DETERMINING THE ROLE OF IRF6 IN T CELL DEVELOPMENT AND FUNCTIONAL COMMITMENT

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Genetics - Doctor of Philosophy
Quantitative Biology - Dual Major

2014

ABSTRACT

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Interferon regulatory factor (IRF) is a protein family with nine members in mammals known to orchestrate the homeostatic mechanisms of host defense. There are functional and/or developmental defects of immune cells in the knockouts of eight family members. Like other family members, IRF6 is involved in regulating the cell cycle but in keratinocytes and mammary epithelial cell with mutations associated with squamous cell carcinomas. However, *Irf6* is the only IRF known to be involved in morphogenesis. In humans, rare variants in *IRF6* cause autosomal dominant orofacial clefting disorders while common variants contribute risk to non-syndromic forms. *IRF6* is the only IRF family member with an as yet undetermined role in immunity.

Here, we used publically available microarray data to uncover a dynamic expression pattern for *Irf6* during hematopoietic development. We found that *Irf6* is expressed early in hematopoiesis in long term hematopoietic stem cells. Also we identified *Irf6* expression in T cell lineage, including developing and functionally committed stages. Irf1, 2, 4, 8 are indispensable for a normal T cell development and differentiation. Genetic variants in *IRF5*, *IRF7* and *IRF8* are associated to autoimmune disorders of T cells. Furthermore, protein complexes between IRF6/IRF5 and IRF6/IRF8 were described. These data together with DNA conservation among the IRF members and structural homology with IRF5 strongly suggests a role for Irf6 in the immune system, specifically in T-cell development and functional commitment.

We utilized a mouse model to show that *Irf6* was required for the regulation of thymocyte development. We found that Irf6 was expressed in the subcapsular region and medulla of the thymus. We further found that *Irf6* regulated the distribution and proliferation of developing thymocytes. In addition, loss of *Irf6* led to an increase in double negative cells with a concomitant increase in TCRγδ. Loss of *Irf6* also led to a reduction in double positive cells with no corresponding reduction in single positive cell maturation. Also, we found that *Irf6* dose is critical in development of both CD4+ and CD8+ cells in an age-dependent manner. These data suggest a novel gene function for *Irf6* in thymocyte development and indicate further studies of *IRF6* variants that might increase the risk of autoimmune disease.

In the mouse, loss of Irf6 leads to perinatal lethality which hinders the ability to test the necessity of *Irf6* in the functionally committed T helper (Th) subsets. In silico analysis suggested a model for Irf6 role in Th17/Treg balance. To test our hypothesis in vivo and overcome perinatal lethality, we employed an adaptive transfer of Irf6 knockout cells into lethally irradiated mice. Mice receiving Irf6 knockout cells had no deficit in restoration of lymphocyte production. In addition, we used two in vitro models to assess the necessity of Irf6 in the commitment of T helper cells. Using a stromal-free culture we found that naive T cells lacking Irf6 could be differentiated efficiently into Th1, Th2, Th17 and Treg using a specific cytokine cocktail. In vitro differentiation of dendritic cells showed significant increase of MHC-II expression after three days of culture. Irf6 might be involved in post-translational regulation of MHC-II. These data indicate that intrinsic Irf6 expression is not essential for T helper subset differentiation. However, a non-cell autonomous role for Irf6 in T cell differentiation through dendritic cells remains plausible.

ACKNOWLEDGEMENTS

To begin, I would like to extend my deepest gratitude to my mentor, Dr. Brian Schutte. His patience with me and confidence in my intellectual ability has been tantamount to my success. I thank him for teaching my not only how to be a scientist but also how to be a good, fair person. They have both been invaluable lessons. I would also like to thank the members of my committee; Titus Brown, Sungjin Kim, and Sainan Wei for the continued support and scientific contribution to this work.

I would like to thank the current and past members of the Schutte Lab: Bill, Walid, Dan, Krysta, Raeuf, Larissa, Julie, Marissa, David, Nicole, Ari, Silas, Soumya, Erica, and Bryana. I could not have asked for a more outstanding group of people to share with both scientifically and socially. Lastly, I would like to thank Dr. Youssef Kousa and Dr. Arriana smith for helping me to grow scientifically, emotionally, and socially. Your guidance, support, and friendship have been invaluable.

I must thank the 5th floor community, members of the Friderici, Fyfe, and Yuzbasiyan-Gurkan labs for their constant willingness to help and for making the 5th a wonderful work environment. I would also like to that the Genetics Program staff and my fellow colleagues. A special thank you goes to Jeannine Lee, Dr. Barbara Sears and Dr. Cathy Ernst for their support and willingness to help in any and all circumstances. I truly appreciate it.

I would also like to thank the many people who graciously contributed to this work. To Jose Suarez-Martinez who pursed the in vitro differentiation studies of dendritic cells. To Dr. Norbert Kaminski and all his lab members for their help, support

and scientific contribution. I must also thank members of the Histopathology laboratory, for sharing their expertise and warm attitudes, and members of ULAR, for there diligence and help with all things mouse related.

Last, but certainly not least, I would thank my family and friends. I would to thank my parents for a lifetime of love, guidance and support. Without them, none of this would be possible. To my wife Dina and my kids Razan and Adam, thank you for being sources of inspiration and laughter. I owe a great deal of gratitude to all of my friends whom for fear of leaving anyone out, I will refrain from mentioning names. Undoubtedly, you have all been my sanity. I sincerely thank you all for your encouragement, insight, and friendship. This journey would not have been the same without you!

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KEY TO SYMBOLS OR ABBREVIATIONS

IRF - Interferon regulatory factor

Th – T helper

TEC – thymic epithelial cells

CK – cytokeratin

DN - double negative

TCR - T cell receptor

DP - double positive

TGFβ - Transforming Growth Factor β

SP - single positive

Treg - regulatory T-cells

T-bet - T-box expressed in T-cells

APC - Antigen Presenting Cell

GATA3 - GATA-binding protein 3

c-MAF - transcription factor Maf

RORγt - retinoic acid receptor–related orphan receptor γt

Klf4 - Kruppel-like factor 4

SLE - systemic lupus erythematosus

FOXP3 - Forkhead box P3

DBD - DNA-binding domain

IAD – Interferon Association Domain

ChIP - Chromatin immunoprecipitation

HSC - Haematopoietic stem cells

RMA - Robust Multi-array Average algorithm

LT-HSC - long term hematopoietic stem cells

ST-HSC - short term hematopoietic stem cells

LMPP - lymphoid multipotent progenitors

CMP - common myeloid progenitors

CLP - common lymphoid progenitors

MEP - megakaryocyte-erythroid progenitor

GMP - granulocyte monocyte progenitors

BLP - B cell-biased lymphoid progenitor

NK - nature killer cells

iTreg - induced regulatory T cells

nTreg - natural regulatory T cell

DC - dendritic cells

pDC - plasmacytoid dendritic cells (pDC)

tDC - thymic dendritic cells

BM.MPh - bone marrow macrophages

tMPh - thymic macrophages

Nu RBCs - nucleated erythrocytes

cTEC - cortical thymic epithelial cells

mTEC - modularly thymic epithelial cells

BrdU - Bromodeoxyuridine

GM-CSF - granulocyte macrophage-colony stimulating factors

BM – Bone marrow

LC - Langerhans cells

LL - Lymphoid leukosis

ALV - Avian Leukosis Virus

TUSC2 - Tumor suppressor candidate 2

EIF4E - Eukaryotic translation initiation factor 4E

MDV2 - Marek's disease virus serotype 2

CHAPTER ONE

Literature Review

Significance

T-cells are the most abundant subset of blood lymphocytes and serve as the core of the adaptive immune response. Changes in T-cell number or function can lead to autoimmune diseases, immune deficiency, inflammatory disorders and cancer. Evolution of lymphocytes with a cadre of highly diverse antigen-recognition receptors is necessary for immune surveillance but also requires stringent screening for auto-reactive clones. T-cells develop in the thymus, where a highly specialized microenvironment educates the evolving T-cells (Janeway *et al.*, 2001; Paul, 2008).

The Interferon regulatory factor (IRF) family of transcription factors are indispensable for functional and developmental regulation of immune cells. While *Irf6* shares DNA conservation and predicted structural homology with the IRF family members, its role in immunity is unknown. However, we already know that IRF6 is involved in protein-protein interactions with IRF5 and IRF8 (Li *et al.*, 2011), both of which regulate T-cell development and T helper differentiation. Preliminary bioinformatic analysis shows expression of *Irf6* during thymocyte development and functional commitment of T helper subsets.

These preliminary findings support the investigation of *Irf6* in T-cell development. Furthermore, it provides a new diagnostic and therapeutic target in autoimmune disorders such as psoriasis and systemic lupus erythematosus.

Thymus

Evolutionary studies show that thymus development began with jawed vertebrates (Boehm & Bleul, 2007; Litman & Cooper, 2007). Mammals in general, and

humans in particular, have a single thymus located superior to the heart at the thoracic inlet and is composed of bilateral lobes (Dooley *et al.*, 2006; Rodewald, 2008; Terszowski *et al.*, 2006). Histologically, the thymus is composed of an inner medulla and a peripheral cortex surrounded by an outer capsule. Thymus tissue is composed of lymphoid cells (CD45+CD7+) and stromal cells with a ratio of 50 lymphoid cells for each stromal cell (Rodewald, 2008; Singer *et al.*, 1986). Non-hematopoietic stromal cells can be further classified into thymic epithelial cells (TEC, Keratin+) and mesenchymal cells (Keratin−) (Anderson *et al.*, 1993). Dendritic cells and macrophages are CD45+ thymic stromal cells, thus they constitute the hematopoietic component of the stromal mesh (Rodewald, 2008) (Figure1.1).

In the mouse, thymus organogenesis starts around 10.5 days after fertilization (E10.5) when endodermal epithelial cells of the third pharyngeal pouch initiate the thymic primordium (Gordon *et al.*, 2004; Hollander *et al.*, 2006). Neural crest cells migrate into the thymic capsule, interlobular septae, and stromal cell network to regulate the early proliferation and differentiation of immature TECs (Ambrosiani *et al.*, 1996; Itoi *et al.*, 2007; Jenkinson *et al.*, 2003; Jenkinson *et al.*, 2007; Johnston, 1966; Yamazaki *et al.*, 2005). Migration of lymphoid precursors (next section) to the thymus starts by E11.5 (Haynes & Heinly, 1995; Liu *et al.*, 2006; Owen & Ritter, 1969). The interaction between lymphoid cells and TECs is critical for normal development of both the lymphoid and epithelial cell compartments (Anderson & Jenkinson, 2001). By E13.5, two thymic epithelial populations can be appreciated by cytokeratin (CK) markers; cortical epithelium (CK8+CK5−) and medullary epithelium (CK8−CK5+) (Klug *et al.*, 2002).

However, additional expansion of medullary islands is observed as late as E18.5 and corresponds with the emergence of mature T-cells (Irla *et al.*, 2008).

T-cells development

T-cell precursors migrate from either the fetal liver or bone marrow to seed the thymus. T-cell precursors are initially called double negative (DN) thymocytes because they lack expression of both CD4 and CD8 (Godfrey *et al.*, 1993; Pearse *et al.*, 1989). DN thymocytes undergo T cell receptor (TCR) rearrangement under guidance of cortical TECs (Raulet *et al.*, 1985; Shinkai *et al.*, 1992; Takahama, 2006; Tourigny *et al.*, 1997; von Boehmer & Fehling, 1997; Xu *et al.*, 1996). Cells with functional TCRs start to express both CD4 and CD8 and are in turn called double positive (DP) thymocytes (Irving *et al.*, 1998; Michie & Zuniga-Pflucker, 2002). In the subcapsular region, transforming growth factor β (TGFβ) signaling suppresses the proliferation of pre-DP thymocytes to regulate the production of DP cells (Benz *et al.*, 2004). DP cells migrate back through the cortex where positive and negative selections occur. In total, only 3-5% of cells survive and reach the thymic medulla. DP cells lose either CD4 or CD8 to reach the single positive (SP) stage. SP thymocytes, either $CD4^+$ (T helper) cells or $CD8^+$ (T cytotoxic) cells, continue their maturation and central tolerance in the medulla before being shuttled out of the thymus (Blackburn & Manley, 2004; Germain, 2002; Hoffmann *et al.*, 2003; Lind *et al.*, 2001; Plotkin *et al.*, 2003; Prockop & Petrie, 2000) (Figure1.1).

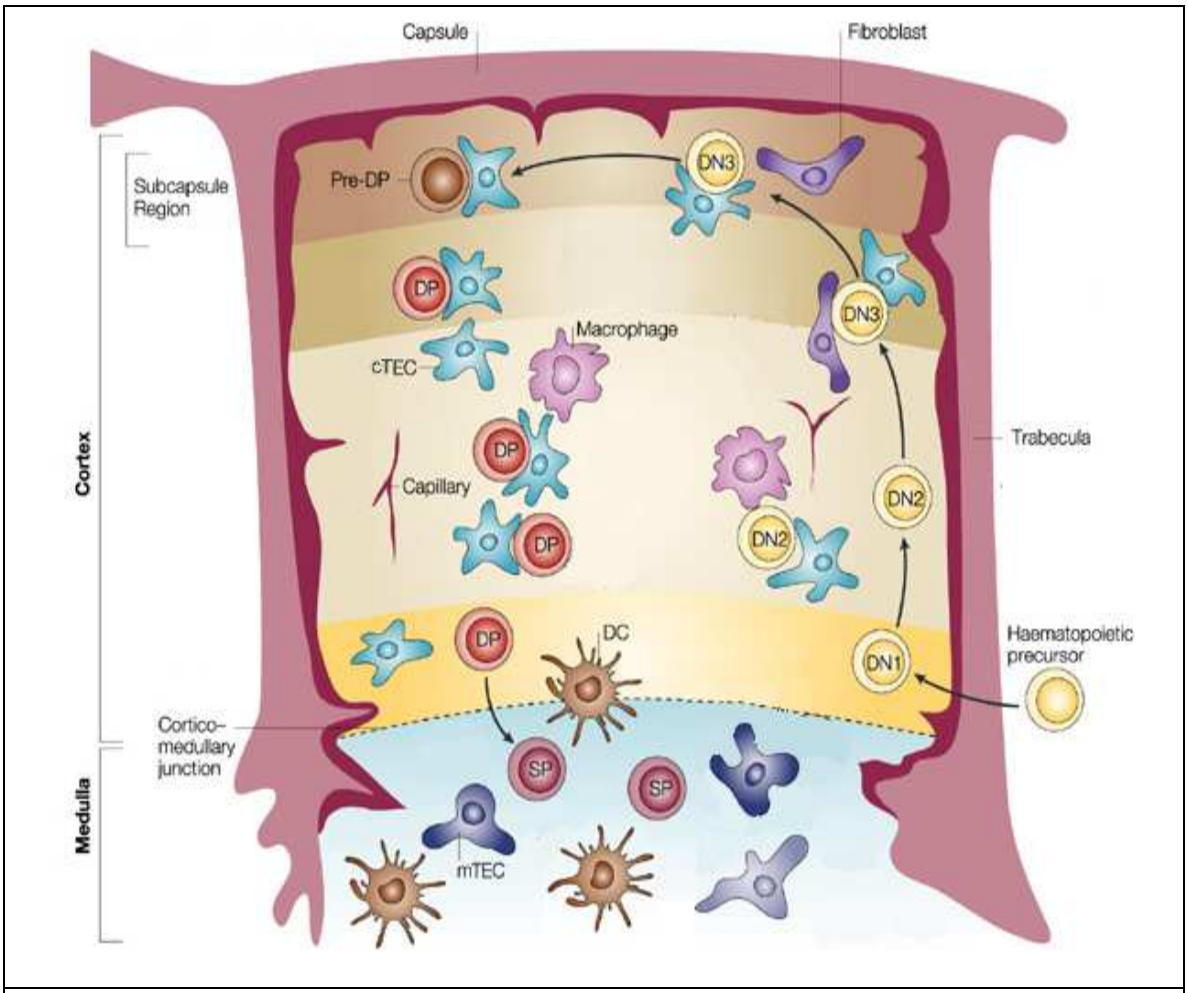

***Figure 1.1**:* **Histological compartments of a thymic lobule with diagramatic representation of T cell development**. Haematopoietic precursors seed the thymus at medullary cortical junction. Recent thymic immigrants lack both CD4 and CD8 and thus called double negative (DN) thymocytes. CD44 and CD25 are two surface markers which mark 4 major developmental sub-populations of DN thymocytes (DN1, $CD44^{+}CD25^{-}$; DN2, $CD44^{+}CD25^{+}$; DN3, $CD44^{-}CD25^{+}$; and DN4, $CD44^{-}CD25^{-}$). Transition from DN4 (also called pre-DP) to DP cells occurs in the subcapsular region. DP cells migrate back through the cortex and reach the thymic medulla. Meanwhile, DP cells lose either CD4 or CD8 to reach the single positive (SP) stage. (Figure is modified from Blackburn and Manley, 2004)

Functional commitment of T helper subsets

T-cells execute their designated functions by either secreting soluble cytokines or through direct cell-cell interaction. T helper (Th) lymphocytes are widely understood to function as the conductors of the adaptive immune orchestra. Upon antigen exposure, T helper cells differentiate into specialized subsets. Each T helper subset differentiates under a unique signaling pathway and lineage-specific transcription factors to produce a characteristic cytokine milieu (Fietta & Delsante, 2009; Hirahara *et al.*, 2011). T helper subsets include Th1, Th2, Th17, regulatory T-cells (Treg), T follicular helper cells, Th9 and Th22 cells (Bluestone *et al.*, 2009; Shevach, 2010). The balance between different T helper cells is most typically defined by mutually exclusive expression of lineage-specific transcription factors.

Relative to all T helper subsets, Th1 and Th2 development and function has been most clearly elucidated. Differentiation of Th1 initially requires expression of a transcription factor called T-box expressed in T-cells (T-bet). Subsequent exposure to IL12 and IL18 among other cytokines produced by Antigen Presenting Cells (APCs) induces completion of the differentiation process. Th1 cells regulate cellular immunity and are essential for the eradication of intracellular pathogens (Matsuoka *et al.*, 2004; Rautajoki *et al.*, 2008). Alternatively, Th2 differentiation necessitates IL4 mediated signaling and expression of the lineage specific transcription factors GATA-binding protein 3 (GATA3) and transcription factor Maf (c-MAF). Th2 cells are responsible for regulating humoral immunity and are implicated in the pathophysiology of allergy (O'Garra & Arai, 2000; Rautajoki *et al.*, 2008).

Th17, a T helper subset producing IL17, has pro-inflammatory effects and protects against bacterial infections in the intestine and the airways (Miossec *et al.*, 2009; Mitsdoerffer *et al.*, 2010). TGFβ in the presence of IL6 can initiate Th17 commitment (Bettelli *et al.*, 2006; Dong, 2006; McGeachy *et al.*, 2009). The Th17 lineage specific transcription factor is retinoic acid receptor–related orphan receptor γt (RORγt) (Ivanov *et al.*, 2006). Kruppel-like factor 4 (Klf4) is another important transcription factor required for full commitment of Th17. T-cell-specific *Klf4*-knockout mice show 24% reduction of IL-17^{+} CD4^{+} T-cells (Botti *et al.*, 2011). Defects in Th17 development can lead to several autoimmune diseases, including rheumatoid arthritis, asthma and systemic lupus erythematosus (SLE) (Maddur *et al.*, 2013; Oukka, 2008; Tesmer *et al.*, 2008). Additional roles for Th17 in graft rejection and inflammatory bowel disease have been described (Agorogiannis *et al.*, 2012; Dong, 2008; Kolls & Linden, 2004).

Treg is a suppressor T helper cell subset that controls the amplitude of the immune response and prevents the development of autoimmune diseases. Impairment of the reciprocal differentiation between Th17 and Treg has been implicated in several autoimmune disorders such as experimental autoimmune encephalomyelitis; a mouse model of multiple sclerosis, and type I diabetes mellitus (Pan *et al.*, 2011). Moreover, Th17/Treg imbalance is associated with tumors (Hu *et al.*, 2011) and graft-versus host rejection(Dander *et al.*, 2009). While there are different subpopulations of Tregs, Forkhead box P3 (FOXP3) is a common marker in this lineage (Green *et al.*, 1983). Two subsets of Tregs are determined by their developmental origin, whereas nTregs arise naturally in the thymus, iTregs are induced peripherally. Treg subtypes might have

similar or overlapping functions but are not identical. For example, Foxp3 deficiency causes fatal systemic autoimmune disease due to preferential Th1 and Th17 induction (Bennett *et al.*, 2001; Brunkow *et al.*, 2001). However selective deficiency of iTreg is associated with Th2 allergic response at mucosal sites when systemic autoimmunity of Th1 or Th17 has not been implicated (Josefowicz *et al.*, 2012). Importantly, prior work showed that TGFβ signaling inhibits both Th1 and Th2 differentiation. More recent work shows that TGFβ signaling is essential for induction of Foxp3 expression and commitment of Treg, either nTreg or iTreg (Chen & Wahl, 2002). The ability of TGFβ signaling to induce FOXP3 while concomitantly suppressing the Th17 cell lineage is mediated by the protein Inhibitor of DNA binding 3 (ID3) (Chen & Wahl, 2002; Maruyama *et al.*, 2011; Pan *et al.*, 2011) (Figure 1.2).

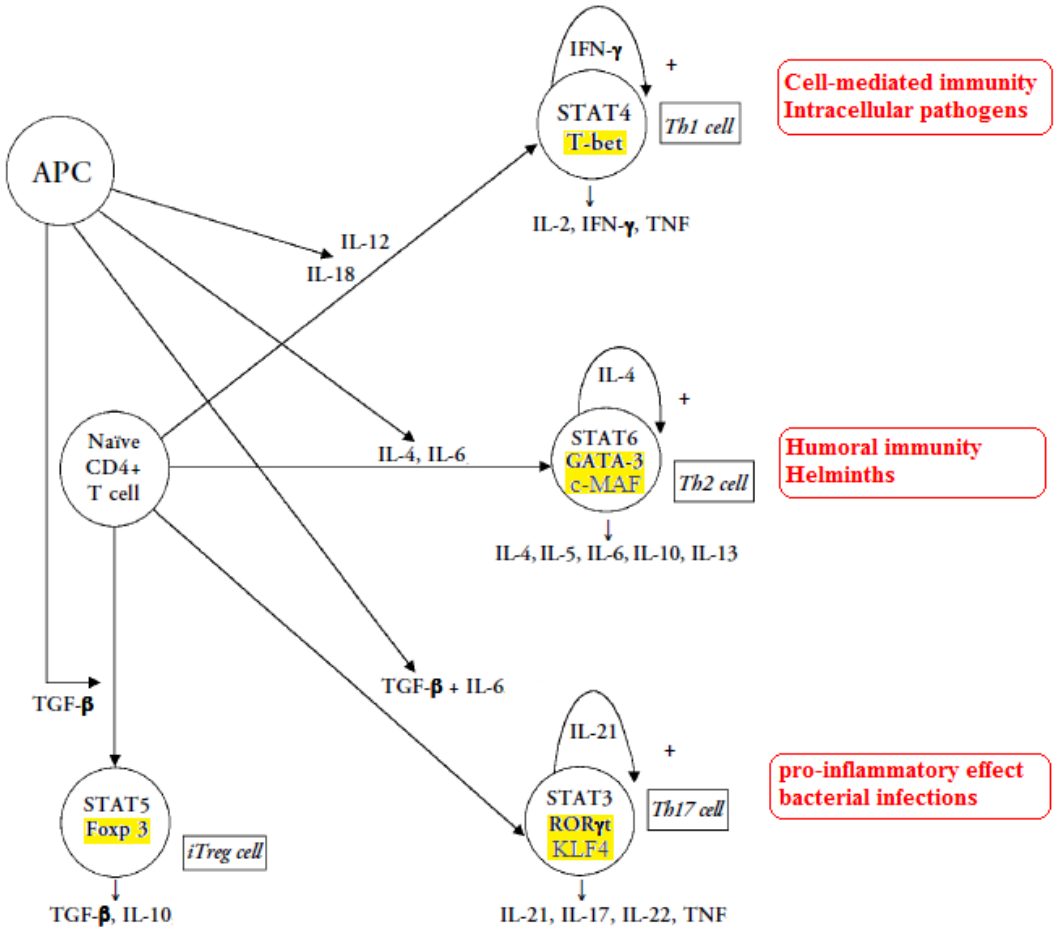

Figure 1.2: **Differentiation of T-helper subsets**. Naïve T cells undergo functional differentiation under cytokine direction form antigen presenting cells (APC).

Figure 1.2 **(cont'd)**: Th1 requires expression of the transcription factor T-box (T-bet). IL12 and IL18 induce STAT4 signaling to induce IFN-γ production to complete the differentiation and expression of IL2, IFN-γ and TNF. Th1 cells invoke cell mediated immunity and induce destruction of intracellular pathogens. IL4 and IL6 induce STAT6 signaling and expression of GATA-binding protein3 (GATA3) and transcription factor Maf (c-MAF) to allow Th2 differentiation. Th2 cells secrete IL4, IL5, IL6, IL10 and IL13 to initiate humoral immunity. Th2 response is required for control of helminthes infection. TGFβ and IL6 initiate STAT3 signaling and expression of retinoic acid receptor–related orphan receptor γt (RORγt) and Kruppel-like factor 4 (Klf4) to induce Th17 commitment. Th17 cells produce IL21, IL17, IL22 and TNF. Th17 cytokines have pro-inflammatory effect and protect against bacterial infections. TGFβ can induce STAT5 signaling and mediate expression of FOXP3 causing Treg commitment. Tregs produce more TGFβ and IL10 to suppress the response. Figure is modified from Fietta and Delsante, 2009.

Interferon regulatory factor family

The interferon regulatory factor (IRF) family of transcription factors has nine members in mammals (Huang *et al.*, 2010; Nehyba *et al.*, 2009). All family members share a highly conserved N-terminal DNA-binding domain (DBD) that possesses five conserved tryptophan residues, each separated by 10–18 amino acids (Kondo *et al.*, 2002; Lohoff & Mak, 2005; Tamura *et al.*, 2008). There is also a shared, but less conserved protein-binding domain at the C-terminus (Kondo *et al.*, 2002; Lohoff & Mak, 2005) (Figure 1.3). The IRF family is known to orchestrate homeostasis of host defense (Tamura *et al.*, 2008; Taniguchi *et al.*, 2001). The functions of different IRFs can be categorized into three main targets. First category is transcriptional regulation of type I interferon responses which is an indispensible downstream target of IRFs (Barnes *et al.*, 2001; Fujita *et al.*, 1989; Honda *et al.*, 2005a; Honda *et al.*, 2005b; Honda *et al.*, 2006; Honda & Taniguchi, 2006; Hoshino *et al.*, 2006; Izaguirre *et al.*, 2003; Marie *et al.*, 1998; Matsui *et al.*, 2006; Matsuyama *et al.*, 1993; Moynagh, 2005; Negishi *et al.*, 2005; Sato *et al.*, 1998; Sato *et al.*, 2000; Tailor *et al.*, 2007; Takaoka *et al.*, 2005; Taniguchi *et al.*, 2001; Taniguchi & Takaoka, 2002; Tsujimura *et al.*, 2004; Yoneyama *et al.*, 1998; Zhao *et al.*, 2006). Second broad category of IRF family functions is their necessary roles in development and function of a cadre of immune cell types, innate immune cells such as phagocytes (Hida *et al.*, 2005; Holtschke *et al.*, 1996; Kamijo *et al.*, 1994; Salkowski *et al.*, 1999; Scheller *et al.*, 1999; Tamura *et al.*, 2000; Tamura *et al.*, 2005b; Testa *et al.*, 2004; Tsujimura *et al.*, 2002) and natural killers (Duncan *et al.*, 1996; Lohoff *et al.*, 2000; Ogasawara *et al.*, 1998; Taki *et al.*, 2005) or adaptive immune cells for example dendritic cells (Gabriele *et al.*, 2006; Honda *et al.*, 2004; Ichikawa *et al.*, 2004; Schiavoni

et al., 2002; Schiavoni *et al.*, 2004; Suzuki *et al.*, 2004; Tamura *et al.*, 2005a; Tsujimura *et al.*, 2003) and lymphocytes (Brien *et al.*, 2011; Brustle *et al.*, 2007; Fragale *et al.*, 2008; Klein *et al.*, 2006; Lee *et al.*, 2006; Lohoff *et al.*, 1997; Lohoff *et al.*, 2002; Lu *et al.*, 2003; Ma *et al.*, 2011; Ma *et al.*, 2006; Mittrucker *et al.*, 1997; Ouyang *et al.*, 2011; Penninger *et al.*, 1997; Penninger & Mak, 1998; Scharton-Kersten *et al.*, 1997; Sciammas *et al.*, 2006; Taki *et al.*, 1997; Tian *et al.*, 2012; Tominaga *et al.*, 2003; White *et al.*, 1996; Zhang *et al.*, 2011; Zheng *et al.*, 2009). Lastly, IRF family members are also involved in the regulation of cell cycle control and oncogenic pathogenesis. *IRF1* (Bouker *et al.*, 2005; Connett *et al.*, 2005; Giatromanolaki *et al.*, 2004; Harada *et al.*, 1993; Kano *et al.*, 1999; Passioura *et al.*, 2005a; Romeo *et al.*, 2002; Tanaka *et al.*, 1994; Tanaka *et al.*, 1996; Yim *et al.*, 2003), *IRF3* (Duguay *et al.*, 2002; Heylbroeck *et al.*, 2000; Kim *et al.*, 1999; Kim *et al.*, 2003), *IRF5* (Barnes *et al.*, 2003; Hu *et al.*, 2005; Mori *et al.*, 2002; Yanai *et al.*, 2007) and *IRF8* (Deng & Daley, 2001; Hao & Ren, 2000; Liu & Abrams, 2003; Yang *et al.*, 2007a; Yang *et al.*, 2007b) are negative regulators of cell proliferation with known pro-apoptotic and tumor suppressor activities. On the other hand, *IRF2* antagonizes the tumor suppressor effect of *IRF1* (Connett *et al.*, 2005; Passioura *et al.*, 2005a; Passioura *et al.*, 2005b; Yim *et al.*, 2003). The role of *IRF4* in oncogenesis is context dependent. For example, *IRF4* is oncogenic in late developmental stages of lymphoid lineage (Hrdlickova *et al.*, 2001; Iida *et al.*, 1997; Ito *et al.*, 2002; Shaffer *et al.*, 2008; Tsuboi *et al.*, 2000) and a tumor suppressor in myeloid leukemia (Jo & Ren, 2011; Ortmann *et al.*, 2005; Schmidt *et al.*, 2000) and B cell malignancies of early developmental stages (Acquaviva *et al.*, 2008; Pathak *et al.*,

2011). Combined deficiency of *IRF4* and *IRF8* produces both myeloid and lymphoid tumors (Jo *et al.*, 2010).

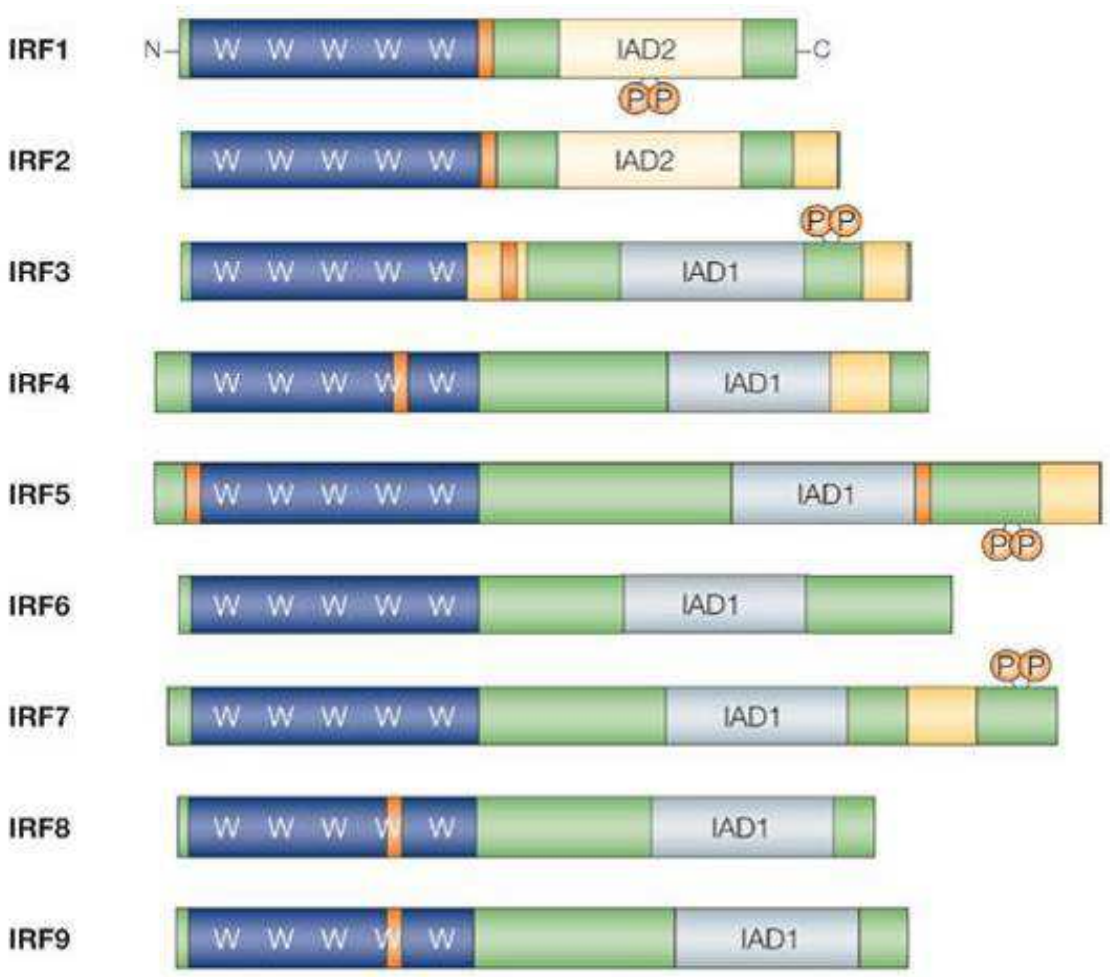

Figure 1.3: **interferon regulatory factor (IRF) protein family in mammals**

(Modified from Lohoff & Mak. Nat.Immunol. 2005). IRF family has nine members in mammals. IRFs are characterized by a highly conserved DNA binding domain (DBD) and a much less conserved protein association domain (regulatory domain; IAD – Interferon Association Domain).

Interferon regulatory factor 6

IRF6 has the canonical family DBD and its protein-binding domain is most closely related to IRF5 (Huang *et al.*, 2010; Nehyba *et al.*, 2009). Like other family members, IRF6 is involved in regulating the cell cycle with an anti-proliferative function in keratinocytes and mammary epithelial cell (Bailey *et al.*, 2008; Ingraham *et al.*, 2006; Richardson *et al.*, 2006). Consistently, mutations in *IRF6* have also been found in patients with squamous cell carcinomas, re-emphasizing tumor suppressor function (Bailey *et al.*, 2009; Botti *et al.*, 2011; Stransky *et al.*, 2011). However, *Irf6* is the only IRF known to be involved morphogenesis (Ingraham *et al.*, 2006; Richardson *et al.*, 2006; Richardson *et al.*, 2009; Thomason *et al.*, 2010). In humans, haploinsufficiency of *IRF6* causes syndromic orofacial clefting (Kondo *et al.*, 2002). Furthermore, a common DNA variant at the *IRF6* locus accounts for 12% of isolated orofacial clefting risk world-wide (Rahimov *et al.*, 2008; Zucchero *et al.*, 2004). Despite sequence conservation and structural similarity, *Irf6* is the only IRF family member with an as yet undetermined role in immunity. In 2005, Lohoff and Mak wondered if *IRF6* is even expressed by haematopoietic cells (Lohoff & Mak, 2005). Furthermore, functional and genetic studies of *Irf6* in the immune system are hindered because the knockout mouse model is perinatal lethal (Ingraham *et al.*, 2006). Thus refined technical strategies are required to test the role of *IRF6* in the immune system.

IRF6 is a putative transcription factor. The DBD of IRF6 binds a sequence highly analogous to the IRF family consensus (Botti *et al.*, 2011; Little *et al.*, 2009). We know that the DBD is critical for *IRF6* function because mutations in this region can lead to more severe forms of orofacial clefting, e.g. popliteal pterygium syndrome (Kondo *et al.*,

2002). Evidence for the transcriptional activity of IRF6 is also shown in Sabel et al. 2009. Expression of the IRF DBD in xenopus embryos results in failure of gastrulation. Co-expression of IRF6 rescues the phenotype suggesting specific competition at the DNA (Sabel *et al.*, 2009). Chromatin immunoprecipitation (ChIP) sequencing for IRF6 binding sites confirmed the direct DNA binding of Irf6 to many genes (Botti *et al.*, 2011). As a result of these studies, we know that *IRF6* binds to and regulates an important transcriptional network

Roles of IRFs in T-cell development and differentiation

IRF family members regulate T-cell biology either by intrinsic transcriptional activities in T-cells or through extrinsic roles in non-T-cells like thymic stromal cells and other immune cells. While IRFs have an important role in immunity, their role in T helper cell development and differentiation is of particular interest for both biological and clinical applications (Figure 1.4).

Although several Irfs are expressed in thymocytes (Colantonio *et al.*, 2011; Hrdlickova *et al.*, 2001; Nordang *et al.*, 2011; Simon *et al.*, 1997), *Irf1* is the only family member with functional studies supporting a role in T-cell development (Lee *et al.*, 1999; White *et al.*, 1996). Non-cell autonomous Irf1 reduces expression of the major histocompatibility complex related genes in the thymic microenvironment (Lee *et al.*, 1999; White *et al.*, 1996). However it is the *Irf1* intrinsic activity in T-cells that is required for development and thymic selection of naïve CD8 T-cells (Matsuyama *et al.*, 1993; Penninger *et al.*, 1997; Penninger & Mak, 1998). In *Irf4* knockout mice, while cell count changes during thymocyte development appear to be unaffected, the proliferative

capacity, antiviral cytotoxicity, allogenic graft rejection, tumor surveillance and cytokine production are markedly impaired (Mittrucker *et al.*, 1997).

Likewise, *Irf1, Irf2* and *Irf8* are critical for mounting a Th1 response (Lohoff *et al.*, 1997; Lohoff *et al.*, 2000; Scharton-Kersten *et al.*, 1997; Taki *et al.*, 1997) mainly through transcriptional activation of Il12 (Coccia *et al.*, 1999; Galon *et al.*, 1999; Giese *et al.*, 1997; Liu *et al.*, 2003; Maruyama *et al.*, 2003; Salkowski *et al.*, 1999); a macrophage derived cytokine mandatory for Th1 differentiation (Murphy & Reiner, 2002). Th1 is further supported by IRF-mediated suppression of IL4 production in Th2 and basophils (Elser *et al.*, 2002; Lohoff *et al.*, 1997; Taki *et al.*, 1997) and activation of APCs (Fantuzzi *et al.*, 2001; Lohoff *et al.*, 2000; Niedbala *et al.*, 2002; Ogasawara *et al.*, 1998; Oppmann *et al.*, 2000). Similarly, *Irf4* is indispensable for a Th2 response (Lohoff *et al.*, 2002; Tominaga *et al.*, 2003). *Irf4* induces intrinsic transcription of Il4 (Hu *et al.*, 2002; Rengarajan *et al.*, 2002) and enhances key transcriptional regulators of Th2 (e.g. GATA3 (Lohoff *et al.*, 2002) and GFI1 (Tominaga *et al.*, 2003)). *Irf4* knockout mice also have abnormal dendritic cell development, potentiating the T-cell defect (Suzuki *et al.*, 2004; Tamura *et al.*, 2005a).

The Irf family also has a prominent role in Th17 commitment. For example, *Irf4* is essential for Th17 differentiation (Brustle *et al.*, 2007; Zhang *et al.*, 2011) by direct transcriptional induction of Il17A and Il21 (Chen *et al.*, 2008; Fanzo *et al.*, 2006). In contrast, *Irf8* is capable of suppressing Th17 differentiation by direct repression of RORγt; the lineage-specific transcription factor of Th17 (Ouyang *et al.*, 2011; Tian *et al.*, 2012). Similar patterns of balanced regulation can be seen in Treg cells. *Irf1* suppresses the production of both nTreg in the thymus and iTreg in the periphery by direct

transcriptional repression of the Foxp3 promoter through a highly conserved Irf binding site (Fragale *et al.*, 2008; Ma *et al.*, 2011). On the contrary, *Irf4* is a direct downstream target of Foxp3 and mediates the immune suppressive effect of Tregs (Zheng *et al.*, 2009). TGF- β1 is known to control the differentiation of both Th17 and Treg (Bettelli *et al.*, 2006; Chen & Wahl, 2002; McGeachy *et al.*, 2009). Previous studies in the palate show *Irf6* as a downstream target to Tgf-β signaling (Le *et al.*, 2012; Xu *et al.*, 2006). However, this interaction has not been tested in T helper subsets.

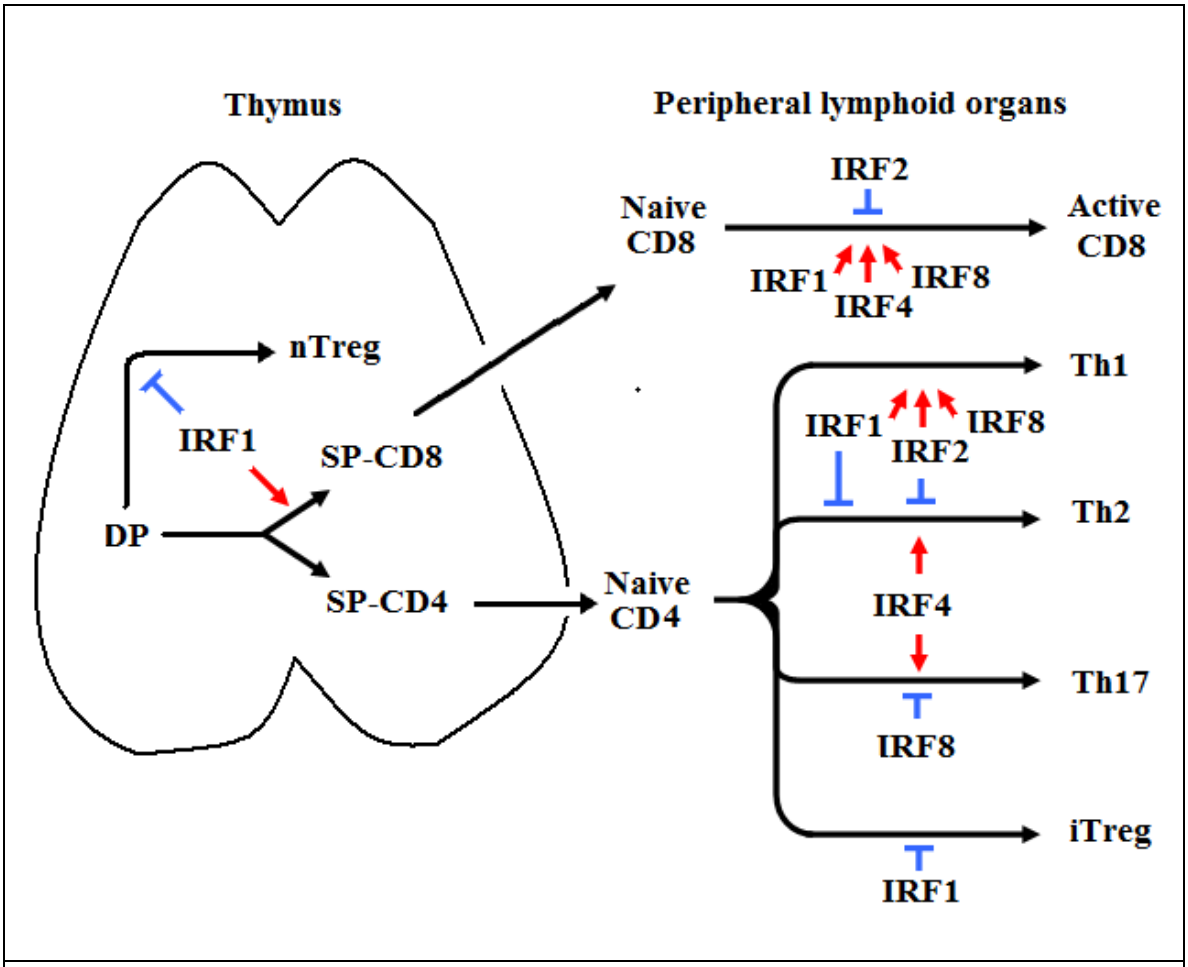

Figure 1.4: **Roles of IRFs in T-cell development and differentiation.** Irfs are required for intra thymic development and functional commitment in peripheral lymphoid organs.

Finally, autoimmune disease association studies have further implicated the IRF family in orchestrating T-cell development and function. Genetic variants in *IRF5*, *IRF7* and *IRF8* are associated to psoriasis, multiple sclerosis and SLE susceptibility (De Jager *et al.*, 2009; Demirci *et al.*, 2007; Gateva *et al.*, 2009; Graham *et al.*, 2006; Harley *et al.*, 2008; Leppa *et al.*, 2011; Patel, 2011; Sanchez *et al.*, 2008). Shitao Li at al 2011 performed a proteomic study to define the candidate protein complexes involved in regulating interferon type I. Interestingly, affinity purification identified protein complex formation between IRF6/IRF5 and IRF6/IRF8, the latter was further confirmed by coimmunoprecipitation in HEK293 cells (Li *et al.*, 2011). Protein-protein interactions between IRF6 and more canonically described immune IRF family members, DNA conservation of the DBD and structural homology with IRF5 strongly suggests a role for Irf6 in the immune system, specifically in T-cell development and functional commitment.

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CHAPTER TWO

Meta-analysis of hematopoietic expression profiles reveals selective Irf6 expression in stem cells and in T-cells

Abstract

The IRF family of transcription factors regulates critical immune functions, including development and differentiation of progenitors and commitment of effecter cells. IRF6 regulates cutaneous, craniofacial and limb development. Here, we used publically available microarray data to uncover a dynamic expression pattern for *Irf6* during hematopoietic development and functional commitment. We found that *Irf6* is expressed early in hematopoiesis, especially in long term hematopoietic stem cells making Irf6 a target for further investigation in long term engraftment studies. Consistent with a role in differentiation, we found abrupt attenuation of Irf6 expression in hematopoietic lineage committed progenitors. Moreover, we observed an age dependent increase of expression in common myeloid progenitor and myeloid erythroid progenitor. Also we identified *Irf6* expression in T cell lineage, including developing and functionally committed stages. Thymic expression of *Irf6* in double positive and single positive thymocytes suggests a possible role in T cell development. We found high Irf6 expression in naïve T cells with persistence in functionally committed T cell subsets except Th1. Considering co-expression and dynamic changes of *Irf6* with other *Irf* family members, this work suggests a complex, interwoven network that regulates T cell lineage. This data further highlights the importance of developing an animal model to study *Irf6* function in hematopoiesis.

Introduction

Haematopoiesis is the process of production of all blood cells from a common pluripotent stem cell. Haematopoietic stem cells (HSC) differentiate into both myeloid and lymphoid lineages. While the myeloid lineages gives rise to erythrocytes, platelets, granulocytes, macrophages, and dendritic cells, the lymphoid lineages gives rise to T-cells, B-cells, and NK-cells. T-cells are the most abundant subset of blood lymphocytes and serve as the core of the adaptive immune response. Changes in T-cell number or function can lead to autoimmune diseases, immune deficiency, inflammatory disorders and cancer (Janeway *et al.*, 2001; Paul, 2008).

The interferon regulatory factor (IRF) family of transcription factors has nine members in mammals (Huang *et al.*, 2010; Nehyba *et al.*, 2009). All family members share a highly conserved N-terminal DNA-binding domain (DBD) and a less conserved C-terminal protein-binding domain (Kondo *et al.*, 2002; Lohoff & Mak, 2005; Tamura *et al.*, 2008). The functions of IRFs in the hematopoietic and immune system are indispensable. IRFs perform their functions by either intrinsic expression in their target cells (Table 2.1) or indirectly by influencing the environment of these cells (Tamura *et al.*, 2008). Intrinsic expression of IRFs are usually constitutive in haematopoietic cells however it can be further induced or activated by external signals (Tamura *et al.*, 2008).

Common and rare *IRF6* variants cause and contribute risk toward craniofacial defects. While rare variants lead to Van der Woude and Popliteal Pterygium Syndrome (Kondo et al., 2002), common *IRF6* variants contribute 12% of orofacial clefting risk (Zucherro et al., 2004). The *Irf6* knockout mouse revealed its role in craniofacial and limb development. Regulation of keratinocyte proliferation and differentiation contributes

to the cutaneous defects. However perinatal mortality hinders our ability to study the role of Irf6 in the haematopoietic system (Ingraham *et al.*, 2006; Richardson *et al.*, 2006; Richardson *et al.*, 2009; Thomason *et al.*, 2010). In 2005, Lohoff and Mak wondered if IRF6 is even expressed by haematopoietic cells (Lohoff & Mak, 2005).

Shitao Li at al 2011 performed a proteomic study to define the candidate protein complexes involved in regulating interferon type I. Interestingly, affinity purification identified protein complex formation between IRF6/IRF5 and IRF6/IRF8, the latter was further confirmed by coimmunoprecipitation in HEK293 cells (Li *et al.*, 2011). Protein-protein interactions between IRF6 and more canonically described immune IRF family members, DNA conservation of the DBD and structural homology with IRF5 strongly suggests a role for Irf6 in the haematopoietic system.

Haematopoietic system is characterized by having different cell lineages each one of them goes through several developmental stages. Many of the developmental stages have rare frequencies and require several markers or cumbersome procedures for isolation. Bench work required for assessment of expression for a given gene throughout the whole haematopoietic system is devastating. In the current era with the explosion of the publically available whole transcriptomic data for almost every cell type, bioinformatic analysis is a very promising approach to replace the classical tedious techniques. In this study, we are trying to profile the expression of Irf6 in different haematopoietic cells to predict the stages likely to be affected by its deficiency. Also we are identifying the pattern of expression of all other family members in the same cell types to identify the possible genetic interactions which are commonly seen between different members of Irf family.

IRF	Intrinsic function in hematopoietic cells
IRF1	* Required for CD8α^{+} (Penninger *et al.*, 1997) and suppress pDCs (Gabriele *et al.*, 2006) * Development of myeloid lineage (Testa *et al.*, 2004) * Required for macrophage functions (Blanco *et al.*, 2000; Brien *et al.*, 2011) * Induction of NK cell-mediated cytotoxicity (Duncan *et al.*, 1996) * Regulation of thymocyte development (Simon *et al.*, 1997) * Induce Th1 differentiation (Kano *et al.*, 2008; Liu *et al.*, 2003; Lohoff *et al.*, 1997; Taki *et al.*, 1997). * Suppress IL4 production by Th2 (Elser *et al.*, 2002) * Suppress Treg cells (Fragale *et al.*, 2008) * Differentiation of CD8+ T cells (Brien *et al.*, 2011)
IRF2	* Self-renewal HSCs (Sato *et al.*, 2009) * Development of epidermal and CD4+ DCs (Ichikawa *et al.*, 2004) * Suppresses basophil expansion (Hida *et al.*, 2005) * Megakaryocytic differentiation (Stellacci *et al.*, 2004) * Regulates macrophage function (Cuesta *et al.*, 2007; Salkowski *et al.*, 1999) * Development of NK cells (Lohoff *et al.*, 2000; Taki *et al.*, 2005) * Regulation of thymocyte development (Simon *et al.*, 1997) * Suppress IL4 production by Th2 (Elser *et al.*, 2002; Zheng *et al.*, 2009) * Possible oncogenic effect in leukemic cells (Passioura *et al.*, 2005) * Suppression of CD8^{+} CTL activity (Hida *et al.*, 2000)

***Table 2.1*: Effects of IRFs intrinsic expression in hematopoietic cells**

Table 2.1 **(cont'd)**

IRF3	* Required for apoptosis of macrophages (Hsu *et al.*, 2004) * Induction of IFN-β in stimulated DCs (Kim *et al.*, 2014; Sakaguchi *et al.*, 2003; Sato *et al.*, 2000) * Prostaglandin E2 production in LPS-primed monocyte (Endo *et al.*, 2014)
IRF4	* Development of $CD11b^{hi}$ $CD8\alpha^{-}$ DCs (Suzuki *et al.*, 2004) and CD4+ DCs (Tamura *et al.*, 2005a) * Induce IL4 production by Th2 (Hu *et al.*, 2002; Lohoff *et al.*, 2002; Rengarajan *et al.*, 2002) * Th17 differentiation (Brustle *et al.*, 2007; Chen *et al.*, 2008; Zhang *et al.*, 2011) * B cell development (Lu *et al.*, 2003; Ma *et al.*, 2006) * Plasma cell differentiation (Klein *et al.*, 2006; Sciammas *et al.*, 2006) * Oncogenic effect in multiple myeloma (Iida *et al.*, 1997; Shaffer *et al.*, 2008) & CLL (Ito *et al.*, 2002; Tsuboi *et al.*, 2000) * Tumor suppressor in early B-cell development (Acquaviva *et al.*, 2008; Pathak *et al.*, 2011) & myeloid transformation (Jo & Ren, 2011)
IRF5	* Production of type I IFNs and IL6 in macrophages after viral infection (Yanai *et al.*, 2007) and pDCs after CpG-A stimulation (Yasuda *et al.*, 2007). * Required in apoptosis in DCs (Couzinet *et al.*, 2008) * Monocytes and B-cells from $Irf5^{-/-}$ mice have an intrinsic defect in their response to pristane-induced lupus (Savitsky *et al.*, 2010; Stone *et al.*, 2012; Yang *et al.*, 2012)

Table 2.1 **(cont'd)**

IRF6	No intrinsic functions identified in hematopoietic cells
IRF7	* Induction of type I IFN in stimulated DCs and macrophage (Honda *et al.*, 2005; Hsieh *et al.*, 2014; Kim *et al.*, 2013; Tamura *et al.*, 2008) * Control monocyte differentiation to macrophage (Lu & Pitha, 2001) * Induction of IL33 in monocytes and macrophages (Sun *et al.*, 2014) * Contradictory effect on antiviral response of CD8 T cells (Gracias *et al.*, 2013; Li *et al.*, 2013; Zhou *et al.*, 2013)
IRF8	* Development of $CD8\alpha^+$ DCs (Schiavoni *et al.*, 2002) and pDCs (Becker *et al.*, 2012; Tamura *et al.*, 2005a) * Development and trafficking of Langerhans cells and dermal DCs (Schiavoni *et al.*, 2004) * Macrophage development (Tamura *et al.*, 2005b) * Suppression of neutrophil production (Becker *et al.*, 2012) * B cell development (Lee *et al.*, 2006; Lu *et al.*, 2003; Ma *et al.*, 2006) * Knock out is associated with CML-like disease (Holtschke *et al.*, 1996) * Suppress Th17 differentiation (Ouyang *et al.*, 2011; Tian *et al.*, 2012).
IRF9	* Regulation of B cell activity and isotype switch in response to self antigen (Thibault *et al.*, 2008) * Modulation of IFN-I and IFN-II responsiveness in macrophages (Farlik *et al.*, 2012; Weiden *et al.*, 2000)

Materials and methods

Gene Expression Omnibus repository (www.ncbi.nlm.nih.gov/geo/), Gene Expression Atlas (www.ebi.ac.uk/gxa/), and the murine haematopoietic data base Blood Express (Miranda-Saavedra *et al.*, 2009) are publically available repositories of microarray experiments. I searched for experiments covering one or more mouse developmental hematopoietic lineages. Raw data of each experiment was analyzed independently using R computing environment (http://www.r-project.org/). The probe intensities were subjected to background correction and quantile normalization using Robust Multi-array Average algorithm (RMA) (Irizarry *et al.*, 2003). Probe numbers for *Irf* genes were identified in the tested microarray platforms using the BioMart ID conversion tool (Kasprzyk, 2011). Relative expression of *Irf* genes was plotted for each experiment. To ensure the reliability of comparison, average expression ranks of *Irf* genes were calculated for every cell type per experiment. To study the co-expression pattern, expression ranks of *Irf* genes in all experiments were pooled and clustered using “gplots” package in R computing environment.

Four Affymatrix (Santa Clara, California, USA) microarray platforms were used in the studied experiments; Murine Genome U74Av2 [MG_U74Av2], Mouse Genome 430 2.0 [Mouse430_2], Mouse Expression 430A [MOE430A], and Mouse Gene 1.0 ST Array [MoGene1]. Studied experiments covered the expression profiles of hematopoietic stem cells (HSC) and its two main subpopulations; long term hematopoietic stem cells (LT-HSC) and short term hematopoietic stem cells (ST-HSC). Several lineage-committed precursors were also compiled, including lymphoid multipotent progenitors (LMPP), common myeloid progenitors (CMP), common lymphoid progenitors (CLP),

megakaryocyte-erythroid progenitor (MEP) and granulocyte monocyte progenitors (GMP). Lymphocyte lineages were heavily covered during early development and after maturation in resting and activated conditions. Early developmental stages included $CD4^+$ $CD8^+$ double negative thymocytes (DN), $CD4^-$ $CD8^-$ double positive thymocytes (DP), B cell-biased lymphoid progenitor (BLP), and pre-ProB cells. Also mature splenic T cells and B cells and nature killer cells (NK) were tested. Resting T cells were identified by being negative for B220 marker and resting B cells were indentified by being negative for CD43 marker. Also naïve and activated T cells with its main subpopulations; T helper and T cytotoxic were analyzed. Functionally committed T helper subpopulations were covered including T-helper 1 (Th1), T-helper 2 (Th2), T-helper 17 (Th17), induced regulatory T cells (iTreg) and natural regulatory T cell (nTreg). BM precursors of myeloid linage were also covered like promyelocytes and myelocytes. Other tested terminally differentiated cells included dendritic cells (DCs), plasmacytoid dendritic cells (pDC), thymic dendritic cells (tDC), monocytes, bone marrow macrophages (BM.MPh), thymic macrophages (tMPh), granulocytes, nucleated erythrocytes (Nu RBCs), and precursor and mature mast cells. Whole thymocytes as well as cortical and modularly thymic epithelial cells (cTEC,mTEC) were included.

Results

Several members of the IRF family of transcription factors are essential for hematopoeisis. However, there are no data on IRF6 because mice that lack Irf6 die shortly after birth from abnormal morphogenesis. As a first step in identifying a potential function for IRF6 in hematopoeisis, we performed a meta analysis on gene expression profiles in murine hematopoietic tissues. We identified 20 such studies (Table 2.2) that included 241 microarrays from 50 unique hematopoietic cell types.

Publication	**lineages**	**Affymetrix Chip**
(Chambers *et al.*, 2007)	LT-HSC, NK, Naïve & activated CD4 & CD8 T-cells, B-cells, Monocytes, Granulocytes, Nu. Erythrocytes	Mouse Genome 430-2
(Holwerda *et al.*, 2013)	Resting and activated T cells and B cell	Mouse Gene 1.0 ST
(Derbinski *et al.*, 2005)	Thymic stromal cells	Murine Genome U74Av2
(Dudziak *et al.*, 2007)	DC, B cells, CD4 T cells. CD8 T cells	Mouse Genome 430-2
(Lin *et al.*, 2014)	Th1,Th2, Th17	Mouse Gene 1.0 ST
(Ficara *et al.*, 2008)	LT-HSC, ST-HSC	Mouse Genome 430-2
(Haddon *et al.*, 2009)	mast cell precursors and mature mast cells	Mouse Expression 430A

Table 2.2: **Mouse expression profiles for hematopoietic cell types**.

Table 2.2 **(Cont'd)**

(Mansson *et al.*, 2007)	HSC	Mouse Genome 430-2
(Robbins *et al.*, 2008)	DCs, NK, B cells, CD8 T cells	Mouse Genome 430-2
(Rodriguez *et al.*, 2007)	Th1 vs Th2	Mouse Genome 430-2
(Tothova *et al.*, 2007)	HSC, MLP	Mouse Genome 430-2
(Venkatraman *et al.*, 2013)	LT-HSC, ST-HSC, MPP	Mouse Genome 430-2
(Vigano *et al.*, 2013)	HSC, pro Tcells, DP T cells	Mouse Gene 1.0 ST
(Wang *et al.*, 2010)	HSC vs CMP and GMP	Mouse Genome 430-2
(Wei *et al.*, 2009)	Naïve T-helper, Th1, Th2, Th17, iTreg, nTreg	Mouse Genome 430-2
(Weischenfeldt *et al.*, 2008)	macrophage vs T-cells	Mouse Genome 430-2
(Beerman *et al.*, 2014)	LT-HSC,ST-HSC, LMPP, CMP, CLP, GMP, MEP, BLP, pre-ProB	Mouse Genome 430-2
(Wong *et al.*, 2014)	HSC, Promyelocytes, Myelocytes, Granulocytes	Mouse Gene 1.0 ST
(Egawa & Littman, 2011)	DN, DP, CD4SP, CD8SP	Mouse Genome 430-2
(Kawazu *et al.*, 2007)	DN cells	Mouse Genome 430-2

Plotting of non-logarithmic normalized data for each study allowed us to compare the level of expression for all 9 *Irf* genes in 50 mouse hematopoietic cells types (Supplementary Figures A1 - A12). However, testing the reproducibility across experiments, following the expression changes along the haematopoietic tree, and prediction of possible family member interactions required a more integrative approach. To facilitate inter-experimental comparison of expression of *Irf* genes, we calculated and ranked the relative expression for each *Irf* gene in each cell type per experiment (Supplementary table A1). We then performed cluster analysis for the average rank expression of *Irf* genes (figure 2.1). We observed two main patterns of differential expression for family members across the studied cell lineages. First, *Irf1*, *Irf3* and *Irf9* have consistently high expression levels in most studied cell lineages with average rank expressions of 93, 83 and 84, respectively. All other family members showed more variable expression among different cell types. *Irf6* was expressed mainly in two stages of hematopoietic development. Early in development, *Irf6* was expressed in HSC and their immediate downstream progeny LMPP. *Irf6* expression was maintained in CLP with apparent down regulation in all myeloid capable progenitors (CMP, MEP, and GMP). However, suppression of *Irf6* expression in CMP and MEP was lost in progenitors obtained from 2 year-old mice (figure 2.2). Regulation of *Irf6* expression was also observed in development of T cells. In two out of three studies, *Irf6* expression was barely detectable in early developing DN thymocytes. We found an increase of Irf6 expression in DP thymocytes and even more in single positive CD4 and CD8 progeny. Expression of *Irf6* peaked to exceed 80% in naïve T-helper and cytotoxic populations. No changes in *Irf6* expression were seen in activated T-helper cells, however the rank

expression profile lost about 10 percentiles in activated T-cytotoxic cells. Terminally differentiated T-helper sub-populations maintained a 63-71% expression rank except TH-1, where *Irf6* rank of expression went down to the 30th percentile for 2 out of 3 ranked studies. Finally, the thymic-derived natural Treg came at the 79th percentile on the rank of expression (Figure 2.3).

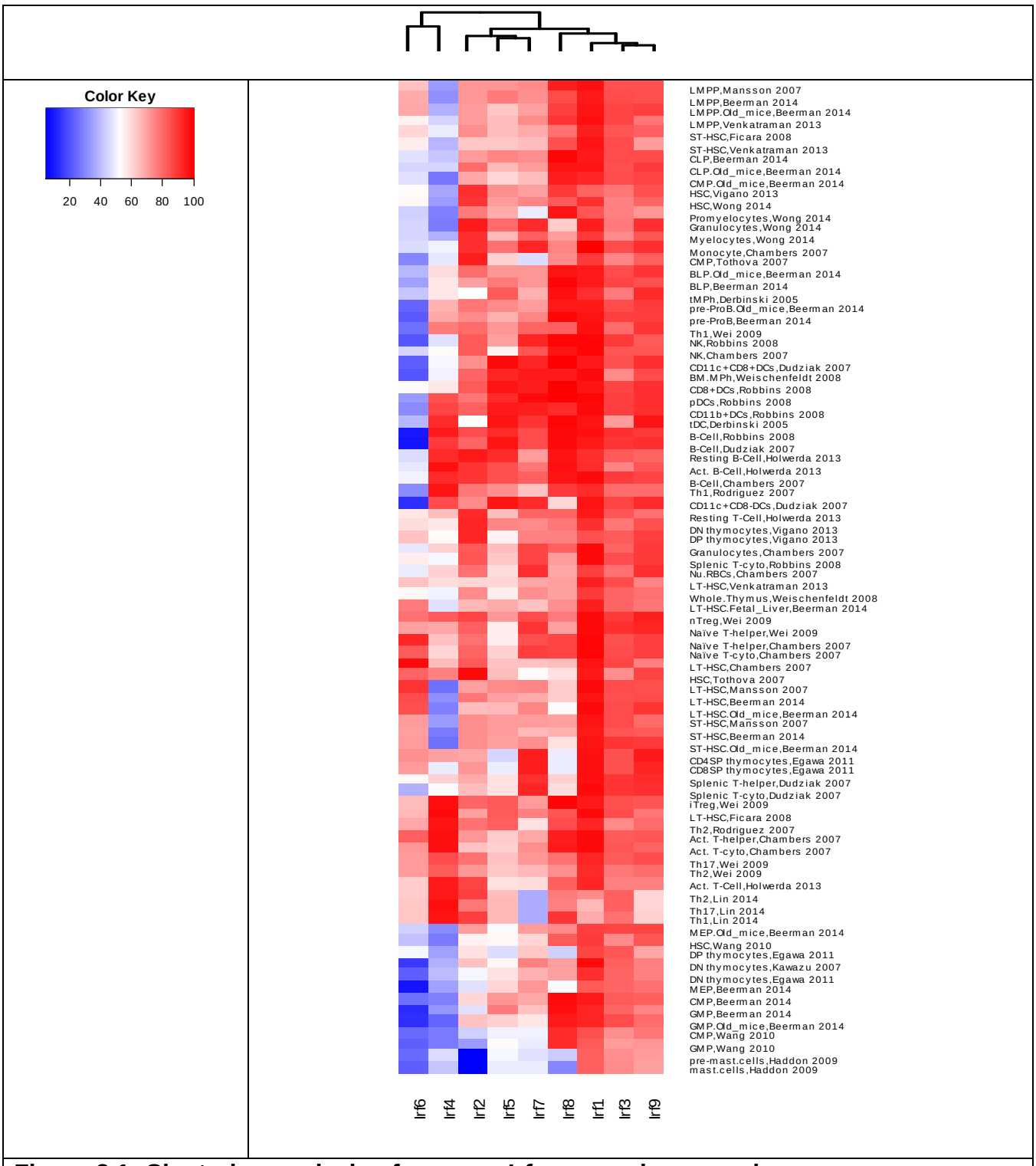

Figure 2.1: Clustering analysis of average *Irf* gene rank expression.

Figure 2.1 (Cont'd): *Irf1*, *Irf3*, and *Irf9* show non-selective high rank of expression in most haematopoietic cell types. The other family members have high variability between cell types, especially *Irf6* and *Irf4*. *Irf6* expression is almost exclusively in early haematopoietic progenitors and T cell developmental and functional cells.

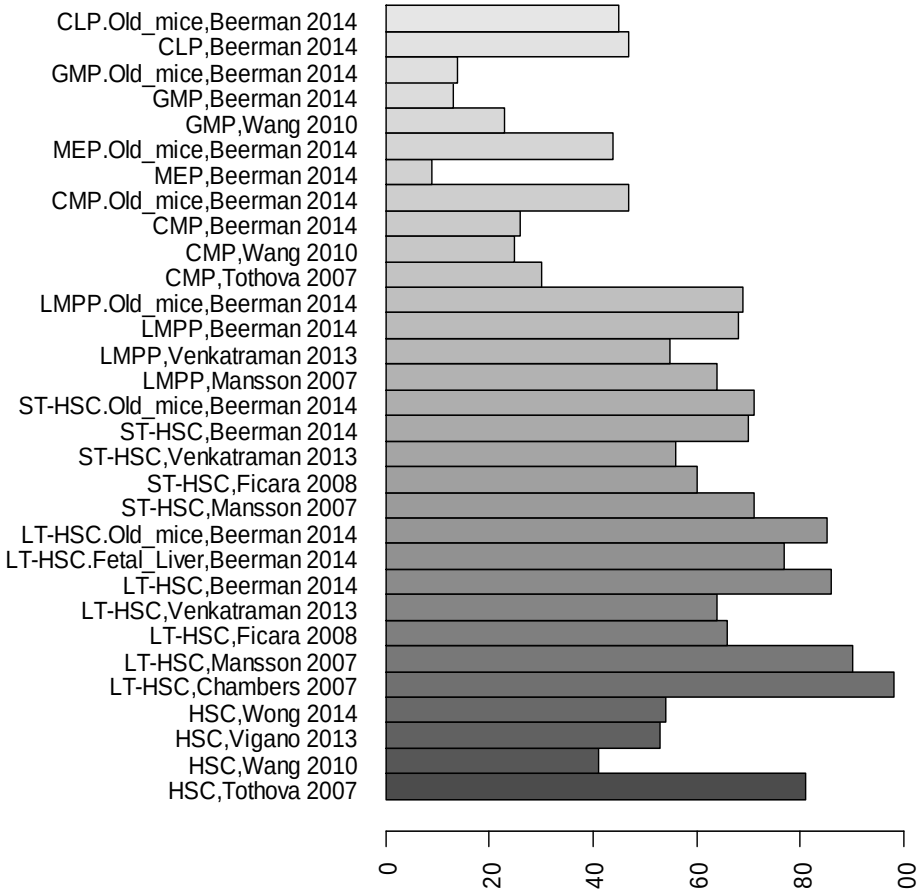

Figure 2.2: Average *Irf6* rank expression in early hematopoietic progenitors. Every bar represents the *Irf6* rank of expression in a given microarray. The horizontal axis represents the percentile rank while the vertical axis shows the names of the studied cell type appended to the names of first author and year of publication. Cells are arranged from the most immature (at the origin of the figure) to the mature stages.

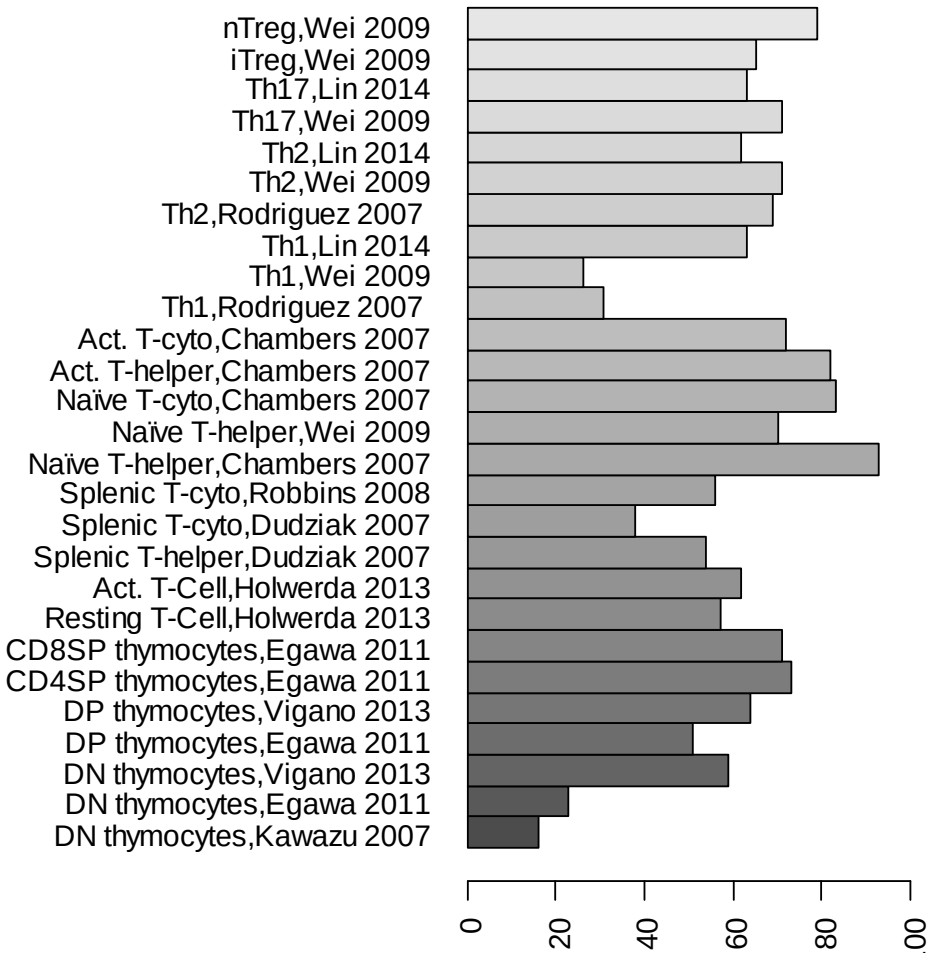

Figure 2.3: Average *Irf6* rank expression in T cell stages. Every bar represents the *Irf6* rank of expression in a given microarray. The horizontal axis represents the percentile rank while the vertical axis shows the names of the studied cell type appended to the names of first author and year of publication. Cells are arranged from the most immature (at the origin of the figure) to the mature stages.

To assess a possible bias of probes across arrays, biological replicates done using different microarray platforms were identified. Average rank expression was calculated for each platform per gene. Paired t-test analysis showed significant bias for *Irf1* and *Irf2* but insignificant changes for the other *Irf* genes (Table 2.3).

Irf1	**Mouse 430-2**	**Mo-Gene1**	**Irf2**	**Mouse 430-2**	**Mo-Gene1**	**Irf3**	**Mouse 430-2**	**Mo-Gene1**
HSC	92	86	HSC	76	90	HSC	73	77
DN	95	91	DN	57	93	DN	81	78
DP	87	85	DP	58	93	DP	84	83
Th1	95	68	Th1	79	88	Th1	80	79
Th2	93	74	Th2	76	88	Th2	76	82
Th17	93	66	Th17	79	78	Th17	83	82
p-value = 0.030*			**p-value =** 0.034*			**p-value =** 0.661		
Irf4	**Mouse 430-2**	**Mo-Gene1**	**Irf5**	**Mouse 430-2**	**Mo-Gene1**	**Irf6**	**Mouse 430-2**	**Mo-Gene1**
HSC	52	35	HSC	59	72	HSC	61	53
DN	39	57	DN	56	75	DN	19	59
DP	35	53	DP	46	55	DP	51	64
Th1	86	96	Th1	73	66	Th1	29	63
Th2	90	95	Th2	72	65	Th2	70	62
Th17	86	97	Th17	64	66	Th17	71	63
p-value = 0.217			**p-value =** 0.319			**p-value =** 0.298		
Irf7	**Mouse 430-2**	**Mo-Gene1**	**Irf8**	**Mouse 430-2**	**Mo-Gene1**	**Irf9**	**Mouse 430-2**	**Mo-Gene1**
HSC	56	72	HSC	70	86	HSC	85	83
DN	71	74	DN	70	78	DN	76	85
DP	63	76	DP	44	76	DP	69	88
Th1	72	37	Th1	86	90	Th1	85	61
Th2	62	37	Th2	79	77	Th2	80	60
Th17	72	37	Th17	79	76	Th17	86	60
p-value = 0.330			**p-value =** 0.149			**p-value =** 0.384		
Table 2.3: **Average rank expression for microarray platforms per gene**								

Discussion

In this study, we uncovered a novel expression pattern for *Irf6* in murine hematopoietic cell lineages. Our results showed that *Irf6* expression is selectively expressed in hematopoietic cell progenitors and terminally differentiated cells. We found that hematopoietic stem cells and T cells are the two main developmental windows where *Irf6* expression was evident.

Highest expression of *Irf6* is seen in LT-HSC which requires further investigation for a possible role of IRF6 in long term BM engraftment. Interferons directly stimulate HSC proliferation and differentiation (Schuettpelz & Link, 2013). Intrinsic expression of *Irf2* was shown to preserve the self-renewal and multilineage differentiation capacity of HSCs by suppression of INF-I signaling (Sato *et al.*, 2009). Our meta-analysis showed that expression of both *Irf2* and *Irf6* were very comparable in HSCs and Irf6 was even higher in the long term compartment. Loss of *Irf6* expression in more differentiated myeloid progenitors with persistent expression in CLP suggested an important role in the lineage commitment of these critical progenitor cells (Figure 2.1). Regulation of early lineage commitment is one of the documented functions in the family. For example, Irf8 promotes DC lineage commitment over neutrophil production in myeloid progenitors (Becker *et al.*, 2012) while Irf4 favors macrophage commitment (Yamamoto *et al.*, 2011). Recently, age dependent increase of activated Irf3 was shown to promote the inflammatory response in aging kidney cells (Xi *et al.*, 2014). The relative increase of *Irf6* in CMP and MEP obtained from old mice might have a similar effect on these progenitors.

In T cells, the expression was evident during thymic development, T-cell activation and terminal functional commitment (Figure 2.2). A possible role of *Irf6* in T cell development can be predicted from intrinsic expression in evolving thymocytes as well as expression in cortical and medullary thymic epithelium. Persistent *Irf6* expression in activated and functionally committed T cell subsets except Th1 suggests a potential role for *Irf6* in functional commitment and terminal differentiation of T cells.

Variability of expression in the same cell type among different experiments was sometimes striking. Two obvious examples were *Irf6* expression in DN thymocytes and Th1 cells, where 2 out of 3 experiments show very low rank of expression (< 30%) while 3rd experiment is uniquely high (> 60%). One possible explanation is a difference between probe sensitivities across the platforms. However comparing all replicates using the two commonly used platforms (Mouse430_2 & MoGene1), we failed to observe a consistent bias. Another possible explanation is that DN cells are a rare population and can be easily contaminated by thymocytes with low expression of CD4 and CD8 (Lucas & Germain, 1996). Finally, induction of Th1 cell *in vitro* is difficult without contamination from other functional subsets. These biological obstacles may explain the high variability we see in our analysis. Non-linear noise signature of microarray experiments is another important factor that always should be considered in these meta-analysis studies (Chen *et al.*, 2011; Leek *et al.*, 2010).

Publically available expression profile data sets are an underutilized tool that can help researchers to understand the expression pattern of their target genes as well as possible interactions with other co-expressed genes. Transcriptional expression of *Irf* members is constitutive and IFN-inducible in most targets tissues (Tamura *et al.*, 2008).

IRFs are considered master regulators of hematopoietic development and differentiation. Until now, the expression pattern of *Irf6* in haematopoiesis has not been studied. Also, perinatal lethality of *Irf6* knockout mouse hindered efforts to study its function in haematopoiesis. We found that *Irf6* expression is selectively expressed early HSC and in various T-cell lineages, including Th1, Th2 and Th17. This work strongly supports a regulatory function of *Irf6* in haematopoiesis.

APPENDIX

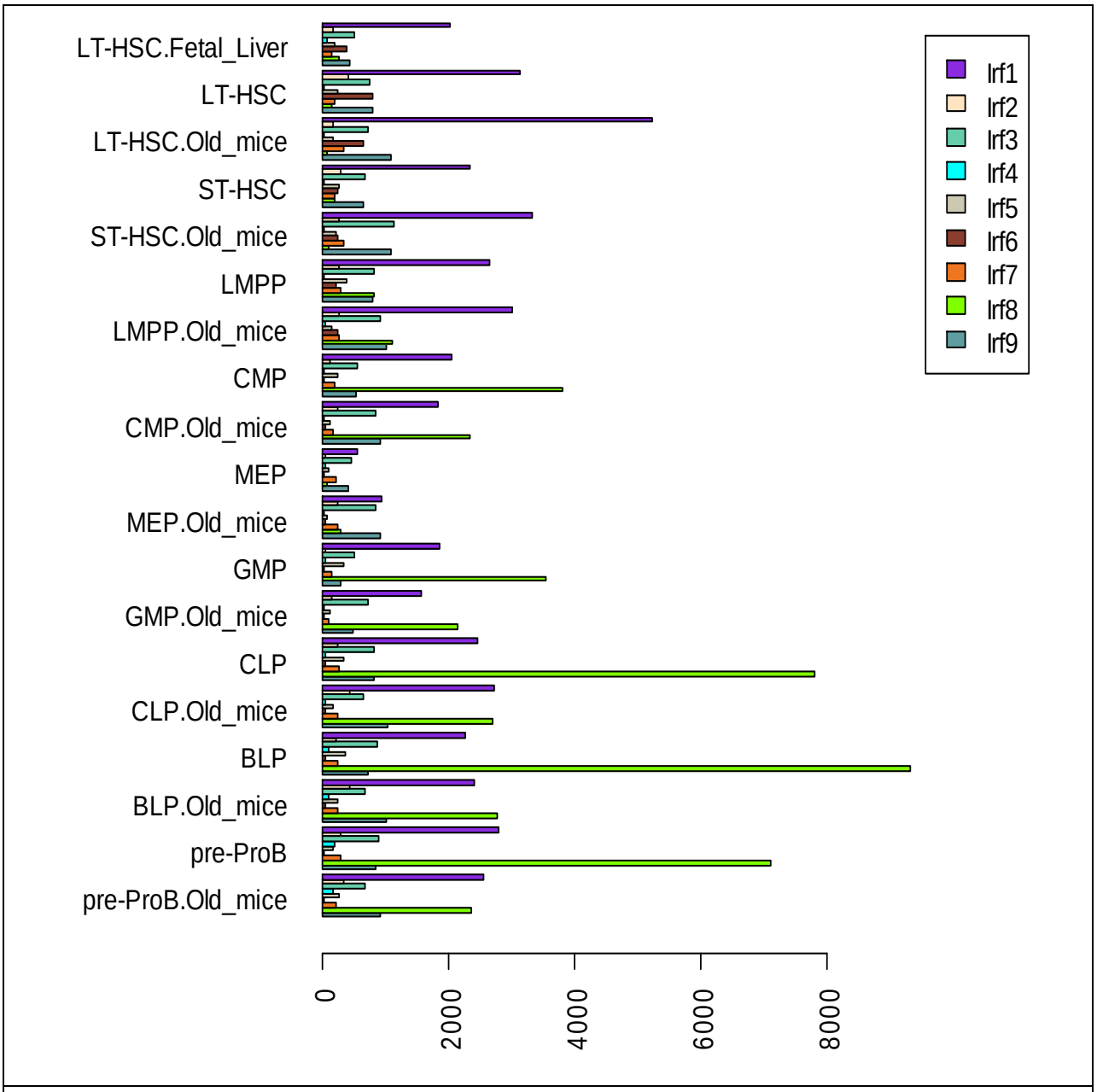

Figure A1: **Expression of *Irf* genes in immature hematopoietic progenitors at 3 months and 2 years of mouse age**. Expression data obtained from Beerman et al (2014).

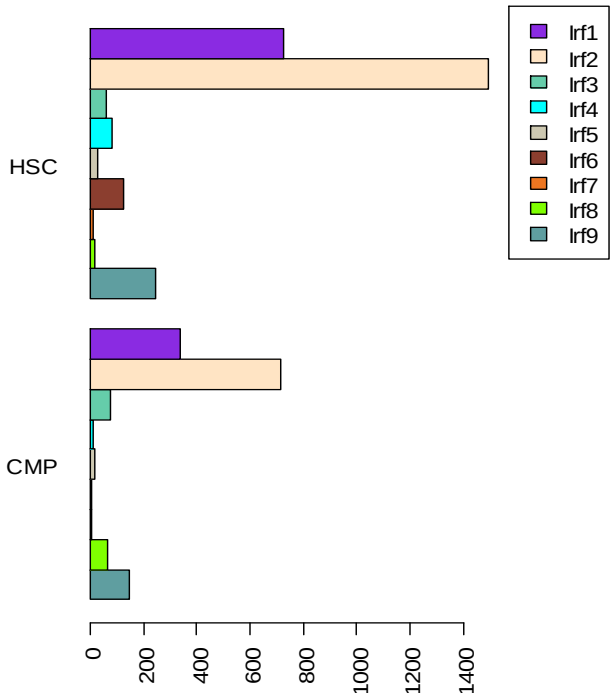

Figure A2a: **Expression of *Irf* genes in immature haematopoietic progenitors**. Expression data obtained from Tothova et al 2007.

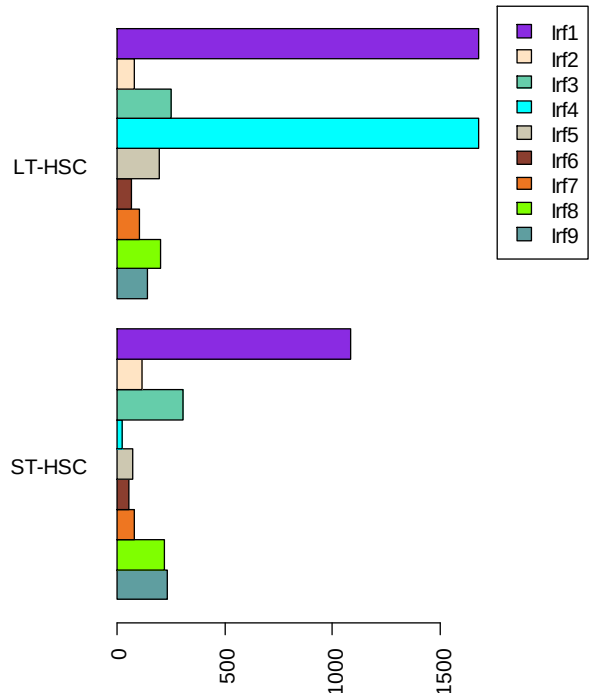

Figure A2b: **Expression of *Irf* genes in immature haematopoietic progenitors**. Expression data obtained from Ficara et al 2008.

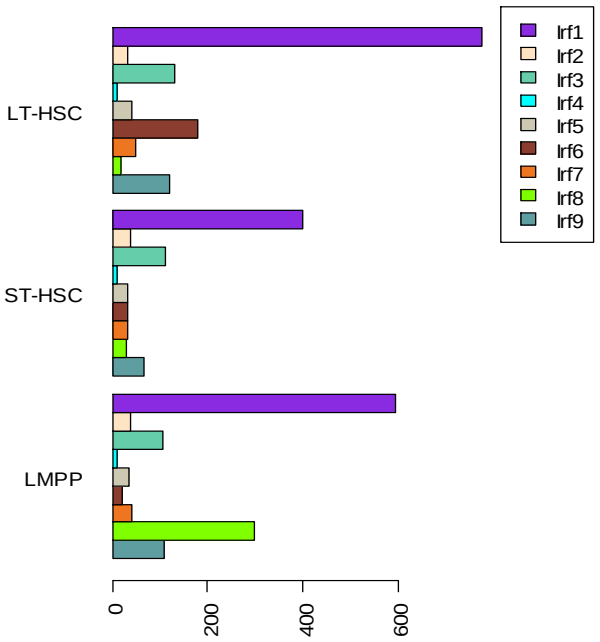

Figure A2c: **Expression of *Irf* genes in immature haematopoietic progenitors**. Expression data obtained from Mansson et al 2007.

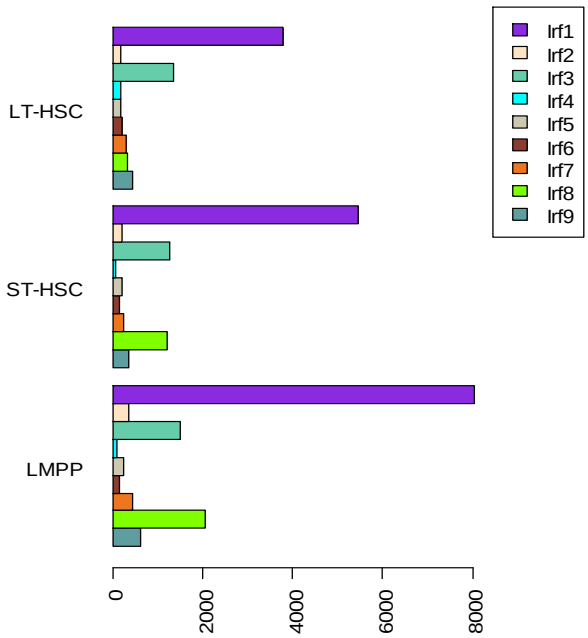

Figure A2d: **Expression of *Irf* genes in immature haematopoietic progenitors**. Expression data obtained from Venkatraman et al 2013.

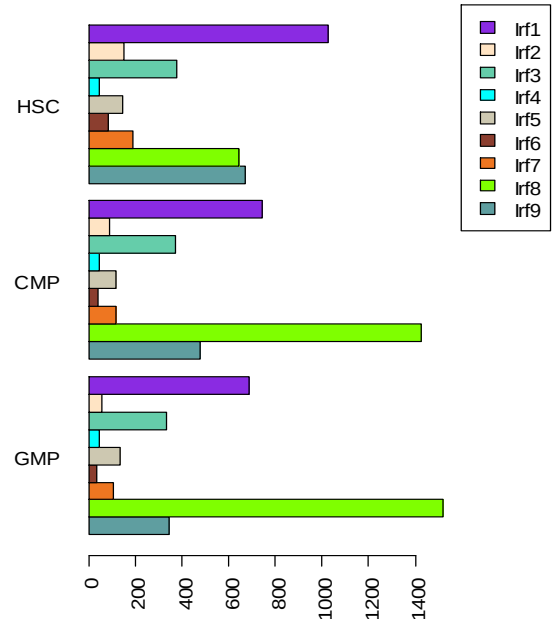

Figure A3: **Expression of *Irf* genes in early myeloid development**. Expression data obtained from Wang et al 2010.

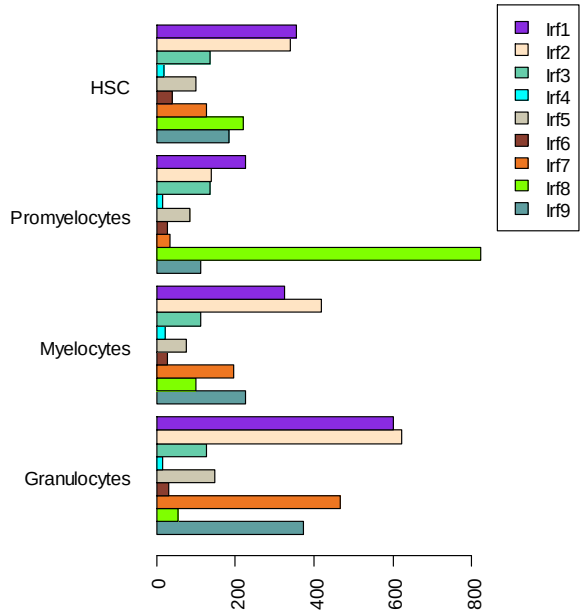

Figure A4: **Expression of *Irf* genes during granulocytes development**. Expression data obtained from Wong et al 2014.

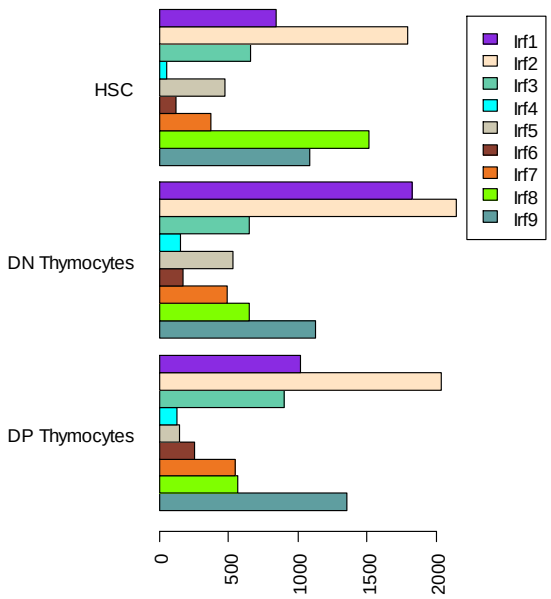

Figure A5a: **Expression of *Irf* genes in thymocyte development**. Expression data obtained from Vigano et al 2013.

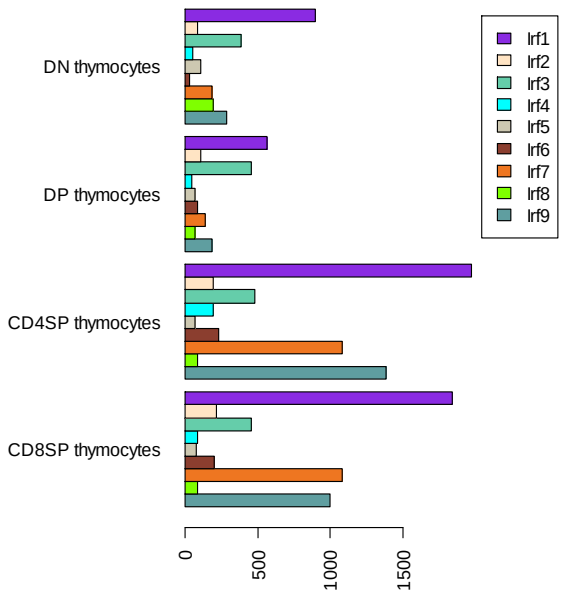

Figure A5b: **Expression of *Irf* genes in thymocyte development**. Expression data obtained from Egawa et al 2011.

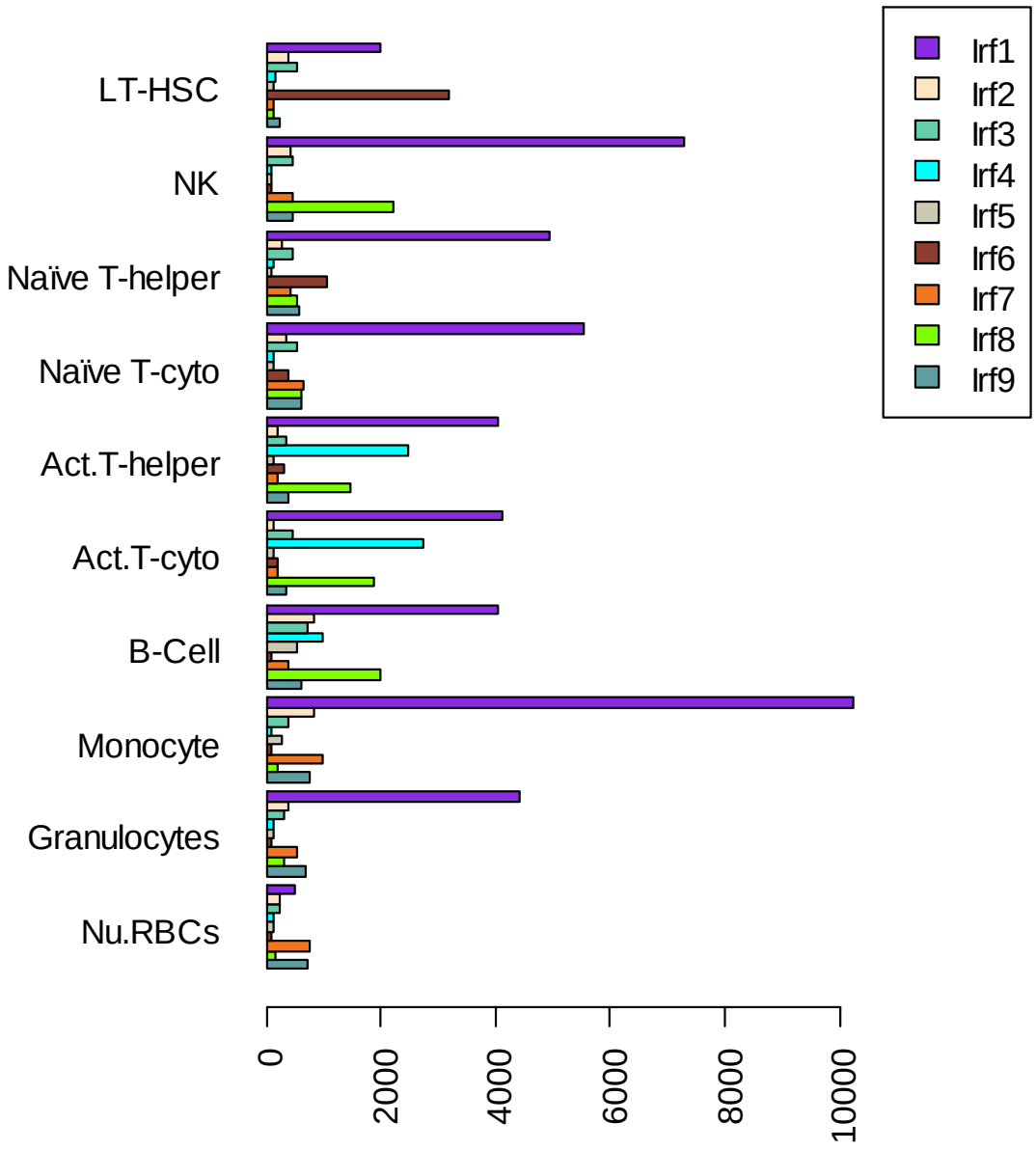

Figure A6: **Expression of *Irf* genes in LT-HSC versus terminally differentiated haematopoietic cells**. Expression data obtained from Chambers et al 2007.

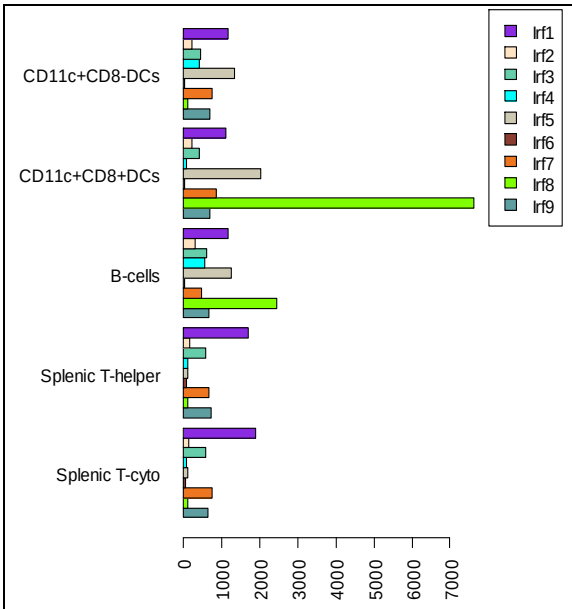

Figure A7a: **Expression of *Irf* genes in lymphocytes and DCs**. Expression data obtained from Dudziak et al 2007.

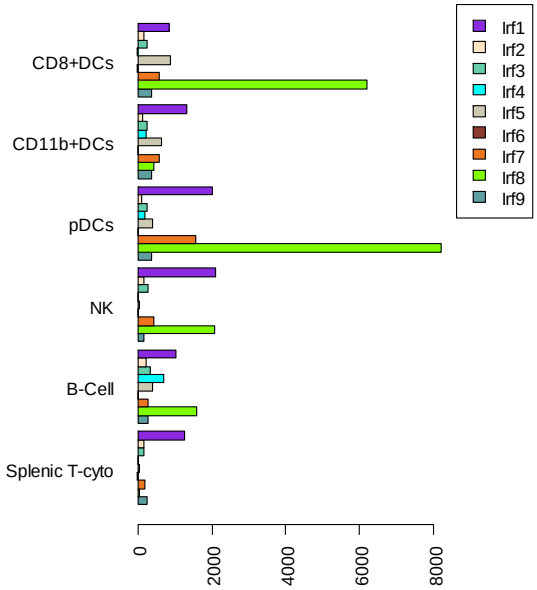

Figure A7b: **Expression of *Irf* genes in lymphocytes and DCs**. Expression data obtained from Robbins et al 2008.

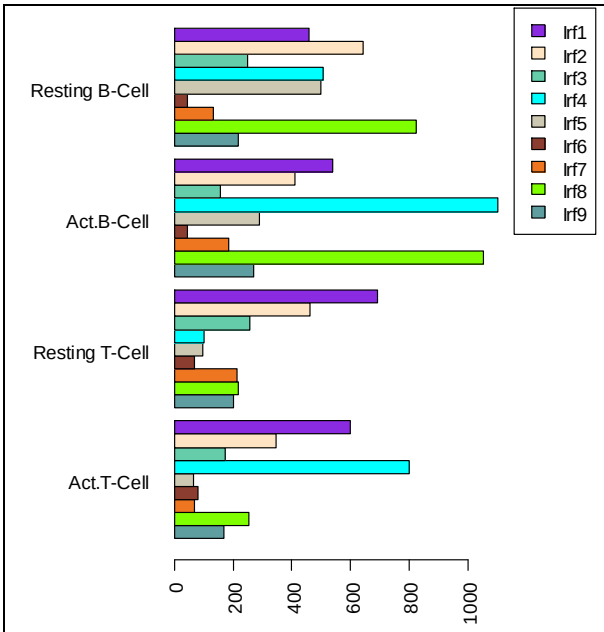

Figure A8: **Expression of *Irf* genes in resting and activated lymphocytes**. Expression data obtained from Holwerda et al 2013.

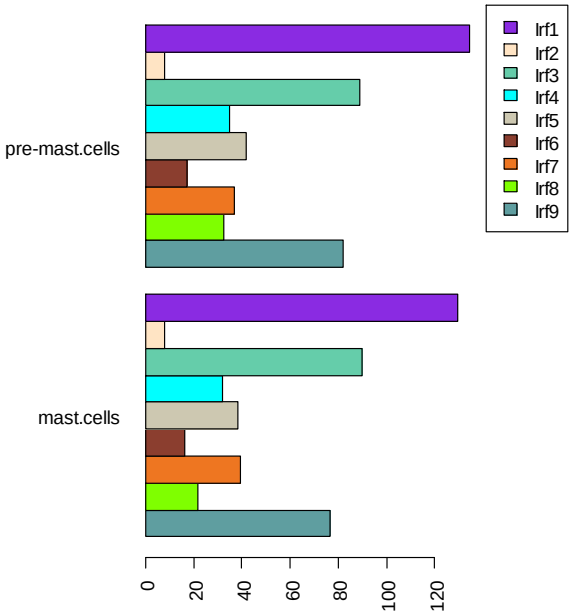

Figure A9: **Expression of *Irf* genes in mast cells and pre-mast cells**. Expression data obtained from Haddon et al 2009.

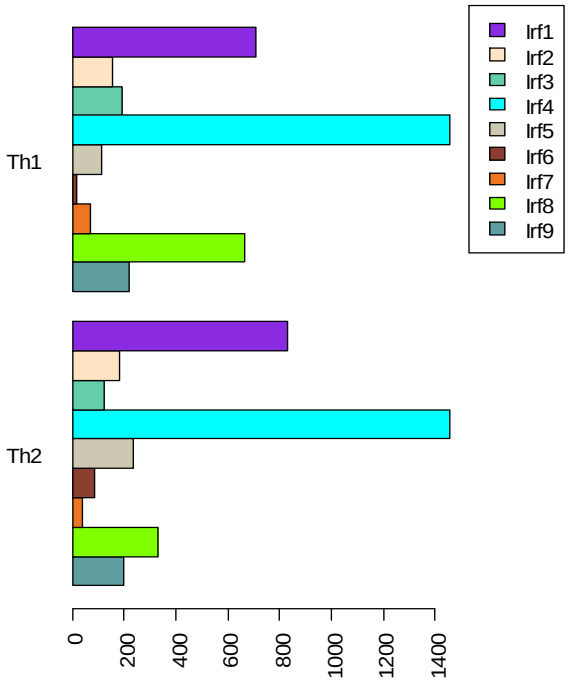

Figure A10a: **Expression of *Irf* genes in T-helper subsets**. Expression data obtained from Rodriguez et al 2007

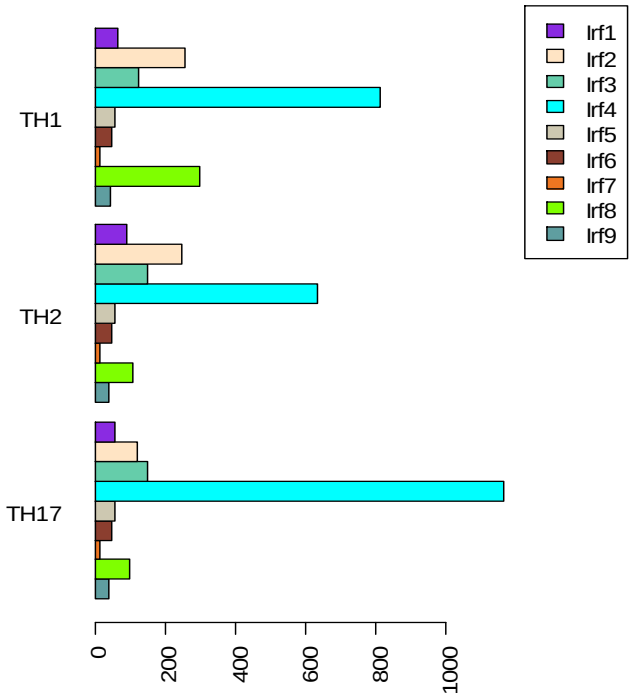

Figure A10b: **Expression of *Irf* genes in T-helper subsets**. Expression data obtained from Lin et al 2014.

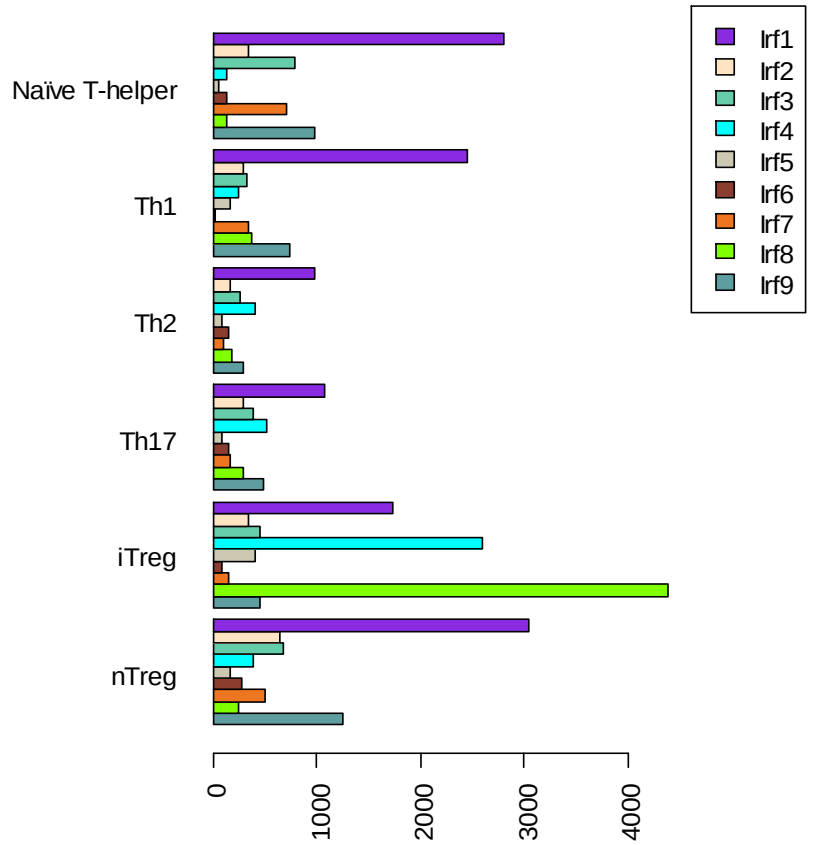

Figure A10c: **Expression of *Irf* genes in T-helper subsets**. Expression data obtained from Wei et al 2009.

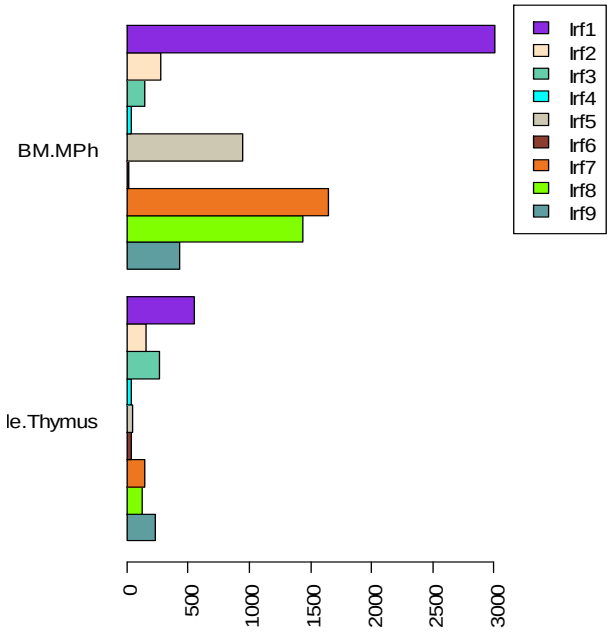

Figure A11: **Expression of *Irf* genes in BM macrophage and whole thymus suspension.** Expression data obtained from Weischenfeldt et al 2008.

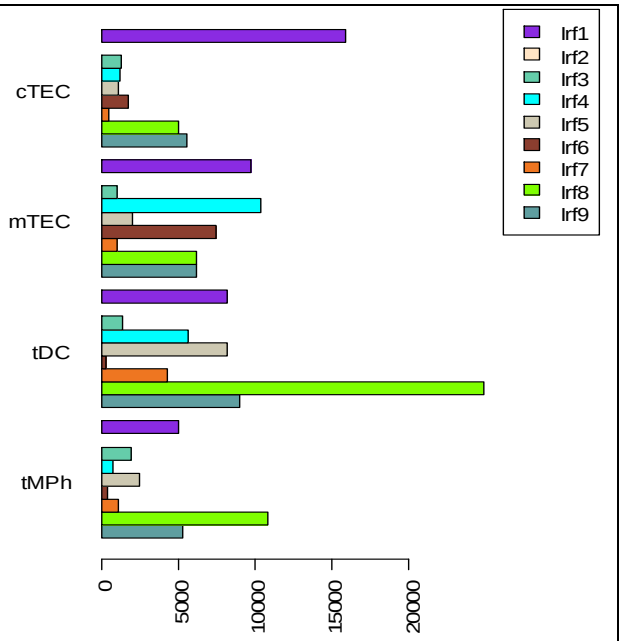

Figure A12: **Expression of *Irf* genes in non-lymphocyte thymic cells**. Expression data obtained from Derbinski et al 2005 (No probes for Irf2).

Author and year	Chip type	Cell lineage	Irf1	Irf2	Irf3	Irf4	Irf5	Irf6	Irf7	Irf8	Irf9
Tothova et al 2007	Mouse430_2	HSC	95	98	73	76	65	81	53	58	87
Wang et al 2010	Mouse430_2	HSC	89	55	74	28	54	41	60	83	84
Vigano et al 2013	MoGene1	HSC	81	91	78	36	73	53	70	89	85
Wong et al 2014	MoGene1	HSC	91	90	76	34	71	54	75	83	81
Chambers et al 2007	Mouse430_2	LT-HSC	96	83	87	65	64	98	64	65	76
Mansson et al 2007	Mouse430_2	LT-HSC	98	70	86	26	74	90	75	62	85
Ficara et al 2008	Mouse430_2	LT-HSC	98	70	86	98	83	66	76	84	78
Venkatraman et al 2013	Mouse430_2	LT-HSC	94	60	86	59	60	64	69	70	75
Beerman et al 2014	Mouse430_2	LT-HSC	96	78	85	32	70	86	68	62	86
Beerman et al 2014	Mouse430_2	LT-HSC (Fetal Liver)	94	66	81	47	68	77	64	73	78
Beerman et al 2014	Mouse430_2	LT-HSC (Old mice)	98	65	86	29	66	85	75	53	90
Mansson et al 2007	Mouse430_2	ST-HSC	96	73	86	34	71	71	71	70	80
Ficara et al 2008	Mouse430_2	ST-HSC	94	73	84	49	65	60	68	79	82
Venkatraman et al 2013	Mouse430_2	ST-HSC	96	63	86	39	63	56	65	85	71
Beerman et al 2014	Mouse430_2	ST-HSC	95	73	84	28	71	70	66	67	83
Beerman et al 2014	Mouse430_2	ST-HSC (Old mice)	96	73	90	26	70	71	73	58	89
Mansson et al 2007	Mouse430_2	LMPP	97	72	85	34	72	64	74	94	85
Venkatraman et al 2013	Mouse430_2	LMPP	97	71	87	45	65	55	74	90	78
Beerman et al 2014	Mouse430_2	LMPP	95	72	86	32	77	68	73	86	85
Beerman et al 2014	Mouse430_2	LMPP (Old mice)	96	71	87	38	63	69	70	88	88
Tothova et al 2007	Mouse430_2	CMP	89	94	75	48	61	30	46	74	82

Table A1: **Average rank of IRFs expression for each cell type per experiment**

Table A1 **(Cont'd)**

Wang et al 2010	Mouse430_2	CMP	85	44	73	28	50	25	50	92	78
Beerman et al 2014	Mouse430_2	CMP	95	60	83	29	72	26	69	98	82
Beerman et al 2014	Mouse430_2	CMP (Old mice)	93	69	86	27	60	47	65	94	87
Beerman et al 2014	Mouse430_2	MEP	83	47	81	35	60	9	72	52	79
Beerman et al 2014	Mouse430_2	MEP (Old mice)	88	71	87	31	52	44	71	74	87
Wang et al 2010	Mouse430_2	GMP	83	34	71	28	53	23	49	92	72
Beerman et al 2014	Mouse430_2	GMP	93	47	81	33	77	13	64	97	75
Beerman et al 2014	Mouse430_2	GMP (Old mice)	93	64	86	24	61	14	57	95	80
Beerman et al 2014	Mouse430_2	CLP	95	71	86	42	75	47	73	99	86
Beerman et al 2014	Mouse430_2	CLP (Old mice)	96	80	85	45	66	45	71	96	89
Beerman et al 2014	Mouse430_2	BLP	95	70	87	57	77	35	72	99	85
Beerman et al 2014	Mouse430_2	BLP (Old mice)	95	80	86	59	72	39	72	96	90
Beerman et al 2014	Mouse430_2	pre-ProB	96	74	88	69	67	22	75	99	88
Beerman et al 2014	Mouse430_2	pre-ProB (Old mice)	95	78	86	67	74	24	71	95	89
Chambers et al 2007	Mouse430_2	B-Cell	98	90	89	92	85	50	82	96	87
Dudziak et al 2007	Mouse430_2	B-Cell	95	81	90	89	96	9	86	98	91
Robbins et al 2008	Mouse430_2	B-Cell	97	86	91	95	92	10	86	98	89
Holwerda et al 2013	MoGene1	Resting B-Cell	91	95	83	92	92	46	71	96	81
Holwerda et al 2013	MoGene1	Activated B-Cell	92	90	75	97	85	48	78	97	84
Weischenfeldt et al 2008	Mouse430_2	Whole Thymus	89	74	81	50	56	54	73	70	79
Derbinski et al 2005	MG_U74Av2	cTEC	98	NA	70	68	67	76	47	91	92
Derbinski et al 2005	MG_U74Av2	mTEC	97	NA	64	97	80	95	65	93	94

Table A1 **(Cont'd)**

Kawazu et al 2007	Mouse430_2	DN thymocytes	98	64	82	38	54	16	76	70	76
Egawa et al 2011	Mouse430_2	DN thymocytes	91	51	81	40	58	23	67	70	76
Vigano et al 2013	MoGene1	DN thymocytes	91	93	78	57	75	59	74	78	85
Egawa et al 2011	Mouse430_2	DP thymocytes	87	58	84	35	46	51	63	44	69
Vigano et al 2013	MoGene1	DP thymocytes	85	93	83	53	55	64	76	76	88
Egawa et al 2011	Mouse430_2	CD4SP thymocytes	97	69	85	70	45	73	94	49	95
Egawa et al 2011	Mouse430_2	CD8SP thymocytes	97	72	85	49	49	71	94	49	93
Holwerda et al 2013	MoGene1	Resting T-Cell	95	92	84	65	65	57	81	81	79
Holwerda et al 2013	MoGene1	Activated T-Cell	93	87	76	95	58	62	59	82	76
Dudziak et al 2007	Mouse430_2	Splenic T-helper	97	68	90	61	58	54	91	61	92
Dudziak et al 2007	Mouse430_2	Splenic T-cytotoxic	98	65	90	52	58	38	93	59	91
Robbins et al 2008	Mouse430_2	Splenic T-cytotxic	98	84	86	51	63	56	87	71	89
Chambers et al 2007	Mouse430_2	Naïve T-helper	99	79	85	64	56	93	85	87	88
Wei et al 2009	Mouse430_2	Naïve T-helper	98	82	91	69	56	70	90	70	93
Chambers et al 2007	Mouse430_2	Naïve T-cytotoxic	99	81	86	60	61	83	88	87	88
Chambers et al 2007	Mouse430_2	Activated T-helper	98	72	83	97	62	82	69	94	84
Chambers et al 2007	Mouse430_2	Activated T-cytotoxic	98	64	84	97	61	72	73	95	81
Rodriguez et al 2007	Mouse430_2	Th1	92	78	80	96	73	31	64	89	80
Wei et al 2009	Mouse430_2	Th1	97	80	80	77	72	26	81	82	90
Lin et al 2014	MoGene1	Th1	68	88	79	96	66	63	37	90	61
Rodriguez et al 2007	Mouse430_2	Th2	93	79	74	96	82	69	58	86	80
Wei et al 2009	Mouse430_2	Th2	92	72	78	83	63	71	66	73	80
Lin et al 2014	MoGene1	Th2	74	88	82	95	65	62	37	77	60

Table A1 **(Cont'd)**

Wei et al 2009	Mouse430_2	Th17	93	79	83	86	64	71	72	79	86
Lin et al 2014	MoGene1	Th17	66	78	82	97	66	63	37	76	60
Wei et al 2009	Mouse430_2	iTreg	95	81	85	97	83	65	71	99	84
Wei et al 2009	Mouse430_2	nTreg	98	87	89	83	72	79	86	77	94
Chambers et al 2007	Mouse430_2	NK	99	83	84	53	55	45	84	96	84
Robbins et al 2008	Mouse430_2	NK	99	83	89	47	70	21	93	99	83
Dudziak et al 2007	Mouse430_2	CD11c+ CD8-DCs	96	74	87	86	96	14	92	60	92
Dudziak et al 2007	Mouse430_2	CD11c+ CD8+DCs	95	73	85	51	98	23	93	100	91
Robbins et al 2008	Mouse430_2	CD11b+DCs	98	82	88	87	95	31	94	92	91
Robbins et al 2008	Mouse430_2	CD8+DCs	96	83	87	57	96	53	94	100	91
Robbins et al 2008	Mouse430_2	pDCs	99	78	88	85	92	34	98	100	91
Derbinski et al 2005	MG_U74Av2	tDC	96	NA	71	92	96	39	90	99	96
Wong et al 2014	MoGene1	Pro-myelocytes	84	77	76	29	68	44	49	96	72
Wong et al 2014	MoGene1	Myelocytes	89	91	74	38	66	45	82	71	84
Weischenfeldt et al 2008	Mouse430_2	BM.MPh	98	81	74	50	93	21	95	95	86
Derbinski et al 2005	MG_U74Av2	tMPh	91	NA	77	57	83	42	67	97	92
Chambers et al 2007	Mouse430_2	Monocyte	100	91	86	50	79	46	93	75	91
Chambers et al 2007	Mouse430_2	Granulocytes	98	83	81	61	65	48	87	82	89
Wong et al 2014	MoGene1	Granulocytes	94	95	77	28	80	45	92	62	91
Chambers et al 2007	Mouse430_2	Nu.RBCs	88	79	79	61	59	49	91	69	91
Haddon et al 2009	MOE430A	mast.cells	82	5	74	42	49	23	49	30	70
Haddon et al 2009	MOE430A	pre-mast.cells	82	5	73	46	51	25	47	43	71

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CHAPTER THREE

The role of *Irf6* in T-cell development in the thymus

Abstract

Rare variants in *IRF6* cause Van der Woude and Popliteal Pterygium Syndromes, two autosomal dominant orofacial clefting disorders. Common *IRF6* variants contribute risk toward non-syndromic orofacial clefting. In addition, rare somatic mutations in *IRF6* are associated with squamous cell carcinoma. However, unlike the other eight members of the *IRF* family, a role for *IRF6* in haematopoietic development has not been described. Previously, we used publically available data to discover dynamic *IRF6* expression in developing thymocytes. Here, we utilized a mouse model to show that *Irf6* was required for the regulation of thymocyte development. We found that Irf6 was expressed in the subcapsular region and medulla of the thymus. We further found that *Irf6* regulated the distribution and proliferation of developing thymocytes. In addition, loss of *Irf6* led to an increase in double negative cells with a concomitant increase in TCRγδ. Loss of *Irf6* also led to a reduction in double positive cells with no corresponding reduction in single positive cell maturation. Finally, we found that *Irf6* dose is critical in development of both CD4+ and CD8+ cells in an age-dependent manner. While perinatal lethality has limited investigation of Irf6 in hematopoiesis, we report here a novel gene function for *Irf6* in thymocyte development. These data suggest that *IRF6* variants may increase risk toward autoimmune disease and that individuals with VWS and PPS may require more rigorous immunological screening. With this work, all Irf family members have an important role in immunity.

Introduction

The thymus gland is a specialized organ necessary for T-cell development. It is composed of an inner medulla and a peripheral cortex surrounded by an outer capsule (Rodewald, 2008; Singer *et al.*, 1986). Thymus tissue is composed of lymphoid cells (CD45+CD7+) and stromal cells with a ratio of 50 lymphoid cells for each stromal cell (Rodewald, 2008; Singer *et al.*, 1986). Non-hematopoietic stromal cells can be further classified into thymic epithelial cells (TEC, Keratin+) and mesenchymal cells (Keratin−) (Anderson *et al.*, 1993). Dendritic cells and macrophages are CD45+ thymic stromal cells, thus they constitute the hematopoietic component of the stromal mesh (Rodewald, 2008). T-cell precursors seed the thymus at the medullary cortical junction. Recent thymic immigrants are called double negative (DN) thymocytes because they lack the expression of both CD4 and CD8 (Godfrey *et al.*, 1993; Pearse *et al.*, 1989). CD44 and CD25 are two surface markers which mark 4 major developmental sub-populations of DN thymocytes (DN1, $CD44^{+}CD25^{-}$; DN2, $CD44^{+}CD25^{+}$; DN3, $CD44^{-}CD25^{+}$; and DN4, $CD44^{-}CD25^{-}$) (Godfrey *et al.*, 1993). DN3 is an obligatory check point where expression of pre-TCR or γδTCR identifies transition from DN3a to DN3b (Michie & Zuniga-Pflucker, 2002). Pre-TCR signaling drives expression of CD4 and CD8 producing double-positive (DP) thymocytes (Hoffman *et al.*, 1996). Developing thymocytes have to migrate through the cortex toward the capsule then back to the medullary space (Takahama, 2006). Transition from DN4 (also called pre-DP) to DP cells occurs in the subcapsular region. Transforming growth factor β (TGFβ) signaling suppresses the proliferation of pre-DP thymocytes to regulate the production of DP cells (Benz *et al.*, 2004). DP cells migrate back through the cortex where positive and negative selections

occur allowing only 3-5% of cells to survive and reach the thymic medulla. DP cells lose either CD4 or CD8 to reach the single positive (SP) stage. SP thymocytes, either $CD4^+$ (T helper) cells or $CD8^+$ (T cytotoxic) cells, continue their maturation and central tolerance in the medulla before being shuttled out of the thymus (Blackburn & Manley, 2004; Germain, 2002; Hoffmann *et al.*, 2003; Lind *et al.*, 2001; Plotkin *et al.*, 2003; Prockop & Petrie, 2000).

Regulation of peripheral CD4:CD8 cell ratios originate in the thymus. Species and strain differences contributed to TCR selection-dependent mechanisms or thymic lineage commitment signaling (Damoiseaux *et al.*, 1999; Rocha *et al.*, 1989; Sim *et al.*, 1998; van Meerwijk *et al.*, 1998). Genetic variations of TCRα loci and MHC haplotypes represent the most important TCR selection-dependent factors (Damoiseaux *et al.*, 1999; Sim *et al.*, 1998). Intrinsic activity of Notch is an example of other factors that influence the thymic lineage commitment and affect the CD4:CD8 T cell ratio (Fowlkes & Robey, 2002; Huang *et al.*, 2003; Robey *et al.*, 1996). Also the interaction of Notch ligand Jagged1 on thymic stroma and DP thymocytes controls the CD4:CD8 ratio (Jimenez *et al.*, 2001). Thymic involution was shown to be induced by Jagged1 expression in thymocytes (Beverly *et al.*, 2006) and is associated with increased CD4:CD8 ratio (Kozlowska *et al.*, 2007).

Although several *Irfs* are expressed in thymocytes (Colantonio *et al.*, 2011; Hrdlickova *et al.*, 2001; Nordang *et al.*, 2011; Simon *et al.*, 1997), *Irf1* is the only family member with functional studies supporting a role in T-cell development. *Irf1* reduces expression of the major histocompatibility complex related genes in the thymic microenvironment (Lee *et al.*, 1999; White *et al.*, 1996). However it is the *Irf1* intrinsic

activity in T-cells that is required for development and thymic selection of naïve CD8 T-cells (Matsuyama *et al.*, 1993; Penninger *et al.*, 1997; Penninger & Mak, 1998). In *Irf4* knockout mice, while cell count changes appear to be unaffected, the proliferative capacity, antiviral cytotoxicity, allogenic graft rejection, tumor surveillance and cytokine production of CD8 cells are markedly impaired (Mittrucker *et al.*, 1997).

Recently, I performed a meta-analysis of microarray experiments on hematopoietic lineages and found that the steady-state level of *Irf6* is induced in the DP and SP thymocytes as well as medullary thymic epithelium. Previous studies in the palatal and skin development show *Irf6* as a downstream target to Tgf-β and notch signaling (Le *et al.*, 2012; Restivo *et al.*, 2011; Xu *et al.*, 2006). However, these interactions have not been tested in T cells.

In this study, we used a mouse model to test for the necessity of *Irf6* in thymocyte development. We found that *Irf6* regulated the distribution and proliferation of developing thymocytes. Also, we found that *Irf6* dose is critical in development of both CD4+ and CD8+ cells in an age-dependent manner. These data suggest that *Irf6* like all other family members is involved in regulation of immune system.

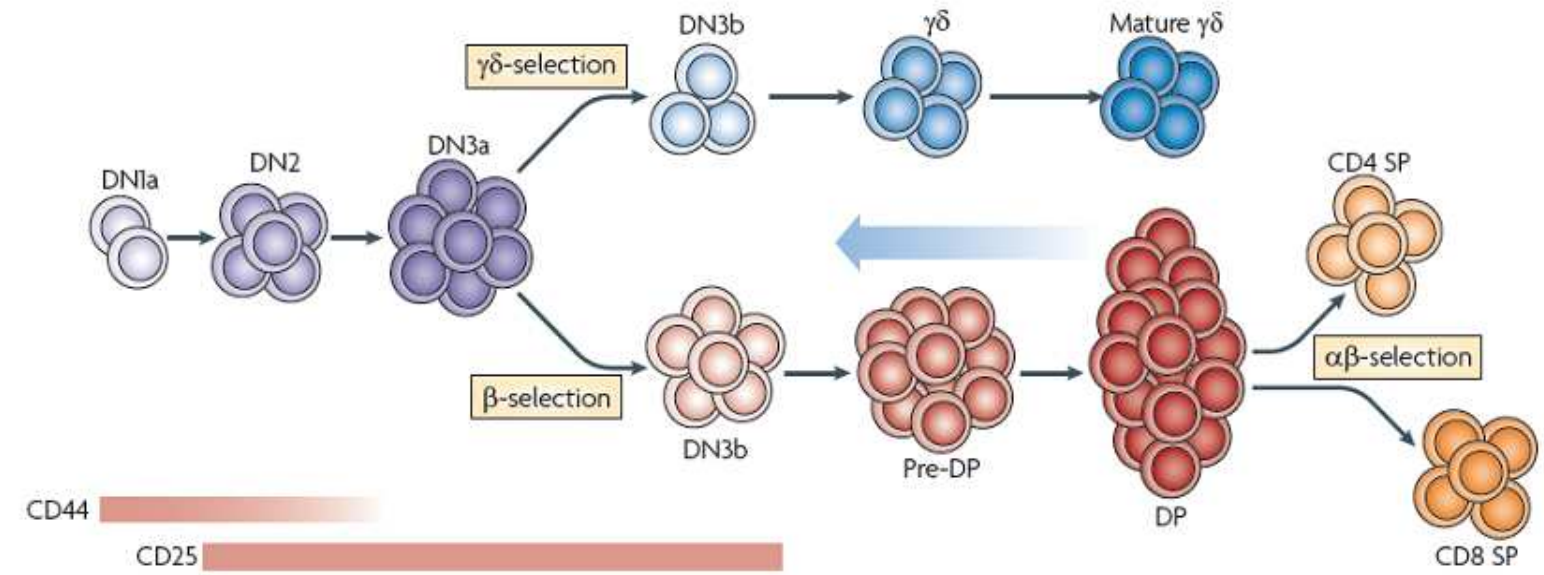

***Figure 3.1**:* **Stages of T cell development in the thymus**. Figure modified from Ciofani and Zuniga-Pflucker, *Nat Rev Immunol* 2010.

Materials and methods

Mice: C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME) or raised in house. Mice with *Irf6* gene trap allele (*Irf6*$^{gt/+}$) were described before (Ingraham *et al.*, 2006). Four week-old wild type C57BL/6 mice were used for initial assessment of *Irf6* expression in the thymus. To compare wild-type and *Irf6*-knockout thymi, *Irf6*$^{gt/+}$ mice were mated to produce wild-type and Irf6-knockout embryos. Pregnant females were sacrificed, embryos were dissected and thymi were collected at E17.5. To study the effect of Irf6 heterozygosity on postnatal thymic proliferation, *Irf6*$^{gt/+}$ and wildtype mice were allowed to mate and heterozygous mice were compared to their wild type littermates at 6-7, 12-13, or 18-20 weeks of age.

Morphological assessment of Irf6 knockout thymi: Relative size of thymi was determined as previously described (Candi *et al.*, 2007). Briefly, thymic gland and heart were dissected using a dissecting microscope. Thymus/heart ratio was defined using the longest dimension of each.

Western blot analysis: Whole cell extracts were prepared by mechanical disruption of thymi in RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) supplemented by protease inhibitors (Complete Protease Inhibitor Cocktail Tablets, Roche). Samples were denatured by incubating at 95°C for 10 minutes. Protein extracts were separated by 10% SDS-PAGE Electrophoresis System and transferred onto PVDF membranes with 1X Tris-Glycine Transfer Buffer. Membranes were blocked with Odyssey blocking solution and probed with polyclonal rabbit antibody against Irf6

(1:250) (Ingraham *et al.*, 2006) and mouse antibody against GAPDH (sc-365062, 1:15000, Santa Cruz Biotechnology, Santa Cruz, CA). Proteins of interest were detected with Infrared IRDye-labeled secondary Donkey anti-Rabbit and goat anti-mouse IgG (H + L) antibodies (1:15000, Thermo Scientific, Rockford, IL) imaged with the Odyssey Infrared Imaging System using the 700 and 800 channels (Thermo Scientific, Rockford, IL), according to the provided protocol.

Immunohistochemistry: Thymi were fixed in 10% formaldehyde, embedded in paraffin and cut into 3 μm-thick transverse sections. To deparaffinize and rehydrate sections, slides were passed through three changes of Xylene, followed by reducing concentrations of ethanol. Antigen retrieval was conducted by boiling in 10mM Sodium Citrate pH6.0 for 10 minutes. Sections were permeabilized in 0.5% Triton X-100 and blocked for one hour in blocking solution (10% normal goat serum, 0.1% Bovine Serum Albumin in 1X Phosphate Buffered Saline) at room temperature. Sections were then incubated in primary antibodies diluted in blocking solution overnight at 4°C. Following incubation, slides were washed three times in 1X PBS and incubated in fluorescent labeled secondary antibodies for 45 min at room temperature. To detect nuclei, slides were incubated in DAPI (Invitrogen, D3571) diluted 1:10,000 in distilled water for 10 minutes. Slides were imaged using the Nikon i90 upright fluorescent microscope. Primary antibodies included rabbit polyclonal anti-Irf6 antibody (Ingraham *et al.*, 2006) and rat monoclonal anti-BrdU (Abcam, Ab6326).. Secondary antibodies included Oregon Green 488 goat anti-rabbit (Molecular Probes, O-6381) and Alexa Fluor 555 Goat Anti-Rat (Molecular Probes, A-21434).

Total cell count and Flow cytometric analysis: Single cell suspension was prepared from dissociated thymi by filtering through a 48-µm nylon mesh. Total thymocyte count was calculated using hemocytometer. Cell count was adjusted to $1x10^6$ followed by FcR blocking with anti-FcR mAb 2.4G2. Subsequent flowcytometric analysis included one of the following:

a) Analysis of the thymocyte subpopulations: To cover most of the developing T-cell populations, cells were co-stained for 6 surface markers; against CD4 (APC, Catalog# 100516, Biolegend), CD8a (FITC, Catalog # 140404, Biolegend), TCRβ (PE-cy7, Catalog# 109222, Biolegend), TCRγδ (PE-cy5.5, Catalog# 118118, Biolegend), CD25 (PE, Catalog# 100610, Biolegend), CD44 (APC-cy7, Catalog# 103028, Biolegend) (figure 3.2). Natural Treg was assessed separately by co-surface staining of CD4 (APC, Catalog# 100516, Biolegend) and CD8a (FITC, Catalog # 140404, Biolegend) with intracellular staining for Foxp3 (PE, Catalog# 12-5773-82, eBioscience) according to the recommended protocol of the company.

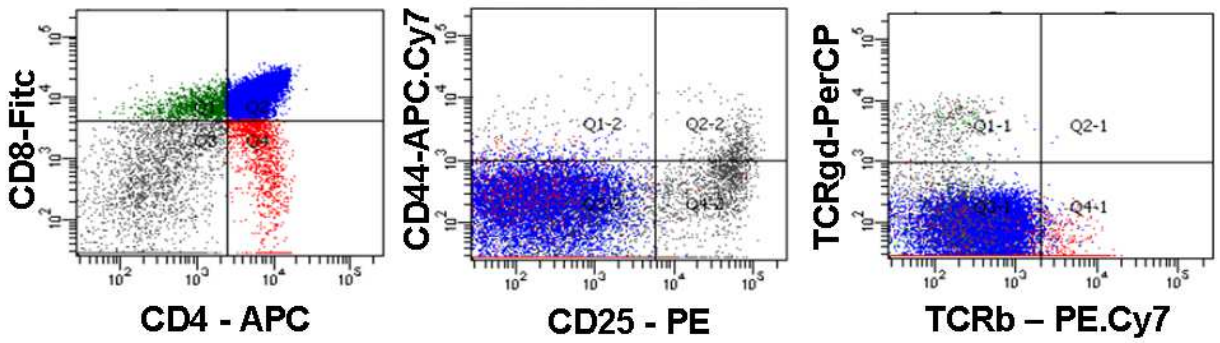

Figure 3.2: **Flowcytometric analysis of the thymocyte subpopulations**. Left scatter diagram plots CD4 and CD8 expression to classify the developing thymocytes into four major groups (DN; black, DP; blue, CD4-SP; red, and CD8-SP; green). Middle scatter diagram plots CD25 and CD44 expression to tease apart the sub-populations of DN thymocytes. Right scatter plots TCRβ and TCRγδ expression.

b) Bromodeoxyuridine (BrdU) incorporation analysis: For embryonic studies, pregnant dams were injected intraperitoneally with BrdU (100ug/gm body weight) one hour before euthanasia. For postnatal studies, each animal was injected intraperitoneally with BrdU (100ug/gm body weight) two hours before euthanasia. After blocking of non-specific binding of Fc receptors, surface staining was done using monoclonal antibodies against CD4 (Alexafluor 700, Catalog# 100536, Biolegend) and CD8a (FITC, Catalog # 140404, Biolegend). Cells were fixed and permeabilized with BD Cytofix/Cytoperm Buffer (Catalog# 554722, BD) for 20 min on ice. For nuclear permeabilization, cells were kept overnight at -80^{o}C in a freezing medium. On the next day, cells were thawed and washed with FACS buffer prior to refixation in BD Cytofix/Cytoperm Buffer for 5min on ice. DNA was partially digested by incubating the cells with 30 µg of DNase/10^{6} cells (Catalog# D-4513, Sigma) for 45min at 37^{o}C. Cells were stained with Anti-BrdU (PerCP-Cy 5.5, Catalog# 560809, BD).

c) Detection of apoptosis was performed using combinations of Annexin V Apoptosis Detection Kit PerCP-eFluor® 710 and Fixable Viability Dye eFluor-780 (eBioscience)

Results

Thymic expression of Irf6: Whole-cell protein extracts were prepared from thymi of 4 week-old mice (n=3). Western blot analysis showed a protein band at the expected size (~53kD) (figure 3.3). To determine in situ localization of Irf6 expression, immunofluorescent staining was done on paraffin embedded tissue (n=3). Irf6 expression was mainly confined to the thymic medulla and sub-capsular compartments (Fig.3.4). The thymic structure did allow delineating the exact cell type expressing Irf6 without co-staining for additional markers. Unlike expected for transcriptions factors, Irf6 expression was confined to the cytoplasmic spaces. However this pattern of Irf6 expression is consistent with previous studies of in other tissues (Bailey *et al.*, 2005; Ingraham *et al.*, 2006).

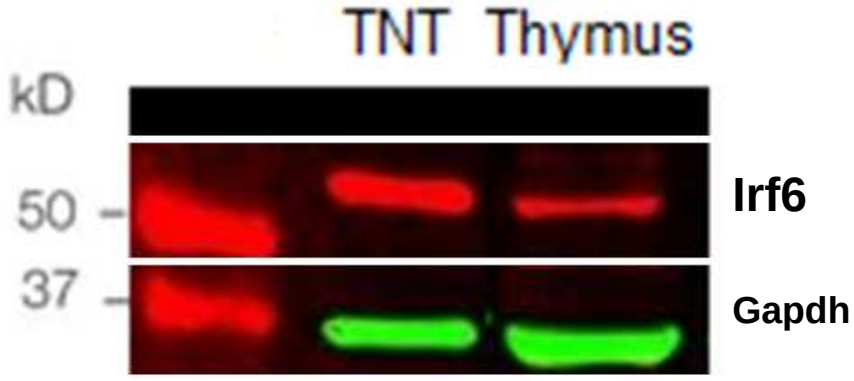

Figure 3.3*:* **Two-color western blot for Irf6 and Gapdh.** Irf6 is Red (~53kD) and Gapdh is green (~37kD). Lanes from left to right represent protein standard, TNT (IRF6 protein synthesized by in-vitro transcription and translation system) and whole-cell protein extract from thymus from a 4 week old mouse (n=3).

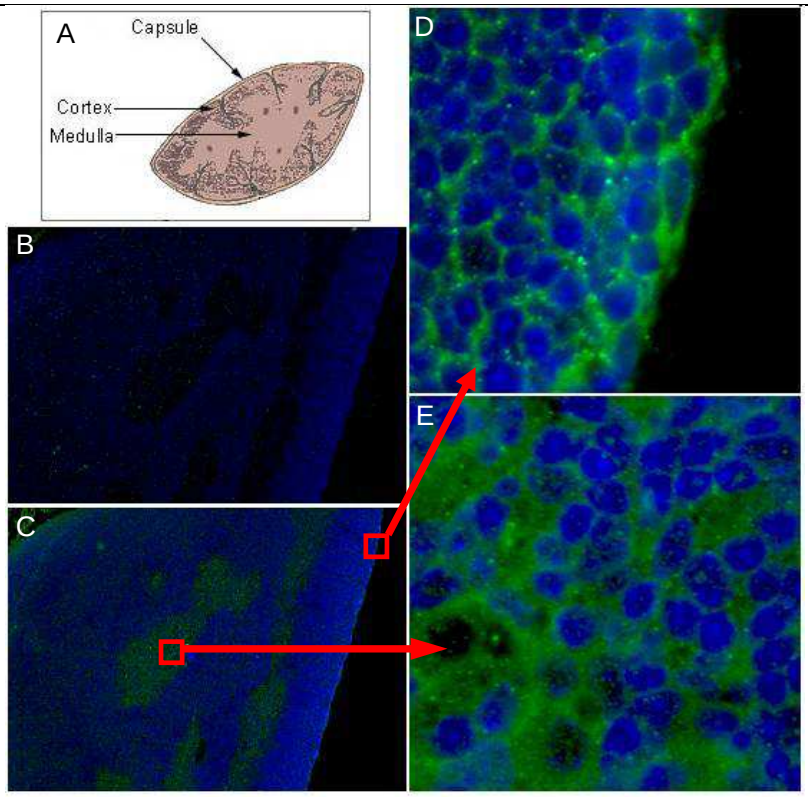

***Figure 3.4**:* **Irf6 expression in the thymus**. (A) Schematic representation of the thymus. (B-E) Immunofluorescent staining of thymic sections obtained from 4 week-old mice. Nuclear DAPI staining (Blue), Irf6 staining (green). The primary Irf6 antibody was pre-incubated with (B) or without (C) blocking peptide and images were taken under 4x objective magnification. 40x magnification is shown for the sub-capsular region (D) and thymic medulla (E) (n=3).

Characterization of thymic changes in Irf6 knock-out embryos: Thymi of E17.5 littermates were compared. No obvious changes of the position, shape or size were detected (figure 3.5). Total thymocyte count showed insignificant changes from wild type littermates by paired t-test analysis. However further analysis of developing thymocyte sub-populations showed significant increase of DN population ($p = 0.017$) and significant decrease of DP population ($p < 0.001$) with insignificant change of single positive populations. We used BrdU staining as a marker of DNA synthesis and cellular proliferation. While the total uptake of BrdU incorporation was not different, we found that DN cells were more proliferative ($p = 0.007$) while DP cells were less proliferative ($p = 0.018$) (figure 3.6 and 3.7). Immunostaining for BrdU in paraffin embedded thymi showed a different distribution of proliferating thymocytes through the cortical space compared to the typical localization in the subcapsular space (Figure 3.8).

Surface staining for TCR showed significant increase of TCRγδ in knockout thymi by a paired t-test analysis between littermates ($p = 0.01$). We did not find a significant difference in TCRβ (Figure 3.9). Testing nuclear expression to mark nTreg showed that embryonic thymi did not start producing Foxp3 +ve T cells at E17.5. We also analyzed Annexin V as a marker of cellular apoptosis and the fixable viability dye to quantify dead cells. Co-staining for both markers showed no change in the total rate of thymocyte apoptosis or cell death in *Irf6* knockout thymocytes (Figure 3.10).

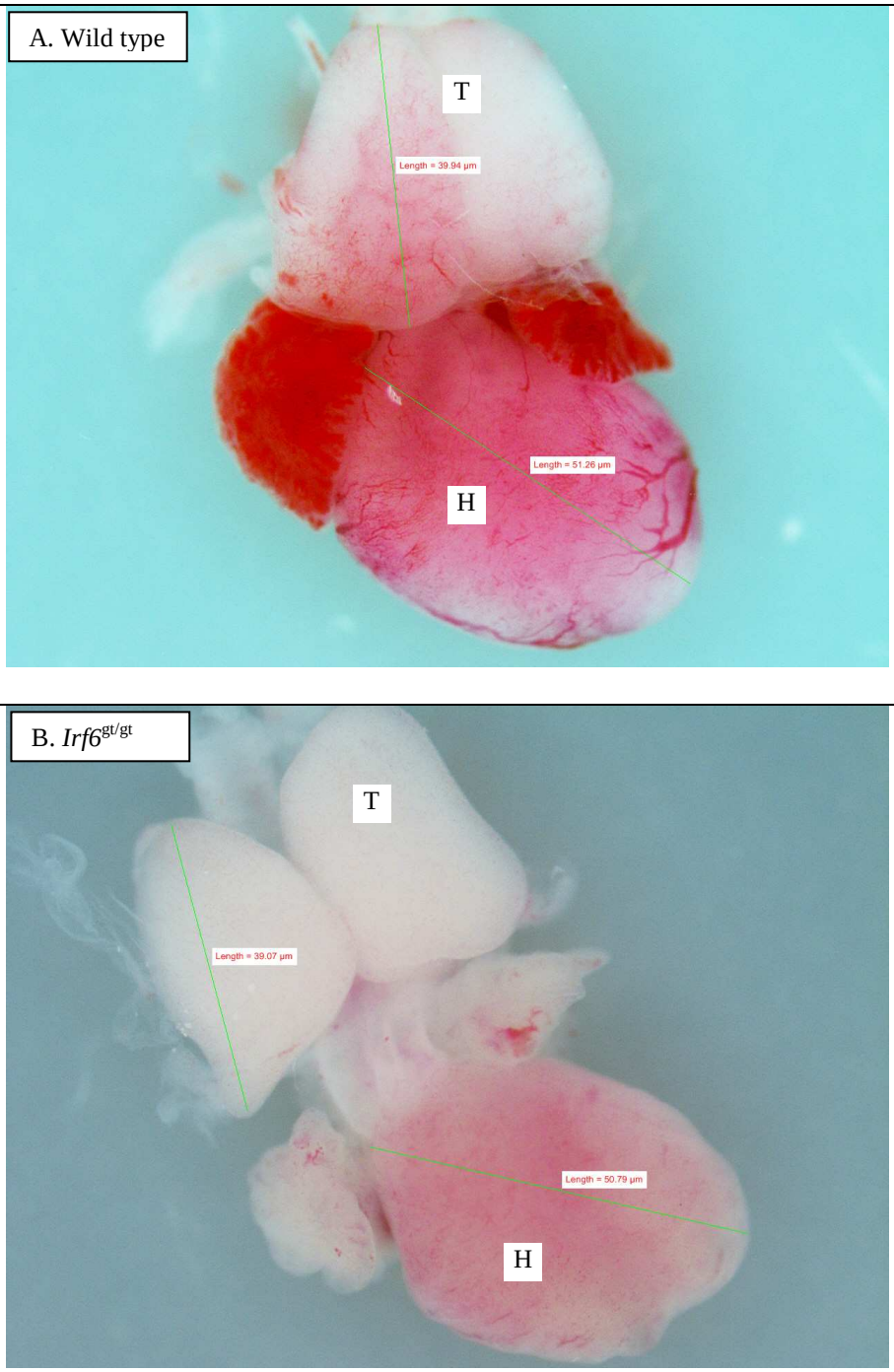

Figure 3.5: T**hymic-cardiac ratio** in A) wild type and B) Irf6$^{gt/gt}$ mice. (T) refers to the thymus and (H) refers to the heart. There was no obvious change in the thymic-cardiac ratio (n=3).

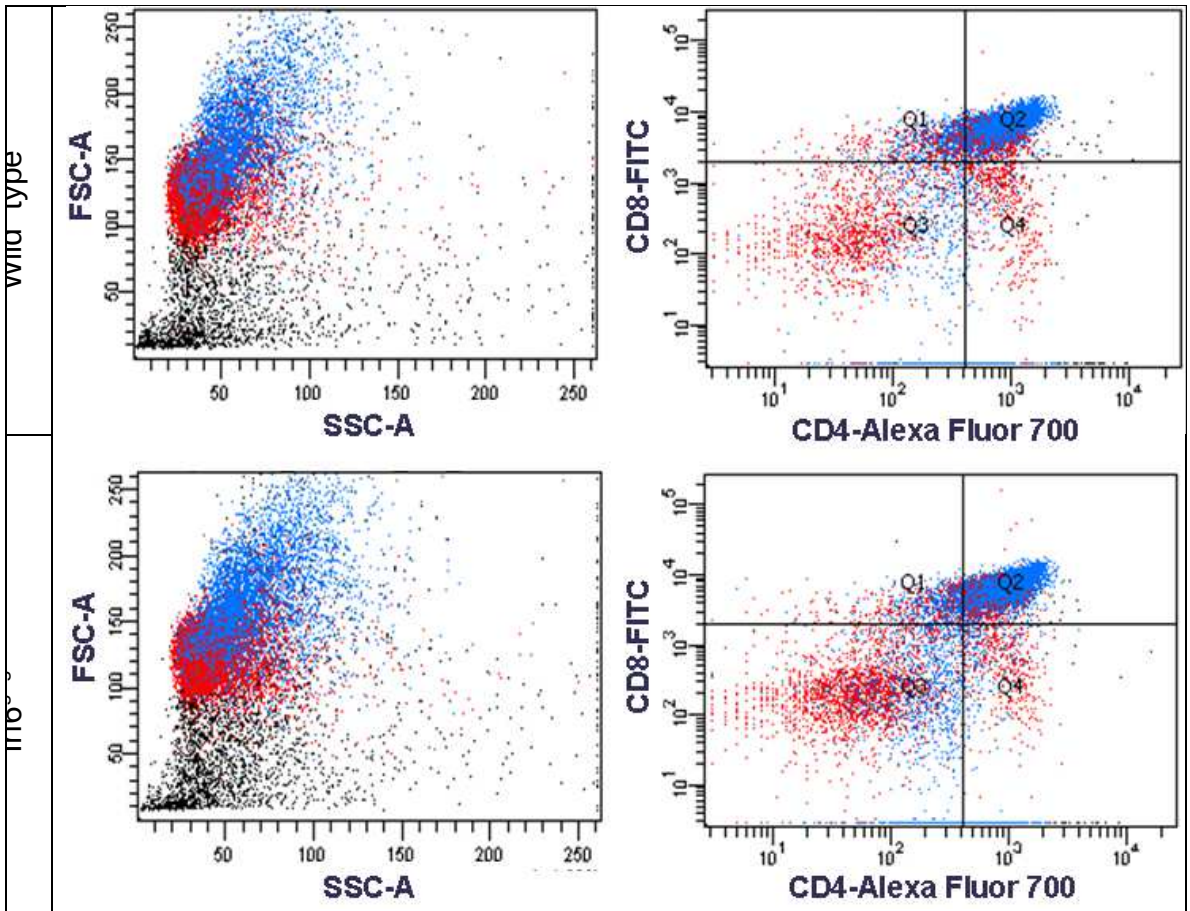

***Figure 3.6**:* **BrdU incorporation in developing thymocytes.** BrdU incorporation in thymocyte populations in wild type (upper panel) and Irf6$^{gt/gt}$ (lower panel) using flowcytometry. Left column scatter plots show the size scatter on x-axes and forward scatter on y-axes. Right side scatter plots show the surface expression of CD4 on the x-axes and CD8 on the y-axes. Red color identifies the thymocytes from black colored debris. Blue color overlay the red color to identify the cells incorporating BrdU. There is no significant difference in total BrdU incorporation. However, we observed a shift of BrdU incorporation from the DP compartment to the DN compartment which compensated the overall proliferation in Irf6 knockout mice.

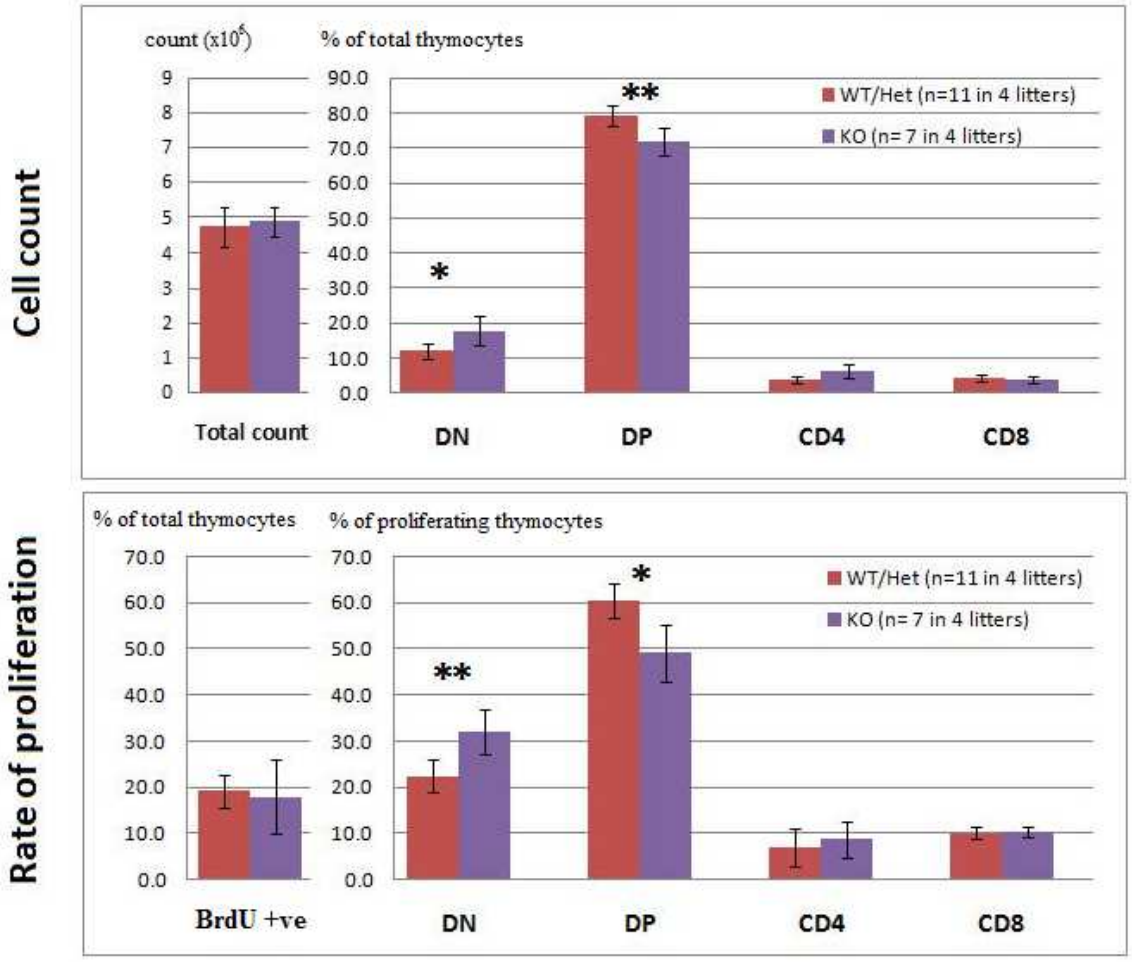

***Figure 3.7: Irf6* regulates proliferation and cell count of thymocytes**.

Statistical analysis of BrdU incorporation and cell counts of total thymocytes and the developing sub-population shows underline the importance of Irf6. In spite of insignificant changes in total proliferation and cell count, there is a significant increase in the count and proliferation of DN cells with a corresponding reduction in DP cells.

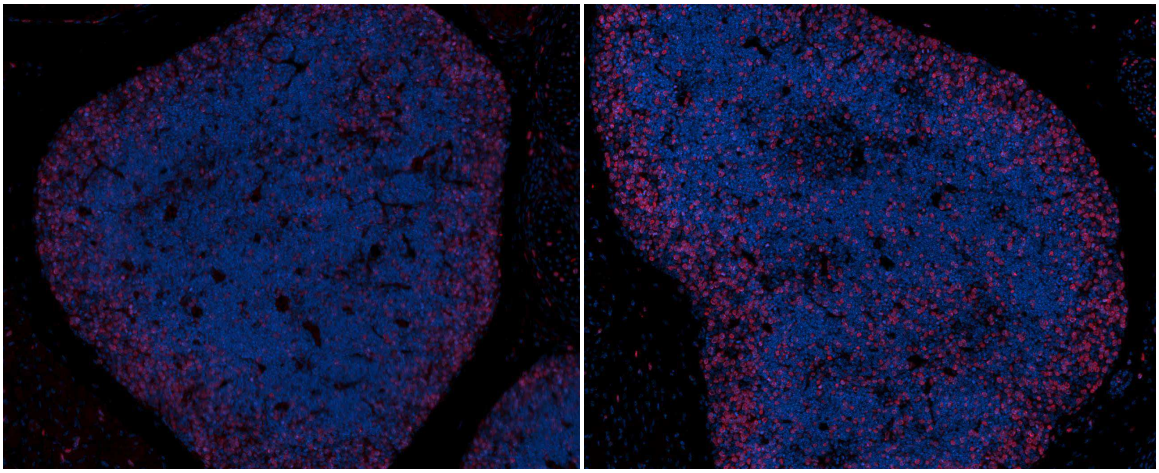

Figure 3.8: **Loss of *Irf6* leads to an abnormal distribution of proliferating cells.** Immunofluorescent staining of thymic sections from E17.5 embryos. Nuclear DAPI staining (Blue), BrdU staining (red). Compared to wildtype embryos, *Irf6* knockout embryos have more cellular proliferation in the thymic medulla (n=3).

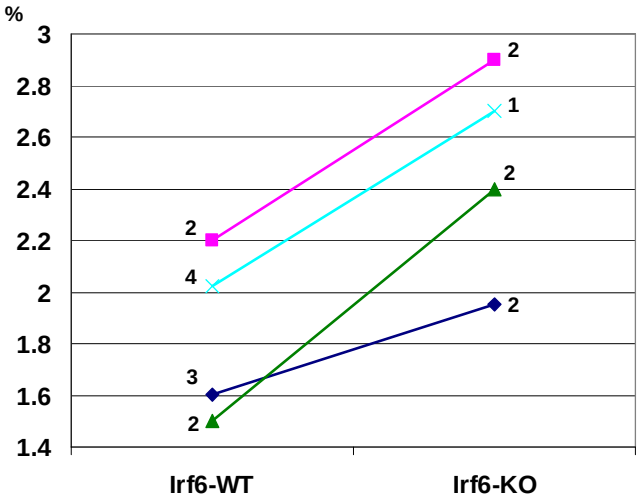

Figure 3.9: **Frequency of TCRγδ.** The figure shows the average frequency of TCRγδ for each genotype per littermates. Each point is labeled by the numbers of embryos. Paired t-test is significant with p = 0.01

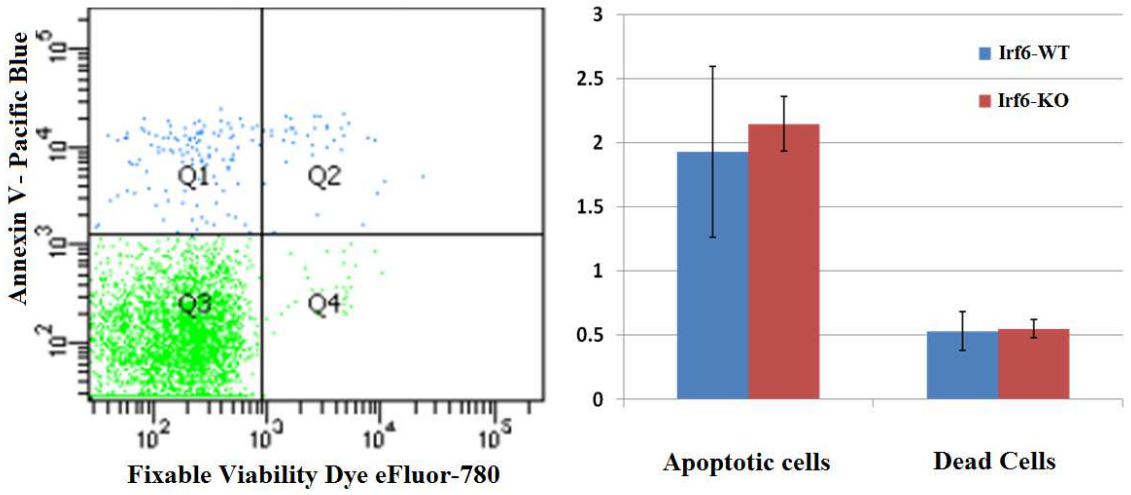

Figure 3.10: **Loss of *Irf6* does not alter total cell death.** Analysis of apoptosis and cell death. Left scatter diagram shows the plotting of the fixable viability dye on the x-axes versus Annexin V on the y-axes where (Q1) square the apoptotic cells and (Q2) square represents the dead cells. Par plot on the right shows insignificant changes in the total count of apoptotic or dead cells in Irf6 knock-outs (n=11 wildtype/heterozygous and 7 knockouts from 4 litters).

Effect of Irf6 heterozygosity in postnatal thymic proliferation: To study the effect of *Irf6* dosage on thymic proliferation over time, we tested mice at post-natal week 6-7, 12-13, or 18-20. At each time point, three heterozygous mice were compared to their matching wild type littermates. BrdU incorporation study showed an insignificant change of total BrdU uptake but the ratio CD4-SP sub-population positive for BrdU shows significant increase ($p = 0.04$) versus a significant reduction in the CD8-SP compartment ($p = 0.03$) at 18-20 weeks of age (figure 3.11).

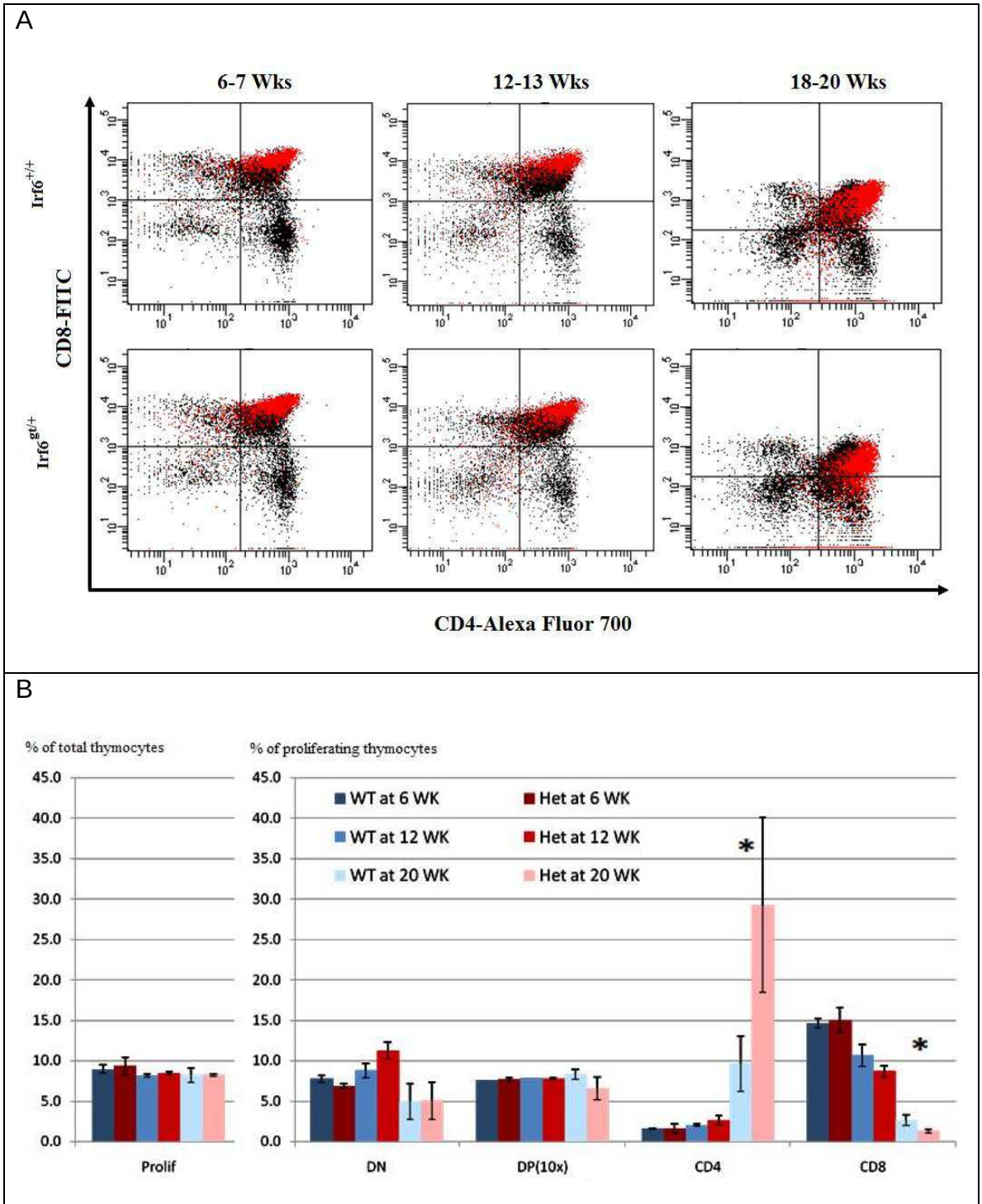

Figure 3.11: **Effect of *Irf6* dosage on BrdU incorporation in thymocyte populations**.

***Figure 3.11* (Cont'd)** (A) Flowcytometric analysis of thymi from Irf6 wild type (Upper panel) and $Irf6^{gt/+}$ (lower panel). Scatter plots shows the surface expression of CD4 on x-axes and CD8 on y-axes at 6-7, 12-13, or 18-20 weeks of age (from left to right). Red color identifies the thymocytes incorporating BrdU. (B) Bar diagram represents the changes of total thymocytes proliferation and the four major sub-populations (mean and SD) at 6, 12 and 20 weeks of age in $Irf6^{+/+}$ and $Irf6^{gt/+}$ thymi (n=3 for each time point and genotype). There is a significant increase of CD4 population with a significant reduction of CD8 thymocytes after 20 weeks of age.

Discussion

The IRF family of transcription factors has been widely studied and regarded in hematopoiesis as master regulators. While *IRF6* is a paralog, sharing a highly conserved DNA and protein binding domains, a role in immunity has not been reported. Instead, previous and ongoing *IRF6* studies have focused on other important roles in cutaneous, limb and craniofacial development. In humans, common variants in *IRF6* lead to common, complex diseases, including cleft lip and palate and squamous cell carcinoma. Furthermore, rare variants in *IRF6* lead to Van der Woude (VWS) and Popliteal Pterygium Syndromes (PPS). Most proximally, these data suggest that individuals with common and rare *IRF6* variants are at increased risk for immunological diseases.

Like cutaneous development, we found that *Irf6* regulates proliferation in thymocytes. In skin and thymus, loss of *Irf6* leads to an expansion of the progenitor cells; germinative and double negative cells respectively. Also a concomitant reduction in downstream daughter cells (keratinizing and double positive cells). However, in contrast to skin which loses the cornified cells, DP cells are not lost. Also we did not see changes in the terminally differentiated single positive thymocytes. Therefore, while some parallels exist, a more complex relationship in the thymus plausible.

Importantly, we found that counts of single positive cells were not altered despite a significant reduction in double positive cells. Our analysis did not show a change in the total number of apoptotic or dead cells. However, further analysis for the thymic sub-population is mandatory. These data can be

explained by either enhanced survival of single positive cells or thymic retention of naïve lymphocytes. The enhancement of survival of single positive cells may occur at the expense of negative selection and predisposition for autoimmune responses. We further observed that Irf6 was expressed in the subcapsular cortex and medulla. However, we have not delineated if this expression was in the thymic epithelium or thymocytes or both. Considering that negative selection is a product of the interaction between the thymic epithelium and double positive thymocytes, enhanced survival could be a cell-autonomous mechanism or results from a milieu of factors. Retention of thymic naïve lymphocytes can be presented as reduced peripheral lymphocyte count. Neonatal lymphopenia itself was shown to predispose for autoimmune disease (Gleeson *et al.*, 1996; Sakaguchi & Sakaguchi, 1989). While an association between VWS/PPS and autoimmune diseases or neonatal lymphopenia has not been described in the literature, multiple factors are likely involved. For example, VWS/PPS are rare congenital anomalies and subclinical manifestations of immunological diseases can be missed. Also considering the role of the Irf family members in T cell development, redundant function is possible. Interactions between *Irf6* and other *Irf* family members seem plausible, if not likely. Pursuing this gene regulatory network may provide novel gene functions for Irf6 in T cell subsets.

Thymic involution is a very controlled process which starts early in life and considered as a central driver of T cell aging (Goronzy & Weyand, 2013) (Aspinall *et al.*, 2010). Aging of thymus is associated with altered distribution of thymocyte populations. Whereas the DN cells start to accumulate, the DP cell

counts decrease with a significant increase of CD4:CD8 ratio (Kozlowska *et al.*, 2007). Embryonic absence of Irf6 is associated with similar changes of DN and DP populations but without significant changes of CD4:CD8 ratio. However, by the age of 6 month, animals with reduced dose of Irf6 start to show significant increase of CD4:CD8 ratio. . Two transgenic lines with constitutively active form of Notch in DP cells showed a decrease in CD4 SP thymocytes and a corresponding increase in CD8 SP thymocytes (Fowlkes & Robey, 2002). IRF6 is a known mediator of Notch in keratinocytes (Restivo *et al.*, 2011). Irf6 heterozygosity in thymocytes might be associated with decreased downstream signals of Notch pathway causing increased CD4:CD8 ratio. Also Expression of the Notch ligand Jagged1 in thymocytes results in thymic involution by inducing apoptosis of thymic stromal epithelial cells (Beverly *et al.*, 2006). This might explain the altered CD4:CD8 observed in mice heterozygous for Irf6 as a sign of early thymic involution.

In summery, we describe a novel gene function for *Irf6* in hematopoietic development. To our knowledge, this is the first study to ascribe immunological function to *Irf6*, completing the family so that all nine paralogs now have a role in haematopoiesis.

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CHAPTER FOUR

The role of *Irf6* in functional commitment of T-cell subsets

Abstract

The IRF family of transcription factors is essential in the differentiation of T helper subsets. In contrast, IRF6 is canonically known for critical roles in craniofacial, limb and cutaneous development. In the mouse, loss of Irf6 leads to perinatal lethality. Recently, we showed that Irf6 is also involved in the regulation of thymic proliferation. However, little is known about the necessity of *Irf6* in the development of functionally committed T helper subsets. Here, we used in silico, in vivo and in vitro assays to determine the role of Irf6 in T cell differentiation. Using in silico analysis, we found and propose a model for Irf6 function in Th17/Treg balance. To test our hypothesis in vivo and overcome perinatal lethality, we employed an adaptive transfer of Irf6 knockout cells into lethally irradiated mice. We observed a 100% survival of chimeric mice receiving Irf6 knockout fetal liver, and mice receiving Irf6 knockout cells had no deficit in restoration of lymphocyte production. In addition, we used two in vitro models to assess the necessity of Irf6 in the commitment of T helper cells. Using a stromal-free culture we found that naive T cells lacking Irf6 could be differentiated into Th1, Th2, Th17 and Treg using a specific cytokine cocktail. We found no differences in cell frequency and mean fluorescence intensity of intracellular cytokines between wild type and Irf6 knockout cells. In vitro differentiation of dendritic cells showed significant increase of MHC-II expression after three days of culture. Irf6 might be involved in post-translational regulation of MHC-II. In conclusion, we found that intrinsic Irf6 expression was not essential for T helper subset differentiation. However, a non-cell autonomous role for Irf6 in T cell differentiation through dendritic cells remains plausible.

Introduction

T helper (Th) lymphocytes function as the conductors of the adaptive immune orchestra. Upon antigen exposure, T helper cells differentiate into specialized subsets. Each T helper subset differentiates under a unique signaling pathway and lineage-specific transcription factors to produce a characteristic cytokine milieu (Fietta & Delsante, 2009; Hirahara *et al.*, 2011). T helper subsets include Th1, Th2, Th17, regulatory T-cells (Treg), T follicular helper cells, Th9 and Th22 cells (Bluestone *et al.*, 2009; Shevach, 2010). The balance between different T helper cells is most typically defined by mutually exclusive expression of lineage-specific transcription factors. In vitro activation of T cells can be done using specific antibodies against the T-cell receptors. The combination of anti-CD3 and anti-CD28 is used to mimic antigenic stimulation (Bjorndahl *et al.*, 1989; Verwilghen *et al.*, 1991). Cytokine cocktails can be formulated to simulate the physiological signaling pathways and drive the differentiation of naïve CD4 T cells into one of several lineages of T helper cells (Constant & Bottomly, 1997; Zhu *et al.*, 2010). Differentiation of naïve cells can be analyzed by measuring their secreted cytokine profile. As such, stromal free models of in-vitro differentiation enable identification of cell autonomous mutant T cell effects.

Normal differentiation of T cells requires antigen presentation and cytokine guidance, two essential functions of Dendritic cells (DCs). DCs are a heterogeneous population of hematopoietic cells known as professional antigen presenting cells (APCs) that display different anatomical localizations, cell surface phenotypes and functions. They all come from CD34 bone marrow stem cells and express CD11c (Merad *et al.*, 2013; Miller *et al.*, 2012). In their immature state they have the ability to respond to

danger signals, engulf antigen, mature and migrate to lymphoid organs. Once the antigen, foreign or self, is internalized they are degraded and presented in the surface of the DCs in the context of major histocompatibility complex class I (MHC-I) and class II (MHC-II). DC maturation involves an increase in surface expression of MHC-II and co-stimulatory molecules like CD86 (Banchereau *et al.*, 2000). There are four major categories of DCs: Conventional DC, Langerhan cells, plasmacytoid DCs and monocyte-derived DCs. All except for the Langerhan cells are derived from bone marrow cells. Conventional DCs are specialized for antigen presentation and have two further subdivisions, CD11b+ and CD103+ DCs (Belz & Nutt, 2012). DCs can differentiate in vitro from bone marrow and blood using a combination of growth factors like granulocyte macrophage-colony stimulating factors (GM-CSF), IL-4, Flt3 ligand and tumor necrosis factor-α (Lipscomb & Masten, 2002).

We have shown abnormal thymic development in *Irf6* deficient mice (Chapter 3). With abnormal thymic development, we can predict abnormal counts of mature T cell subsets in the peripheral blood and/or biased T cell immune response (Tanigaki *et al.*, 2004). *Irf1, 2, 4, 8* are indispensable for normal T helper differentiation (Kano *et al.*, 2008; Lohoff *et al.*, 2000; Lohoff & Mak, 2005; Tamura *et al.*, 2008; Tominaga *et al.*, 2003). Also, variants in *IRF5*, *IRF7* and *IRF8* are associated with psoriasis, multiple sclerosis and systemic lupus erythematosus (SLE) (De Jager *et al.*, 2009; Demirci *et al.*, 2007; Gateva *et al.*, 2009; Graham *et al.*, 2006; Harley *et al.*, 2008; Leppa *et al.*, 2011; Patel, 2011; Sanchez *et al.*, 2008). Conservation of IRF family members (Lohoff & Mak, 2005) and documented protein-protein interactions between IRF6 and both IRF5 and IRF8 (Li *et al.*, 2011) suggest a role of *IRF6* in T helper cell commitment. My meta-

analysis of microarray studies in T helper subsets reveals a significant reduction of *Irf6* expression in Th1 compared to Th2, Th17 and Treg suggesting an intrinsic role for Irf6 in development of T helper subsets (Chapter 1).

Materials and methods

Bioinformatic analysis: Publically available microarray studies were meta-analyzed using R computing environment (http://www.r-project.org/) to predict the possible roles of *Irf6* in T-helper commitment. We examined two microarray studies altering *Irf6* expression; 1) *Irf6* knockout mouse skin (Ingraham *et al.*, 2006) and 2) *IRF6* knockdown in human keratinocytes (Botti *et al.*, 2011). We also analyzed two microarray studies that altered Foxp3 expression; 1) knockout (Williams & Rudensky, 2007) and 2) over-expression (Fontenot *et al.*, 2005) of *Foxp3* in CD4+CD25+ cells. Data were integrated with Chip-seq analysis of *IRF6* binding sites in human keratinocytes (Botti *et al.*, 2011).

Mice and adoptive transfer: Mice heterozygous for the *Irf6* gene trap allele ($Irf6^{gt/+}$) were mated to produce wild-type ($Irf6^{+/+}$) and Irf6-knockout ($Irf6^{gt/gt}$) embryos. Fetal livers were harvested from E12.5 embryos and suspended in Iscove's modified Dulbecco's medium supplemented by 2% fetal calf serum. A single-cell suspension was prepared by passage through a 26-gauge needle. Crude DNA extraction was done and rapid PCR-based genotyping was performed. Recipient mice were congenic strain of C57BL/6 mice that carry the differential B cell antigen designated CD45.1 (NCI, Washington, DC. NY). Ten-week old recipient mice were lethally irradiated using X-RAD320 Irradiation System (PXi, North Branford, CT). Recipient mice received two doses of irradiation (5.5 Gy each) with a three hour interval. Irradiated mice were

injected retro-orbitally with the liver cell suspensions after 6 hours from the first dose of irradiation. Host mice were maintained on autoclaved water containing trimethoprim-sulfamethoxazole (0.65-1.6 mg/ml). All mice were maintained at the Michigan State University pathogen-free facility.

Th1, Th2, Th17 and Treg differentiation in vitro: Single cell suspensions of the spleens were prepared under sterile conditions. Naïve T-cells were purified by negative selection using magnetic beads from EasyStep Mouse Naïve CD4+ T Cell Isolation Kit (STEMCELL Technologies Inc, Vancouver, BC, Canada). Unwanted cells were targeted for removal with biotinylated antibodies directed against non-naïve CD4+ T cells (CD8, CD11b, CD11c, CD19, CD24, CD25, CD44, CD45R, CD49b, TCRγ/δ, TER119) then captured by streptavidin-coated magnetic particles. Naïve T-cells were cultured in X-vivo medium supplemented by 1 mM sodium pyruvate, nonessential amino acids, and L-glutamine. Culture plates were coated with anti-CD3ε antibody (clone 145-2C11, BD, Cat# 553058) 5µg/mL. Soluble anti-CD28 antibody (clone 37.51, BD, Cat# 553295) 2µg/ml, 2-mercaptoethanol 50 µM and 1X Penicillin-Streptomycin were added to the culture medium. Medium was enriched by the differentiation cocktail of Th1 [IL2 (20ng/ml, R&D, cat# 202-IL-010) + IL12 (20ng/ml, R&D, cat# 419-ML-010) + anti- IL4 (10µg/ml, R&D, cat# AB-404-NA)], Th2 [IL2 (20ng/ml, R&D, cat# 202-IL-010) + IL4 (100ng/ml, R&D, cat# 404-ML-010) + anti-IFN-γ (10µg/ml, BD, cat# 554408) + anti-IL12 (10µg/ml, Biolegend, cat# 505304)], Th17 [TGFβ1 (1ng/ml, R&D, cat# 7666-MB-005) + IL6 (100ng/ml, R&D, cat# 406-ML-005) + anti- IL4 (10µg/ml, R&D, cat# AB-404-NA) + anti-IFN-γ (10µg/ml, BD, cat# 554408) + FICZ (300 nM, Santa cruz, cat# sc-300019A)], or iTreg [IL2 (20ng/ml, R&D, cat# 202-IL-010) + TGFβ1 (5ng/ml, R&D, cat# 7666-MB-

005)] or under non-skewing conditions [only IL2 (20ng/ml, R&D, cat# 202-IL-010)]. Cells were cultured for four days, washed, and re-suspended in new wells at 10^6 cells/ml in the presence of 10ng/ml PMA and 1µg/ml ionomycin (extra supplementation with 2-mercaptoethanol and IL2 or IL6 enhance the cells' viability). After 1 hour, 1x Monensin was added for another four hours to block the cytokine secretion. Ice-cold EDTA was added to a final concentration 2 mM and incubated for 15 min at room temperature to decrease the cellular clumping. All cells were collected, washed and stained for viability with the fixable viability dye eFluor 780 as recommended (eBioscience, cat# 65-0865). Each cell type was intracellularly stained for CD4 and the appropriate differentiation marker (INF-γ for Th1, IL4 for Th2, IL17 for Th17 and Foxp3 for iTreg) (Bettelli *et al.*, 2006; Ghoreschi *et al.*, 2010; Huh *et al.*, 2011; Maruyama *et al.*, 2011; McKenzie *et al.*, 1999; Nurieva *et al.*, 2009; Rodriguez *et al.*, 2007; Zhou *et al.*, 2003).

In vitro generation of bone marrow–derived DC: Femurs and tibias of adoptively transferred mice were flushed for bone marrow, and 5 x 10^6 cells were grown in a 6-well plate (BD Falcon Franklin Lakes, NJ). We used 4 ml of RPMI media supplemented with 10% serum containing 100 units/ml penicillin and 100 µg/ml streptomycin (Gibco, Invitrogen). This media was applied for 9 days in the presence of granulocyte-macrophage colony stimulating factor (20 ng/ml; Peprotech, Rocky Hill, NJ). In total, 50% of the media was replaced on days 3, 6, and 8. After 9 days, bone marrow–derived DC (BM-DC) were left unstimulated (naive) or stimulated with LPS (1 µg/ml) in 2% fetal bovine serum RPMI. After 24 h, BM-DC were stained for CD8 (53–6.7), CD103 (2E7), CD11b (M1/70), CD11c (N418), CD86 (GL-1), Gr-1 (RB6-8C5), MHC-I (H-$2K^b$ AF6-

88.5), and MHC-II (I-A/I-E, M5/114.15.2) and analyzed by flow cytometry for analysis of DC populations. Total $CD11c^+$ were counted to represent the total DCs. We attempted to identify three subsets of DCs, including CD8 DCs, CD103 DCs and CD11b DCs (for gating scheme, see figure 4.1)

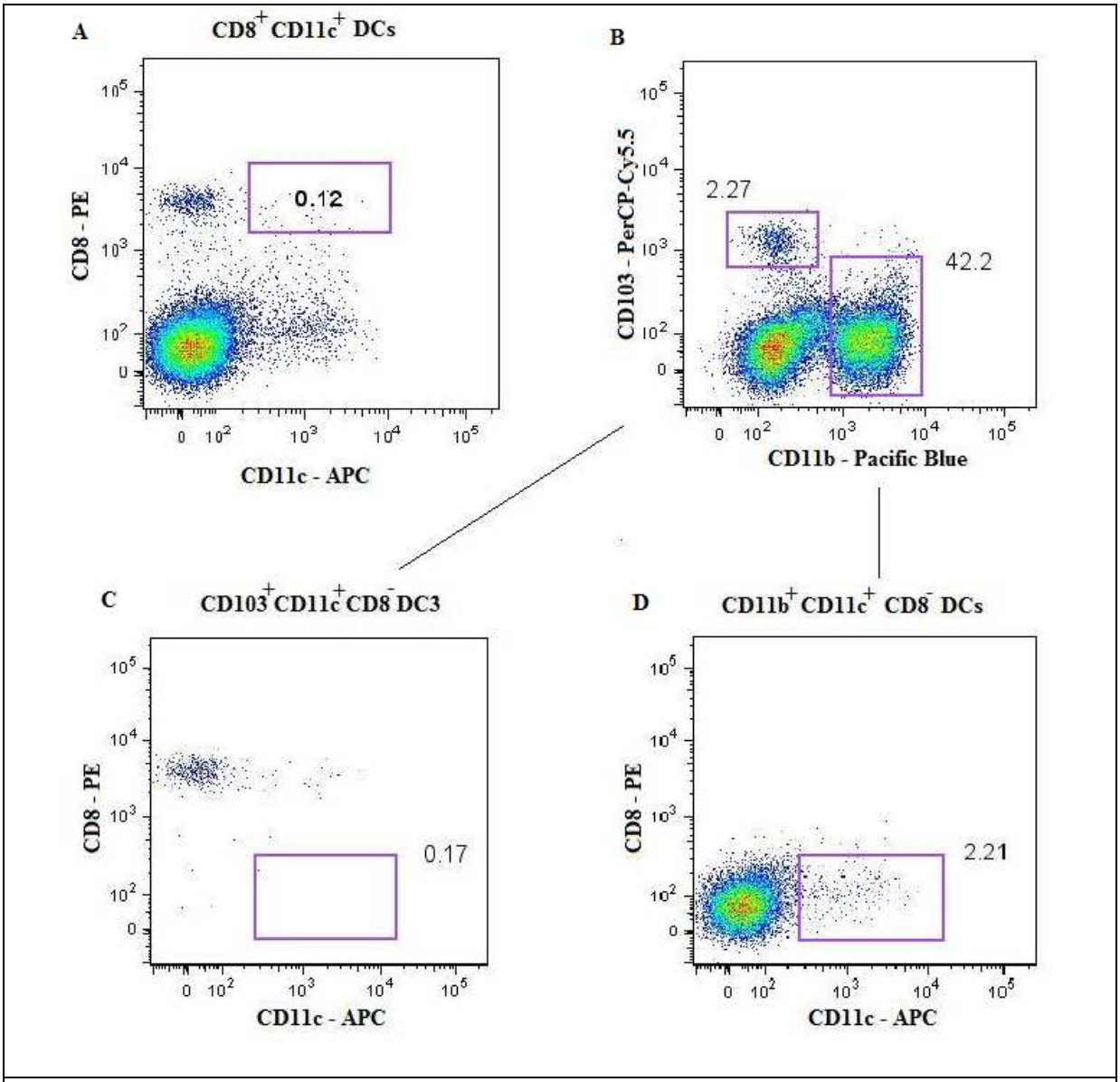


Figure 4.1: **Gating scheme for identification of DC populations in BM**. Living cells were either gated for $CD8^{+}CD11c$ DCs (A) or gated to identify $CD103^{+}$ and $CD11b^{+}$ cell populations (B). $CD103^{+}$ cell population were further gated to identify $CD103^{+}CD11c^{+}CD8^{-}$ DCs (C). $CD11b^{+}$ cell population were further gated to identify $CD1b^{+}CD11c^{+}CD8^{-}$ DCs (D)

Results

Bioinformatic prediction of possible roles of Irf6 in T-helper commitment:

Analysis of microarray data from *Irf6*-knockout skin (Ingraham et al., 2006) showed a 13-fold reduction of *Il18*. However, no significant change of *IL18* was found when *IRF6* was knocked down in keratinocytes (Botti et al., 20xx). Microarray analysis of the *Foxp3* knockout cells showed a 2-fold reduction of *Irf6*, *Foxp3* overexpression increases *Irf6* transcription (citation). Therefore, *Foxp3* regulates *Irf6* expression in $CD4^+$ $CD25^+$ T-cells. Integration of gene expression profiling and Chip-seq analysis of *IRF6* in keratinocytes showed significant effects on several T cell transcription factors. There was significant up-regulation of Id3 ($p=0.000584$) and down-regulation of GATA3 ($p=0.015595$) and Klf4 ($p=0.006814$). ChIP sequencing for IRF6 binding sites showed six candidate binding sites in a gene desert downstream to GATA3. In addition, *IRF6* bound to and was required for the regulation of Klf4 (Botti et al., 20xx). Thus, bioinformatic analyses of publicly-available data suggest that *Irf6* is involved in a gene regulatory network during T-helper commitment.

Efficiency of adoptive transfer:

As expected, 100% mortality was observed in untreated control animals 11-14 days after irradiation. Post-mortem examination showed severe pallor of the internal organs and gastrointestinal bleeding. On the other hand, all irradiated mice treated with adoptive transfer survived beyond this time point. To further confirm the efficiency of adoptive transfer, peripheral blood lymphocytes of recipient mice were stained for CD45.1 (the native marker of the recipient mice) and CD45.2 (marker of donor mice).

Flowcytometric analysis of living lymphocytes showed a predominance of the donor marker in recipient mice (Figure 4.2).

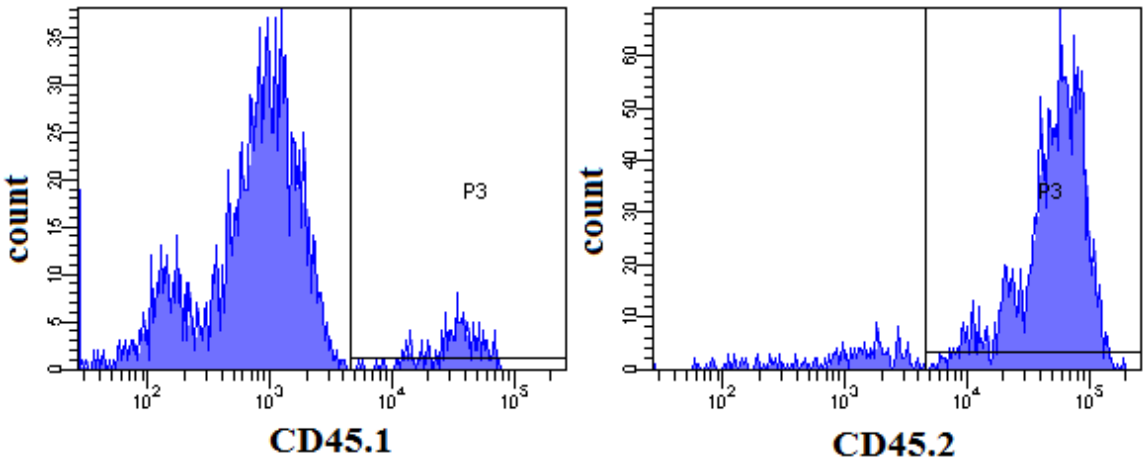


Figure 4.2: **Efficiency of adoptive transfer**. The histograms show the level of CD45.1 expression (marker of the recipient mice) and CD45.2 expression (marker of donor mice) in peripheral blood lymphocytes of adoptively transferred mice.

Th1, Th2, Th17 and Treg differentiation in vitro:

We cultured naïve CD4 cells isolated from the spleens of recipient mice after adoptive transfer from either wild type or *Irf6* knockout fetal livers. Cultured CD4 cells originating from wild type or *Irf6* knockout cells were not different in both non-skewing (Il2 only) or differentiation conditions specific for Th1, Th2, Th17 or Treg (Figure 4.3).

A

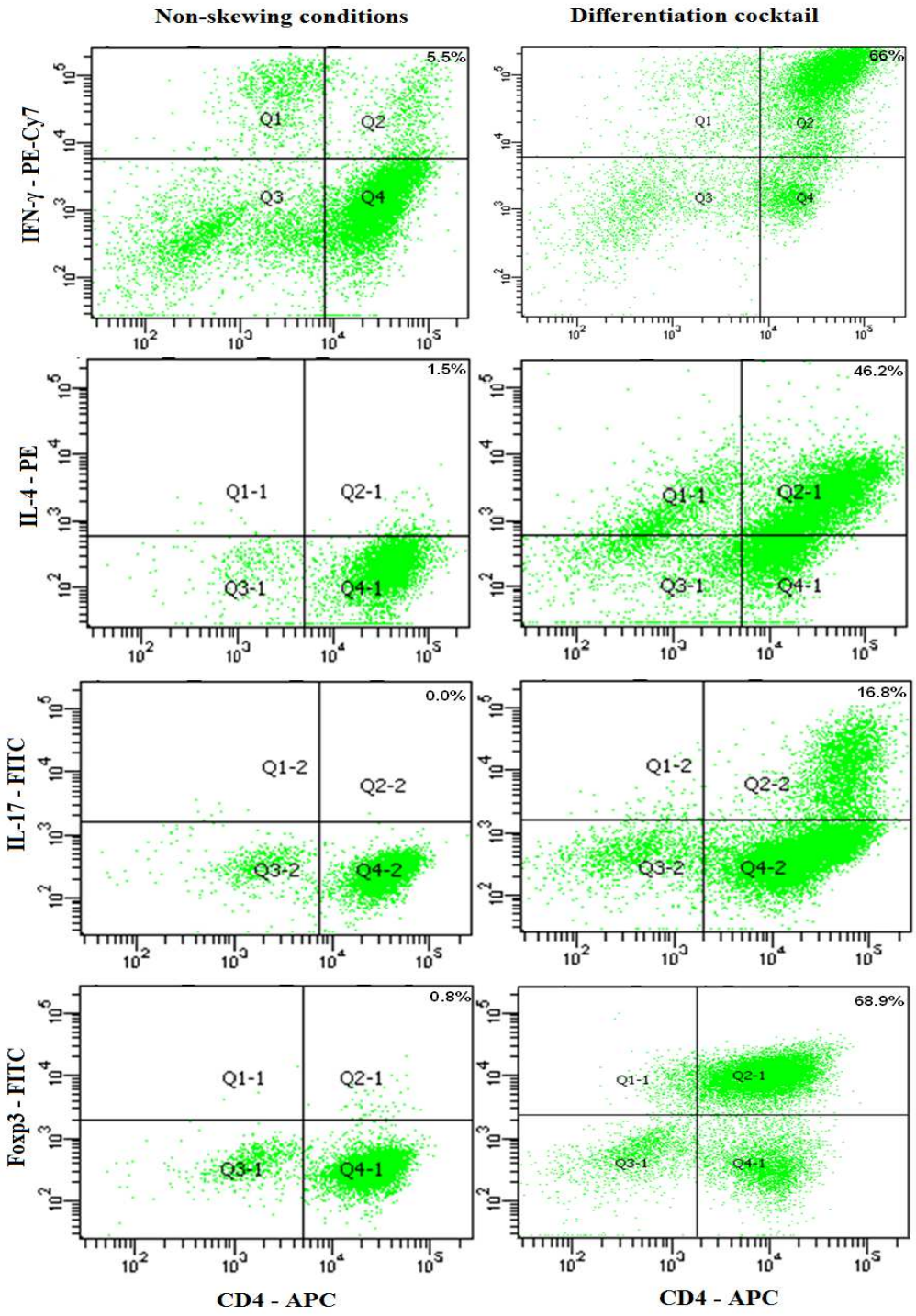
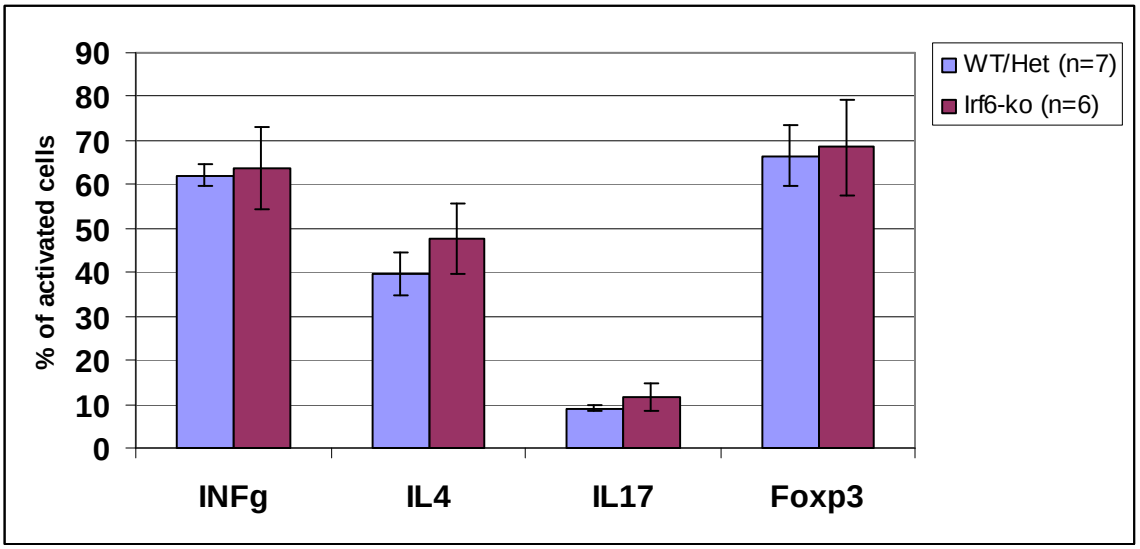


Figure 4.3: **Th1, Th2, Th17 and Treg differentiation in vitro.**

Figure 4.3 **(Cont'd)**

B



Naïve T-cells were cultured with plate-bound anti-CD3 and soluble anti-CD28 in X-vivo medium enriched by differentiation cocktails for Th1, Th2, Th17, Treg or under non-skewing conditions for 4 days. Cultured cells were reactivated with PMA and ionomycin for 5 hours and cytokine expression was blocked by Monensin. Living cells were determined by the viability dye assay. Differentiated cells were identified by co-expression of intracellular CD4 and either INF-γ, IL4, IL17, or Foxp3 as markers of Th1, Th2, Th17 and iTreg populations. Thresholds were identified by comparison to matching populations grown under non-skewing conditions (Panel A from top to bottom respectively shows a representative sample). Mean and standard error of Irf6 expression in non-mutants and Irf6 knockouts were blotted in the bar graph (B).

Changes of bone marrow DCs in Irf6 adoptively transferred mice:

Bioinformatic analysis suggested a possible role for *Irf6* in regulating the functions of DCs. Flowcytometric analysis of living cells in bone marrow (BM) from adoptively transferred animals showed ~ 3.5 % $CD11c^+$ cells in both wild type and *Irf6* knockout samples. $CD8^+CD11c^+$ cells were barely detected (< 0.2%). The two main DCs sub-populations identified were $CD103^+CD8^-$ and $CD11b^+CD8^-$ cells. Subpopulation size did not allow for a statistical analysis (Figure 4.4).

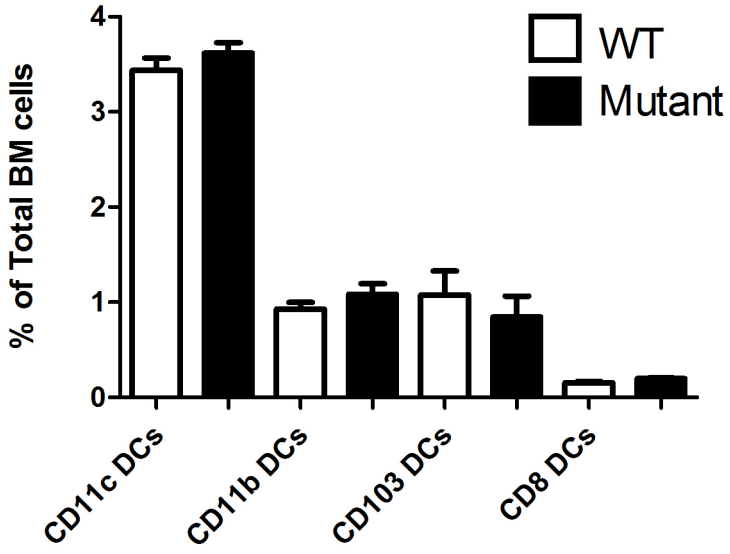


Figure 4.4: **Frequency of total DCs and their sub-populations in bone marrow**. About 3.5 % are positive for $CD11c^+$. $CD8^+CD11c^+$ cells were barely detected (< 0.2%). The two main DCs sub-populations identified were $CD103^+CD8^-$ and $CD11b^+CD8^-$ cells (n= 3 for each genotype).

In GM-CSF supplemented culture, the cells soon became adherent to the plates. Cells were sampled for flowcytometric analysis at days 3, 6, and 9 of culture. The cultures were sampled after another 24 hours under normal culturing conditions or under stimulation with LPS. The frequency of $CD11c^{+}$ cells increased gradually with selective differentiation into $CD11b^{+}CD8^{-}$ sub-population. We also found a $CD11b^{hi}$ population between days 3 to 6 day that was lost by the ninth day. We saw a relatively faster increase in the frequency of $CD11c^{+}$ and $CD11b^{+}$ at day 9 of culture in *Irf6* knockout mice but this was not statistically significant (Figure 4.5).

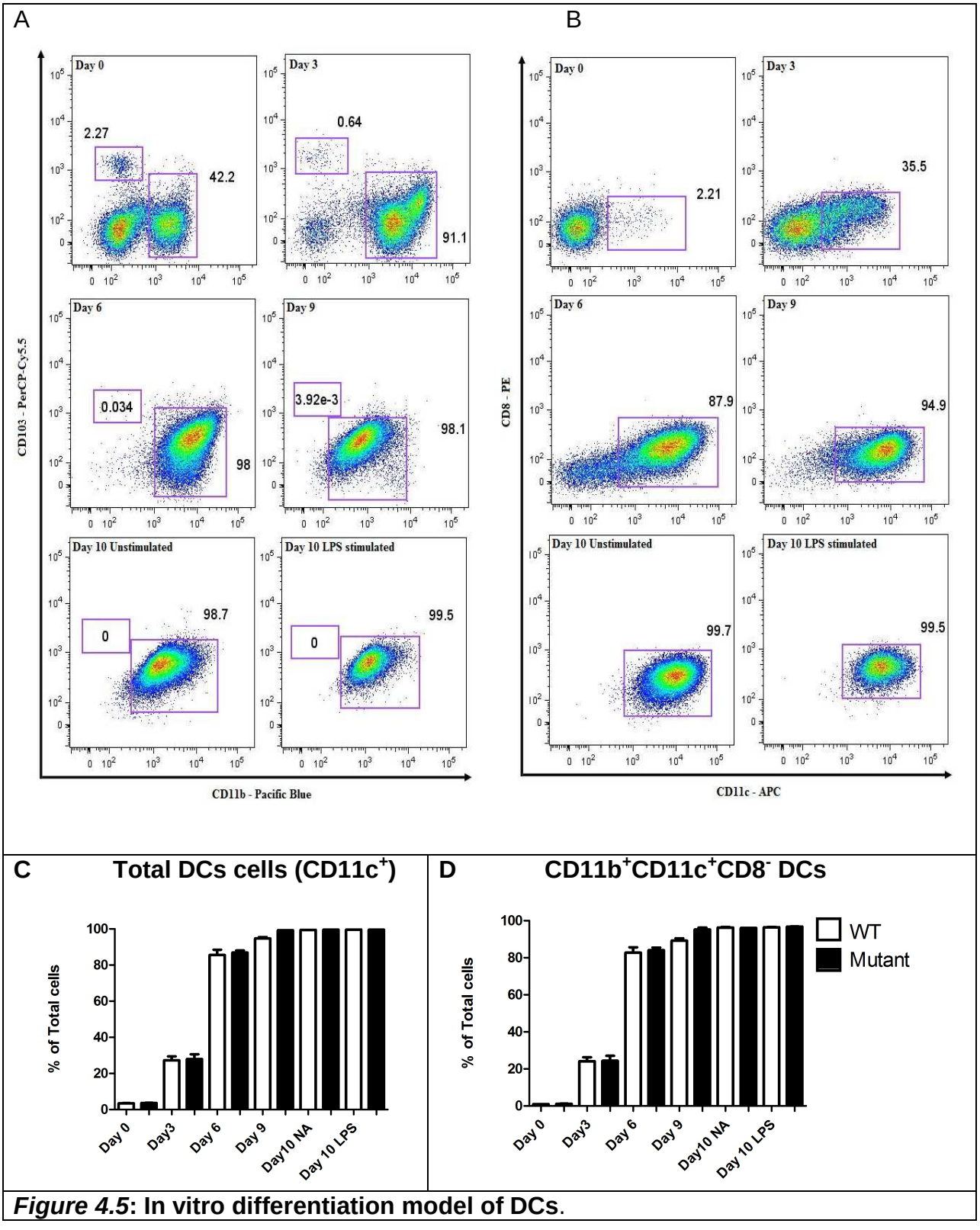
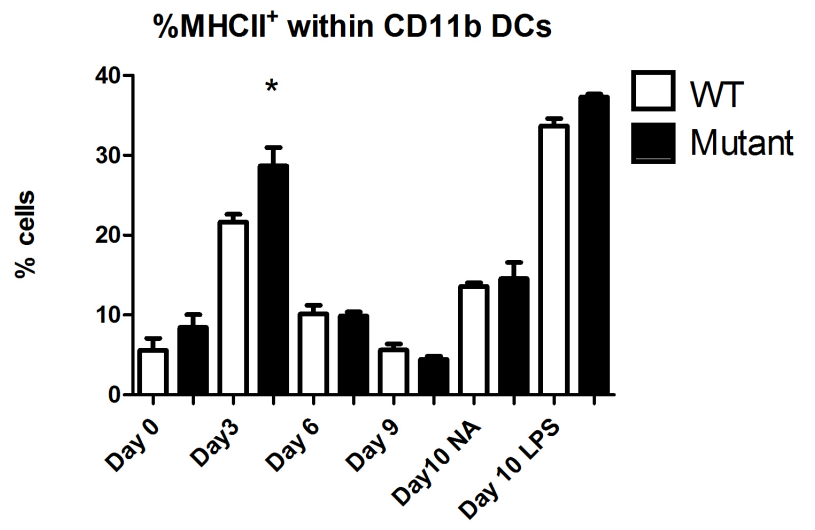


***Figure 4.5*: In vitro differentiation model of DCs.**

***Figure 4.5* (Cont'd):** Cells were cultured with GM-CSF and sampled at culture days 0, 3, 6 and 9. Cells were than cultured for another 24 hours either unstimulated or under stimulation with LPS. (A) A representative sample showing the expression of CD11b and CD103 on living cells. The cells gradually progressed towards CD11b positive phenotypes. There is a $CD11b^{hi}$ population seen between days 3 to 6 that was than lost by the end of day 9. (B) The expression of CD11c and CD8 on $CD11b^{+}$ cells from panel A. There is gradual increase in $CD11c^{+}$ cells through the days of cultures until reaching almost 100%. (C,D) Bar diagrams showing the changes in the frequency of total DCs (All $CD11c^{+}$ cells) (C) and changes in the $CD11b^{+}CD11c^{+}CD8^{-}$ DCs sub-population (D) in wild type and Irf6 knockout cells. GM-CSF skewed the differentiation of the bone marrow cells into $CD11c^{+}$ $CD11b^{+}$DC. By day 3, almost all cells that are $CD11c^{+}$ are $CD11b^{+}$ as well. There is no significant difference between the cell frequencies of wild type and Irf6 knockout mice (n= 3 for each genotype)..

MHC-II and the co-stimulatory molecule CD86 are surface markers of DCs associated with maturity and functional commitment (Banchereau *et al.*, 2000). We followed up the expression of maturation markers at the time of BM sampling and throughout the in vitro differentiation course. We saw an initial increase of both MHC-II and CD86 in knockout *Irf6* DCs at the time of BM collection, however it was not statistically significant. Cultured cells showed a marked increase of maturation markers at day 3 followed by gradual decrease until day 9. Interestingly, the level of MHC-II expression was significantly higher in knockout *Irf6* DCs at day 3 (p value = 0.016). As expected, there was a marked increase in the expression of MHC-II and CD86 after 24 hours of LPS stimulation but there was no significant difference between wild type and knockout *Irf6* cells (Figure 4.6).

A



B

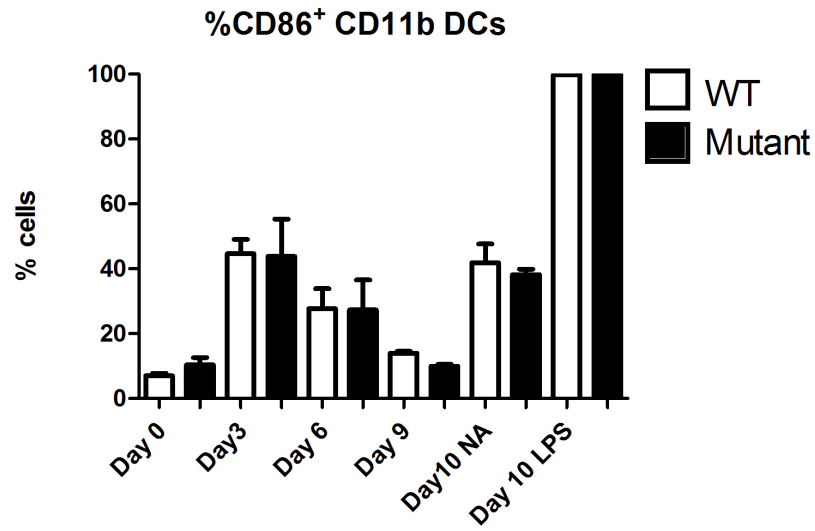


Figure 4.6: **Expression of maturation markers on DCs in culture**. Mean fluorescence intensity of MHC-II (A) and CD86 (B) expression from DCs following in vitro differentiation cultures of wild type and *Irf6* knockout mice. Cells were sampled at days 0, 3, 6 and 9. Then further sampled 24 hours after culturing either unstimulated or under stimulation with LPS. A significant increase in MHC-II expression was observed at day 3 between *Irf6* wild type and knockout mice (n= 3 for each genotype).

Discussion

Irf family members are either indispensable for normal T helper differentiation or have known variants associated with autoimmune disorders of T cells (Lohoff & Mak, 2005; Patel, 2011). *Foxp3, Id3*, *Gata3* and *Klf4* are a group of transcription factors responsible for developmental regulation and balance between Th17 and Treg. Microarray analysis of *Foxp3* altered expression in $CD4^+$ $CD25^+$ suggested that *Irf6* is a downstream target of Foxp3. Botti et al 2011 performed gene expression profiling in primary human keratinocytes after siRNA-mediated *IRF6* depletion. Expression analysis showed a significant increase of ID3 and significant reduction of GATA3 and KLF4. In the same study, genome-wide analysis of IRF6 binding sites suggested direct transcriptional regulation with both GATA3 and KLF4. These data favor an intrinsic role of *Irf6* in regulating the balance of Th17 and Treg with less Th17 differentiation relative to Tregs in the *Irf6* knockout (Figure 4.7)

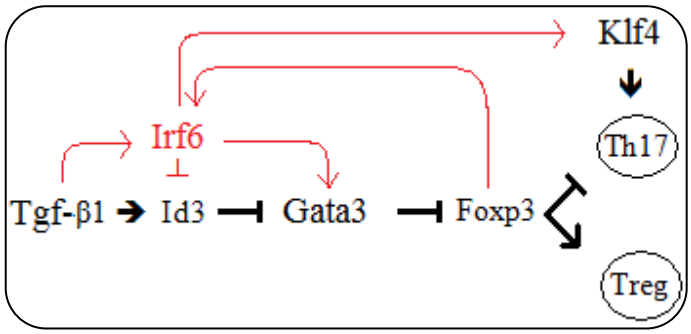


Figure 4.7: **Proposed model of *Irf6* in Th17/Treg balance**. Bold black lines represent the well-known pathway of Th17/Treg. The red lines represent the predicted genetic interactions with Irf6

Adoptive transfer is a successful tool to overcome the problem of perinatal lethality. One major advantage compared to the tissue specific knockout approaches is the ability to study the effect of gene deficiency in the whole haematopoietic system with the known extensive cell-cell interactions. However, adoptive transfer experiments generate animal chimeras which reduce but do not eliminate the effect of the target gene. Using adoptive transfer, we found that *Irf6* knockout fetal livers were sufficient to replenish the bone marrow of lethally irradiated mice. Lymphocytes in the peripheral blood of chimeric mice were shown to be mostly derived from donor stem cells indicating functional competency of *Irf6*-deficient stem cells.

We successfully completed *in vitro* culture to yield different T helper subsets using conditioned medium. The rate and efficiency of differentiated T helper subsets seen here was highly comparable to prior work. We compared the frequency and mean fluorescence intensity of cytokine production with and without *Irf6.* Remarkably, *in vitro* differentiation of *Irf6* knockout cells successfully produced all T helper subsets targeted in this work. In fact, the production of these T helper subsets was not different from wild type cells. Therefore, we conclude that intrinsic *Irf6* expression is not essential for the differentiation of Th1, Th2, Th17 or Treg.

Bioinformatic analysis showed a 13-fold reduction of *Il18* in *Irf6*-deficient skin but no change after knocking down *IRF6* in human keratinocytes. Because keratinocytes and APCs are the predominate source of Il18 in skin (Huising *et al.*, 2004), these findings favor APCs as the primary driver for changes in Il18 expression in the *Irf6*-deficient skin (Nicklin *et al.*, 1994; Suttles *et al.*, 1990). Il18 is a powerful Th1-polarizing

cytokine (Fietta & Delsante, 2009) so that systemic deficiency of APC-derived Il18 in *Irf6* knockout would be expected to prevent the appropriate polarization of Th1. To test this hypothesis we studied the *Irf6*-deficient bone marrow DCs from adoptively transferred mice. We tested for any changes in counts and adopted an in vitro approach to test their development and functional commitment Direct analysis of bone marrow from adoptively transferred animals showed that 3.5% of bone marrow cells stained positive for the DC marker CD11c independent of the genotype. Most of $CD11c^+$ cells were almost equally divided between CD103 and CD11b sub-populations. The paucity of this subpopulation did not allow for a valid statistical analysis. For in vitro differentiation with GM-CSF, we saw a gradual increase in $CD11c^+$ cells until reaching almost 100%. GM-CSF skewed the differentiation of the bone marrow cells into $CD11c^+$ $CD11b^+$ DC. By day 3, CD103 DCs were lost and almost all cells that are $CD11c^+$ became $CD11b^+$ as well.

It is interesting to note the appearance of the CD11b-high population between days 3 to 6. However, the magnitude of CD11b expression decreased by day 9. CD11b, also known as Integrin alpha M and macrophage-1 antigen (Mac-1), is a member of the β_2-integrin family of adhesion molecules. It plays a role in cell adhesion, phagocytosis and extravasation (Springer, 1990). Initially, in our *in vitro* model, the seeded bone marrow cells that were non-adherent become adherent upon persistent GM-CSF addition. This observation might explain the requirement of such high levels of integrin expression. High confluence in the plates at the later time points of the culture could trigger a reduction in expression.

As markers of maturation, we assessed the expression of MHC-II antigens and the co-stimulatory molecule CD86 throughout the days of culture. Significantly, we found a trend toward increased MHC-II expression in knockout $CD11b^{+}$ DCs that became statistically significant at culture day 3. MHC-II expression in APCs is tightly regulated (Pai *et al.*, 2002). In mouse immature dendritic cells, MHC-II beta-chain cytoplasmic tail is ubiquitinated which is partly required for the sequestration of MHC-II (Shin *et al.*, 2006; Tze *et al.*, 2011). Interestingly, *Irf6* is known to negatively regulate transcriptional factor P63 (Tp63) by targeting it for proteasome-mediated degradation *(Thomason et al., 2010)*. Proteasomal degradation of Tp63 is also ubiquitin-mediated (Li *et al.*, 2008; Westfall *et al.*, 2005). Irf6 might be required at least partially for ubiquitin-mediated regulation of MHC-II in immature DCs. This finding supports our bioinformatic suggestion for a non-cell autonomous role of Irf6 in T cell differentiation through dendritic cells. Normal counts and overall developmental pattern of *Irf6*-deficient bone marrow DCs suggests functional redundancy of *Irf6*. However, our bioinformatic proposal is mainly pointing to Langerhans cells, a DC population that predominate the skin. Considering that *Irf6* function has most clearly been delineated in skin, specific analysis of Langerhans cells might be a valuable determination.

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CHAPTER FIVE

Summary and Future Directions

Summary and Future Directions

IRF protein family has nine members in mammals (Huang et al., 2010; Nehyba et al., 2009). All family members share a highly conserved N-terminus DBD and a less conserved protein-binding domain at the C-terminus (Kondo *et al.*, 2002). The IRF family is known to orchestrate homeostasis of host defense. They are mandatory for regulation of type I interferon responses, development and function of a cadre of immune cell types, and cell cycle control and oncogenic pathogenesis (Tamura *et al.*, 2008; Taniguchi *et al.*, 2001). *Irf6* is the only IRF known to be involved in skin and craniofacial development. Haploinsufficiency of human IRF6 cause two Mendelian clefting disorders (Kondo *et al.*, 2002). Furthermore, a common DNA variant at the *IRF6* locus contributes risk for isolated cleft lip and palate (Rahimov *et al.*, 2008). Mice deficient for Irf6 display severe skin, limb, and craniofacial defects while heterozygosity for Irf6 null allele is associated with oral adhesions (Ingraham *et al.*, 2006; Richardson *et al.*, 2006). However, IRF6 shares some native functions of its family. IRF6 is involved in regulating the cell cycle with an anti-proliferative function in keratinocytes and mammary epithelial cell (Bailey *et al.*, 2008; Ingraham *et al.*, 2006; Richardson *et al.*, 2006). Also, mutations in *IRF6* have also been associated with oncogenesis (Bailey *et al.*, 2009; Botti *et al.*, 2011; Stransky *et al.*, 2011). Regulation of the immune system, a major function of all family member, is yet undetermined for *IRF6*. Proteomic studies showed IRF6/IRF5 and IRF6/IRF8 as candidate protein complexes involved in regulating interferon type I. Despite DNA conservation of the DBD, and structural homology and interactions between IRF6 and more canonically described immune IRF family members, expression data of *IRF6* in haematopoietic system is completely lacking. Cost and labor required for assessment of expression for a given gene through

out the whole haematopoietic system is devastating. We decided to use a bioinformatic approach to examine the expression of Irf6 thought out the developing stages of haematopoietic cells using the publically available whole transcriptomic data for almost every cell type.

We found that *Irf6* is expressed early in haematopoiesis specially in long term hematopoietic stem cells with abrupt attenuation of *Irf6* expression in hematopoietic lineage committed progenitors. Common myeloid progenitor and myeloid erythroid progenitor isolated from old mice showed relative increase of *Irf6*. Also we identified *Irf6* expression in T cell lineage, including developing and functionally committed stages. Future studies should confirm the expression of Irf6 in these predicted populations. Flowcytometric sorting of targeted populations from bone marrow, thymus and splenic suspensions followed by RT-PCR and western plot for Irf6 would be ideal.

In our bioinformatic analysis, Variability of expression of in the same cell type among different experiments was sometimes striking. Unavoidable biological variability and using different probes in different microarray chips are valid reasons for variability. However non-linear noise signature of microarray experiments (also called batch effect) is another important factor that always hinders similar meta-analysis studies. Batch effect can be defined as the systematic error introduced when samples are processed in multiple batches. Several approaches have been developed for removing batch effects from microarray data (Scherer, 2009). However, having different cell types running in separate batches is the worst possible experimental design. Chen et al said “No way to correct for poor experimental design. If cases and controls are run in separate batches, genuine biological variation can be entirely confounded by batch effects. No method

was able to reduce the batch effects sufficiently without also removing the variation caused by case-control differences" (Chen *et al.*, 2011). I am proposing a new approach to deal with similar conditions. If we can collect enough number of microarray profiles done in multiple batches for a given cell type, we can assume that only true biological data is shared among all profiles. Simple data decomposition approaches can discriminate between batch effects and biological variance. Once we identify the batch effect for every experiment, we can correct the profiles of other cell types tested in these experiments. Working on development of such technique that might allow us to correct for batch effects in cases of poor experimental design would open a new horizon for utilizing the tremendous resources of publically available data.

Selective Expression of *Irf6* in HSC and T cell lineage suggested its functional requirement. However perinatal mortality of *Irf6* knockout mouse hindered our ability to study the role of Irf6 in the haematopoietic system (Ingraham *et al.*, 2006). To test if expression of Irf6 expression is essentially required for development of these cells, *Irf6* knockout fetal livers were adoptively transferred to lethally irradiated adult mice. Chimeric mice were able to recover and lymphocytes in their peripheral blood were mostly derived from donor stem cells. This indicates the ability of Irf6 knockout stem cells to replenish the bone marrow and develop into mature lymphocytes.

Chimeric mice are particularly useful because they enable us to test the effect of Irf6 deficiency in adult mice. Furthermore, it enables the study of the gene effect in the whole haematopoietic system with the known extensive cell-cell interactions. Future studies should employ the Chimeric mice to explore the function of *Irf6* in HSC. LT-HSC should be able to repopulate the bone marrow for life while ST-HSC has a

repopulation potential not exceeding 8-12 weeks (Passegue *et al.*, 2005). High level of Irf6 expression in LT-HSC suggests a possible role in long term engraftment studies. Chimeric mice should be followed up for more than 3 moths to detect later complete or partial failure of engraftment. One alternative approach to test for this is serial transplantation, in which donor HSCs are engrafted into a primary host then subsequently isolated and engrafted into secondary hosts (Weissman, 2000). Serial transplantation of HSC can replenish the recipient bone marrow in successive but limited transplants. The number of transplants correlates with the efficiency of HSC. Furthermore, the stem cell exhaustion in serial transplantations mimics an accelerated aging process, thus we can use this to test if deprivation of exhausted progenitors of Irf6 would accelerate their aging process (Ramkumar *et al.*, 2013). Competitive repopulation is another assay to detect the minimal defects by mixing Irf6 deficient HSC with wild type HSC. Blood cells produced by each genotype should be proportional to the mixing ratio unless the Irf6 deficient progenitor is sub-optimally efficient for one or more lineage commitment (Harrison, 1980).

To test for the effect of Irf6 expression in developing lymphocytes, we characterized the thymi in Irf6 knockout embryos at E17.5 in comparison to their wild type littermates. We found that *Irf6* regulates proliferation in thymocytes where loss of *Irf6* leads to an expansion of DN thymocytes and reduction of DP thymocytes. This effect is parallel to some extend to what Irf6 does in the skin. In contrast to the pro-apoptotic effect of Irf6 in the skin, we did not see change in the total no of apoptotic or dead cells. Further analysis for the no of apoptotic cells in the thymic sub-popualtion is mandatory to identify any hidden balanced disturbances. Our rate of apoptosis of

embryonic thymocytes is about 2% which comparable to those observed by others in adult mice (Ismael *et al.*, 1998; Jung *et al.*, 2004). However this rate of spontaneous apoptosis might not be able to detect saddle effect. Apoptosis induction experiments (e.g. Anti-CD3 inducted apoptosis) should be done to verify the effect of Irf6 deficiency on thymic apoptosis (Chrest *et al.*, 1995). In vitro apoptosis assays can be used as well to test for activation-induced cell death by culturing thymocytes with anti-Fas, plate-bound anti-CD3, or dexamethasone for 24 h to induce apoptosis (Shui *et al.*, 2007).

We observed sustained counts of single positive thymocytes in spite of the reduction of their immediate precursors. This was not an effect of increased proliferation as shown by normal level of BrdU incorporation. We still have 2 possible explanations; enhanced survival of single positive cells and thymic retention of naïve lymphocytes. We need to test for the efficiency of negative selection to see how if it is reduced to compensate for lower rate of proliferation in DP cells. We can breed The Irf6 heterozygous mice onto a background expressing alloreactive TCR (e.g. BM3.6 transgenic mice). Efficient negative selection would eliminate all the DP thymocytes (Sponaas *et al.*, 1994). To test the hypothesis of thymic retention, we can mate the $Irf6^{gt/+}$ with Rag2p-GFP mice. Boursalian et al created these mice to identify recent emigrants of the thymus. GFP is expressed at a high level in DP thymocytes under the Rag2 promoter. Because GFP is a relatively stable protein, cells recently emigrating from the thymus can be identified by a “shoulder” of GFP expression (Boursalian *et al.*, 2004). McCaughtry et al calculated the half-life of GFP protein in RAG2p-GFP transgenic mice to calculate the thymic retention time (McCaughtry *et al.*, 2007).

We showed that Irf6 is expressed in the subcapsular cortex and medulla. However, we did not delineate if this expression is in the thymic epithelium or thymocytes or both. One of the important future directions is to determine the exact site of expression underlying the reported altered proliferation. One of the best genetic approaches would be the conditional knockouts. Floxed Irf6 strain can be crossed with different tissue specific Cre recombinase strains to induce Irf6 excision only in these target tissues. Lck-Cre and CD2-Cre transgenic mice are typically used to generate T-cell-specific conditional knockout mice. Regulatory sequences of the two stains drive the Cre recombinase enzyme expression very early in DN thymocytes, however CD2-Cre target the B cells as well (Garvin *et al.*, 1988; Wildin *et al.*, 1991; Zhumabekov *et al.*, 1995). CD4-Cre mouse is another transgenic strain which expresses the Cre recombinase under the control of a CD4 minigene. The CD4 minigene is composed of the proximal enhancer, the promoter and the silencer of CD4 gene. These regulatory sequences start the Cre expression in DN3 ($CD44^{-}CD25^{+}$) stage (Wolfer *et al.*, 2001). To target developing thymocytes starting from the DP stage, Rorc-Cre strain would drive the expression of Cre recombinase in DP thymocytes and their single positive progeny whereas DN precursors would be untouched (Eberl & Littman, 2004).

By the age of 6 month, we saw significant increase of CD4:CD8 ratio in *Irf6* heterozygous mice. A more prolonged study is required to test the effect of Irf6 heterozygocity in older mice. We proposed impaired notch signaling in developing thymocytes mediating the altered CD4:CD8 ratio. Notch suppression can be assessed by measuring its canonical target gene HES1 transcription.

Adoptively transferred mice is another approach to discriminate between haematopoietic form non-haematopoietic mechanisms. In chimeric mice, the haematopoietic elements of the thymus should be Irf6 deficient while thymic epithelium would be wild type. Careful analysis should be considered because of the possible left over recipient Irf6 wild type cells. Also we should consider the possible differences between embryonic developmental events we are trying to replicate and the adult thymic microenvironment we have in chimeric mice. Whereas the adoptively transferred mice is a very suitable model to study the Irf6 dosage effect on the postnatal thymic changes of CD4:CD8 ratio.

Future studies should be designed to determine the underlying molecular changes in Irf6 knockout thymocytes. Previous transcriptomic studies tried to figure out transcriptional networks regulated by Irf6 but non of them was completely successful. There are 3 microarray studies on mammalian tissues aimed to profile the transcriptional signature of *IRF6.* The earliest study examined murine knockout skin. An important caveat is that skin includes both the epidermis and dermis. Considering that Irf6 is only expressed in the epidermis, the dermal tissue added unavoidable noise to the expression profile. The other studies were done by knocking down IRF6 in primary human keratinocytes and erythroid progenitors (Botti et al., 2011; Xu et al., 2012). Silencing efficiency limits the generalizability of these results. Microarray experiment utilizing a homogenous cell population and a knockout model would be the most sensitive assays at detecting transcriptional regulation by IRF6. We need to identify beyond doubt the primary cell type causing the altered proliferation of developing

thymocytes. The ease of sorting the thymic population would provide an almost homogenous population where transcriptional analysis would be very valuable.

Common variants in IRF6 contribute risk toward orofacial clefting. Rare variants in *IRF6* lead to Van der Woude and Popliteal Pterygium Syndromes. This data suggests that individuals with common and rare *IRF6* variants are at increased risk for immunological diseases. Future studies should be designed to screen for the neonatal lymophocyte counts in those patients. Furthermore, old patients with Irf6 variants should be tested for early thymic involution.

Irf family members are either indispensable for normal T helper differentiation or have known variants associated with autoimmune disorders of T cells (Lohoff & Mak, 2005; Patel, 2011). We tried to utilize the publically available microarray studies to suspect the possible roles of Irf6 in T-helper commitment. We predicted a possible role of intrinsic Irf6 expression in induction of Th17 differentiation on the expense of Treg differentiation. Also we expected that Irf6 regulates the DC functions to mediate a non-cell autonomous polarization of Th1.

To test our hypothesis about the necessity of intrinsic Irf6 expression for regulation of T helper differentiation, we adopted an in vitro differentiation model. Naive T cells lacking Irf6 could be differentiated into Th1, Th2, Th17 and Treg using a specific cytokine cocktail. This experiment proves for the first time that intrinsic Irf6 expression is not essential for T helper subset differentiation. There is no difference in cell frequency and mean fluorescence intensity between wild type and Irf6 knockout cells. However, the sensitivity of the in vitro model to detect saddle changes is questionable. The

concentrations of cytokines used in culture to induce differentiation are too high to saturate the signaling pathways. Also the signaling pathways essential for in vitro differentiation are not always the same those invoked in vivo. For example, IL-4 is essential for in vitro Th2 differentiation and naive T cells with mutant genes in the IL-4/STAT6 signaling pathway can not produce Th2 cells. By contrast, in vivo Th2 differentiation can occur in mice that have deletions in IL-4, IL-4Rα or STAT6 (van Panhuys *et al.*, 2008). In the future, in vivo differentiation analysis of Irf6 deficient T cells should complement our current work. Adoptively transferred mice can be used in these experiments; however it will not be able to differentiate between the autonomous T cell defects and non-autonomous defects mediated by other Irf6 deficient haematopoietic cells. The lineage specific conditional knockout would be the best model for such experiments. Irf6 floxed strain needs to be crossed with Cre transgenic mice expressing the recombinase enzyme under the regulatory sequences of lineage specific promoters. Tbet-Cre, Gata3-Cre, RORγt-Cre, and Foxp3-Cre have been published before (Eberl & Littman, 2004; Francius *et al.*, 2013; Haddad *et al.*, 2013; Rubtsov *et al.*, 2008).

For the analysis of the possible role of Irf6 in DC functions, we tested the bone marrow of adoptively transferred mice for DC populations. Irf6 deficiency has no effect total DC count in BM. In vitro differentiation of BM in GM-CSF enriched medium, showed the same differentiation pattern of DCs of both wild type and Irf6 knock chimeric mice. There is a trend toward increased MHC-II expression in knockout CD11b+ DCs that became statistically significant at culture day 3. This finding supports our bioinformatic suggestion for a non-cell autonomous role of Irf6 in T cell differentiation through dendritic cells. Future studies should cover detailed analysis of DC functions

under Irf6 deficiency. In vitro differentiated DCs should be tested for cytokine production including IL6, IL12p40 and IL10. Flowcytometric analysis if intracellular cytokines is on approach and direct measurement of cytokines in culture supernatants is another one (Said *et al.*, 2014). Migration assay is another important functional determination. The assay is usually done in transwell plates where activated DCs are cultured in the upper wells and allowed to migrate to the lower wells under the guidance of chemotactic agents e.g. CCL5 and CCL21 (Gibbs *et al.*, 2013). T cell co-culture is one of the most canonical experiments to assess the antigen presenting capacity of DCs. To standardize the response, MHC restricted T cells with antigen specific TCRs are used for co-culture. For example, MHC class I-restricted, ovalbumin-specific, CD8+ T cells can be co-cultured with the target DCs after being pulsed with OVA peptide. The Co-culture should be harvested after 4 days and CD8 T cell proliferation and IFN gamma secretion would be measured (Clarke *et al.*, 2000).

Langerhans cells (LC) are distinguishable from other DC populations. LCs are characterized by lower MHC-II levels, and very high levels of the C-type lectin langerin (Merad *et al.*, 2008). *Irf8* controls the trafficking of LCs to the regional lymph nodes (Schiavoni *et al.*, 2004) while *Irf2* is required to maintain normal counts of LCs (Ichikawa *et al.*, 2004). Our bioinformatic analysis suggests DCs as the main source of IL8 deficiency in Irf6 knockout skin. If this holds true, *Irf6* would have an important non-cell autonomous role in T cell differentiation. Considering that Irf6 function has most clearly been delineated in skin, specific analysis LCs should be considered in future studies. Most LCs develop from fetal liver monocytes and self-renew throughout life independently from the BM (Hoeffel et al., 2012; Merad et al., 2002). Importantly, with

severe LC depletion, LCs are repopulated by blood-borne monocytes (Ginhoux et al., 2006). These data indicate that we can study *Irf6* deficient LCs in our adoptively transfer mice. One technical problem that usually faces the analysis of LCs is the contamination of cell preparations with significant numbers of conventional dermal dendritic cells. Initial digestion of the skin with dispase enzyme allows mechanical separation of epidermis without any attached dermal component. Trypsinization of epidermal cells allows the formation of single cell suspension for subsequent sorting of LCs (Pena-Cruz *et al.*, 2001). Culturing and functional studies can be done the same way as described above with conventional DCs.

In conclusion, this research tried for the first time to connect *Irf6* to the canonical roles of the other family members in the immune system. We made use of publically available microarray data to predict the possible roles of *Irf6* throughout the haematopoietic system. Successfully, we were able to confirm a novel role of Irf6 in the proliferation of developing thymocytes. Unlike other family members, intrinsic Irf6 expression is not essential for T helper subset differentiation. However, a non-cell autonomous role for Irf6 in T cell differentiation through dendritic cells remains plausible.

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CHAPTER SIX

Role of endogenous Avian Leukosis Virus in the pathophysiology of spontaneous ALV-like tumors in chickens

Abstract

Lymphoid leukosis (LL) is a B cell tumor of the chicken bursa with a huge economic impact on the poultry industry. The tumors are induced by infection with Avian Leukosis Virus (ALV). However, endogenous ALVs is known to have little or no oncogenic potential. Marek's disease (MD) is another viral neoplastic disorder with hogh mortality rate in chicken. Serotype 2 MD vaccine (MDV2) is an attenuated virus and naturally non-oncogenic but has been shown to enhance the development of both exogenous ALV-induced and spontaneous lymphoid leukosis. AF-227 is a new field strain of subgroup E endogenous ALV (ALV-E). AF-227 has been isolated from commercial chicken experiencing spontaneous ALV-like LL. Although ALV-E viruses are known to be non-oncogenic, the influence of ALV-E and its possible interaction with MDV2 on the enhancement of spontaneous ALV-like LL are still unclear. In this study we used RNA-Seq to generate an expression profile from spontaneous ALV-like LL obtaine from chickens inoculated with strain AF-227 of ALV-E in conjunction serotype 2 MD vaccine to uncover potential molecular oncogenic events.. We identified the absence of Tumor suppressor candidate 2 (*TUSC2*) in all tumor samples. *TUSC2* is a tumor suppressor gene which is considered as a molecular link between inflammatory response and mitochondrial homeostasis (Hood *et al.*, 2013; Uzhachenko *et al.*, 2012). *TUSC2* can influence and complement the PI3K/AKT and p53 pathways. Our pathway enrichment analysis showed significant dysregulation of both pathways. We also identified overexpression of another important proto-oncogene called Eukaryotic translation initiation factor 4E (*EIF4E*). While *EIF4E* overexpression could be a downstream effect of *TUSC2* loss and abnormal Akt signaling, another possible

scenario is the convergence of the both genetic defects to induce the B cell transformation. In this work we identified candidate molecular targets of LL in chickens infected by a new endogenous ALV in conjunction with MDV2.

Introduction

Lymphoid leukosis (LL) is a B-cell lymphoma of chickens. LL usually appears in chickens of about 4 months of age and older. Tumors typically involve the liver, spleen, and bursa of Fabricius (Fadly & Nair, 2008). Tumors are usually composed of aggregates of lymphoblasts of B-cell origin and characterized by monoclonal production of IgM (Payne & Rennie, 1975). The primary lesion usually presents as a well defined mass at the site of the bursa, but at a time the normal bursa tissue is usually gone (Fadly & Nair, 2008).

LL can be induced by transmissible strains of retroviruses called Avian Leukosis Virus (ALV). These strains are defined as exogenous for being transmitted as infectious virus particles. Exogenous ALVs multiply in most tissues and organs of the body but the infection persists longer in bursal lymphocytes, the target cells of neoplastic transformation (Baba & Humphries, 1985). Exogenous non-defective ALVs do not harbor any oncogene; they have been shown to induce lymphoid leukosis by activation of cellular myc oncogene. Only Defective exogenous ALV harbor an oncogene such v-myc, v-src, v-myb, etc. and have been shown to induce acute tumors in susceptible host (Fadly & Nair, 2008). On the other hand, spontaneous ALV-like LL has been shown to develop in certain lines of chickens at one year of age or older. These spontaneous ALV-like LL tumors are detected in certain genetic lines of chickens in absence of infection with any of the subgroups of exogenous ALV, and hence the name spontaneous is used to describe such tumors (Crittenden *et al.*, 1979)

Based on envelope glycoproteins, ALVs that occur in chickens are classified into six groups, A, B, C, D, E and J (Payne *et al.*, 1991; Vogt, 1997; Weiss *et al.*, 1982). Unlike exogenous ALVs, subgroup E viruses are avian retrovirus-like elements that are transmitted genetically in a Mendelian fashion and are termed endogenous viruses (Fadly & Nair, 2008). Domestic chicken genome carries at least 16 endogenous ALV proviral loci (ev-1 through ev-16) (Rovigatti & Astrin, 1983). Many endogenous viruses are genetically defective and incapable of giving rise to infectious virions (Crittenden & Astrin, 1981), whereas others are not and may be expressed in an infectious form (Crittenden *et al.*, 1983). In this form, they then are transmitted similarly to exogenous viruses, although most chickens are genetically resistant to such exogenous infection (Fadly & Nair, 2008). Rous-associated virus type-0 (RAV-0), a subgroup E endogenous virus had little or no oncogenic potential (Motta *et al.*, 1975). However RAV-60, subgroup E recombinants of endogenous and exogenous viruses enhanced the development of lymphoid leukosis (Crittenden *et al.*, 1980; Robinson *et al.*, 1980). Endogenous ALVs also influence the response of the bird to infection by exogenous ALV (Crittenden *et al.*, 1982; Smith & Fadly, 1988). Recently, a field strain of endogenous ALV-E, termed AF-227 was isolated from blood from commercial chickens.

The ability of serotype 2 Marek's disease virus serotype 2 (MDV2) to enhance the development of LL after ALV exposure was reported (Bacon *et al.*, 1989; Fadly & Witter, 1993). Bursa cells co-infected with ALV and MDV2 are more likely to be transformed (Fynan *et al.*, 1992). MDV2 was shown to increase ALV gene expression and virus production (Pulaski *et al.*, 1992). This interaction presents a huge danger because it holds true with the marek’s vaccine attenuated viruses (Marsh *et al.*, 1995).

Furthermore, vaccination of exogenous ALV-free chicken against Marek's disease increased the chance of developing LL (Crittenden *et al.*, 1979). The interaction of MDV with endogenous ALVs in these birds is not well understood. In this study, in order to uncover the molecular oncogenic events of spontaneous ALV-like LL, we used RNA-Seq to generate an expression profile from tumor tissues collected from chickens embryonically inoculated with strain AF-227 of ALV-E and vaccinated with MDV2 at hatch.

Materials and methods

Experimental treatment of chicken: RFS is a strain of chicken that is free of endogenous virus but is susceptible to ALV infection (Zhang *et al.*, 2005). Studied chicken were categorized into 4 groups; control group of 24 chickens injected with phosphate buffered saline (PBS), 36 chickens injected with AF227 alone, 24 chickens treated with MDV2 vaccine alone, and 36 chickens treated with AF227 plus MDV2 vaccination. The PBS and AF227 were given via yolk-sac inoculation at 7 days of embryonation. The MDV2 vaccination was given at hatch intra-abdominally. Chickens were followed up closely for the appearance of the tumors at 4-6 month of age. After euthanasia, tumor samples were collected as well circumscribed masses from the anatomical site of the bursa.

Next generation sequencing of RNA: Six tumor samples from the group treated simultaneously with AF-227 inoculation and MDV2 vaccination were used for the

analysis. As controls, we collected 3 whole bursa samples from 3-week old chickens, and we collected 3 B cell samples from spleens of age matched chicken. We extracted mRNA from tissue homogenates as recommended (Qiagen, RNeasy Kit, Valencia, CA). RNA-seq libraries were prepared using the Illumina TruSeq Stranded mRNA kit following the manufacturer's instructions (Illumina, San Diego, CA). Sequencing was performed on an Illumina HiSeq 2500, run in High Output mode in a paired end 2x100bp format using Illumina TruSeq PE Cluster Kit (v3) and TruSeq SBS Kit (v3). Samples were divided on 3 lanes (4 samples per lane). Raw reads were filtered to remove adaptors, ambiguous reads and low-quality reads. Approximately 661 million clean paired reads (132 Gbp) were obtained (Table 6.1).

Lane	Sample	PF clusters	% per lane	R1 %≥ Q30	R2 %≥ Q30	R1 Ave Q-Score	R2 Ave Q-Score	Insert Size (bp)	Yield (Gbp)
1	Tumor 1	49,249,019	21.6	91.1	68.1	35.7	27.9	484	9.85
1	Tumor 2	37,972,904	16.6	91.0	69.3	35.6	28.4	521	7.59
1	Tumor 3	93,542,998	41.0	91.0	67.9	35.6	27.9	506	18.71
1	Tumor 4	43,807,926	19.2	91.0	68.8	35.6	28.2	530	8.76
2	Tumor 5	38,709,590	17.2	88.4	62.2	34.9	26.0	547	7.74
2	Tumor 6	90,399,233	40.1	87.9	62.1	34.7	25.9	503	18.08
2	B cells 1	52,096,499	23.1	87.7	62.3	34.7	26.0	495	10.42
2	B cells 2	40,697,155	18.1	86.2	58.1	34.2	24.5	540	8.14
3	B cells 3	72,903,292	33.2	86.5	62.2	34.3	26.1	542	14.58
3	Bursa 1	76,772,884	35.0	86.1	61.3	34.2	25.7	541	15.35
3	Bursa 2	34,194,285	15.6	87.0	64.0	34.5	26.7	524	6.84
3	Bursa 3	30,856,288	14.0	86.4	63.0	34.3	26.3	532	6.17
Total		661,202,073							132.2

Table 6.1: **Yield and quality of NGS**. PF clusters: Paired end clusters passing filter. R1: first strand read (the antisense or 3' read). R2: The sense or the 5' read. Q-scores: quality score (A property that is logarithmically related to the base call error probability P where $Q=-10 \log_{10} P$). Q30: quality score of 30

Bioinformatic analysis: Initial exploration of the data quality was done using the FASTX toolkit (FASTX Toolkit by Hannon lab). Quality checking showed abnormally rapid deterioration of the quality scores of the reverse reads (Figure 6.1a). Also there was abnormal distribution of the nucleotide content of the initial 13 bases of either forward or revere reads (Figure 6.1b).

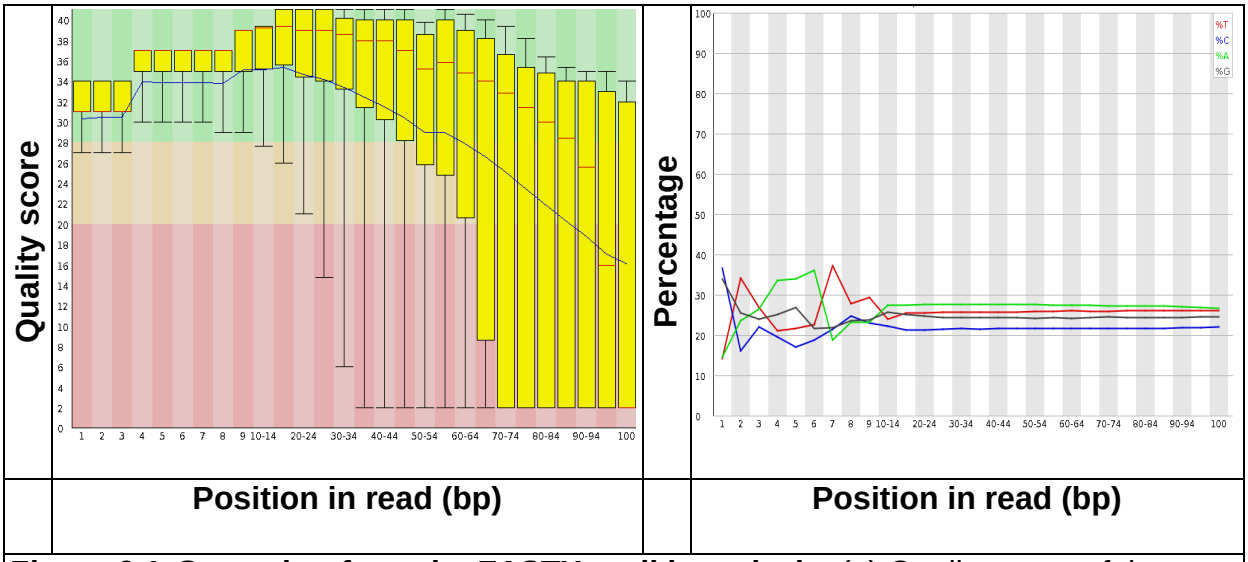

***Figure 6.1*: Snap shot from the FASTX toolkit analysis**. (a) Quality score of the reverse reads samples. (b) Sequence content across all bases

Trimmomatic software was used with its palindromic approach to remove read-through adaptor sequences and isolate orphans from true paired reads (Bolger *et al.*, 2014). Simultaneous head cropping of the first 13 bases was done. Three approaches of data processing were compared to identify the optimal gene model with minimal loss of data. The first two approaches used the Trimmomatic built-in functions. 1) The SlidingWindow method scans from the 5' end and clips the read once the average quality within the window falls below a threshold. 2) The MaxInfo method is an adaptive

quality trimmer that balances read length and error rate to maximize the value of each read. Short reads (less than 36 bases) were excluded after both trimming approaches. 3) We used the pre-processing method (FASTX toolkit) which trims the last 20 bases then reads were filtered out if they fell below a threshold.

TopHat v2.0.10 Software was used to map the reads back to the chicken genome (Kim *et al.*, 2013). The most updated versions of the chicken genome assembly and annotation (Galgal4, release 74) were obtained from the ensemble Genome Browser (ftp.ensembl.org/pub/release-74). Ensemble annotation was preferred over the reference annotation of the Genebank because it covered more genes in our transcriptome (Figure 6.2).

***Figure 6.2**:* **Snap shot of a genome browser (IGV) comparing the annotations of different assemblies**. A representative stretch of the Genebank reference annotation (1st lane), ensemble annotation (2nd lane), and our assembly (3rd lane). The ensemble annotation covered our transcriptome more completely.

One of the known issues about the current chicken assembly is the possibility of replication of some genes between chromosomal and non-chromosomal sequences. To ensure appropriate alignment of the reads to their corresponding genes, we performed two rounds of mapping. For the first round, we mapped all reads against the chromosomal sequences only and limited the initial transcriptome indexing to the chromosomal annotation. The unmapped reads were used for another round of assembly using the non-chromosomal sequences and annotation.

Transcriptome assembly and subsequent differential expression analysis was done using the Cufflinks package v2.1.1 as shown before (Figure 6.3) (Trapnell *et al.*, 2012)

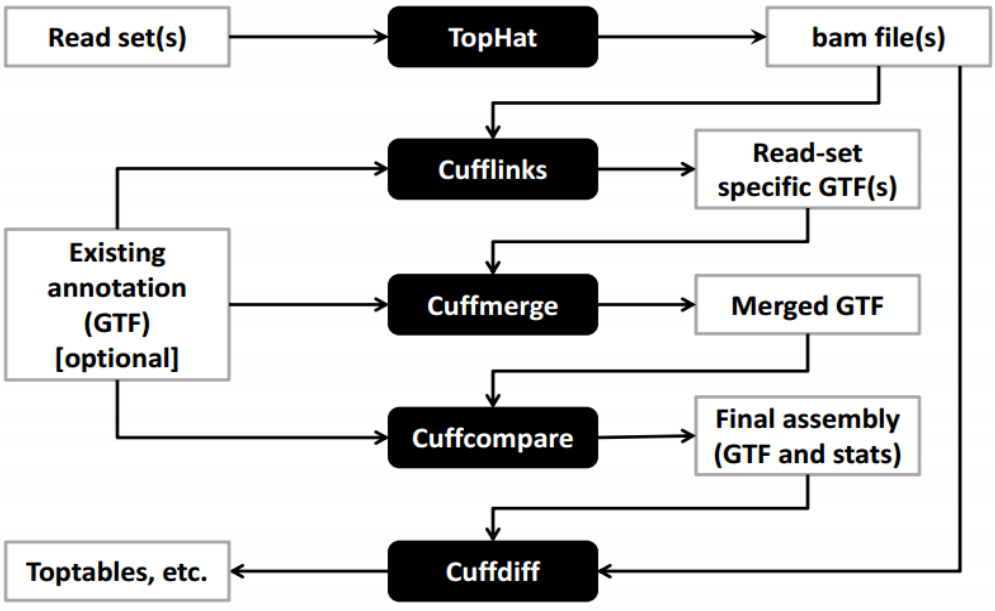

Figure 6.3: **Diagrammatic representation of the TopHat/Cufflinks analysis** (Trapnell *et al.*, 2012)

Downstream clustering analysis was done using R software. For pathway analysis, DAVID annotation tool was used (Figure 6.4 summarizes the whole pipeline).

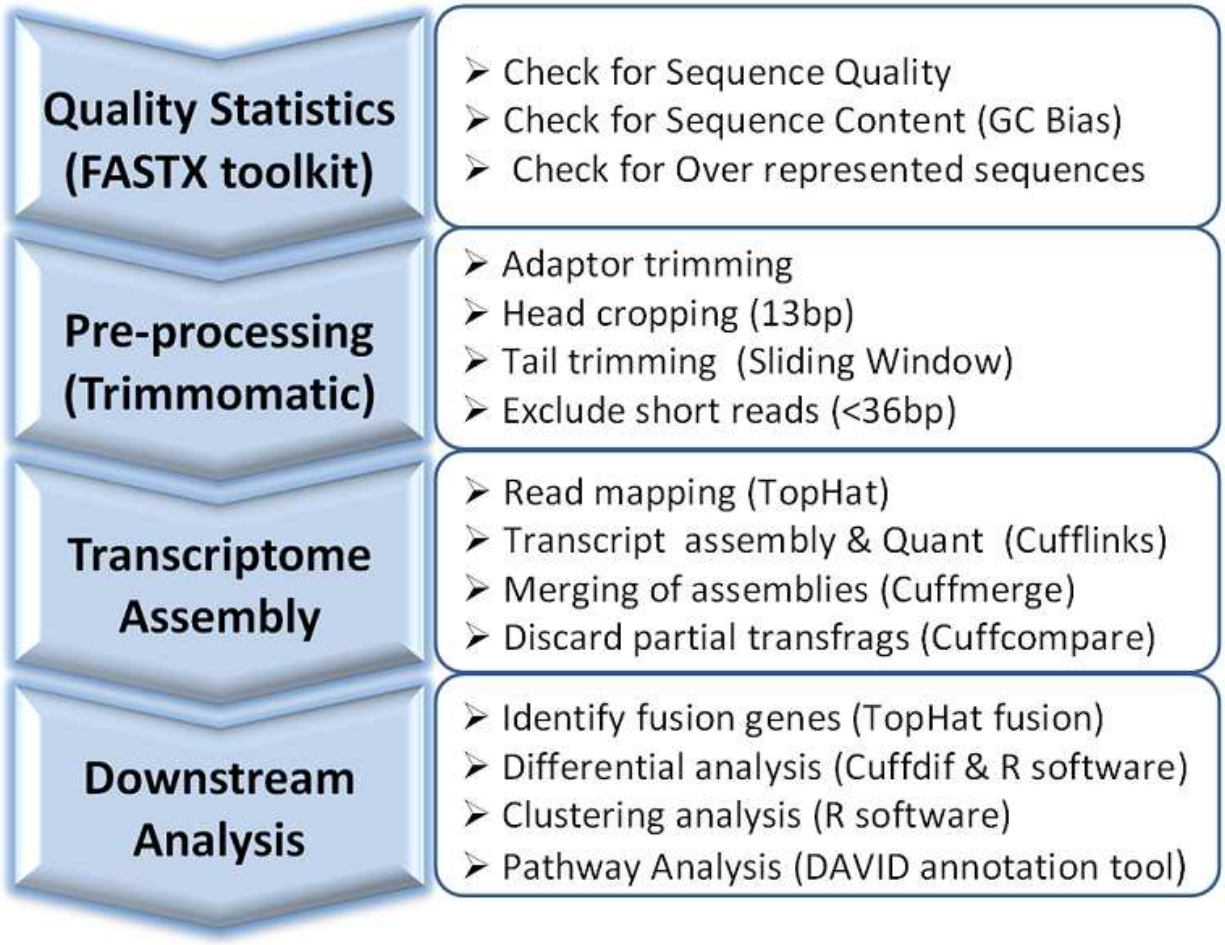

Figure 6.4: Work flow for the pipeline of NGS analysis

Results

Tumor incidence in the studied groups: In the experiment, there are four experimental groups; PBS control group, a group injected with AF227 alone, a group treated with MDV2 vaccine alone, and a group treated with AF227 plus MDV2 vaccination. The incidence of LL-like tumors was 8% in the PBS control group, 14% with chicken injected with AF227 alone, 17% in the group of MDV2 vaccination alone, and 42% in the group treated with both AF227 injection and MDV2 vaccination.

The performance of different preprocessing approaches: The MaxInfo approach caused the minimal loss of data during pre-processing. This allowed the preservation of the highest number of paired ended clusters. However most of these data were lost during the mapping with TopHat. The SlidingWindow and Tail trimming approaches were almost equal when we compared the no of dropped reads after quality filtering. However, SlidingWindow was more successful in keeping longer reads giving the highest average read length after pre-processing. Furthermore, SlidingWindow showed a slightly better sensitivity compared to the other two techniques. Interestingly, the SlidingWindow approach was the least no to map reads outside the annotated genome giving the smallest number of isoforms of new genes. This allowed the SlidingWindow approach to achieve the highest locus specificity (Table 6.2). Considering the quality of the assembly and the accompanying annotation, we recognize that this comparison is not fair and we do not have the appropriate control to judge the actual specificity of

these techniques. In this analysis, our original plan did not include identification of new genes so adding more non-annotated transcripts to our assembly would do nothing but decreasing the statistical power. Thus we selected the SlidingWindow technique to be our preprocessing approach. Future study to compare pre-processing approaches should consider using of a simulated data.

	MaxInfo	SlidingWindow	Tail trimming
Statistics after sample pre-processing			
paired end	75,377,436	59,487,520	57,131,448
singletons	280,950	7,676,613	8,536,476
Total reads	75,658,386	67,164,133	65,667,924
# of bases	5,583,750,709	5,493,544,077	4,596,335,379
Average read length	74	82	70
no of alignments and unmapped reads after TopHat analysis			
no of alignments	44,900,716	42,669,372	41,734,076
unmapped-qc passed	31,102,767	25,340,970	25,375,868
unmapped-qc failed	31,536	5,115	11,067
Total	76,035,019	68,015,457	67,121,011
Quality of mapping			
properly paired	22,512,228	20,867,419	18,671,936
improperly paired	16,213,828	13,747,268	13,719,707
singletons	6,174,660	8,054,685	9,342,433
Comparison to reference annotation (Cuffcompare stat)			
All assembled isoforms	48,353	48,609	51,395
Ref isoform: Complete match	12,393	12,465	12,438
Isoforms of new genes	7,174	5,852	7,539
Locus Sensitivity	78.7	79	78.8
Locus Specificity	51	57.6	52.2

Table 6.2: **Comparing the performance of different preprocessing approaches**.

Results shown represent the smallest sample in size (Tumor sample 2).

Expression profile of Tumor samples is closer to bursa tissue than mature splenic B cells: The normalized read counts mapped to cufflink genes were used for analysis. Testing the rate of gene expression across all the studied samples showed unequal depth of sequencing (Figure 6.5a). To overcome the sequencing depth effect, genes that showed up uniquely due to high sequencing depth were excluded. Ward Hierarchical Clustering was done using Euclidean distance to generate the distance matrix. The new clustering analysis showed low biological variability between samples of the same biological origin and also showed a clear distinction of tumor and bursa samples from splenic B cells (Figure 6.5b)

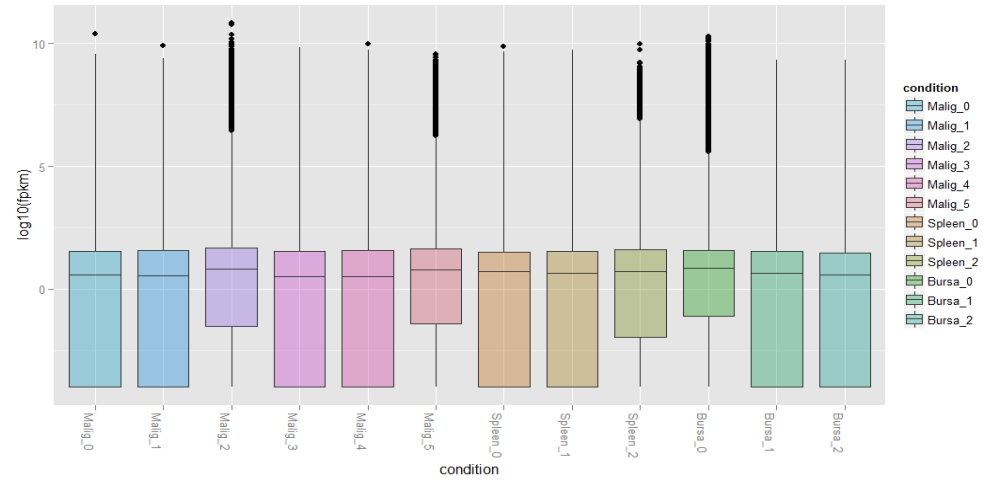

Figure 6.5a: Boxplot of expression profiles

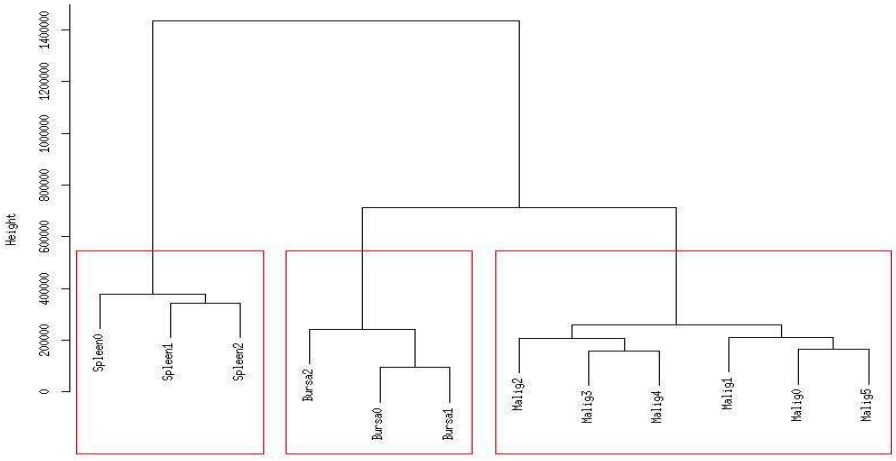

Figure 6.5b: **Ward Hierarchical Clustering using Euclidean distance**

Differential expression analysis: We performed pairwise comparisons for each of the three groups. Significant differential expression was considered with p value < 0.05 after correction for multiple analyses (Figure 6.6). To identify genes relevant to tumorigenesis, we chose genes that showed significant change in tumor tissues when compared to both bursa and splenic B cells (385 went up and 395 went down; supplementary table A2 and A3). Among the differentially expressed genes *TUSC2* is a tumor suppressor gene that was completely lost in all tumor samples but was the highest expressed gene in both bursa and splenic B cells (Kondo *et al.*, 2001; Meng *et al.*, 2013). On the other hand, the oncogene EIF4E ranked 2nd among the up regulated genes in malignant samples compared to being undetected in the other tissues (Carroll & Borden, 2013; Culjkovic-Kraljacic *et al.*, 2012; Mamane *et al.*, 2004)

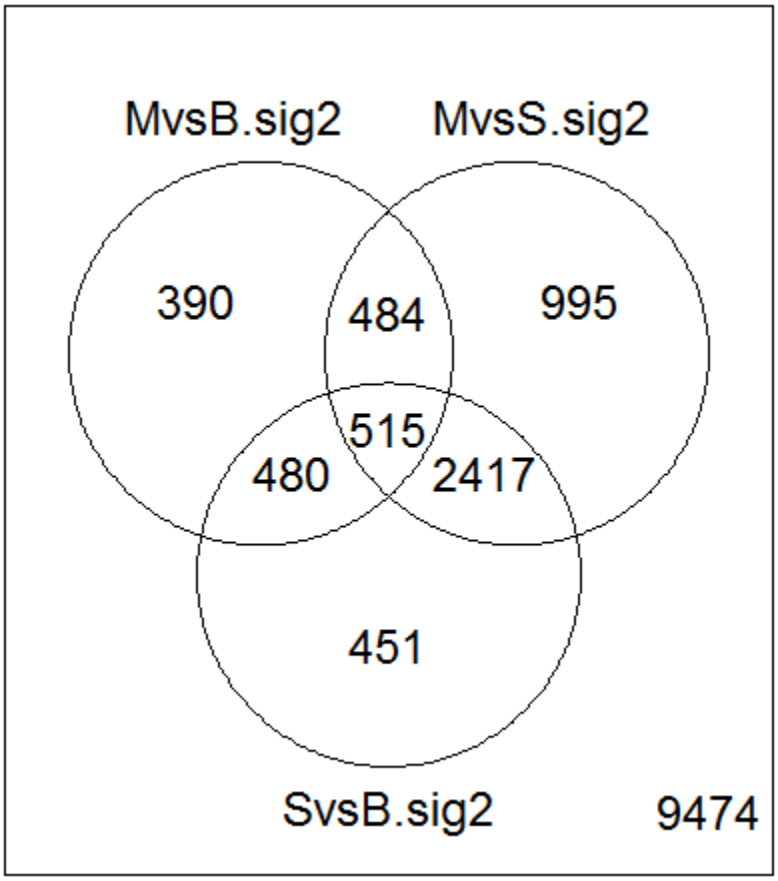

Figure 6.6: **Differential expression Analysis***:* The differentially expressed genes between every two studied tissues are counted in a circle. (a) MvsB.sign: Significant differentially expressed genes between malignant and bursa tissue. (b) MvsS.sign: Significant differentially expressed genes between malignant and splenic B cells. (c) SvsB.sign: Significant differentially expressed genes between splenic B cells and bursa tissue. Intersections count the genes that appeared significant in more than one comparison

Pathway enrichment analysis: For pathway analysis, significant hits were further enriched by moving the p value threshold to 0.01 and excluding genes with low level of expression (20 FPKM was used as an arbitrary cut off). With these criteria we had 228 differentially expressed genes (144 went up and 84 went down). To overcome the poor annotation of chicken genome and low resources of their genetic pathways, we identified 218 human orthologs. KEGG pathway analysis showed significant enrichment of several cancer pathways (Table 6.3). PANTHER Pathway analysis ensured the involvement of p53 signaling pathway.

KEGG Pathways	**PValue**	**Genes**
Prostate cancer	0.013	E2F3, PIK3CD, PIK3R5, RB1,TCF7L2, CTNNB1
p53 signaling pathway	0.023	BID, CD82, FAS, CCNG1, THBS1
Chronic myeloid leukemia	0.031	E2F3, HDAC1, PIK3CD, PIK3R5, RB1
Small cell lung cancer	0.044	E2F3, ITGA6, PIK3CD, PIK3R5, RB1
Alzheimer's disease	0.045	BID, APP, PSEN1, SDHD, IL1B, FAS, ATP5A1
Apoptosis	0.049	BID, PIK3CD, IL1B, PIK3R5, FAS
Endometrial cancer	0.049	PIK3CD, PIK3R5, TCF7L2, CTNNB1
Non-small cell lung cancer	0.054	E2F3, PIK3CD, PIK3R5, RB1
Cytosolic DNA-sensing	0.057	POLR1D, IL18, IL1B, IRF3
Cell adhesion molecules	0.058	ALCAM, SDC1, PTPRF, ITGA6, PVRL3, SDC4
Pathways in cancer	0.075	BID, E2F3, HDAC1, ITGA6, PIK3CD, PIK3R5, RB1, FAS, TCF7L2, CTNNB1

Table 6.3: **Pathway enrichment analysis** (DAVID Functional annotation tool 6.7)

***Table 6.3* (Cont'd):**

Toll-like receptor signaling	0.077	MAP2K3, PIK3CD, IL1B, PIK3R5, IRF3
Glioma	0.078	E2F3, PIK3CD, PIK3R5, RB1
PANTHER Pathways	**PValue**	**Genes**
p53 feedback loops 2	0.000	E2F3, PIK3CD, PIK3R5, RB1, CCNG1, TPTE, CTNNB1
p53 pathway	0.000	E2F3, HDAC1, CD82, PIK3CD, PIK3R5, FAS, CCNG1, THBS1, TPTE

Discussion

Avian tumor viruses of economic importance include Marek's disease virus and avian retroviruses, namely avian leukosis virus (ALV) and reticuloendotheliosis virus (Witter, 1997). Avian retroviruses are associated with neoplastic diseases that represent a serious burden in poultry industry. Lympohoid leukousis is a B cell tumor that usually arises from the bursa tissue and is known to be enhanced by ALV (Baba & Humphries, 1985). ALV mortality and morbidity has a huge economic burden estimated to be in millions of U.S. dollars each year (Fadly & Nair, 2008). Marek's vaccine is routinely used in poultry industry; however the attenuated vaccine strains were shown to enhance the development of LL after ALV exposure (Fynan *et al.*, 1992; Marsh *et al.*, 1995).

Understanding the molecular events of oncogenesis mediated by interactions of ALV and MDV2 is critical for development of better strategies of prevention and control. The development of the RFS, a strain of chicken which is endogenous virus free but still susceptible to endogenous AVL infection, represents a proper negative control to the virally infected chicken. However, the late onset of the disease was an obstacle for the study design. By the time the tumors develop, there was no visible bursa tissue in the non-infected controls. To have the appropriate control, we compared the tumor samples to bursa tissues from 3 month old chicken as well as B cells sorted from the spleens of the age matched chicken.

Bursa of Fabricius is known as the primary site of LL (Fadly & Nair, 2008). In our analysis, the phylogenic convergence of tumor samples with bursa samples confirms the bursa as a tissue of origin for these tumors. The homogeneity of tumor samples

seen in the clustering analysis suggests a common underlying molecular pathogenesis in all samples. Exogenous ALV was shown to enhance the B cell transformation by activation of the c-myc oncogene by adjacent integration of ALV provirus (Kung & Liu, 1997). In our experiment, we did not find a change of *Myc* expression in any tumor samples. Instead, *TUSC2* (*FUS1*) is a tumor suppressor gene that was completely lost in all tumor samples. Among the differentially expressed genes, *TUSC2* was the highest expressed gene in both normal bursa and splenic B cells. The *TUSC2* gene resides in the 3p21.3 human chromosomal region. Chromosomal abnormalities in the 3p21.3 region are observed in lung, breast, cervical, and other cancers (Lerman & Minna, 2000; Senchenko *et al.*, 2003; Zabarovsky *et al.*, 2002). The impaired expression of *TUSC2* is a pathognomonic feature in most types of lung cancers (Ivanova *et al.*, 2009; Prudkin *et al.*, 2008) and an effective therapeutic target also in these tumors (Meng *et al.*, 2013). Mice lacking one or both copies of the *Tusc2* gene develop a chronic inflammatory autoimmune disorder and produce tumors at the sites of chronic inflammation (Ivanova *et al.*, 2007). *TUSC2* is considered as a molecular link between inflammatory response and mitochondrial homeostasis (Hood *et al.*, 2013; Uzhachenko *et al.*, 2012). *TUSC2* was shown to influence and complement the PI3K/AKT and p53 pathways (Figure 6.7) (Ji & Roth, 2008; Meng *et al.*, 2013). Our pathway enrichment analysis showed significant dysregulation of both pathways.

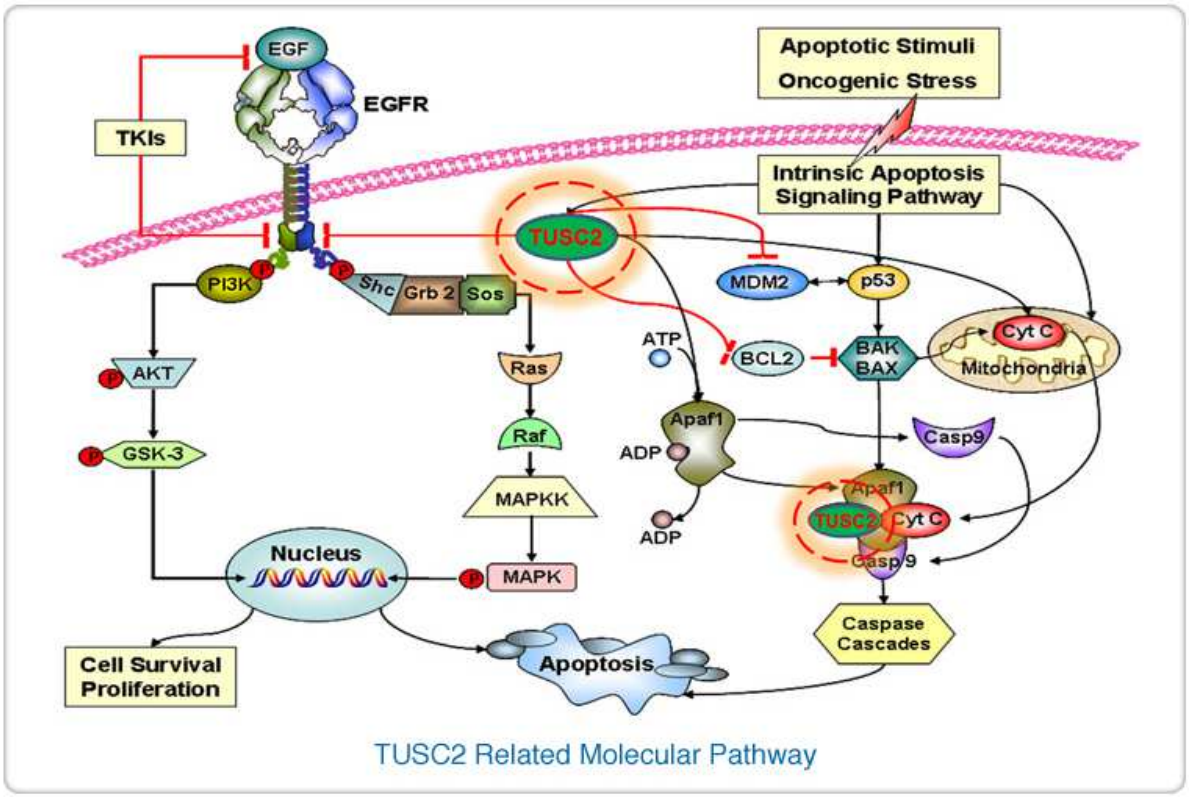

Figure 6.7: TUSC2 Molecular pathway (Ji & Roth, 2008)

We also observed increased expression of *EIF4E*, a the proto-oncogene associated with many cancers including colon (Zimmer *et al.*, 2000), head and neck (Franklin *et al.*, 1999; Nathan *et al.*, 1997; Nathan *et al.*, 1999), and breast cancers (Larsson *et al.*, 2007; Soni *et al.*, 2008). Transgenic mice overexpressing *Elf4e* by the ubiquitous β-actin promoter show high incidence of tumors including B-cell lymphomas, angiosarcomas, lung adenocarcinomas and hepatocellular adenomas (Ruggero *et al.*, 2004). Importantly, a cooperation between *Elf4e* and *c-Myc* in B-cell lymphomagenesis was shown. In this co-operation, *Elf4e* suppresses the c-Myc-induced apoptosis while c-Myc antagonized *Elf4e*-induced growth arrest (Ruggero *et al.*, 2004). The *Elf4e*

expression is usually secondary to apparent phosphoinositide-3 kinase and Akt signaling (Culjkovic *et al.*, 2008; Ruggero *et al.*, 2004; Zimmer *et al.*, 2000). EIF4E overexpression seen in our tumor sample could be a downstream effect of TUSC2 loss and abnormal Akt signaling. Another possible scenario is the convergence of the both genetic defects to induce the B cell transformation

Future studies should confirm the transcriptional changes of TUSC2 and EIF4E in the tumor samples. We also need to confirm this association in more independent ALV-like tumors. Transgenic mice overexpressing EIF4E by the ubiquitous β-actin promoter have been published before (Ruggero *et al.*, 2004). This mouse is good source to test the interaction between EIF4E and TUSC2. We can knock down TUSC2 in non-transformed bursa cells obtained from these mice and observe for transformation. Both TUSC2 and EIF4E are known therapeutic targets in cancer (Meng *et al.*, 2013; Soni *et al.*, 2008). Meng et al transfected the lung cancer cells with vectors expressing TUSC2 to increase the susceptibility of the cells to apoptotic agents. Similarly, Soni et al. knocked-down EIF4E by small interfering RNA to inhibit growth in different breast cancer cell subtypes. We should implement similar experiments on malignant lymphoblasts obtained from LL tumors to prove the necessity of these genes in the oncogenic changes of LL.

APPENDIX

Gene	Normalized expression			q value		
	Malig	Bursa	Spleen	Malig vs Bursa	Malig vs Spleen	Spleen vs Bursa
SLC25A37	2.47E+09	3.63E+08	0	0.006024	0.000508	0.000508
EIF4E	490456	0	0	0.009843	0.009843	1
FAM117A	235448	0	0	0.0281	0.0281	1
ENSGALG00000022685	82305.1	19629.8	7658.55	0.000859	0.000859	0.101893
RPS15	7755.37	5058.15	3563.76	0.028294	0.000508	0.137016
RPS25	5165.21	3229.19	2909.53	0.013846	0.003083	0.692084
RPL17L	5141.41	1722.61	993.123	0.015006	0.000859	0.434835
RPL7A	4936.85	3163.14	1930.87	0.042044	0.000508	0.040248
RPS4	3878.47	2553.37	2268.59	0.046946	0.010375	0.662805
ENSGALG00000027261	3439.99	600.394	1154.29	0.000859	0.035828	0.393214
ATP5A1W	2978.79	674.247	415.53	0.000859	0.000859	0.507745
GNG4	2542.9	769.284	15.0153	0.000508	0.002755	0.007117
RPS12	2093.8	1344.21	1231.73	0.026069	0.008392	0.758037
FAM13B	1786.25	292.282	48.545	0.000508	0.000508	0.000508
EEF1D	1724.06	1151.83	961.716	0.049562	0.002755	0.50061
CRIP2	1716.01	836.72	672.093	0.000508	0.000508	0.398886
BTF3	1572.08	252.772	293.264	0.000859	0.001511	0.857955
RPL35A	1525.8	1027.51	918.353	0.040248	0.007615	0.671937
POLN	1447.48	247.656	98.4941	0.000508	0.000508	0.018625
SLC39A14	1327.44	202.748	135.206	0.000859	0.000859	0.394509

Table A2: Genes significantly up regulated in malignant samples over both bursa and spleen samples

Table A2 **(Cont'd)**

HSPD1	874.058	551.08	215.93	0.021405	0.000508	0.000508
MYBL1	736.129	271.897	7.88936	0.002755	0.000508	0.000508
EIF2S3	692.205	441.462	270.228	0.032432	0.000508	0.038893
ENSGALG00000028307	656.52	42.5198	233.911	0.000859	0.03681	0.005736
DMTN	649.783	97.4209	79.6317	0.000859	0.000859	0.764011
ENSGALG00000014584	614.533	122.83	128.524	0.003299	0.005347	0.958976
IL18	605.775	140.243	171.42	0.000508	0.000508	0.578448
LDHA	594.428	330.738	172.09	0.003402	0.000508	0.004024
ENSGALG00000000140	572.084	81.5445	127.872	0.000859	0.009905	0.610247
ENSGALG00000013312	569.394	51.6126	233.934	0.000859	0.03933	0.001511
SC4MOL	552.336	288.086	225.925	0.000939	0.000508	0.39884
ACTR3B	539.3	311.391	67.1201	0.004604	0.000508	0.000508
PIWIL2	509.391	9.13099	22.159	0.000859	0.000859	1
VMP1	483.036	231.505	80.8812	0.000508	0.000508	0.000508
PDIA6	477.388	275.772	153.555	0.004889	0.000508	0.004314
TPI1	418.364	280.348	217.931	0.036676	0.000508	0.317232
FKBP4	406.854	229.886	188.823	0.003707	0.000508	0.454642
WDR66	395.587	156.601	4.3107	0.000508	0.000508	0.000508
RSU1	392.048	245.256	99.3984	0.018835	0.000508	0.000508
CSDE1	367.353	233.261	234.746	0.019233	0.020418	0.980924
FAM60A	354.441	201.146	164.332	0.003707	0.000508	0.468064
TM9SF2	350.555	217.29	132.598	0.01606	0.000508	0.027709
SEPT6	341.85	198.31	179.967	0.006845	0.000508	0.733828

Table A2 **(Cont'd)**

ENSGALG00000020895	338.071	193.897	124.225	0.003707	0.000508	0.091393
BLNK	333.235	224.644	169.254	0.049562	0.000508	0.249674
SPINW	332.045	42.6419	101.209	0.000859	0.010258	0.177605
DHDH	319.533	145.858	35.3754	0.042556	0.000859	0.031897
ENSGALG00000019602	315.064	74.458	143.549	0.000508	0.002413	0.014947
SNORA16	312.335	171.836	142.492	0.024147	0.004024	0.639895
IPO7	308.907	199.58	94.4668	0.033302	0.000508	0.000508
LGALS1	306.282	92.8542	38.9417	0.000508	0.000508	0.05016
BID	298.275	162.666	46.1011	0.000508	0.000508	0.000508
ENSGALG00000022335	293.607	36.9159	29.3242	0.009485	0.005736	0.838977
gga-mir-762	291.055	148.323	85.3646	0.000508	0.000508	0.009893
GPI	284.194	190.053	81.8	0.039549	0.000508	0.000508
UBE2E1	283.805	159.514	116.691	0.002061	0.000508	0.217859
HSPA4L	278.009	169.702	97.8684	0.023951	0.000508	0.032086
BZW2	269.658	181.166	97.6183	0.041552	0.000508	0.006024
PNN	268.402	171.811	150.208	0.020792	0.003402	0.618302
CCNC	266.684	136.492	58.9468	0.000508	0.000508	0.000508
EIF3B	264.604	171.512	109.77	0.021596	0.000508	0.042792
GMPR	263.33	107.439	18.6393	0.000508	0.000508	0.000508
SDHD	260.804	110.21	64.9439	0.000508	0.000508	0.021
DUSP22	257.983	115.374	78.0294	0.010857	0.000508	0.326545
PRKCD	248.53	138.264	53.4495	0.004314	0.000508	0.000508
CCND3	247.497	166.089	121.128	0.042792	0.000508	0.203596

Table A2 **(Cont'd)**

ODC1	239.001	114.771	63.6004	0.000508	0.000508	0.016719
PURH	229.909	150.336	111.574	0.032991	0.001337	0.258521
RBM38	229.787	151.976	7.25138	0.032086	0.000508	0.000508
FAM103A1	227.108	136.682	64.5182	0.006301	0.000508	0.002755
RAB9A	219.558	107.797	41.7997	0.000508	0.000508	0.000508
PKNOX1	214.933	29.8019	37.4982	0.000508	0.000508	0.501767
NUCB2	203.125	91.7936	63.5541	0.000508	0.000508	0.255855
C18orf25	199.863	23.698	23.4113	0.000859	0.001511	0.991141
PRKD3	198.314	98.7271	65.1583	0.001337	0.000508	0.162981
TPD52L2	195.664	99.5018	129.402	0.000508	0.035035	0.303313
CXCL13L2	191.823	109.278	18.7992	0.012919	0.000508	0.000508
NFI1	188.678	52.1044	72.4373	0.000508	0.000508	0.344425
HBS1L	188.626	117.347	78.164	0.046054	0.000508	0.180928
LRMP	187.84	49.8108	42.2991	0.000508	0.000508	0.56758
DNAJB6	182.228	101.845	96.1518	0.000939	0.000939	0.842609
CENPW	177.263	102.599	9.06266	0.007117	0.000508	0.000508
RCC2	176.647	85.701	114.035	0.000508	0.023544	0.2502
RCSD1	176.273	83.8259	81.6743	0.000508	0.000508	0.92688
SRD5A3	173.525	78.4872	49.0575	0.000508	0.000508	0.08419
STOM	172.502	55.8967	36.2095	0.000508	0.000508	0.089407
RASGRP3	171.546	73.8425	97.1626	0.000508	0.003707	0.30215
PAFAH1B2	171.149	102.004	64.456	0.033141	0.000508	0.165353
CCNG1	166.591	86.5334	42.4034	0.000508	0.000508	0.003707

Table A2 **(Cont'd)**

HSPH1	165.808	58.8905	62.9261	0.000508	0.000508	0.839923
NMT2	160.332	87.4131	92.5816	0.001707	0.004604	0.850306
HDAC1	159.336	78.9645	80.4277	0.000508	0.000939	0.948897
WEE1	156.792	104.255	13.4923	0.042377	0.000508	0.000508
MND1	156.462	90.6355	70.8982	0.034519	0.002755	0.500816
ANXA5	152.907	90.9782	96.2058	0.019233	0.038529	0.859449
VGLL4	152.286	69.2409	59.312	0.000508	0.000508	0.566259
IMPAD1	148.816	80.0987	28.648	0.031558	0.000508	0.010375
HSPA4	147.318	81.99	84.4473	0.001337	0.003083	0.919397
USP10	146.244	96.6553	68.995	0.032259	0.000508	0.169499
POLR1D	145.753	67.6363	57.0256	0.003402	0.000508	0.665455
NCOA7	141.926	86.2869	61.679	0.013626	0.000508	0.172718
TEC	140.088	70.6503	9.15593	0.000508	0.000508	0.000508
SASS6	131.796	77.3956	38.1112	0.014731	0.000508	0.009636
C19ORF12	131.184	60.3329	72.1503	0.000508	0.008392	0.562318
GGA.31975	129.111	84.9572	40.5779	0.03415	0.000508	0.000939
LYAR	127.994	83.0236	30.6556	0.025695	0.000508	0.000508
R3HDM1	126.274	54.4965	38.6039	0.000508	0.000508	0.163572
METTL14	125.206	75.238	27.3061	0.009143	0.000508	0.000508
LRRC4	124.358	8.82336	33.1291	0.001511	0.007037	0.133254
SRPK1	123.492	74.8857	62.7456	0.016284	0.000508	0.540002
MED7	122.227	67.8779	41.24	0.008904	0.000508	0.093999
MOSPD2	121.27	56.1096	59.1198	0.000508	0.000508	0.854879

Table A2 **(Cont'd)**

SEPT11	120.431	57.4969	11.085	0.004314	0.000508	0.000939
URI1	118.785	78.1492	52.5295	0.037726	0.000508	0.114861
PPIF	118.176	73.6934	41.4491	0.019233	0.000508	0.015839
ZNF644	118.023	53.6857	26.3529	0.029363	0.000508	0.023367
CD1A1	118.021	19.2832	61.4972	0.000508	0.004889	0.000939
FAM129A	117.796	55.668	46.4483	0.000508	0.000508	0.487016
USP15	117.323	67.2571	56.5604	0.000508	0.000508	0.49222
WAPAL	116.197	78.5842	75.9481	0.048049	0.027354	0.907135
SDR16C5	113.738	44.0725	52.0214	0.000508	0.000508	0.546921
MTR	109.581	61.3958	52.282	0.027354	0.001707	0.66036
ENSGALG00000006723	108.964	67.3431	41.6608	0.029707	0.000508	0.081169
P4HA2	108.706	42.9481	27.6161	0.000508	0.000508	0.117276
EXOC6	107.899	67.9605	61.3801	0.024758	0.004604	0.728121
TMEM248	107.301	68.3728	59.4079	0.035516	0.005742	0.638185
RNF139	106.856	57.1786	40.3825	0.003083	0.000508	0.222124
MAP2K3	106.519	48.9303	52.8649	0.001707	0.001707	0.828237
TMEM65	102.539	48.417	17.1679	0.001707	0.000508	0.003083
FAS	100.487	40.2208	15.6239	0.006024	0.000508	0.021791
STAMBPL1	98.5653	56.5554	14.698	0.010626	0.000508	0.000508
MRPL3	97.395	56.7945	32.6014	0.030653	0.000508	0.054967
ZEB1	92.5795	55.8862	60.1504	0.029363	0.045323	0.831729
DTNBP1	91.8084	43.069	36.0961	0.020792	0.003402	0.676834
EHBP1	90.6004	57.3104	20.4566	0.046498	0.000508	0.000508

Table A2 **(Cont'd)**

TRIP12	87.9382	52.68	59.2868	0.004889	0.040878	0.65692
BEND7	87.3775	54.7902	44.5491	0.045615	0.004604	0.511324
MTSS1	86.1399	33.4998	49.5651	0.000508	0.049102	0.252313
IL20RA	85.2251	13.2447	13.7549	0.000508	0.000508	0.933827
HDAC11	85.0971	33.0563	25.9839	0.000508	0.000508	0.433117
BLOC1S4	85.0875	47.8756	38.9432	0.046792	0.004604	0.619534
SLC20A2	83.7792	36.4321	14.754	0.000508	0.000508	0.000508
INPP5K	83.4341	45.9041	34.41	0.004024	0.000508	0.293996
CHORDC1	82.6696	50.9036	36.955	0.008392	0.000508	0.180993
DDRGK1	82.5846	38.1112	48.2685	0.000508	0.018195	0.420128
SCN4B	82.3832	17.9699	4.2067	0.000508	0.000508	0.019045
PLS3	81.0464	39.4989	6.53773	0.000508	0.000508	0.000508
SCIN	80.5355	52.5698	24.6277	0.035705	0.000508	0.003083
LPCAT3	80.169	47.095	33.5928	0.013846	0.000508	0.264015
FABP4	78.3716	2.08767	1.12654	0.001337	0.003402	0.59548
NFYA	78.2558	50.3369	33.5311	0.024561	0.000508	0.096047
GGA.46369	76.9525	32.6288	19.9279	0.018409	0.000508	0.292991
DNAJC10	74.7917	46.3469	28.5099	0.035705	0.000508	0.081406
CA13	73.5144	15.4671	7.89494	0.015156	0.003707	0.508024
LIN7C	73.11	45.1682	34.8197	0.013626	0.000508	0.316899
SLC7A10	72.2439	4.85704	0.506395	0.000508	0.000508	0.014287
VLDLR	70.5922	19.8233	11.9741	0.000508	0.000508	0.171605
C12ORF35	70.535	32.3002	32.5506	0.000508	0.000508	0.980794

Table A2 **(Cont'd)**

RIF1	70.3058	45.3766	14.7656	0.035035	0.000508	0.000508
SERHL2	68.804	22.1829	39.1919	0.000508	0.033141	0.115818
NUDCD1	68.3359	39.6472	18.4469	0.046498	0.000508	0.021596
RRN3	67.9998	35.1339	36.232	0.000939	0.001707	0.920274
PGAM5	65.5674	42.8371	39.9614	0.045615	0.021178	0.827901
HIVEP2	65.0191	33.9471	33.0092	0.000508	0.001337	0.924921
PSEN1	64.5392	16.969	21.6597	0.000508	0.000508	0.529285
ZCCHC10	62.9052	37.6818	23.9699	0.016936	0.000508	0.106616
SLC25A12	62.8992	38.8356	29.5155	0.029707	0.000508	0.364586
RECQL5	61.5121	14.4256	5.69456	0.000939	0.000508	0.068378
TPK1	61.4578	23.2101	16.919	0.010133	0.001337	0.555679
ENSGALG00000027836	61.2314	11.8349	12.4052	0.019632	0.004833	1
LYRM1	60.4049	25.6746	30.0922	0.010375	0.040248	0.758453
PIT54	59.1434	4.81754	2.2398	0.006625	0.043255	1
MAP2K2	58.011	32.9457	33.5946	0.030281	0.046645	0.960897
OSBPL3	56.6664	17.8905	13.8551	0.000508	0.000508	0.397079
KCNK17	56.5455	15.8311	20.4354	0.000508	0.000508	0.501887
GGA.31495	56.3451	19.7133	14.4842	0.000508	0.000508	0.357609
CCDC34	56.03	18.7015	21.4638	0.007615	0.010857	0.816414
FAM125B	55.8808	31.6062	24.6963	0.003083	0.000508	0.367547
NOC3L	55.1614	34.3804	22.0094	0.023367	0.000508	0.103494
COL6A1	54.2246	34.0331	0.340008	0.034691	0.000508	0.000508
GMPS	54.0928	32.0966	22.1008	0.010133	0.000508	0.139349

Table A2 **(Cont'd)**

ACSL5	53.9811	33.1821	26.4646	0.023181	0.000508	0.454946
MOXD1	53.6435	14.8233	0.253233	0.000508	0.005742	0.012254
TOMM34	53.4712	24.6449	18.191	0.002755	0.000508	0.429937
MB21D1	53.3292	25.9723	12.8736	0.009387	0.000508	0.073075
KIAA1430	52.8945	27.9942	23.5556	0.005455	0.001337	0.616729
PGPEP1	52.5872	20.3683	18.9546	0.004314	0.002413	0.892303
E2F3	52.2599	24.161	6.26593	0.003083	0.000508	0.004889
FKBP7	52.1913	26.6998	5.84127	0.026647	0.000508	0.002061
TPTE	51.8425	13.1802	2.25835	0.000508	0.000508	0.000508
SLCO4A1	51.6363	23.5946	1.47743	0.000508	0.000508	0.000508
SNX10	51.4705	28.2791	31.1594	0.004604	0.020593	0.764273
ABCF2	50.9524	32.9192	24.0782	0.029178	0.000508	0.240839
ACSL3	50.9399	26.1913	11.2004	0.000508	0.000508	0.002755
ARID1B	50.6929	33.42	28.4761	0.032786	0.003083	0.549158
AK1	50.607	22.2764	1.78904	0.01756	0.000508	0.004604
GZMA	50.4736	7.8734	11.696	0.000508	0.000508	0.40661
MAMDC2	50.1389	12.7447	2.81786	0.000508	0.000508	0.001337
HSF2	49.8087	29.6242	18.9939	0.019233	0.000508	0.123308
RB1	49.5251	26.7261	22.8285	0.002755	0.000939	0.625532
ZNF598	49.3741	31.3705	32.6548	0.023737	0.04055	0.894454
ANKRD27	48.2475	28.6898	17.8925	0.012919	0.000508	0.076479
ENSGALG00000027080	47.4958	10.2974	10.7812	0.009485	0.028262	0.964009
LCA5L	47.4075	9.8274	2.95464	0.000508	0.000508	0.012919

Table A2 **(Cont'd)**

RAG2	47.3762	26.5306	0.219988	0.025695	0.038204	0.065085
RFLB	46.9476	2.0684	3.19626	0.002061	0.000508	0.653075
LSS	46.0031	21.4339	24.1398	0.002755	0.029556	0.78243
NAA25	45.7087	30.3296	25.4397	0.036199	0.001337	0.517014
ENSGALG00000021862	45.6503	14.2771	6.47071	0.037877	0.005455	0.367625
LRRCC1	45.4312	25.1816	8.99862	0.005742	0.000508	0.000508
PITRM1	44.9262	22.7592	18.0313	0.001707	0.000508	0.440357
TNFRSF1B	44.0166	13.1469	27.0104	0.000508	0.04055	0.008648
FGF12	43.8994	18.8334	4.47353	0.015617	0.000508	0.007875
ZFP92	43.794	24.4881	7.88216	0.024147	0.000508	0.001337
DDX47	43.5566	24.7253	23.9979	0.010133	0.006301	0.930252
JMJD6	43.3195	20.7141	27.1286	0.000508	0.023181	0.335209
C3H2ORF43	43.2789	21.2175	19.7676	0.000508	0.000508	0.822123
DHX40	43.0061	20.6415	19.8732	0.006571	0.002755	0.927894
TADA1L	42.8508	24.6315	22.821	0.048785	0.022381	0.852897
G0S2	40.9724	1.22642	4.25318	0.002061	0.000939	0.199004
CD200	40.9574	20.6454	21.9917	0.001337	0.027037	0.869328
ANKRD60	40.7367	25.1179	23.0967	0.032596	0.014519	0.807934
TRABD	40.5668	25.6072	15.4532	0.029178	0.000508	0.044542
MTHFD1	40.2411	23.3022	10.1842	0.004889	0.000508	0.001707
PGS1	39.4513	17.8118	13.3978	0.000939	0.000508	0.475205
GLT1D1	39.3647	13.3234	7.87353	0.000508	0.000508	0.116698
BTBD11	39.3412	18.2841	7.07037	0.000508	0.000508	0.002061

Table A2 **(Cont'd)**

NCAPD3	39.2565	24.5125	4.12427	0.023544	0.000508	0.000508
DENND3	38.9517	3.77331	10.8735	0.000508	0.000508	0.005162
PTPRF	38.931	18.485	1.1627	0.000508	0.000508	0.000508
FBXO38	38.848	22.2507	13.0786	0.008648	0.000508	0.046792
GFER	38.4681	20.0902	14.8312	0.012919	0.000939	0.432814
TNFSF4	38.2446	3.50097	1.72807	0.001337	0.006024	0.552373
WDR4	38.2083	22.1554	12.5673	0.024758	0.000508	0.07635
COL6A2	37.8591	24.4129	0.406533	0.034691	0.000508	0.000508
GUCY1B3	36.5347	16.6291	10.5002	0.003083	0.000508	0.228148
FAM160B1	36.1975	20.6031	18.1704	0.004889	0.000508	0.663588
FAF1	35.1842	21.1093	20.0421	0.019843	0.009893	0.877255
IFIH1	34.6964	12.5105	19.0742	0.000508	0.007357	0.129485
NEK4	33.7175	18.1395	5.38546	0.022381	0.000508	0.000508
WDHD1	33.4796	21.5096	3.32527	0.041371	0.000508	0.000508
TTPAL	33.0189	14.7241	8.14469	0.012919	0.028453	0.364262
MAPK11	32.9836	16.3064	1.62906	0.032432	0.000508	0.000508
RNF111	32.9516	19.5606	17.5657	0.032786	0.006571	0.758753
C4ORF21	32.9371	21.9377	0.87406	0.017772	0.000508	0.000508
PVRL3	32.775	8.46255	0.265238	0.000508	0.000508	0.000508
PCM1	32.7401	18.9889	18.9329	0.003707	0.004024	0.992666
ENSGALG00000006092	32.3991	13.9438	12.3499	0.000508	0.000508	0.748264
ARFGAP1	31.7584	19.4565	20.5426	0.021178	0.043084	0.865256
SYT1	31.0921	1.16967	0.238788	0.000508	0.000508	0.051824

Table A2 **(Cont'd)**

PUS1	30.584	17.5488	14.9951	0.018195	0.003707	0.649421
GPHN	30.0662	9.25647	6.73085	0.000508	0.000508	0.40091
LRRK2	29.1594	11.5437	15.7136	0.000508	0.003707	0.285162
PAX3	28.1347	13.0398	0.32904	0.000508	0.000508	0.000508
FADS1	28.0537	7.39644	6.88725	0.000508	0.000508	0.884774
SH3GL3	27.9784	3.8012	2.63069	0.000508	0.000508	0.562974
SELO	27.9732	8.76824	8.94975	0.000508	0.000508	0.956393
MAD1L1	27.9508	15.3401	15.0505	0.012707	0.012482	0.960504
THBS1	27.4234	8.26698	0.58576	0.000508	0.000508	0.000508
ENSGALG00000006966	27.2309	15.2322	13.0741	0.007875	0.000939	0.620797
DNAJB9	26.9246	16.7054	12.0064	0.028632	0.000508	0.252139
TCF7L2	26.7849	8.60307	9.09137	0.000508	0.000508	0.894998
STK32C	26.3767	3.2101	0.453204	0.000508	0.000508	0.040086
MED13	25.6803	15.2209	15.6017	0.010133	0.015839	0.936589
CALB1	25.3831	3.99522	0.226361	0.000508	0.000508	0.000508
ENSGALG00000014126	25.2239	11.2387	0.440083	0.048785	0.000508	0.005455
FAM114A1	24.762	13.2319	13.2114	0.009143	0.008392	0.996596
FNIP2	24.7337	5.55651	5.60829	0.000508	0.000508	0.983789
DSCAM	24.5632	1.38692	0.239229	0.000508	0.000508	0.007875
TUBE1	23.8774	12.0403	13.3382	0.016936	0.041552	0.812206
RNF157	23.849	12.4548	0.539129	0.043558	0.000508	0.000508
MYOM1	22.8251	1.02126	0.262186	0.000508	0.000508	0.046366
CIP1	22.6267	4.85745	9.41451	0.000508	0.032432	0.244025

Table A2 **(Cont'd)**

ORAI2	21.508	11.0767	9.72237	0.011088	0.003707	0.745265
CEP192	21.288	12.3918	2.61824	0.025695	0.000508	0.000508
ENSGALG00000021106	21.1327	2.37149	1.10897	0.026458	0.040406	0.580822
FAM217B	21.0359	9.85268	9.38811	0.000508	0.000508	0.889108
SNAP47	20.9185	12.3021	12.7205	0.027709	0.034867	0.925882
NLE1	20.1023	10.0715	6.48369	0.016284	0.001337	0.286941
GPSM1	19.9191	11.8862	14.4637	0.003402	0.043422	0.582877
FN1	19.697	11.2927	9.11708	0.012254	0.002413	0.519092
BAG3	19.5765	9.61523	8.35796	0.004889	0.006301	0.729573
DHX35	18.8492	9.35007	8.2523	0.010375	0.005162	0.765197
METTL4	18.735	11.2722	10.0986	0.048939	0.016508	0.776927
TVB	18.4069	7.27926	2.97432	0.004889	0.000508	0.103013
MOCOS	17.9925	0.933332	3.0796	0.000508	0.000508	0.035868
MYCN	17.5292	1.05757	1.79772	0.000508	0.000508	0.498167
ASPG	17.3655	9.10193	5.39033	0.018195	0.000508	0.139711
SLC25A33	17.1409	8.93389	3.0606	0.045917	0.000508	0.02791
gga-mir-1815	16.7175	1.26115	0.873254	0.003083	0.002413	0.732404
DPYSL4	16.5991	4.54763	0.247559	0.001337	0.001707	0.012021
NEK3	16.4941	6.51483	1.55727	0.018625	0.000508	0.017772
THNSL1	16.4157	7.04515	7.33576	0.004889	0.006024	0.933537
RGS6	16.3934	3.0426	0.126573	0.000508	0.005742	0.022785
RHOBTB1	16.1637	8.78056	1.72698	0.029556	0.000508	0.000508
ENSGALG00000006123	15.9964	6.82803	4.8052	0.000508	0.000508	0.265098

Table A2 **(Cont'd)**

EPHA7	15.9705	0.870556	0.216215	0.000508	0.000508	0.073203
SULT4A1	15.9019	8.53686	6.81603	0.036028	0.006301	0.611525
PRSS12	15.7126	0.706388	0.368459	0.000508	0.002413	0.583741
PGF	15.6582	4.49558	0.409219	0.011573	0.004604	0.033141
CLASP1	15.6231	9.1885	8.76924	0.025882	0.010857	0.89444
LATS2	15.5634	8.09807	9.69152	0.004314	0.046792	0.574291
ENSGALG00000014441	15.5108	6.24225	5.81998	0.01756	0.014947	0.911365
STAM	15.3471	8.41972	8.63155	0.006845	0.008392	0.94356
ICOS	15.1298	5.19889	3.15919	0.000508	0.000508	0.264839
RGS2	15.0883	3.47913	2.14216	0.000508	0.000939	0.454777
RTTN	14.9542	8.57434	5.56366	0.008648	0.000508	0.141326
SYBU	14.6854	3.02304	1.381	0.000508	0.000508	0.13819
FIG4	13.5863	6.75678	7.9271	0.009893	0.041024	0.674637
COL6A3	13.5388	2.04746	0.210838	0.000508	0.000508	0.000508
PARM1	13.1774	1.89581	1.66977	0.009387	0.028816	0.914599
SLC35F2	13.1249	6.45205	1.14313	0.046366	0.000508	0.006845
ERI2	12.941	7.84044	1.76924	0.043084	0.000508	0.000508
AEN	12.9359	4.91742	4.76232	0.01315	0.009893	0.960825
ZNF407	12.9052	6.47408	7.63422	0.002061	0.021	0.610378
ASIC2	12.6336	0.186888	3.14368	0.002413	0.003083	0.011573
DIAPH3	11.932	6.61767	0.803416	0.025324	0.000508	0.000508
KIAA1009	11.68	6.25508	6.39541	0.008648	0.012482	0.953462
PLCH2	11.3226	3.19749	0.216423	0.000508	0.000508	0.000508

Table A2 **(Cont'd)**

ALPL	11.3097	2.07703	0.229626	0.003707	0.025512	0.093999
BBS9	11.1582	5.31855	0.349019	0.017363	0.000508	0.001707
BDKRB1	11.1482	1.13409	0.085925	0.000939	0.036028	0.072279
SLMO1	11.0455	5.55538	4.23482	0.037726	0.012254	0.594906
DOC2B	10.7208	2.40364	0.237793	0.007117	0.000508	0.018625
SCNN1B	10.7128	1.79812	0.123963	0.000508	0.001337	0.005742
FAM171A1	10.2727	5.25634	0.515851	0.049562	0.000508	0.000508
FAM184B	10.202	2.98471	0.811926	0.001337	0.000508	0.017363
CHRDL2	10.0199	0.110684	0.29021	0.035035	0.001707	1
AFAP1	9.8054	4.43864	0.276595	0.001707	0.000508	0.000508
OLFM3	9.73988	3.42697	0.377979	0.005162	0.000508	0.003083
TPCN3	9.40088	3.21536	2.99359	0.010375	0.008139	0.91364
ANXA10	9.27805	0.772904	0.43548	0.009143	0.006571	0.656324
ANKRD50	9.2712	3.15949	0.512016	0.001707	0.000508	0.000508
RGS9BP	9.1278	4.34957	1.58088	0.009143	0.000508	0.018409
MMS22L	8.14548	4.89063	2.08418	0.032596	0.000508	0.009893
OSBPL6	7.84098	2.15542	0.124638	0.000508	0.000508	0.002755
CRYM	7.57204	2.50579	3.35413	0.008139	0.042516	0.624648
PTCHD1	7.39944	1.74242	0.429777	0.000508	0.000508	0.029707
GPR37	7.30112	3.28242	0.027359	0.003707	0.015384	0.029707
C10orf71	7.04963	1.12007	0.658535	0.000508	0.000508	0.368281
CNKSR2	6.83599	0.616383	2.98556	0.000508	0.045323	0.023367
TTC8	6.80802	2.47346	3.78663	0.000939	0.034519	0.261578

Table A2 **(Cont'd)**

MAGI2	6.70258	0.465019	0.151336	0.000508	0.000508	0.141028
LAG3	6.39963	1.0365	2.01941	0.006845	0.042921	0.423992
SDR42E2	6.22889	0.913805	0.726087	0.000508	0.000508	0.778251
C7	6.11809	2.60805	0.15911	0.020418	0.000508	0.000508
FHL5	6.11332	1.37099	0.033903	0.001707	0.03838	0.062552
PLCH1	6.01695	0.293417	1.57899	0.000508	0.000508	0.002061
ITGA8	5.80644	2.53176	0.205959	0.035868	0.000508	0.000508
RFWD3	5.57267	2.90438	1.54593	0.019233	0.000508	0.117474
MYO3A	5.37341	0.158732	0.030312	0.000508	0.013846	1
ENSGALG00000005470	5.1624	0.067117	0.774846	0.000508	0.000508	0.011088
ENSGALG00000002318	4.78461	1.7871	0.403331	0.004024	0.000508	0.019233
NXPH2	4.69216	0.556137	0.6655	0.001337	0.001707	0.843259
TMEM136	4.56306	1.21256	1.41621	0.008648	0.017363	0.836256
HTR4	4.21403	0.589149	0.814369	0.006571	0.009143	0.74288
SLC1A6	3.77089	1.27315	0.919667	0.041194	0.046199	0.716556
RUNX2	3.72993	1.42735	1.71663	0.009636	0.036839	0.732349
KIAA1107	3.62648	1.07242	0.332517	0.000939	0.000508	0.033623
GLRB	3.61473	0.140573	0.022559	0.000508	0.002755	1
ENSGALG00000019276	3.43791	1.00268	0.224975	0.007117	0.001707	0.065488
ATRNL1	3.25328	1.23281	0.271235	0.01315	0.000508	0.018625
RALY	3.07879	0.545744	0.463026	0.000939	0.000508	0.835098
ANGPTL4	2.94338	0.400201	0.085569	0.003707	0.019638	1
ENO2	2.91482	0.392882	0.734461	0.005162	0.014287	0.451768

Table A2 **(Cont'd)**

TLE1	2.8866	1.03826	0.928909	0.010133	0.004024	0.856261
COL28A1	2.67343	0.239752	0.018897	0.000508	0.003707	1
LRRC2	2.48313	0.123282	0.09928	0.000939	0.001337	1
CORIN	2.32654	0.089815	0.126155	0.000508	0.000508	1
CDKL1	1.93031	0.830798	0.713016	0.041024	0.027354	0.81552
SCML4	1.84969	0.089224	0.039091	0.002413	0.002061	1
SYCP2	1.84611	0.64382	0.366739	0.015617	0.000939	0.374654
BICD1	1.70598	0.23625	0.109174	0.000508	0.000508	1
PLXNA2	1.53473	0.471503	0.381628	0.006845	0.001337	0.741867
NPAS3	1.46558	0.28029	0.123925	0.004314	0.000939	1
CD109	1.44851	0.413856	0.051065	0.020225	0.001707	0.033141
THBS2	1.36734	0.451123	0.023163	0.037211	0.045761	0.086943
ATP7B	1.29929	0.059637	0.218104	0.006845	0.005162	1
RBP2	1.27597	0	0	0.000508	0.000508	1
NPFFR2	1.17549	0	0	0.000508	0.000508	1
CMYA5	1.13088	0.140566	0.205191	0.000508	0.002061	1
SYPL1	1.09834	0.248076	0.153447	0.010626	0.017989	1
GGA.46638	0.768885	0	0	0.020593	0.020593	1
GPR123	0.662402	0.16424	0.037028	0.031014	0.024758	1
GPR179	0.574987	0.060694	0.033828	0.006571	0.010133	1

Gene	Normalized expression			q value		
	Malig	Bursa	Spleen	Malig vs Bursa	Malig vs Spleen	Spleen vs Bursa
TUSC2	0	99564600	28623200	0.000508	0.009143	0.366455
TMEM194B	0	343911	359023	0.005742	0.013846	0.976893
ENSGALG00000027490	0	11363	4.38036	0.000508	0.000508	0.000508
GGA.13329	0	59.6019	14.739	0.000508	0.000939	0.140236
GRP	0	1.37458	37.4668	0.000508	0.000508	0.007117
PTPRO	0.031932	0.636413	2.64164	0.037019	0.033808	0.007875
CPAMD8	0.067003	0.883522	0.898642	0.004604	0.004604	0.979558
TSPAN7	0.070848	3.17931	4.88668	0.03838	0.038204	0.47944
ENSGALG00000000584	0.091068	1.59459	0.829181	0.000508	0.000508	0.178746
CASZ1	0.091848	1.06893	0.708934	0.006845	0.017158	0.500045
PCSK5	0.096798	0.895686	0.387093	0.004314	0.045917	0.145658
RASGEF1C	0.10418	2.00911	2.55017	0.015156	0.012021	0.751984
NCAM1	0.108176	1.04421	2.52137	0.008904	0.000939	0.05016
PAMR1	0.110381	0.878291	0.905252	0.032432	0.030281	0.971404
LAPTM4B	0.110602	5.93472	3.35264	0.001707	0.002061	0.185641
DTNA	0.113291	1.79288	0.771049	0.003083	0.022785	0.217859
GPR64	0.121855	2.28681	33.2029	0.001337	0.000508	0.000508
MB21D2	0.133389	0.838088	3.24251	0.022182	0.001337	0.012254
ZBTB42	0.145226	3.81675	4.88122	0.034324	0.033302	0.717333
SLCO6A1	0.146579	2.65498	1.46056	0.000939	0.001707	0.182957

Table A3: Genes significantly down regulated in malignant samples over both bursa and spleen samples

Table A3 **(Cont'd)**

SVEP1	0.156212	1.81515	0.501278	0.000508	0.036367	0.004604
SEMA5B	0.162812	0.880199	2.64042	0.016508	0.000508	0.041371
APCDD1	0.165404	5.56992	1.03581	0.002755	0.041876	0.028632
PCDH1	0.184728	1.03515	1.64086	0.005742	0.000508	0.363706
SLC16A7	0.187369	1.00218	7.13907	0.006845	0.000508	0.000508
NT5E	0.201322	15.8623	1.99502	0.011088	0.044686	0.000508
ILDR1	0.20429	2.74872	1.84065	0.000508	0.002755	0.357978
PLCB4	0.205287	1.58669	1.65557	0.001337	0.001337	0.939069
ZNF618	0.230157	1.53125	1.97447	0.000939	0.000508	0.66241
MISP	0.243856	1.48565	5.44347	0.041024	0.002755	0.10673
K60	0.244816	4.90364	12.8634	0.011573	0.006301	0.045323
AMOTL1	0.247764	2.55799	1.26129	0.003083	0.027183	0.241526
ZNF385B	0.260418	1.03713	2.33625	0.041552	0.004604	0.163572
CDS1	0.267813	3.5723	2.94819	0.000939	0.001707	0.685291
ENSGALG00000014946	0.275816	2.24047	2.70998	0.000939	0.000508	0.800608
SLC2A9	0.277643	13.3941	3.54365	0.000508	0.000508	0.000508
RAB11FIP1	0.284042	5.70401	1.7216	0.000508	0.007875	0.022381
DOCK5	0.288369	0.902696	4.29257	0.036367	0.000508	0.001337
ENSGALG00000020561	0.300321	3.05807	4.94227	0.000508	0.000508	0.216986
ADAM12	0.302022	0.968187	5.41723	0.03397	0.000508	0.000508
P2RY2	0.30577	7.29672	8.87752	0.014062	0.012707	0.758476
NFATC2	0.306909	1.13348	9.42593	0.040248	0.000508	0.000508
EPHB2	0.314025	2.64524	3.18688	0.002061	0.000939	0.734497

Table A3 **(Cont'd)**

HUNK	0.32346	1.38466	1.51163	0.009387	0.007117	0.888702
TLR5	0.326069	1.93421	1.49421	0.009636	0.023544	0.677327
PTGS1	0.329113	2.77142	8.7778	0.019436	0.002061	0.072681
PKD1	0.33125	1.50511	2.80855	0.000939	0.000508	0.082469
PPL	0.333797	10.0143	1.4017	0.000508	0.015839	0.000508
ME3	0.336629	2.92409	2.07601	0.012021	0.02626	0.607226
KCNK5	0.353873	5.85075	6.0595	0.000508	0.000508	0.932756
SH3TC2	0.365139	3.61872	6.31777	0.002061	0.000508	0.283832
PKIB	0.365324	2.44848	2.53344	0.002755	0.002061	0.953462
ENC1	0.372791	1.8575	3.83025	0.037019	0.007615	0.214132
SEMA3C	0.38573	5.91293	1.66328	0.000508	0.005455	0.001337
BEND5	0.391377	1.57968	2.04879	0.01315	0.005162	0.624454
CNFI-A4	0.392051	3.09856	1.57607	0.003083	0.031908	0.257037
ARHGEF17	0.407019	1.93121	1.26498	0.006024	0.045323	0.472769
ACOX2	0.413346	3.82857	2.90633	0.002755	0.006301	0.628671
DAB1	0.427216	1.43655	6.36677	0.032596	0.000508	0.000508
GATA3	0.43529	3.54972	3.10249	0.000508	0.000939	0.776628
ABI3BP	0.440246	2.00226	5.98381	0.006845	0.000508	0.012482
PCTP	0.446443	1.68886	2.06064	0.040878	0.021178	0.775497
KANK1	0.447502	1.33973	9.90361	0.009636	0.000508	0.000508
MAT1A	0.44817	2.66387	2.14173	0.006024	0.013626	0.708843
INADL	0.453643	3.67865	1.58889	0.000508	0.013386	0.035381
OSGIN1	0.456752	4.06742	4.14297	0.003402	0.003083	0.975903

Table A3 **(Cont'd)**

KLF4	0.457453	4.11843	4.44655	0.000508	0.000508	0.899002
GIPC2	0.466792	16.878	9.15438	0.001707	0.003402	0.201631
NIPAL1	0.470516	2.93558	5.2957	0.000939	0.000508	0.145118
SHROOM2	0.488595	9.49892	3.3803	0.000508	0.000508	0.013846
ENSGALG00000011717	0.527863	2.22384	1.913	0.008904	0.017989	0.807146
SLC6A8	0.545285	4.02394	4.25168	0.000939	0.000508	0.928389
ENSGALG00000006325	0.548728	3.3864	6.34173	0.005742	0.000939	0.214199
PTPN13	0.555312	3.00328	6.98901	0.000508	0.000508	0.008392
MAML3	0.559631	1.98999	4.44928	0.048939	0.003707	0.154647
EDN3	0.577767	3.03137	4.30061	0.002755	0.000508	0.42898
ER81	0.583483	4.149	1.78656	0.000508	0.030653	0.041717
GOLPH3L	0.621384	39.7059	6.37631	0.000508	0.007117	0.001337
CHTL1A	0.625205	5.27514	3.06649	0.004024	0.027709	0.343902
OTUD7A	0.632015	4.35121	17.2045	0.003083	0.000508	0.000508
MAGI1	0.665257	2.73304	4.46245	0.007357	0.000508	0.335038
ARHGAP6	0.70358	3.98005	4.65347	0.000508	0.000508	0.709315
HELIOS	0.738464	3.02799	11.5467	0.017363	0.000508	0.007357
ENSGALG00000011930	0.740705	216.926	4.34613	0.000508	0.009893	0.000508
ENPP2	0.74349	2.80555	3.83038	0.043558	0.012482	0.665226
KALRN	0.745573	6.52853	10.1921	0.001337	0.000508	0.331392
IRK1	0.754172	5.58922	2.86682	0.001337	0.021791	0.184982
HIC2	0.767319	3.08165	6.69065	0.009387	0.000508	0.104579
ODZ3	0.800241	4.48102	18.6009	0.000508	0.000508	0.000508

Table A3 **(Cont'd)**

NFATC1	0.801019	2.40936	21.9287	0.042202	0.000508	0.000508
PLD1	0.820285	4.26886	4.49194	0.000508	0.000508	0.898116
SEMA6D	0.8324	2.48963	2.21298	0.009893	0.020418	0.803342
TLR3	0.832801	7.75684	2.17355	0.000508	0.043902	0.000939
MAPK13	0.847589	5.66231	6.29015	0.009387	0.006571	0.871348
PLCD1	0.861296	9.24695	3.76277	0.000508	0.000939	0.004024
IL8	0.878176	6.55452	7.2487	0.000508	0.000508	0.831875
PPM1H	0.890929	12.4084	8.43123	0.003083	0.011088	0.654779
HOMER2	0.896948	3.86327	3.72386	0.038204	0.043558	0.960148
ARHGEF16	0.919259	4.193	4.40153	0.011088	0.008904	0.943239
STON2	0.923557	3.55556	8.60936	0.006301	0.000508	0.040248
SOCS2	0.928653	5.7905	11.0307	0.024561	0.004889	0.306597
CABLES1	0.94884	2.71065	4.45746	0.035705	0.000939	0.3128
MLKL	0.953769	7.04383	3.69207	0.003083	0.023544	0.221607
EML1	0.965554	5.16482	3.64939	0.000508	0.003083	0.422559
SSBP2	0.969702	5.67496	4.77462	0.007357	0.014062	0.798599
MAPKAPK3	0.971674	12.6696	3.57895	0.000939	0.047893	0.006301
RXRA	0.97844	3.04846	15.3345	0.043558	0.000508	0.000508
FAM135A	0.9896	4.01014	5.68699	0.000508	0.000508	0.300943
LRRC16A	0.996079	4.62987	5.59665	0.000508	0.000508	0.638122
TMEM116	1.02607	8.94275	3.22859	0.000939	0.046054	0.048181
RBM47	1.03047	9.2107	26.7843	0.000508	0.000508	0.004314
IL17REL	1.0513	27.0148	9.85616	0.000508	0.000508	0.007875

Table A3 **(Cont'd)**

ENSGALG00000019861	1.05626	10.9267	10.7654	0.000508	0.000508	0.973018
PLEKHA5	1.06136	3.77473	4.65813	0.001337	0.000508	0.569319
BACE2	1.06673	16.3356	9.0057	0.000508	0.000508	0.047893
BAAT	1.10777	5.504	5.16292	0.013846	0.019233	0.929253
EPHA3	1.11099	3.42788	4.95961	0.005162	0.000508	0.352751
MEIS1	1.11402	27.4519	5.38298	0.000508	0.010133	0.004889
SMAD6	1.12881	4.20661	19.2271	0.025134	0.000508	0.011803
UGT8	1.15209	31.7668	15.0632	0.000508	0.000508	0.012919
DOCK1	1.15404	4.09638	4.78731	0.000508	0.000508	0.730117
IQSEC1	1.15571	4.40534	6.71355	0.002413	0.000508	0.304986
BEAN1	1.17527	7.51839	7.33904	0.007615	0.007615	0.969601
HS1BP3	1.17598	3.32188	4.11694	0.030281	0.009387	0.673603
ARHGAP20	1.20295	3.18119	5.13516	0.020024	0.000939	0.250639
RGN	1.22335	6.0976	5.13665	0.029178	0.049102	0.791728
CSRP2	1.22801	21.5894	4.16787	0.000508	0.003707	0.000508
CAMKK1	1.24793	3.95076	5.2606	0.019045	0.004889	0.581677
FAM134B	1.26981	6.78977	5.36433	0.000508	0.000939	0.514154
IRF6	1.29576	20.0255	8.48929	0.000508	0.004314	0.024352
GNAL	1.29741	10.7448	7.62893	0.001337	0.007875	0.509265
CCBP2	1.30546	6.55851	4.69991	0.007357	0.024561	0.558356
KIAA1598	1.3269	3.7982	6.80223	0.047893	0.001707	0.242418
ENSGALG00000002326	1.33972	6.4645	4.7818	0.000508	0.000508	0.395182
AASS	1.34746	13.0536	3.31588	0.000508	0.023367	0.000508

Table A3 **(Cont'd)**

EPHX2	1.35934	4.31111	8.24317	0.028294	0.000939	0.203465
ABCB1	1.3627	6.29988	19.4842	0.000508	0.000508	0.000508
MBOAT2	1.36666	4.80178	24.4073	0.012021	0.000508	0.000508
SRC	1.38908	5.73228	24.9429	0.011328	0.000508	0.002061
CDC42EP1	1.40803	7.30381	11.7118	0.020792	0.006301	0.511948
GAB1	1.42744	4.08284	5.57697	0.004024	0.000508	0.413481
SH3RF3	1.43413	9.60105	5.17429	0.000508	0.001337	0.052287
SLC41A3	1.44033	4.4492	5.19179	0.021596	0.009636	0.785341
ENSGALG00000012808	1.44155	3.29659	5.45497	0.028453	0.000939	0.160928
SRD5A2	1.44869	15.7273	7.11396	0.000508	0.006024	0.046366
TFEC	1.45138	5.14745	17.7414	0.000508	0.000508	0.000508
SATB1	1.47683	5.74643	7.94949	0.001707	0.000508	0.380986
FAM117B	1.4802	3.66993	12.5907	0.019233	0.000508	0.000508
CCR7	1.50697	3.83858	32.2082	0.023181	0.000508	0.000508
MICALL2	1.55982	6.74082	4.66559	0.001707	0.01606	0.452392
KLF9	1.56472	6.55789	7.65751	0.009387	0.005455	0.780665
PDE5A	1.59226	4.86121	4.56863	0.010857	0.014947	0.902645
DDAH1	1.60018	8.89949	12.4112	0.043084	0.020593	0.736545
FARP1	1.61098	5.96495	6.94414	0.000508	0.000508	0.686913
DYRK2	1.65168	7.3765	19.2492	0.001337	0.000508	0.003402
IRF2BPL	1.66892	5.8896	6.80751	0.043246	0.023181	0.846017
PCGF5	1.71267	7.82354	27.8981	0.014287	0.000508	0.004314
CPNE2	1.71884	9.4985	4.79694	0.000939	0.03738	0.151019

Table A3 **(Cont'd)**

AFAP1L2	1.72013	6.95002	8.24132	0.000508	0.000508	0.66036
KLF3	1.72173	7.14715	13.457	0.000508	0.000508	0.022588
CASS4	1.72638	6.18142	109.568	0.007615	0.000508	0.000508
TNRC18	1.74806	6.55669	6.06989	0.000508	0.000508	0.822839
ARRDC1	1.74908	5.0054	4.48179	0.014287	0.032786	0.832429
MGLL	1.75663	8.69318	27.0603	0.000508	0.000508	0.000508
LAMP5	1.76243	22.534	38.597	0.000508	0.000508	0.285486
PARD6G	1.78558	4.67688	24.6255	0.02791	0.000508	0.000508
WWTR1	1.84592	5.44693	6.83097	0.042792	0.017772	0.697029
TMEM37	1.847	6.50435	6.08477	0.032259	0.043422	0.924091
ARHGEF10L	1.87624	7.25919	16.5192	0.000939	0.000508	0.019233
SPIC	1.87633	24.1935	105.371	0.000508	0.000508	0.000508
LRP5	1.87986	5.38799	13.969	0.000508	0.000508	0.001337
TSPAN15	1.90083	24.7261	6.37061	0.000508	0.009893	0.001707
IGF2	1.91937	6.52536	49.4515	0.010375	0.000508	0.000508
PLK2	1.94176	5.44003	6.83741	0.004889	0.000508	0.551246
LAMA3	1.96362	9.81279	23.9708	0.000508	0.000508	0.000508
TIAM1	1.96809	5.03917	23.7046	0.030477	0.000508	0.000508
NOX1	1.98415	9.28745	9.17865	0.008648	0.009143	0.982039
KIAA1462	2.03437	4.99135	44.7799	0.007357	0.000508	0.000508
ABCA3	2.04775	4.21727	16.9987	0.036676	0.000508	0.000508
ANKRD6	2.07171	6.69452	14.0766	0.026069	0.000508	0.12758
EMB	2.08889	6.70126	11.1727	0.009893	0.000508	0.098245

Table A3 **(Cont'd)**

SULF2	2.08961	9.0529	46.7473	0.000508	0.000508	0.000508
TRIO	2.1088	5.19878	13.6031	0.014731	0.000508	0.006024
ENSGALG00000011008	2.1592	5.33329	5.90774	0.028816	0.016719	0.829355
HAAO	2.21228	9.76345	16.4075	0.010857	0.001337	0.363627
CRIM1	2.22432	6.98092	5.98069	0.004024	0.013626	0.730172
TMEM51	2.25335	9.94368	6.94165	0.003707	0.0281	0.498167
WDFY3	2.30125	5.42357	11.8498	0.000508	0.000508	0.000508
RNF144A	2.31835	4.87521	26.0959	0.019233	0.000508	0.000508
TSPAN9	2.32859	7.75289	7.96248	0.004314	0.003402	0.957816
TREM-B2V2	2.35699	8.34008	24.2048	0.023367	0.000508	0.023367
IGF2BP2	2.3577	8.21414	22.5546	0.002061	0.000508	0.000939
GNA11	2.37698	7.18044	17.4372	0.035516	0.000508	0.049562
SYT8	2.38611	13.1117	15.7214	0.000508	0.000508	0.664939
CHST9	2.40234	12.2818	17.7513	0.007615	0.001707	0.461785
TRAM2	2.40576	6.17187	25.4709	0.032086	0.000508	0.000508
FAM65B	2.45146	5.424	133.542	0.034691	0.000508	0.000508
CD36	2.56963	9.67458	117.539	0.004604	0.000508	0.000508
SERPINB1	2.57356	6.29345	6.67028	0.020225	0.012707	0.892507
C3H8ORF80	2.69404	20.8806	16.3182	0.000508	0.000508	0.446232
ALCAM	2.75823	127.507	20.4103	0.000508	0.000508	0.000508
TNFAIP2	2.75853	7.71761	102.071	0.011803	0.000508	0.000508
TAAR1	2.77091	26.0546	9.51468	0.000508	0.020792	0.019045
SLCO2B1	2.77915	14.05	26.7004	0.000508	0.000508	0.113746

Table A3 **(Cont'd)**

C1orf198	2.78438	8.417	7.74482	0.002061	0.005742	0.840492
IL13RA2	2.79102	7.89643	59.5155	0.025695	0.000508	0.000508
ENSGALG00000026622	2.84781	6.31692	7.62765	0.020024	0.003707	0.651155
DCLK3	2.87625	8.71786	67.4573	0.004314	0.000508	0.000508
FNDC3B	2.90721	5.53627	13.5541	0.021	0.000508	0.001337
IDUA	2.94622	5.73965	10.1385	0.037877	0.000508	0.063686
TOM1	2.96555	6.65738	11.872	0.041717	0.000939	0.137252
TCN2	2.99622	7.7554	17.166	0.043558	0.000508	0.087339
GPR126	3.00488	14.0896	12.9421	0.000508	0.000508	0.793093
GSTA3	3.00787	61.8514	34.6994	0.000508	0.000508	0.086553
CMPK2	3.01488	10.1194	6.68449	0.000508	0.016284	0.209266
XDH	3.01609	14.616	10.2905	0.000508	0.000508	0.215215
ADIPOQ	3.02201	7.79763	31.0603	0.044819	0.000508	0.001707
gga-mir-1723	3.04244	8.47628	11.7999	0.005455	0.000508	0.368638
TMEM55A	3.08481	5.79507	15.8879	0.048472	0.000508	0.000939
F10	3.16978	16.7194	23.6763	0.000508	0.000508	0.297434
SMAD7A	3.17377	23.2823	7.90604	0.000508	0.046498	0.014287
TRIM25	3.18202	9.43226	22.0807	0.006571	0.000508	0.012707
ARHGAP21	3.25739	6.65078	6.65756	0.014062	0.014287	0.997074
KIAA0284	3.30032	6.37151	6.19441	0.032596	0.046498	0.945797
JUP	3.3069	15.4853	12.9723	0.000508	0.000508	0.658316
PMP22	3.35261	11.3209	21.0397	0.004604	0.000508	0.119606
MBNL2	3.48825	8.89566	8.91137	0.004314	0.004314	0.995875

Table A3 **(Cont'd)**

ARRDC4	3.51399	14.5759	11.5788	0.000508	0.000508	0.453215
LCAT	3.52257	24.0232	15.2853	0.000508	0.000508	0.133525
KLF4	3.63126	11.7424	418.52	0.001707	0.000508	0.000508
SLC46A2	3.65269	13.9485	23.8908	0.023367	0.001337	0.372919
K123	3.74981	38.4522	45.4698	0.000508	0.000508	0.54621
OLFML2A	3.76175	10.4987	22.559	0.007875	0.000508	0.028453
IL34	3.77065	33.7574	25.9901	0.000508	0.003083	0.646678
CRIP2	3.842	14.1945	18.8438	0.024939	0.008648	0.651942
GAS6	3.86588	9.10347	9.46873	0.017158	0.014731	0.929763
IGSF1	3.88626	8.15236	19.9483	0.042516	0.000508	0.008139
PLEKHG3	3.92112	9.48711	9.37216	0.032596	0.030101	0.981859
TNFRSF11A	3.93804	9.61676	20.5085	0.003707	0.000508	0.007875
EFNB2	3.99331	18.9242	26.2589	0.000508	0.000508	0.245044
CHST2	4.04376	19.8605	57.621	0.000508	0.000508	0.000508
CYP46A1	4.13707	8.11952	16.4152	0.049259	0.000508	0.029707
DENND4C	4.23717	7.63147	17.4612	0.036199	0.000508	0.003402
CX3CR1	4.27272	47.8291	100.933	0.000508	0.000508	0.003083
MAOA	4.36015	17.5283	136.06	0.001337	0.000508	0.000508
LHFP	4.46778	11.7735	38.4283	0.0281	0.000508	0.003707
ATP11A	4.51971	11.3786	34.7579	0.015384	0.000508	0.003707
LAMB1	4.54205	16.7902	22.5519	0.000508	0.000508	0.307152
ENSGALG00000026188	4.58184	11.7172	14.2484	0.001337	0.000508	0.565392
REV-ERBB	4.72928	10.7032	19.8868	0.028294	0.000508	0.063937

Table A3 **(Cont'd)**

TGM4	4.75004	68.7104	294.359	0.000508	0.000508	0.000508
MERTK	4.91421	11.5692	62.3949	0.002755	0.000508	0.000508
TESC	5.06102	20.2355	31.7507	0.005455	0.000939	0.400379
ST14	5.18044	19.941	15.431	0.000508	0.000508	0.44242
SASH1	5.4587	13.0119	40.6049	0.002413	0.000508	0.000508
AHR	5.51759	27.2075	14.5191	0.000508	0.000939	0.007615
VNN1	5.84053	77.7306	15.2045	0.000508	0.003083	0.000508
MAP4K4	5.88045	40.107	24.2251	0.000508	0.000508	0.164038
RUNX3	6.01675	13.6743	39.3157	0.002061	0.000508	0.000508
CBFA2T3	6.02418	20.1362	16.4519	0.000508	0.005455	0.624648
SOWAHC	6.07657	16.9537	170.749	0.007615	0.000508	0.000508
ENSGALG00000008755	6.08812	15.2984	22.1987	0.028453	0.003707	0.360152
ENSGALG00000010336	6.10817	76.9616	778.603	0.000508	0.000508	0.000508
ETS2	6.22195	34.9152	74.1951	0.000508	0.000508	0.002755
PLA2G4A	6.42729	14.5428	32.1337	0.002061	0.000508	0.001337
ATP2B4	6.6352	23.8871	77.9922	0.009893	0.000508	0.011803
AAED1	6.64458	20.0226	29.5981	0.040248	0.006571	0.446072
CPD	6.71025	16.3524	14.6723	0.000508	0.001707	0.732428
CLSTN1	6.7327	13.8602	26.6486	0.007615	0.000508	0.020792
ITGA6	6.77825	21.7023	37.8627	0.000508	0.000508	0.027709
HPGD	6.89161	23.3354	225.344	0.02626	0.000508	0.000508
SVIL	6.96261	12.8806	42.8868	0.0281	0.000508	0.000508
ENSGALG00000028304	6.98649	12.2243	37.6404	0.01606	0.000508	0.000508

Table A3 **(Cont'd)**

EDNRA	7.16296	13.8902	13.9197	0.030101	0.029363	0.995352
GNG10	7.25267	66.5739	102.052	0.000508	0.000508	0.143124
BACH2	7.31154	14.2218	18.7513	0.031558	0.002413	0.43691
MARK1	7.33468	16.2673	16.3064	0.003083	0.003083	0.994128
SESTD1	7.39224	16.9444	43.6512	0.004314	0.000508	0.000508
INPP5A	7.39908	13.5694	21.2495	0.036676	0.000939	0.128116
ADAM9	7.53558	14.0712	21.5508	0.035868	0.000508	0.177888
DNASE1L3	7.63404	17.6706	84.9297	0.044542	0.000508	0.000508
HES4	7.76319	29.602	61.8236	0.001337	0.000508	0.037211
ENSGALG00000008518	7.94889	59.4623	46.1109	0.000508	0.000508	0.507657
FOXI1	7.94947	18.1078	26.1027	0.007117	0.000508	0.259917
ABCC3	8.06548	19.8229	35.7362	0.046946	0.000508	0.227132
PIK3IP1	8.23974	34.201	101.858	0.000508	0.000508	0.000508
HIC1	8.28923	18.492	30.8397	0.008648	0.000508	0.128215
MCF2L	8.41862	16.1075	58.257	0.033141	0.000508	0.000508
SMPD1	8.49132	28.4941	74.3114	0.000508	0.000508	0.000508
CHDZ	8.77642	14.099	35.7552	0.047277	0.000508	0.000508
ANTXR2	9.04841	17.9383	42.7212	0.047112	0.000508	0.004024
MADH2	9.1565	21.433	18.3724	0.000939	0.007875	0.627012
ST3GAL2	9.1924	21.7255	53.9813	0.038701	0.000508	0.023951
AGPAT2	9.41875	23.4749	27.0785	0.031197	0.011573	0.771527
SULT1B1	9.4671	35.8784	37.6757	0.000508	0.000508	0.909989
BATF3	9.54049	32.4843	37.8953	0.002755	0.000508	0.734885

Table A3 **(Cont'd)**

KAT2B	9.59892	18.7419	50.6204	0.004889	0.000508	0.000508
MCCC2	9.90912	24.542	22.6469	0.001707	0.007357	0.834804
UGCG	9.96857	18.9801	40.8877	0.008392	0.000508	0.001337
SCARB2	10.3897	22.9079	39.6122	0.002755	0.000508	0.045154
TRIM3	10.4111	20.1098	31.4078	0.044542	0.000508	0.204185
SDC1	10.4561	34.821	33.3641	0.000508	0.000508	0.897981
FYB	10.4835	22.8407	129.24	0.000508	0.000508	0.000508
GNPTAB	10.8809	27.0677	52.5856	0.000508	0.000508	0.003402
ST3GAL6	11.0098	34.544	21.161	0.000508	0.035207	0.112432
TPRN	11.3196	24.8641	64.7043	0.003402	0.000508	0.000508
TNFSF11	11.9016	26.3119	34.0054	0.002755	0.000508	0.367069
S100A9	12.32	37.9278	50.3173	0.024352	0.005455	0.574868
SMPD1	12.4333	27.9909	84.0121	0.019233	0.000508	0.001707
CLCN5	12.5258	21.1835	53.5637	0.046199	0.000508	0.000508
ELF3	12.7239	58.9684	44.3292	0.000508	0.000508	0.440149
FABP7	13.3715	49.0606	440.517	0.000939	0.000508	0.000508
IRF-3	13.6281	33.7315	51.8144	0.007615	0.000508	0.254839
NFKBIE	13.8619	27.5447	69.07	0.017158	0.000508	0.000939
BDH2	13.8849	25.3178	42.8935	0.022785	0.000508	0.049562
PPDPF	14.0051	69.0516	143.646	0.000508	0.000508	0.025324
TMBIM1	14.1906	32.8311	56.4239	0.009387	0.000508	0.088765
PLOD2	14.2848	28.5609	28.1654	0.003402	0.005162	0.966534
SNTB1	14.7221	32.5636	51.9525	0.000508	0.000508	0.077064

Table A3 **(Cont'd)**

PLAU	14.8344	34.9595	149.627	0.000939	0.000508	0.000508
RASGEF1A	15.1064	30.2156	56.8098	0.004889	0.000508	0.007357
TBC1D1	15.2898	29.97	28.1608	0.003707	0.010375	0.848791
B3GNT2	15.4667	47.576	52.3391	0.000508	0.000508	0.751878
TSPAN12	15.6636	32.2947	34.4499	0.007357	0.002413	0.858718
MPP1	16.2099	33.2076	53.3024	0.003083	0.000508	0.076905
SYDE1	16.6019	51.4967	535.765	0.029356	0.000859	0.000859
VDAC1	16.9436	46.7329	93.2086	0.003083	0.000508	0.027183
ZDHHC9	17.7021	32.6889	30.5284	0.009893	0.022182	0.842465
BRT-1	18.3421	63.9373	87.8242	0.000508	0.000508	0.430863
DUSP7	18.583	62.6784	79.7746	0.000508	0.000508	0.363473
ENSGALG00000026611	19.0873	109.709	93.5736	0.000859	0.000859	0.773962
TCP11L2	19.7596	32.3972	69.5213	0.03738	0.000508	0.001337
NFE2L2	19.9299	44.8117	56.0434	0.000939	0.000508	0.416203
S100A16	19.9739	72.0528	203.875	0.022182	0.000508	0.025324
SUB1	20.7937	35.206	56.0618	0.015156	0.000508	0.052287
FAM3C	20.9968	42.1351	71.7236	0.005162	0.000508	0.038204
PDLIM5	21.2027	38.6722	45.6607	0.004314	0.000508	0.545845
SGK3	21.5071	38.5786	59.721	0.004604	0.000508	0.066133
PLEKHB2	22.1496	61.6623	37.139	0.000508	0.014947	0.029006
TUBB6	22.2051	43.1896	63.5819	0.008904	0.000508	0.17283
CTNS	22.6631	41.255	68.6362	0.012919	0.000508	0.04743
LTB4R	23.4651	84.2222	160.847	0.000508	0.000508	0.015156

Table A3 **(Cont'd)**

SAMHD1	23.6977	61.057	42.2576	0.000508	0.004889	0.129821
TGIF1	23.7211	46.0039	52.9727	0.021596	0.001707	0.687532
ADD3	24.7541	93.2358	110.55	0.000508	0.000508	0.541107
TAGAP	25.3725	42.189	73.8918	0.017989	0.000508	0.015384
CFD	25.3762	871.565	88.8297	0.000508	0.000508	0.000508
ASS1	26.0674	97.1358	226.195	0.000508	0.000508	0.000508
LTF	27.4783	1381.72	175.752	0.000508	0.000508	0.000508
CTNNB1	27.6302	100.267	62.8923	0.000508	0.000508	0.036839
PLSCR4	28.525	92.5301	120.117	0.000508	0.000508	0.393532
DRAM1	29.3442	53.5489	75.6942	0.009143	0.000508	0.184697
EPSTI1	31.2086	55.3298	70.2693	0.030653	0.001337	0.442132
ENSGALG00000011190	31.3789	164.558	1878.44	0.000939	0.000508	0.000508
HEXB	31.9978	68.2679	213.893	0.001707	0.000508	0.000508
LITAF	32.5128	69.7379	331.117	0.017772	0.000508	0.000508
C4BPA	32.5148	116.301	284.629	0.028816	0.000508	0.06968
LYZ	33.8154	5986.69	452.803	0.000508	0.000508	0.000508
CD82	34.6493	76.1211	163.19	0.000508	0.000508	0.000508
gga-mir-147	36.0413	93.5739	66.9866	0.000508	0.007615	0.183878
GNE	36.1216	58.6151	73.1789	0.014062	0.000508	0.3883
VCAM1	37.2466	58.9571	289.64	0.039913	0.000508	0.000508
PIK3CD	37.3971	75.4274	81.9943	0.009143	0.001707	0.80464
GPR137B	38.7829	66.8007	177.188	0.043246	0.000508	0.000508
FAM49A	41.6386	90.4122	364.548	0.000508	0.000508	0.000508

Table A3 **(Cont'd)**

S100A11	42.144	178.771	109.105	0.000508	0.011803	0.161481
TAX1BP3	50.3501	88.4422	149.038	0.008392	0.000508	0.026069
APP	50.3658	243.817	161.855	0.000508	0.000508	0.075505
SMAP2	55.0645	90.871	313.284	0.037538	0.000508	0.000508
ISG12(2)	58.855	156.207	472.858	0.006301	0.000508	0.000508
HADH	61.5091	107.228	125.483	0.012919	0.001707	0.584976
SLC40A1	69.1872	291.578	1925.16	0.000508	0.000508	0.000508
gga-mir-3526	73.1726	145.732	119.473	0.000508	0.016284	0.43925
SDC4	77.3579	167.166	224.543	0.006301	0.000508	0.361273
TIMD4	77.5062	562.83	393.011	0.000508	0.000508	0.162875
ITM2B	77.9683	187.133	273.665	0.000508	0.000508	0.142615
ATP1B1	78.6643	150.195	138.533	0.005455	0.016936	0.797835
NADK	81.5953	155.52	141.061	0.001337	0.003402	0.720354
CREG1	82.046	135.421	687.759	0.011088	0.000508	0.000508
ACVRL1	99.2499	244.363	2377.99	0.015801	0.000859	0.000859
ANXA11	99.3072	168.218	247.286	0.014519	0.000508	0.144181
ANKRD13D	115.107	362.664	518.865	0.002181	0.000859	0.480855
TSPAN3	127.301	215.206	257.599	0.011088	0.000939	0.538153
MYH9	164.506	244.164	488.105	0.043558	0.000508	0.007357
ENSGALG00000026970	179.44	574.432	645.614	0.000508	0.000508	0.673321
PNRC1	184.523	345.639	581.711	0.000939	0.000508	0.01756
IL-1BETA	192.66	710.049	650.191	0.000859	0.000859	0.873133
SH3BGRL3	220.355	395.444	366.86	0.001707	0.009387	0.781003

Table A3 **(Cont'd)**

AKR1B10	287.807	476.292	689.201	0.046199	0.000508	0.234754
C3D	294.952	1090.11	1118.3	0.000859	0.000859	0.954525
IGJ	305.666	646.79	688.118	0.000508	0.001337	0.858204
LAPTM4A	327.313	533.039	836.049	0.008904	0.000508	0.044394
PIK3R5	382.61	976.528	1588.47	0.001511	0.000859	0.203152
ENSGALG00000015398	530.181	1095.66	1125.56	0.008139	0.006301	0.927452
SP1	777.066	1503.76	2167.92	0.028769	0.000859	0.350366

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