447 | 1.19E-004 |
| NM_013835.2 | chr7 | 108543030 | 108544097 | Olfr513 | 9.12E-005 |
| NM_009480.2 | chr9 | 37494636 | 37495602 | Olfr874 | 7.45E-004 |
| NM_009480 | chr9 | 38273088 | 38274176 | Olfr909 | 7.70E-004 |

| | | | | | |
|---|---|---|---|---|---|
| N/A | chr9 | 38736694 | 38738609 | Olfr934 | 1.05E-007 |
| NM_019745.3 | chrX | 103288473 | 103289370 | P2ry10 | 1.30E-004 |
| NM_019745 | chr18 | 37076697 | 37077764 | Pcdha4 | 5.88E-005 |
| NM_009250 | chrX | 162728136 | 162729335 | Prps2 | 5.05E-006 |
| NM_009250.1 | chr8 | 108826617 | 108828237 | Psmb10 | 7.25E-004 |
| N/A | chr14 | 6886963 | 6888353 | Pxk | 1.29E-004 |
| N/A | chr16 | 23524490 | 23525950 | Rtp4 | 7.82E-007 |
| N/A | chr14 | 29592704 | 29594053 | Sfmbt1 | 6.77E-004 |
| NM_028934 | chr12 | 73836896 | 73838084 | Six6os1 | 5.93E-004 |
| NM_028934.2 | chr16 | 45453210 | 45454363 | Slc9a10 | 1.88E-004 |
| NM_177864 | chr16 | 10698765 | 10699910 | Socs1 | 9.42E-005 |
| NM_177864.2 | chr10 | 23725481 | 23726571 | Taar7e | 5.70E-005 |
| NM_178277.1 | chr6 | 132724193 | 132725776 | Tas2r102 | 2.10E-005 |
| NM_178277 | chr10 | 104982572 | 104983738 | Tmtc2 | 5.41E-004 |
| NM_018811 | chr8 | 10005740 | 10006903 | Tnfsf13b | 9.25E-004 |
| NM_018811.6 | chr11 | 98839678 | 98841006 | Top2a | 3.26E-005 |
| NM_025630 | chr9 | 107833198 | 107834394 | Ube1l | 1.49E-009 |
| NM_025630.2 | chrX | 148836162 | 148837506 | Ubqln2 | 1.37E-004 |
| NM_011785.3 | chrX | 22846606 | 22847892 | Wdr44 | 3.41E-004 |
| NM_011785 | chr16 | 64730360 | 64731599 | Zfp654 | 8.22E-004 |
| NM_011786.1 | chrX | 131612591 | 131614119 | Ngfrap1 | 9.88E-005 |
| NM_011786 | chr8 | 28504835 | 28506082 | Erlin-2 | 3.92E-004 |
| NM_178114 | chr8 | 28504835 | 28506082 | Prosc | 3.92E-004 |
| NM_178114.3 | chr1 | 59987628 | 59989433 | Ica1l | 5.44E-004 |
| NM_029847.4 | chrX | 107013232 | 107014468 | Pou3f4 | 5.64E-004 |
| NM_029847 | chr14 | 60589767 | 60591028 | Dleu2 | 5.07E-004 |
| NM_025338 | chr6 | 51454330 | 51455847 | Snx10 | 2.81E-005 |
| NM_025338.3 | chr19 | 34706021 | 34707458 | | 3.63E-006 |
| NM_145943 | chr19 | 34706021 | 34707458 | Ifit1 | 3.63E-006 |
| NM_145943.1 | chrX | 49514558 | 49515514 | Fam122b | 2.52E-004 |
| N/A | chrX | 49514558 | 49515514 | Fam122c | 2.52E-004 |
| NM_172502 | chr8 | 74465996 | 74467365 | Bst2 | 1.08E-006 |
| NM_172502.3 | chr8 | 74465996 | 74467365 | Fam125a | 1.08E-006 |
| NM_025950 | chr10 | 126668263 | 126669492 | Kif5a | 4.44E-005 |
| NM_025950.2 | chr10 | 126668263 | 126669492 | Dctn2 | 4.44E-005 |
| NM_001099688 | chr8 | 69620920 | 69622217 | Npy1r | 1.94E-004 |
| NM_001099688.2 | chr8 | 69620920 | 69622217 | Npy5r | 1.94E-004 |
| NM_020488 | chr1 | 157799122 | 157800480 | Ifrg15 | 4.61E-005 |
| NM_020488.1 | chr1 | 157799122 | 157800480 | Tor1aip1 | 4.61E-005 |

**Supplementary Table 2: IRF8 binding targets identified by ChIP on Chip**

| *IRF8 targets by ChIP on Chip* | | | | | |
|---|---|---|---|---|---|
| ***Refseq DNA*** | ***Chromosome*** | ***Start*** | ***End*** | ***Gene ID*** | ***P[Xbar]*** |
| NM_181407.2 | chr19 | 12867824 | 12868834 | Lpxn | 2.33E-015 |
| NM_007568 | chr11 | 121044112 | 121045813 | BC017643 | 1.79E-014 |
| NM_009068 | chr7 | 92753171 | 92755123 | Prcp | 4.78E-014 |
| N/A | chr7 | 18735951 | 18737614 | Zfp296 | 2.36E-013 |
| NM_008659.2 | chr11 | 74644091 | 74645744 | Mnt | 5.53E-013 |
| NM_010442 | chrX | 6945944 | 6947664 | Gripap1 | 9.74E-013 |
| NM_146789 | chr2 | 80379299 | 80380984 | Nckap1 | 1.15E-012 |
| NM_025773.1 | chr6 | 13558784 | 13560376 | Tmem168 | 2.40E-012 |
| NM_027539 | chr4 | 126054918 | 126056413 | Clspn | 8.25E-012 |
| NM_146010.1 | chr6 | 40542457 | 40543831 | Tas2r138 | 1.03E-011 |
| NM_207670 | chr3 | 142466861 | 142468479 | Gbp6 | 1.41E-011 |
| NM_010462.2 | chr17 | 34986844 | 34988266 | H2-gs10 | 4.57E-011 |
| NM_182806 | chr7 | 45325244 | 45326314 | Ftl1 | 7.40E-011 |
| NM_021397.1 | chr1 | 173248244 | 173250036 | Usf1 | 1.96E-010 |
| NM_175523.4 | chr14 | 68910118 | 68911311 | Pdlim2 | 7.48E-010 |
| NM_029756.1 | chr7 | 35043436 | 35044953 | Rhpn2 | 9.45E-010 |
| NM_021384.2 | chr5 | 93037039 | 93038456 | Rchy1 | 1.04E-009 |
| N/A | chr6 | 50387163 | 50388237 | | 1.30E-009 |
| NM_009848.3 | chr12 | 84999833 | 85001358 | Dnalc1 | 3.14E-009 |
| NM_001013371.1 | chr1 | 99489566 | 99490618 | D1Ertd622e | 3.67E-009 |
| NM_027552.1 | chr11 | 48714157 | 48715620 | Irgm | 4.01E-009 |
| NM_025779 | chr17 | 33291901 | 33294025 | C3HC4 | 5.61E-009 |
| NM_146442.1 | chr13 | 55327558 | 55328837 | Nsd1 | 1.17E-008 |
| NM_177852.3 | chr11 | 79902836 | 79905170 | Crlf3 | 1.58E-008 |
| N/A | chr11 | 79902836 | 79905170 | | 1.58E-008 |
| NM_008049 | chr19 | 40714004 | 40715394 | Entpd1 | 1.74E-008 |
| NM_030887.2 | chr3 | 89996423 | 89998094 | Il6ra | 2.84E-008 |
| NM_146178 | chr17 | 33954816 | 33956066 | Btnl2 | 3.74E-008 |
| NM_012051 | chr17 | 33954816 | 33956066 | Ea | 3.74E-008 |
| NM_172732 | chr3 | 106917741 | 106918664 | Cd53 | 3.86E-008 |
| NM_022993.3 | chr3 | 107165584 | 107167214 | Kcna3 | 3.89E-008 |
| NM_027689.2 | chr5 | 20699845 | 20700832 | Pion | 4.10E-008 |
| NM_153175.3 | chr11 | 100931213 | 100932577 | Fam134c | 4.73E-008 |
| N/A | chr13 | 118147033 | 118148502 | | 4.88E-008 |
| N/A | chr2 | 167196048 | 167197311 | Zfp313 | 7.31E-008 |
| NM_172923 | chr5 | 105226856 | 105227798 | Abcg3 | 9.05E-008 |
| NM_023044 | chr18 | 31462463 | 31463823 | Rit2 | 9.20E-008 |
| NM_007435 | chr17 | 22570497 | 22571839 | A630033E08Rik | 1.92E-007 |
| NM_019927.1 | chr9 | 50526073 | 50527304 | Alg9 | 2.08E-007 |
| NM_027897.2 | chr6 | 57464914 | 57466175 | Ppm1k | 2.56E-007 |
| NM_172291.1 | chr14 | 64551884 | 64552937 | Elp3 | 2.87E-007 |

| | | | | | |
|---|---|---|---|---|---|
| XR_003956 | chr5 | 37118409 | 37119936 | Man2b2 | 3.15E-007 |
| NM_033622.1 | chr11 | 95231719 | 95232987 | Spop | 3.60E-007 |
| NM_007969.3 | chr9 | 20730107 | 20731192 | Edg5 | 4.38E-007 |
| NM_198166 | chr5 | 130359367 | 130360524 | Tpst1 | 4.41E-007 |
| NM_013554 | chr17 | 35628266 | 35629479 | H2-T24 | 5.16E-007 |
| NM_133672 | chr15 | 76204043 | 76205254 | Tssk5 | 6.20E-007 |
| NM_008579.4 | chr14 | 53417093 | 53418317 | Lrp10 | 6.60E-007 |
| NM_001033245.1 | chr14 | 121051182 | 121052260 | Gpr18 | 7.08E-007 |
| NM_133981.1 | chr17 | 34781167 | 34782220 | Aif1 | 7.25E-007 |
| NM_023738.2 | chrX | 102341490 | 102343145 | Tlr13 | 7.74E-007 |
| NM_030239.2 | chrX | 69970919 | 69972128 | Abcd1 | 7.95E-007 |
| NM_026557.2 | chr3 | 98422539 | 98423733 | Phgdh | 8.31E-007 |
| NM_001033632.1 | chr8 | 77985273 | 77986854 | Hmox1 | 1.30E-006 |
| NM_008533 | chr6 | 17585334 | 17586274 | Capza2 | 1.65E-006 |
| NM_013837.1 | chr19 | 34259621 | 34261293 | Stambpl1 | 1.74E-006 |
| NM_016966 | chr15 | 36551830 | 36553098 | Pabpc1 | 1.87E-006 |
| NM_025287.2 | chr16 | 23524490 | 23525950 | Rtp4 | 2.15E-006 |
| NM_177584.3 | chr8 | 32615275 | 32616712 | BC019943 | 2.24E-006 |
| NM_010388.2 | chr19 | 21543550 | 21545133 | Gda | 2.71E-006 |
| NM_009853.1 | chr3 | 129960735 | 129961940 | Ccdc109b | 3.98E-006 |
| N/A | chr10 | 61880831 | 61882146 | Vps26a | 4.14E-006 |
| NM_133815.1 | chr13 | 116053416 | 116054733 | Itga2 | 4.33E-006 |
| NM_008292.2 | chr3 | 90085186 | 90086680 | Hax1 | 4.80E-006 |
| NM_019467 | chr9 | 55125334 | 55126581 | AI118078 | 5.12E-006 |
| NM_178057.2 | chr12 | 101286348 | 101287487 | Gpr68 | 5.40E-006 |
| NM_016686.3 | chr10 | 115219996 | 115221500 | Tspan8 | 5.98E-006 |
| NM_183099.2 | chr9 | 37405519 | 37406989 | Tbrg1 | 6.79E-006 |
| NM_130454.1 | chr11 | 62277018 | 62278170 | Pigl | 7.14E-006 |
| NM_011798 | chr9 | 107833198 | 107834394 | Ube1l | 7.26E-006 |
| NM_010874.2 | chr4 | 49536631 | 49537934 | Mrpl50 | 7.73E-006 |
| NM_170778.2 | chrX | 158245890 | 158247267 | Ctps2 | 7.89E-006 |
| NM_175683.2 | chr17 | 34256978 | 34257941 | Crebl1 | 8.02E-006 |
| NM_146042.2 | chr13 | 55031950 | 55033312 | Hk3 | 8.65E-006 |
| NM_019549.1 | chr13 | 75551707 | 75553118 | Pcsk1 | 1.00E-005 |
| NM_139270 | chr13 | 6647925 | 6649266 | Pfkp | 1.05E-005 |
| NM_145976.2 | chrX | 159783410 | 159784701 | Asb11 | 1.13E-005 |
| NM_146874 | chr15 | 34624350 | 34625274 | Npal2 | 1.18E-005 |
| NM_017406.2 | chr2 | 72274619 | 72275729 | Cdca7 | 1.24E-005 |
| NM_009065.2 | chr13 | 14405535 | 14406484 | Psma2 | 0.000014475 |
| NM_012000.3 | chr11 | 69481687 | 69483154 | Cd68 | 1.49E-005 |
| NM_009035.3 | chr19 | 12494025 | 12495696 | Pfpl | 1.51E-005 |
| NM_177767 | chr8 | 70423343 | 70424604 | Nat2 | 1.58E-005 |
| NM_030743.4 | chr5 | 93211787 | 93213069 | Vdp | 1.85E-005 |
| NM_011768.2 | chr11 | 87886240 | 87887398 | Vezf1 | 1.95E-005 |

| | | | | | |
|---|---|---|---|---|---|
| NM_010813 | chr13 | 37352490 | 37353717 | Ly86 | 1.95E-005 |
| NM_134071 | chr5 | 78087575 | 78088779 | AK086399 | 2.07E-005 |
| NM_019490.1 | chr1 | 145534302 | 145535779 | Trove2 | 2.07E-005 |
| NM_007651.2 | chr7 | 4662932 | 4664288 | Ccdc106 | 2.26E-005 |
| NM_145545 | chr3 | 87606820 | 87608039 | Etv3 | 2.46E-005 |
| NM_018737.2 | chr6 | 129372872 | 129374112 | Clec9a | 2.50E-005 |
| N/A | chr2 | 73327485 | 73328692 | Wipf1 | 2.53E-005 |
| NM_175554.3 | chr18 | 60928150 | 60929912 | Cd74 | 2.62E-005 |
| NM_008550.1 | chr1 | 183682150 | 183683302 | Lbr | 2.80E-005 |
| NM_133825.2 | chr8 | 14889593 | 14890877 | Cln8 | 2.86E-005 |
| NM_025866.1 | chr5 | 17366084 | 17367127 | Cd36 | 2.90E-005 |
| NM_133217.2 | chr12 | 102359798 | 102360780 | Atxn3 | 2.93E-005 |
| NM_079835.1 | chr13 | 56190698 | 56191732 | BC027057 | 3.15E-005 |
| NM_207676.2 | chr15 | 102791090 | 102792809 | Hoxc10 | 3.20E-005 |
| NM_181817.1 | chr10 | 84266834 | 84268141 | Rfx4 | 3.23E-005 |
| NM_025289.2 | chr1 | 109355476 | 109356796 | Serpinb10 | 3.39E-005 |
| NM_177730 | chr18 | 50251058 | 50252657 | Hsd17b4 | 3.42E-005 |
| NM_145732.1 | chr8 | 89751850 | 89752911 | chr8:089752389-089752448 | 3.63E-005 |
| NM_178603.2 | chr18 | 70694906 | 70696210 | Mbd2 | 3.74E-005 |
| NM_010581.3 | chr9 | 5298422 | 5299591 | Casp1 | 4.00E-005 |
| NM_010773.1 | chr19 | 59127301 | 59128765 | LOC71653 | 4.25E-005 |
| NM_021548.4 | chr1 | 59858025 | 59859161 | Als2cr13 | 4.31E-005 |
| N/A | chr14 | 54839785 | 54841136 | | 4.33E-005 |
| NM_144832 | chr9 | 59285301 | 59286767 | Arih1 | 4.47E-005 |
| N/A | chr1 | 173246618 | 173247843 | | 4.53E-005 |
| NM_023386 | chr11 | 115744892 | 115745853 | Recql5 | 4.57E-005 |
| NM_028811.1 | chr2 | 180632814 | 180634057 | Dido1 | 4.65E-005 |
| NM_011284.3 | chr5 | 53876462 | 53877593 | Rbpsuh | 4.66E-005 |
| NM_145469.2 | chr16 | 97689649 | 97690659 | Mx2 | 4.85E-005 |
| N/A | chr9 | 123711706 | 123712769 | Xcr1 | 4.94E-005 |
| NM_146614.1 | chr2 | 90473327 | 90474794 | Nup160 | 4.98E-005 |
| NM_008418.1 | chr4 | 4720997 | 4722526 | Impad1 | 5.63E-005 |
| NM_198106 | chr4 | 132032107 | 132033439 | Rpa2 | 5.67E-005 |
| NM_010510.1 | chr17 | 79974453 | 79975657 | Hnrpll | 6.50E-005 |
| NM_022993 | chr12 | 86486600 | 86487876 | Jundm2 | 6.77E-005 |
| NM_008331.2 | chr10 | 119768728 | 119770018 | Hmga2 | 6.81E-005 |
| NM_007604 | chr9 | 50306625 | 50307990 | Bcdo2 | 6.96E-005 |
| N/A | chr11 | 68831318 | 68832566 | | 6.98E-005 |
| NM_011826 | chr8 | 98523079 | 98524172 | Gins3 | 7.26E-005 |
| NM_001042605 | chr13 | 103808285 | 103809414 | Cd180 | 7.38E-005 |
| NM_025821.2 | chr5 | 92477328 | 92478658 | Btc | 7.47E-005 |
| NM_145978.1 | chr9 | 38127589 | 38128912 | Olfr900 | 7.69E-005 |
| NM_022409 | chr16 | 27289185 | 27290546 | Uts2d | 7.81E-005 |
| NM_008774 | chr8 | 96926882 | 96928127 | Ogfod1 | 7.86E-005 |

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|---|---|---|---|---|---|
| NM_010559.2 | chr2 | 74491063 | 74492576 | Hoxd10 | 7.89E-005 |
| NM_001033245 | chr3 | 59193390 | 59194479 | Gpr171 | 8.20E-005 |
| NM_030198 | chr10 | 4536728 | 4538014 | Fbxo5 | 9.16E-005 |
| NM_010745 | chr2 | 43377039 | 43378625 | Kynu | 9.20E-005 |
| NM_009480.2 | chr8 | 10005740 | 10007422 | Tnfsf13b | 9.94E-005 |
| N/A | chr5 | 136273816 | 136274756 | | 0.000102787 |
| NM_029682 | chr3 | 88736992 | 88738423 | Rxfp4 | 0.000105755 |
| NM_010252.3 | chrX | 98359505 | 98360555 | Ercc6l | 0.000107276 |
| NM_019703.2 | chr9 | 39587696 | 39589015 | Olfr971 | 0.00010855 |
| NM_010260.1 | chr5 | 121711491 | 121712384 | Erp29 | 0.000111969 |
| NM_021524.1 | chr2 | 89096178 | 89097999 | Olfr1230 | 0.000117107 |
| NM_011284 | chr1 | 65243212 | 65244827 | Pthr2 | 0.000119005 |
| NM_009192.2 | chr13 | 34039934 | 34041315 | Ripk1 | 0.000127667 |
| NM_010333.2 | chr3 | 87007566 | 87008938 | Dcamkl2 | 0.000128399 |
| NM_018776.1 | chr3 | 106349131 | 106350748 | Chi3l4 | 0.000129748 |
| NM_028990 | chr2 | 142751272 | 142752314 | Snrpb2 | 0.000136428 |
| NM_026853.1 | chr13 | 77594376 | 77595543 | Ankrd32 | 0.000138462 |
| NM_205820.1 | chr16 | 45453210 | 45454571 | Slc9a10 | 0.000138913 |
| NM_008326.1 | chr19 | 34706021 | 34707458 | Ifit1 | 0.000143846 |
| NM_021512 | chr11 | 75469334 | 75470501 | Myo1c | 0.000145683 |
| NM_144927 | chr9 | 74839741 | 74841025 | Arpp19 | 0.000150361 |
| NM_029705.1 | chr7 | 123159071 | 123160678 | Arhgap17 | 0.000151 |
| NM_175493 | chr3 | 142557472 | 142558590 | Gbp2 | 0.000156544 |
| NM_001001451 | chr1 | 178646578 | 178647768 | Sdccag8 | 0.000160205 |
| N/A | chr16 | 90614677 | 90615933 | | 0.000171056 |
| NM_001039536.1 | chr12 | 33405977 | 33406967 | Pbef1 | 0.000181596 |
| NM_009061.2 | chr11 | 16912334 | 16913537 | Plek | 0.000187134 |
| NM_025995.2 | chr11 | 3097458 | 3098366 | Eif4enif1 | 0.00019228 |
| NM_001037725.1 | chr14 | 113927053 | 113928140 | AK017164 | 0.000200727 |
| NM_173398 | chr9 | 34959460 | 34960847 | Foxred1 | 0.000212201 |
| NM_028821.1 | chr6 | 77913853 | 77914881 | Ctnna2 | 0.000218159 |
| NM_012057.1 | chr13 | 47200535 | 47201624 | Ibrdc2 | 0.000233804 |
| NM_016965 | chr9 | 107766515 | 107767781 | Mst1r | 0.000236383 |
| NM_009807.2 | chr16 | 45143592 | 45144692 | Btla | 0.000263914 |
| NM_010266 | chr11 | 83519516 | 83520979 | Expi | 0.000287842 |
| NM_172508.2 | chr1 | 145764635 | 145765969 | Rgs2 | 0.000288533 |
| NM_008396 | chr4 | 87993891 | 87995065 | Ifnb1 | 0.000307929 |
| NM_026129.2 | chr16 | 35858184 | 35859410 | Dtx3l | 0.000309642 |
| NM_010745.1 | chr8 | 13155360 | 13156690 | Lamp1 | 0.000312045 |
| NM_009388.2 | chr15 | 66645402 | 66646694 | Sla | 0.000314195 |
| NM_205820 | chr19 | 10908826 | 10910003 | Slc15a3 | 0.000316033 |
| NM_013628.2 | chr7 | 108044485 | 108045662 | Olfr488 | 0.000317786 |
| N/A | chrX | 90378305 | 90379681 | Zfx | 0.000341645 |
| NM_008207.2 | chr6 | 48637330 | 48638375 | Gimap6 | 0.000375059 |

| | | | | | |
|---|---|---|---|---|---|
| N/A | chr7 | 30300903 | 30302212 | Zbtb32 | 0.000409263 |
| NM_144802 | chr17 | 33753291 | 33754646 | H2-DMb1 | 0.000413952 |
| NM_008739.3 | chr10 | 88034112 | 88035255 | Mybpc1 | 0.000431414 |
| NM_007643.2 | chr16 | 8589194 | 8590253 | Carhsp1 | 0.000436489 |
| NM_028243 | chr16 | 13814514 | 13815322 | Pdxdc1 | 0.00044447 |
| NM_023743.1 | chr2 | 3340458 | 3341757 | Dclre1c | 0.000444604 |
| NM_013606 | chr2 | 3340458 | 3341757 | Meig1 | 0.000444604 |
| NM_144529.1 | chr3 | 96145359 | 96147119 | AK139516 | 0.000465703 |
| NM_146235.2 | chr3 | 118551235 | 118552555 | Dpyd | 0.000505259 |
| NM_013835 | chr6 | 125196888 | 125198062 | Tapbpl | 0.000513151 |
| NM_134152 | chr16 | 96552791 | 96554263 | Itgb2l | 0.000517309 |
| NM_020583.4 | chr7 | 140867863 | 140869119 | Ifitm6 | 0.000528754 |
| NM_028303.1 | chr9 | 38736694 | 38738064 | Olfr934 | 0.000550114 |
| NM_010684.2 | chr7 | 78785243 | 78786771 | Isg20 | 0.000560275 |
| NM_145391.1 | chr10 | 33899120 | 33900490 | Sart2 | 0.00058303 |
| NM_008405.2 | chr9 | 47279229 | 47280405 | Igsf4a | 0.000620825 |
| NM_009074.1 | chr7 | 89503604 | 89504798 | Me3 | 0.000668159 |
| NM_021335 | chr12 | 27046088 | 27047134 | Rsad2 | 0.000691066 |
| NM_153138 | chr12 | 27046088 | 27047134 | Tyki | 0.000691066 |
| NM_027552 | chr6 | 29473978 | 29475104 | Irf5 | 0.000710311 |
| NM_182806.1 | chr5 | 71119921 | 71121259 | Gabrg1 | 0.000725055 |
| N/A | chr17 | 73852260 | 73853837 | Xdh | 0.000836132 |
| NM_145126 | chr16 | 49776733 | 49777979 | Cd47 | 0.000926232 |
| NM_011723.2 | chr1 | 16606202 | 16607397 | Ube2w | 0.000936509 |
| NM_020557.4 | chr14 | 29378950 | 29380194 | Tkt | 0.000945949 |
| NM_146732 | chr7 | 121893235 | 121894573 | Ndufab1 | 0.000960224 |
| NM_028177.2 | chr4 | 137704304 | 137705554 | Mul1 | 0.000960986 |
| NM_008944.1 | chrX | 96828379 | 96829638 | Pdzd11 | 0.000967589 |

**Supplementary Table 3**:Genes regulated by IRF8 in response to *Legionella pneumophila* infection.
Fold change cut-off : 1.5, P-value < 0.05, Benjamini Hochberg correction.

| **Genes unique to LP02 set** | | | | |
|---|---|---|---|---|
| ***Accession No*** | ***Gene Name*** | ***Gene ID*** | ***GO*** | ***KEGG*** |
| AK040740 | Interleukin 7 Receptor | CD127/IL-7Ralpha | immune system process, immune system development, cell communication, signal transduction | cytokine-cytokine receptor signalling, JAK STAT signalling pathway |
| AK083237 | Von Willebrand Factor homolog | Vwf | Blood coaggulation, cell adhesion, hemostasis | |
| NM_001005423 | Dilute suppressor protein | Mreg | Melanocyte differentiation, pigmentation | |
| NM_001033922 | Triggering receptor expressed on myeloid cells 4 | | | |
| NM_008207 | Histocompatibility 2, T locus 24 | H2-T24 | | Type I diabetes mellitus, antigen processing and presentation, Cell adhesion molecules |
| NM_010317 | Guanine nucleotide binding protein, gamma 4 subunit | | | |
| NM_010356 | Glutathione-S transferrase a3 | Gsta3 | Metabolic process | Glutathione transferrase activity |
| NM_010495 | Inhibitor of DNA binding1 | Id1 | BMP signalling, regulation of transcription, regulaiton of angiogenesis | TGF-beta signalling |
| NM_011990 | Solute carrier family 7 | Scl7a11 | amino acid transport | |
| NM_016846 | Ral guanine nucleotide dissociation stimulator,1 | | | |
| NM_017370 | Haptoglobin | Hp | proteolysis, Hb binding, serine-type endopeptidase activity | |
| NM_021407 | Triggering receptor expression on myeloid cells 3 | Trem3 | protein binding, receptor activity, signalling transducer activity | |
| NM_026855 | ARV1 homolog (yeast) | Arv1 | lipid metabolic process | |
| NM_032418 | Dystrophia-myotonica protein kinase | Dmpk | protein amino acid phosphorylation, regulation of sodium ion transport | ATP binding, kinase activity |
| NM_145508 | dual specificity tyrosine-(Y)-phosphorylation regulated kinase | Dyrk3 | erythrocyte differentiation, protein amino acid phosphorylation | |
| NM_178754 | Rho GTPase activation protein 6 | Arhgap6 | actin filament organization, signal transduction, regulation of GTPase activity | |
| NM_201518 | Fibronectin leucine rich transmembrane protein 2 | Flrt2 | integral to membrane | |

| | | | | |
|---|---|---|---|---|
| AK046483 | Gamma-aminobutyric acid (GABA-A) receptor | Gabrg3 | chloride transport, gamma-aminobutiric acid signalling pathway, ion transport | Neuroactive ligand-receptor interaction |

| **Genes unique to LP02 set** | | | | |
|---|---|---|---|---|
| ***Accession No*** | ***Gene Name*** | ***Gene ID*** | ***GO*** | ***KEGG*** |
| NM_007393 | Actin, beta (Actb), mRNA | Actb | ACTIN, BETA, CYTOPLASMIC | Direct protein sequencing, Methylation, acetylation, atp-binding, blocked amino end, cell motility, cytoplasm, cytoskeleton, methylated amino acid, microfilament, mitosis, nitration, nucleotide-binding, phosphoprotein, structural protein, |
| NM_009609 | Actin, gamma, cytoplasmic 1, mRNA (cDNA clone MGC:30279 IMAGE:3499390) | Actg1 | ACTIN, GAMMA, CYTOPLASMIC 1 | Direct protein sequencing, Methylation, acetylation, atp-binding, blocked amino end, cell motility, cytoplasm, cytoskeleton, methylated amino acid, microfilament, mitosis, nucleotide-binding, structural protein, |
| NM_146036 | AHA1, activator of heat shock protein ATPase homolog 1 (yeast), mRNA (cDNA clone MGC:36618 IMAGE:5346745) | Ahsa1 | AHA1, ACTIVATOR OF HEAT SHOCK PROTEIN ATPASE HOMOLOG 1 (YEAST) | chaperone, cytoplasm, endoplasmic reticulum, stress response, |

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|---|---|---|---|---|
| NM_009741 | B-cell leukemia/lymphoma 2 (Bcl2), transcript variant 1, mRNA | Bcl2 | B-CELL LEUKEMIA/LYMPHOMA 2 | |
| NM_177410 | B-cell leukemia/lymphoma 2 (Bcl2), transcript variant 1, mRNA | Bcl2 | BCL2-LIKE 1 | Mitochondrion, alternative splicing, apoptosis, endoplasmic reticulum, membrane, nucleus, phosphoprotein, transmembrane, |
| NM_009743 | BCL2-like 1 (Bcl2l1), nuclear gene encoding mitochondrial protein, mRNA | Bcl2l1 | | |
| XM_354599 | cannabinoid receptor interacting protein 1 | - | CARBONIC ANHYDRASE 9 | 3d-structure, Mitochondrion, alternative splicing, apoptosis, cytoplasm, membrane, transmembrane, |
| XM_131981 | carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase | - | | |
| NM_139305 | Carbonic anhydrase 9 (Car9), mRNA | Car9 | | |
| NM_007800 | Cathepsin G (Ctsg), mRNA | Ctsg | CATHEPSIN G | alternative splicing, cell projection, glycoprotein, lyase, membrane, metal-binding, signal, transmembrane, zinc, |

| | | | | |
|---|---|---|---|---|
| BC003476 | CD74 antigen (invariant polypeptide of major histocompatibility complex, class II antigen-associated), mRNA (cDNA clone MGC:6 | Cd74 | CD74 ANTIGEN (INVARIANT POLYPEPTIDE OF MAJOR HISTOCOMPATIBILITY COMPLEX, CLASS II ANTIGEN-ASSOCIATED) | Direct protein sequencing, Zymogen, glycoprotein, hydrolase, intermediate filament, membrane, protease, serine protease, serine proteinase, signal, |
| NM_021443 | Chemokine (C-C motif) ligand 8 (Ccl8), mRNA | Ccl8 | CHEMOKINE (C-C MOTIF) LIGAND 8 | |
| NM_021274 | Chemokine (C-X-C motif) ligand 10, mRNA (cDNA clone MGC:41087 IMAGE:1446589) | Cxcl10 | CHEMOKINE (C-X-C MOTIF) LIGAND 10 | Secreted, chemotaxis, cytokine, heparin-binding, inflammatory response, signal, |
| NM_008599 | Chemokine (C-X-C motif) ligand 9, mRNA (cDNA clone MGC:6179 IMAGE:3257716) | Cxcl9 | CHEMOKINE (C-X-C MOTIF) LIGAND 9 | Secreted, chemotaxis, cytokine, glycoprotein, heparin-binding, signal, |
| NM_028071 | Coactosin-like 1 (Dictyostelium) (Cotl1), mRNA | Cotl1 | | |
| NM_139117 | Cold shock domain protein A (Csda), transcript variant 2, mRNA | Csda | COLD SHOCK DOMAIN PROTEIN A | Transcription, Transcription regulation, activator, alternative splicing, atp-binding, leucine-rich repeat, nucleotide-binding, nucleus, |
| NM_007782 | Colony stimulating factor 3 receptor (granulocyte) (Csf3r), mRNA | Csf3r | COLONY STIMULATING FACTOR 3 RECEPTOR (GRANULOCYTE) | Transcription, Transcription regulation, alternative splicing, cytoplasm, dna-binding, nucleus, phosphoprotein, repressor, rna-binding, |

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|---|---|---|---|---|
| NM_016748 | Cytidine 5'-triphosphate synthase, mRNA (cDNA clone MGC:5946 IMAGE:3498717) | Ctps | CYTIDINE 5'-TRIPHOSPHATE SYNTHASE | 3d-structure, cell adhesion, glycoprotein, immunoglobulin domain, membrane, receptor, signal, transmembrane, transmembrane protein, |
| NM_201408 | deoxyhypusine synthase | - | DEOXYHYPUSINE SYNTHASE | Glutamine amidotransferase, ligase, phosphoprotein, pyrimidine biosynthesis, |
| NM_010049 | Dihydrofolate reductase, mRNA (cDNA clone MGC:11577 IMAGE:3707582) | Dhfr | DIHYDROFOLATE REDUCTASE | |
| AK002850 | Disabled homolog 2 (Drosophila), mRNA (cDNA clone MGC:18401 IMAGE:4242174) | Dab2 | DISABLED HOMOLOG 2 (DROSOPHILA) | 3d-structure, Direct protein sequencing, nadp, one-carbon metabolism, oxidoreductase, |
| NM_001008702 | Disabled homolog 2 (Drosophila), mRNA (cDNA clone MGC:18401 IMAGE:4242174) | Dab2 | | |
| NM_001047433 | DPH3 homolog (KTI11, S. cerevisiae), mRNA (cDNA clone MGC:36233 IMAGE:4920779) | Dph3 | | |
| NM_176933 | Dual specificity phosphatase 4, mRNA (cDNA clone MGC:143691 IMAGE:40092218) | Dusp4 | DUAL SPECIFICITY PHOSPHATASE 4 | Coiled coil, alternative splicing, initiation factor, phosphoprotein, protein biosynthesis, translation regulation, wd repeat, |

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|---|---|---|---|---|
| NM_026268 | Dual specificity phosphatase 6, mRNA (cDNA clone MGC:6625 IMAGE:3491528) | Dusp6 | DUAL SPECIFICITY PHOSPHATASE 6 | hydrolase, nucleus, protein phosphatase, |
| NM_010058 | Dystrophia myotonica-containing WD repeat motif, mRNA (cDNA clone MGC:37679 IMAGE:5054380) | Dmwd | DYSTROPHIA MYOTONICA-CONTAINING WD REPEAT MOTIF | cytoplasm, hydrolase, protein phosphatase, |
| AK053245 | E030050A11Rik | - | DYSTROPHIA MYOTONICA-CONTAINING WD REPEAT MOTIF | phosphoprotein, wd repeat, |
| NM_023794 | Ets variant gene 5, mRNA (cDNA clone MGC:28414 IMAGE:4036564) | Etv5 | | |
| AK044865 | eukaryotic translation initiation factor 2a | - | | |
| NM_027773 | Family with sequence similarity 57, member A (Fam57a), mRNA | Fam57a | | |
| NM_008013 | Fibrinogen-like protein 2, mRNA (cDNA clone MGC:19044 IMAGE:4189071) | Fgl2 | FIBRINOGEN-LIKE PROTEIN 2 | |
| NM_010228 | FMS-like tyrosine kinase 1, mRNA (cDNA clone MGC:36074 IMAGE:5368921) | Flt1 | FMS-LIKE TYROSINE KINASE 1 | Coiled coil, Secreted, cytolysis, glycoprotein, signal, |

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|---|---|---|---|---|
| NM_008039 | Formyl peptide receptor 2 (Fpr2), mRNA | Fpr2 | FORMYL PEPTIDE RECEPTOR, RELATED SEQUENCE 2 | ATP, Developmental protein, Direct protein sequencing, angiogenesis, atp-binding, differentiation, glycoprotein, immunoglobulin domain, kinase, membrane, nucleotide-binding, phosphoprotein, receptor, signal, transferase, transmembrane, tyrosine-protein kinase, |
| AF114382 | Four and half LIM domain protein 3 (Fhl3) | Fhl3 | FOUR AND A HALF LIM DOMAINS 3 | receptor, |
| NM_022420 | G protein-coupled receptor, family C, group 5, member B (Gprc5b), mRNA | Gprc5b | G PROTEIN-COUPLED RECEPTOR, FAMILY C, GROUP 5, MEMBER B | lim domain, metal-binding, zinc, zinc-finger, |
| NM_178389 | Galactose-4-epimerase, UDP, mRNA (cDNA clone MGC:36852 IMAGE:4224021) | Gale | GALACTOSE-4-EPIMERASE, UDP | g-protein coupled receptor, glycoprotein, membrane, receptor, signal, transducer, transmembrane, |
| NM_177367 | Gem (nuclear organelle) associated protein 4, mRNA (cDNA clone MGC:28146 IMAGE:3982927) | Gemin4 | GEM (NUCLEAR ORGANELLE) ASSOCIATED PROTEIN 4 | NAD, carbohydrate metabolism, galactose metabolism, isomerase, |
| XM_358631 | gene model 1673, (NCBI) | - | | |
| XM_130416 | GINS complex subunit 1 (Psf1 homolog), mRNA (cDNA clone MGC:41228 IMAGE:3466154) | Gins1 | | |

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|---|---|---|---|---|
| NM_011824 | Gremlin 1, mRNA (cDNA clone MGC:19137 IMAGE:4216669) | Grem1 | GREMLIN 1 | |
| NM_007836 | Growth arrest and DNA-damage-inducible 45 alpha, mRNA (cDNA clone MGC:18457 IMAGE:4035898) | Gadd45a | GROWTH ARREST AND DNA-DAMAGE-INDUCIBLE 45 ALPHA | Secreted, cytokine, glycoprotein, signal, |
| NM_010260 | Guanylate binding protein 2, mRNA (cDNA clone MGC:41173 IMAGE:1230883) | Gbp2 | GUANYLATE NUCLEOTIDE BINDING PROTEIN 2 | DNA damage, cell cycle, growth arrest, |
| NM_153564 | Guanylate binding protein 5 (Gbp5), mRNA | Gbp5 | GUANYLATE NUCLEOTIDE BINDING PROTEIN 5 | GTP-binding, lipoprotein, membrane, nucleotide-binding, prenylation, |
| NM_175654 | Histone cluster 1, H4d, mRNA (cDNA clone IMAGE:40061655) | Hist1h4d | HISTONE 1, H4H | GTP-binding, lipoprotein, membrane, nucleotide-binding, prenylation, |
| NM_001013828 | Interferon-inducible GTPase-like, mRNA (cDNA clone MGC:28308 IMAGE:4013233) | Iigp1 | INTERFERON-INDUCED PROTEIN WITH TETRATRICOPEPTIDE REPEATS 3 | 3d-structure, Chromosomal protein, DNA binding, Methylation, Nucleosome core, acetylation, dna-binding, nucleus, phosphoprotein, ubl conjugation, |
| M64404 | Interleukin 1 receptor antagonist (Il1rn), transcript variant 1, mRNA | Il1rn | INTERLEUKIN 1 RECEPTOR ANTAGONIST | 3d-structure, Direct protein sequencing, Secreted, cytokine, immunoregulation, inflammation, inflammatory response, lymphokine, macrophage, mitogen, pyrogen, |

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|---|---|---|---|---|
| NM_031167 | Interleukin 1 receptor antagonist (Il1rn), transcript variant 1, mRNA | Il1rn | INTERLEUKIN 1 RECEPTOR ANTAGONIST | Secreted, alternative splicing, cytokine receptor, cytoplasm, glycoprotein, receptor, signal, |
| NM_146165 | JTV1 gene (Jtv1), mRNA | Jtv1 | JTV1 GENE | hydrolase, |
| NM_145416 | KRI1 homolog (S. cerevisiae) (Kri1), mRNA | Kri1 | | |
| NM_153388 | Leucine rich repeat and fibronectin type III domain containing 4 (Lrfn4), mRNA | Lrfn4 | LEUCINE RICH REPEAT AND FIBRONECTIN TYPE III DOMAIN CONTAINING 4 | |
| NM_010688 | LIM and SH3 protein 1, mRNA (cDNA clone MGC:5975 IMAGE:3490532) | Lasp1 | LIM AND SH3 PROTEIN 1 | glycoprotein, immunoglobulin domain, leucine-rich repeat, membrane, signal, transmembrane, |
| XM_193524 | LOC270589 | - | | |
| NM_008608 | Matrix metallopeptidase 14 (membrane-inserted) (Mmp14), mRNA | Mmp14 | MATRIX METALLOPEPTIDASE 14 (MEMBRANE-INSERTED) | SH3 domain, acetylation, actin-binding, cytoplasm, cytoskeleton, ion transport, lim domain, metal-binding, phosphoprotein, transport, zinc, |
| NM_009616 | Meltrin beta | Adam19 | | |
| AK009261 | mevalonate kinase | - | MEVALONATE KINASE | Cleavage on pair of basic residues, Secreted, Zymogen, calcium, collagen degradation, extracellular matrix, hydrolase, membrane, metal-binding, metalloprotease, metalloproteinase, protease, signal, transmembrane, zinc, |

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|---|---|---|---|---|
| AK040723 | MHC class II transactivator CIITA form IV | Ciita | | |
| NM_007575 | MHC class II transactivator CIITA form IV | Ciita | | |
| NM_026246 | Mitochondrial ribosomal protein L49, mRNA (cDNA clone MGC:35661 IMAGE:5368499) | Mrpl49 | MITOCHONDRIAL RIBOSOMAL PROTEIN L49 | atp-binding, cholesterol biosynthesis, cytoplasm, kinase, lipid synthesis, nucleotide-binding, peroxisome, steroid biosynthesis, sterol biosynthesis, transferase, |
| NM_010824 | Myeloperoxidase (Mpo), nuclear gene encoding mitochondrial protein, mRNA | Mpo | MYELOPEROXIDASE | Mitochondrion, ribonucleoprotein, ribosomal protein, |
| XM_283793 | myosin, light polypeptide 9, regulatory | - | | |
| NM_172742 | Myotubularin related protein 10, mRNA (cDNA clone IMAGE:3964696) | Mtmr10 | | |
| NM_010846 | Myxovirus (influenza virus) resistance 1, mRNA (cDNA clone MGC:18532 IMAGE:4011821) | Mx1 | MYXOVIRUS (INFLUENZA VIRUS) RESISTANCE 1 | Peroxidase, calcium, chromoprotein, glycoprotein, heme, hydrogen peroxide, iron, lysosome, metal-binding, metalloprotein, oxidation, oxidoreductase, signal, |
| NM_025998 | Na+/K+ transporting ATPase interacting 1 (Nkain1), mRNA | Nkain1 | | |

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|---|---|---|---|---|
| NM_178421 | Nanos homolog 1 (Drosophila), mRNA (cDNA clone MGC:102288 IMAGE:6849859) | Nanos1 | NANOS HOMOLOG 1 (DROSOPHILA) | GTP-binding, antiviral defense, nucleotide-binding, nucleus, |
| NM_138747 | NOP2 nucleolar protein homolog (yeast), mRNA (cDNA clone MGC:6746 IMAGE:3592148) | Nop2 | | |
| NM_008885 | Peripheral myelin protein 22, mRNA (cDNA clone MGC:18564 IMAGE:4225316) | Pmp22 | PERIPHERAL MYELIN PROTEIN | |
| NM_019932 | Platelet factor 4 (Pf4), mRNA | Pf4 | PLEXIN DOMAIN CONTAINING 2 | cell cycle, disease mutation, glycoprotein, growth arrest, membrane, transmembrane, transmembrane protein, |
| AK090072 | Predicted gene, OTTMUSG00000016644 (OTTMUSG00000016644), transcript variant 2, mRNA | OTTMUSG00000016644 | | |
| NM_008788 | Procollagen C-endopeptidase enhancer protein (Pcolce), mRNA | Pcolce | PROCOLLAGEN C-ENDOPEPTIDASE ENHANCER PROTEIN | glycoprotein, membrane, signal, transmembrane, |
| NM_011072 | Profilin 1, mRNA (cDNA clone MGC:6236 IMAGE:3490976) | Pfn1 | PROFILIN 1 | Direct protein sequencing, Secreted, collagen, glycoprotein, signal, |

| | | | | |
|---|---|---|---|---|
| NM_028295 | Protein disulfide isomerase associated 5, mRNA (cDNA clone MGC:11961 IMAGE:3600849) | Pdia5 | PROTEIN DISULFIDE ISOMERASE ASSOCIATED 5 | Direct protein sequencing, acetylated amino end, acetylation, actin binding, actin-binding, cytoplasm, cytoskeleton, phosphoprotein, |
| NM_011178 | Proteinase 3 (Prtn3), mRNA | Prtn3 | PROTEINASE 3 | endoplasmic reticulum, isomerase, redox-active center, signal, |
| AK042036 | Quiescin Q6 sulfhydryl oxidase 2 (Qsox2), mRNA | Qsox2 | QUIESCIN Q6-LIKE 1 | Zymogen, collagen degradation, glycoprotein, hydrolase, protease, serine protease, signal, |
| NM_009005 | RAB7, member RAS oncogene family (Rab7), mRNA | Rab7 | RAB7, MEMBER RAS ONCOGENE FAMILY | |
| NM_015811 | Regulator of G-protein signaling 1, mRNA (cDNA clone MGC:41114 IMAGE:1328269) | Rgs1 | REGULATOR OF G-PROTEIN SIGNALING 1 | Direct protein sequencing, GTP binding, GTP-binding, Methylation, P-loop, cytoplasmic vesicle, endosome, lipoprotein, lysosome, nucleotide binding, nucleotide-binding, phosphoprotein, prenylation, protein transport, transport, |
| NM_029879 | Regulator of G-protein signalling 7 binding protein, mRNA (cDNA clone MGC:143795 IMAGE:40093423) | Rgs7bp | | |
| NM_133982 | Ribonuclease P 25 subunit (human) (Rpp25), mRNA | Rpp25 | RIBONUCLEASE P 25 SUBUNIT (HUMAN) | phosphoprotein, signal transduction inhibitor, |

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| AK052480 | ribosomal protein S24 | - | RIBOSOMAL PROTEIN S24 | hydrolase, nucleus, phosphoprotein, rna-binding, trna processing, |
| NM_025598 | RIKEN cDNA 2700038C09 gene (2700038C09Rik), mRNA | 2700038C09Rik | RIKEN CDNA 2700038C09 GENE | glycoprotein, membrane, transmembrane, |
| XM_148080 | RIKEN cDNA 2700045P11 gene | - | RIKEN CDNA 2700045P11 GENE | |
| NM_172998 | Ring finger protein, transmembrane 2 (Rnft2), transcript variant 2, mRNA | Rnft2 | RIKEN CDNA 2810418N01 GENE | |
| NM_029036 | S100P binding protein, mRNA (cDNA clone MGC:48235 IMAGE:1548746) | S100pbp | S100P BINDING PROTEIN | |
| NM_009242 | Secreted acidic cysteine rich glycoprotein, mRNA (cDNA clone MGC:6232 IMAGE:3586402) | Sparc | SECRETED ACIDIC CYSTEINE RICH GLYCOPROTEIN | |
| NM_011340 | Serine (or cysteine) peptidase inhibitor, clade F, member 1, mRNA (cDNA clone MGC:29977 IMAGE:5123884) | Serpinf1 | SERINE (OR CYSTEINE) PEPTIDASE INHIBITOR, CLADE F, MEMBER 1 | Direct protein sequencing, EF hand, Secreted, basement membrane, calcium, calcium binding, collagen binding, copper, extracellular matrix, glycoprotein, hydroxyapatite binding, phosphoprotein, signal, |
| NM_009171 | Serine hydroxymethyltransferase 1 (soluble), mRNA (cDNA clone MGC:14007 IMAGE:4160579) | Shmt1 | SERINE HYDROXYMETHYL TRANSFERASE 1 (SOLUBLE) | Direct protein sequencing, Secreted, glycoprotein, signal, |

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| NM_026796 | SET and MYND domain containing 2, mRNA (cDNA clone MGC:28724 IMAGE:4458755) | Smyd2 | | |
| NM_172507 | SH3 domain binding glutamic acid-rich protein like 2, mRNA (cDNA clone MGC:46986 IMAGE:5008777) | Sh3bgrl2 | SH3 DOMAIN BINDING GLUTAMIC ACID-RICH PROTEIN LIKE 2 | 3d-structure, cytoplasm, methyltransferase, one-carbon metabolism, phosphoprotein, pyridoxal phosphate, transferase, |
| NM_181590 | SHQ1 homolog (S. cerevisiae) (Shq1), mRNA | Shq1 | SHQ1 HOMOLOG (S. CEREVISIAE) | Direct protein sequencing, nucleus, sh3-binding, |
| XM_139078 | similar to nucleolar protein 5A | - | SIMILAR TO INTERFERON-INDUCIBLE GTPASE | alternative splicing, |
| NM_024254 | Solute carrier family 12, member 6, mRNA (cDNA clone IMAGE:5359319) | Slc12a6 | | |
| AK011893 | SPC25, NDC80 kinetochore complex component, homolog (S. cerevisiae), mRNA (cDNA clone MGC:40860 IMAGE:5369405) | Spc25 | | |
| NM_009272 | Spermidine synthase, mRNA (cDNA clone MGC:11495 IMAGE:3966710) | Srm | SPERMIDINE SYNTHASE | |
| NM_016964 | Stromal antigen 3 (Stag3), mRNA | Stag3 | STROMAL ANTIGEN 3 | Coiled coil, alternative splicing, cell cycle, cell division, centromere, mitosis, nucleus, |
| NM_179203 | TOB3 | Atad3a | | |

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| NM_031178 | Toll-like receptor 9 (Tlr9), mRNA | Tlr9 | TOLL-LIKE RECEPTOR 9 | Meiosis, cell cycle, chromosome partition, nucleus, |
| NM_001025106 | Transmembrane protein 201 (Tmem201), transcript variant 1, mRNA | Tmem201 | | |
| NM_021793 | Transmembrane protein 8 (five membrane-spanning domains), mRNA (cDNA clone MGC:11794 IMAGE:3595205) | Tmem8 | TRANSMEMBRANE PROTEIN 8 (FIVE MEMBRANE-SPANNING DOMAINS) | metal-binding, zinc, zinc-finger, |
| AK020775 | Tripartite motif-containing 35 (Trim35), mRNA | Trim35 | TRIPARTITE MOTIF-CONTAINING 35 | alternative splicing, cell adhesion, egf-like domain, glycoprotein, membrane, signal, transmembrane, |
| NM_011677 | Uracil DNA glycosylase, mRNA (cDNA clone MGC:13929 IMAGE:4009947) | Ung | URACIL DNA GLYCOSYLASE | Coiled coil, alternative splicing, apoptosis, cytoplasm, metal-binding, nucleus, zinc, zinc-finger, |
| AK079112 | Wolf-Hirschhorn syndrome candidate 1 (human), mRNA (cDNA clone IMAGE:9007381) | Whsc1 | | |
| XM_109956 | WW, C2 and coiled-coil domain containing 1 | - | | |
| NM_182996 | Zinc finger protein 692, mRNA (cDNA clone MGC:179040 IMAGE:9054032) | Zfp692 | ZINC FINGER PROTEIN 692 | DNA damage, DNA repair, Mitochondrion, alternative splicing, glycosidase, hydrolase, nucleus, phosphoprotein, transit peptide, |