IDENTIFICATION OF CYTOKINE INDUCED CHANGES TO THE PANCREATIC
ISLET EPIGENOME

by

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B.Sc. Biology, Memorial University of Newfoundland and Labrador, 2009

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in
THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES

(Cell and Developmental Biology)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

August 2015

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Abstract

The initial onset of type 1 diabetes, as well as islet graft rejection, is characterized by the autoimmune assault on the β-cells of the pancreatic islets of Langerhans. Resident and infiltrating immune cells secrete a cocktail of cytokines, such as IFNγ, IL-1β, and TNFα, which in turn, signal the β-cells to produce and secrete various chemokines and cytokines that lead to the recruitment of additional immune cells, eventually leading to β-cell failure and death. During these processes the expression of many genes becomes altered within β-cells, and we hypothesized that alterations to the chromatin states of β-cell cis-regulatory regions underlies these gene expression changes. The chromatin state of a given cis-regulatory region can be identified by the pattern of post-translational histone modifications on adjacent nucleosomes. For this study we focused on 4 histone modifications: Histone 3 Lysine 4 monomethylation (H3K4me1) and trimethylation (H3K4me3), Histone 3 Lysine 9 trimethylation (H3K9me3) and Histone 3 Lysine 27 trimethylation (H3K27me3); with a particular focus on H3K4me1 that is associated with active or poised enhancers and promoters. Our ChIP-Seq analysis revealed that, upon IFNγ, IL-1β, and TNFα exposure, many genomic regions in β-cells acquire de novo H3K4me1, despite being initially marked by the repressive histone modification H3K27me3. Many chemokine and cytokine genes were associated with these de novo enhancer regions, and the expression of many of these chemokine and cytokine genes is induced in islets exposed to IFNγ, IL-1β, and TNFα. We identified the Trithorax group (TrxG) complexes as likely candidates involved in the generation of these de novo enhancers, as they can contain proteins with H3K4 methyltransferase and H3K27me3 demethylase activity. To confirm the involvement of these complexes we attempted to block their activity by using an adenovirus expressing shRNAs
targeting the core TrxG complex subunit Wdr5, and by using a small molecule selective inhibitor (GSK-J4) of the H3K27me demethylases Utx and Jmjd3. Both approaches resulted in blunting of the IFNγ, Il-1β, and TNFα induced expression of proinflammatory cytokines, with GSK-J4 having a more pronounced effect.
Preface

I designed, conducted and analyzed all experiments in this project in consultation with my supervisor Dr. Brad Hoffman.

A version of chapter 2 and 3 has been submitted for publication. In consultation with my supervisor and lab mates Amol Gill, Cheryl Whiting and Bryan Tennant, we designed, conducted and analyzed all the experiments and Dr Hoffman wrote the manuscript.
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<tr>
<td>Abcc8</td>
<td>ATP-Binding Cassette Sub-Family C</td>
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<tr>
<td>Ar</td>
<td>Androgen receptor</td>
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<tr>
<td>Ash2l</td>
<td>Ash2 (absent, small, or homeotic)-like</td>
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<td>BAF</td>
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<td>DAVID</td>
<td>Database for Annotation, Visualization and Integrated Discovery</td>
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<td>DMEM</td>
<td>Dulbecco's Modified Eagle's medium</td>
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<td>DNA</td>
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<td>hESC</td>
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<td>XIST</td>
<td>X Inactive Specific Transcript</td>
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Acknowledgements

I am extremely grateful to have been given this opportunity to be on the cutting edge of scientific discovery. Thank you Dr Brad Hoffman for providing me guidance, knowledge, patience and chances to learn.

I would like to thank the entire Hoffman lab for all of their insight, assistance and entertaining conversations throughout the years. Bryan has provided so much technical and intellectual mentorship and has contributed greatly to the completion of this thesis. Special thanks for all the great work Amol did to assist this project. Thanks to Cheryl and Stephanie for so much support at the bench and grabbing coffee.

Thank you to my committee members, Dr Jan Ehses, Dr Francis Lynn and Dr Bruce Verchere for your time and direction. You have greatly helped shape this work.

I have to thank my family and friends, without you this wouldn’t have been possible. You have given me such tremendous support and encouragement throughout this entire process.

Finally I would like to thank Nicole. Your support and encouragement have been a huge part of getting this finished and looking good.
Dedication

To Mom, Dad, Grampa and Nana.

Your encouragement made this happen.
Chapter 1  Introduction

1.1.  Thesis overview

The field of gene regulation has undergone dramatic expansion in recent years, with the identification of several exciting new players, such as non-coding RNAs and histone modifications. It is becoming increasingly clear that transcription factors alone are not the master regulators of gene expression. Rather, proper gene expression relies on an intricate interplay of RNAs, transcription factors and chemical modifications to DNA and to the histones it intertwines. The impact of histone modifications are profound, as chemical changes of various residues in the histone tails can alter the accessibility of nearby DNA, helping demarcate different types of regulatory elements, and influencing gene expression at a level prior to transcription.

Proper gene expression is fundamental for virtually all cellular events. Dysregulation of gene expression can lead to dysfunction at cellular, organelle and organismal levels, and is involved in the pathogenesis of many diseases. One example is type 1 diabetes mellitus (T1D), which is characterized by the autoimmune assault of insulin producing β-cells in the pancreatic islets of Langerhans. During the pathogenesis of this disease, the β-cells undergo widespread changes in gene expression, including the down-regulation of genes involved in β-cell function, and the up-regulation of genes involved in apoptosis and inflammation. Over the last few years a growing body of evidence has emerged showing that changes to chromatin, such as histone modifications, underlie such gene expression changes. Given this, we focus on two major
questions in how proinflammatory cytokines produced by immune-cells during T1D pathogenesis induce gene expression changes in β-cells: 1) What are the underlying changes to histones modifications that occur around genes whose expression is altered, and 2) Is it possible to prevent such gene expression changes by blocking these histone modification changes? In this thesis, we attempt to address these questions by examining the impact of a simulated autoimmune assault on the epigenetic landscape of pancreatic islets, and determining the role that the Trithorax group (TrxG) complex plays in this process.

1.2. Pancreatic Islets of Langerhans

The pancreatic Islets of Langerhans are heterogeneous clusters of five cell types that comprise the endocrine functions of the pancreas; including: Glucagon producing α-cells; Insulin producing β-cells; Somatostatin producing δ-cells; Ghrelin producing ε-cells; and Pancreatic polypeptide producing PP-cells, with the majority of cells being β-cells\textsuperscript{18}. In addition to the endocrine cells, each islet also contains resident macrophages, dendritic cells, and endothelial cells\textsuperscript{19,20}. Islet morphology varies somewhat between species. For instance, mouse islets tend to have an increased proportion of β-cells within their core, with the other 4 cell types forming a mantle around them. Human islets, on the other hand, have a more mosaic arrangement with the cells dispersed throughout the islet. Moreover, mouse islets tend to have an increased quantity of β-cells, approximately 80%, whereas human islets contain about 65%\textsuperscript{18}. Regardless of these differences, the overall morphology and function of both human and mouse pancreatic islets share far more in common, than they differ.
1.3. Diabetes mellitus

Diabetes mellitus is a family of metabolic diseases, commonly referred to as diabetes, which are characterized by prolonged elevated levels of glucose in the blood, or hyperglycemia\(^1\,^2\). Diabetes can be subdivided into five primary types: Type 1 diabetes (T1D), Type 2 diabetes (T2D), gestational diabetes, neonatal diabetes, and mature onset diabetes of the young (MODY)\(^2\). Although all result in similar metabolic and physiological syndromes the underlying causes and therefore, the treatment of these five types are rather different.

Recent estimates indicate that as of 2013 382 million people worldwide are diagnosed with diabetes, with ~90% of the cases being T2D\(^3\). T2D, previously referred to as non-insulin-dependent diabetes mellitus is characterized by insufficient insulin production or insulin resistance\(^2\). In T2D, the body is unable to produce enough insulin and unable to properly sense and respond to insulin. Lifestyle factors, such as obesity, lack of physical exercise and high fat, high sugar dietary choices, as well as genetic predisposition are the primary causes of T2D\(^1\,^2\). In the case of insulin resistance, the body becomes resistant to insulin, resulting in increased insulin demand on the \(\beta\)-cells. This causes an increase in \(\beta\)-cell mass to a point, at which time the islets are no longer able to compensate for the increased demand and hyperglycemia ensues\(^1\,^2\). As such, the treatment of T2D varies depending on the cause. In many cases, patients are able to maintain normal blood glucose levels with diet and exercise alone. In other cases, patients may be prescribed a variety of medications that increase insulin sensitivity, decrease glucose
production by the liver\textsuperscript{1}, or enhance insulin secretion by pancreatic beta cells. Eventually, many individuals with T2D also require insulin therapy.

Gestational diabetes mellitus is a condition in which pregnant women experience an inability to maintain normal blood glucose levels\textsuperscript{2}. Although the precise causes of gestational diabetes are not fully understood, it is believed that pregnancy hormones and other factors interfere with the action of insulin and it’s binding to the insulin receptor\textsuperscript{4}. Gestational diabetes can typically be managed with adjustments to diet and exercise during pregnancy, however some cases require the use of medications used in T2D, or even insulin\textsuperscript{1}. The majority of gestational diabetes cases resolve after birth, though, some cases persist with T2D like symptoms. Women who experience gestational diabetes are also at an increased risk of developing T2D, or in rare cases T1D, later in life\textsuperscript{4}.

T1D, formerly referred to as insulin-dependent diabetes mellitus, is characterized by the loss of the insulin producing \(\beta\)-cells within the pancreatic islets of Langerhans. T1D is an autoimmune disorder in which the patient’s own immune system attacks and destroys the insulin producing \(\beta\)-cells\textsuperscript{5-7}. T1D is most commonly treated with insulin therapy, in which patients must monitor their blood glucose levels and inject exogenous insulin to compensate for carbohydrate intake. The invention of the modern insulin pump as well as the continuous glucose monitor have provided patients with tools to maintain normal glucose levels and greatly extend the time before diabetes related complications arise. With the development of the Edmonton protocol, islet transplantation has become a promising treatment for T1D\textsuperscript{9,10}. However, despite promising initial results, the majority of transplant recipients exhibit graft failure and are back on insulin within 5
years\textsuperscript{9}. On top of this, the requirement of immunosuppressive anti-rejection drugs, and limited supply of cadaveric donor islets has restricted the success and availability of islet transplant as a global cure for T1D\textsuperscript{9,10}.

In addition, there are also neonatal diabetes and MODY. In these cases, diabetes is the result of genetic mutations in key genes that are required for β-cell function or development, such as Insulin (Ins), Glucokinase (Gck) or Hepatocyte Nuclear Factor 1-Beta (Hnf1β)\textsuperscript{8}. Depending on the type of mutation, the onset of diabetes can vary. Some mutations result in neonatal diabetes, with symptoms arising within the first 6 months after birth, while other mutations cause MODY, where symptoms typically arise during adolescence or early adulthood.

Recently, several groups have successfully generated glucose-responsive, insulin-secreting β-like cells from hESCs and encapsulated them prior to transplantation to evade the autoimmune response\textsuperscript{11,12}. While β-like cells are continuing to show huge potential for the treatment of people with diabetes, there is a great deal of work being done to identify T1D patients before they become symptomatic. The treatment of these pre-diabetic T1D patients with therapeutics designed to block the loss of their native β-cells would be preferable to β-like cell transplantation. It is our hope that the research done in this thesis will further our understanding of the pathogenesis of T1D and T2D, as well as, identify potential novel targets for future therapeutics.
1.4. Onset of T1D

Although the exact trigger/s that cause the immune system to attack and destroy the β-cells continue to elude us, how the process progresses is largely understood. Primarily, the immune system utilizes multiple cell types including TNFα/Il-1β secreting macrophages and IFNγ secreting auto-reactive T-cells to attack and destroy the β-cells. These cytokines are recognized by the β-cells and reduce expression of transcription factors required for proper β-cell function such as Pdx1, Mafa and Neurod1. Additionally, proinflammatory and proapoptotic transcription factors such as Nfκb, Stat1, and Irf3 become expressed. The induction of these transcription factors leads to β-cell dysfunction by inhibiting important functional genes such as Glut-2 (Slc2a2) and Ins1, as well as inducing the expression and release of cytokines and chemokines from the β-cells. During this process, the β-cells produce and secrete a variety of chemokines, such as Ccl2 (Mcp-1), Ccl20 (Mip-3α), Cxcl9 (Mig) and Cxcl10 (Ip-10), resulting in the recruitment and activation of additional immune cells, amplifying the immune response in a feed forward loop, ending in the death of the β-cells and ultimately diabetes.

1.5. Cis-Regulatory elements

The precise temporal and spatial gene expression pattern of different cells is what allows us to become the complex organisms that we are. Each cell in the body contains the same set of genes, but it is through the action of various cis-regulatory elements that each cell is able to express the correct amount of a specific gene at the required time. These regulatory elements are
scattered throughout the genome and can be categorized into four main roles: promoters, enhancers, insulators, and silencers\textsuperscript{21-23}.

Promoters are the regulatory elements located adjacent to the transcriptional start site (TSS) of genes. They contain binding sites for specific transcription factors that regulate gene expression by assembling the transcriptional pre-initiation complex, and subsequently, recruiting RNA polymerase II to the TSS\textsuperscript{21}.

Enhancer elements are typically comprised of a small cluster of transcription factors binding sites and are interspersed throughout the genome. Some enhancers can be several hundred kb upstream of the promoter, down stream of the promoter, within introns or even past the 3’ end of a gene\textsuperscript{21}. Enhancers act to augment the expression level and the timing of a gene. Despite being located many kilobases away, many enhancers work together, mainly through DNA-looping, to adjust the expression level of one single gene\textsuperscript{21}. To further complicate the matters, different sets of enhancer elements may work to regulate the same gene at different times or in different tissues.

In addition to regular enhancer elements, studies have discovered the existence of stimuli induced latent or \textit{de novo} enhancer elements\textsuperscript{24,25}. These elements are inactive under normal conditions, but become active and regulate expression of subsets of genes in response to various stimuli. Some of these elements persist upon removal of the stimuli and prime the cells to a faster and/or more robust response to restimulation\textsuperscript{24}. \textit{De novo} enhancers can be further broken down into three subcategories: selectively activated, promiscuously activated, and selectively activated
but promiscuous upon restimulation\textsuperscript{24}. As the name suggests, selectively activated \textit{de novo} enhancers are activated by stimulation and restimulation by a specific stimuli. Alternatively, \textit{de novo} enhancers can be promiscuously activated/reactivated by a variety of stimuli. In addition, some \textit{de novo} enhancers are selectively activated but promiscuous upon restimulation, meaning, the enhancers are formed by a specific stimuli, but once formed, can be restimulated by a variety of stressors.

Insulators act as genomic barriers, preventing regulatory elements on one side of them from interacting with regulatory elements on the other side, in a position-dependent manner. In addition to limiting the action of other regulatory elements, they also act to prevent the spread of repressive chromatin, such as heterochromatin\textsuperscript{21}.

Unlike the positive, transcription-promoting nature of promoters and enhancers, silencers act in a negative way to prevent the expression of a given gene. They contain clusters of binding sites for repressive transcription factors and much like enhancers, can act across long genomic distances\textsuperscript{21}.

1.6. Epigenetics

The term “epigenetics” refers to a layer of heritable modifications on top of the genome\textsuperscript{28-30}. These modifications can take place directly on the DNA, such as the methylation of cytosine residues, or on the tails of the histones that the DNA is wrapped around. The full extent of histone modification is still being determined, ranging from acetylation, phosphorylation and
methyltransferase, to ADP ribosylation, ubiquitylation and sumoylation\textsuperscript{31,32}. The effect of the modification is dependent on both the amount of modification, mono-, di-, or tri-methylation for example, and also the particular residue that is modified, lysine 4 or lysine 27 for instance. While the function of some types of modifications is similar, such as acetylation generally being a mark of active regulatory elements, the function of other modifications is dependent on the location and number of modifications. For example, mono-, di, or tri- methylation of Histone 3 Lysine 4 (H3K4me1, H3K4Me2, H3K4me3) are strongly correlated with actively transcribed genes, while trimethylation of Histone 3 Lysine 27 (H3K4me3) is associated with actively repressed chromatin\textsuperscript{29,31,33,34}. Recent studies reveal that the epigenome is rather dynamic, capable of responding to a variety of environmental stimuli to produce rapid changes in gene expression\textsuperscript{24-29}

Taken together, these modifications create a “Histone Code” which can be read to interpret the nature and activity of a given genomic region. H3K4me1/me3 are marks strongly associated with actively transcribed genes. H3K4me3 is found predominantly around promoter elements, while H3K4me1 is associated with promoters and enhancers\textsuperscript{29,35-39}. H3K9me3 is a mark of repressed regions and heterochromatin\textsuperscript{37}. Lastly, H3K27me3 is associated with the polycomb repressive complexes, and actively repressed chromatin\textsuperscript{34,40,41}. Through comparison of the enrichment levels and localization of these four histone modifications we are able to classify chromatin into one of four possible chromatin states: inactive, repressed, bivalent and active/poised (Fig.1.1). Regions that are unmarked by either active or repressive histone modifications are referred to as being in an inactive state. H3K9me3 and/or H3K27me3 mark repressive chromatin states, which are not permissive to gene transcription. Active and poised chromatin states both contain H3K4me1/3, with poised regions requiring the addition of histone
acetylation to become actively involved in transcriptional regulation. Regions with a bivalent chromatin state contain both active (H3K4me1/me3) as well as repressive (H3K27me3) histone marks and are primarily in a transitional state, waiting for the removal of either the active or repressive marks to become transcriptionally active or repressed.\textsuperscript{35,37,39,42-44}
Figure 1.1. Chromatin States classification using H3K4me1, H3K4me3 and H3K27me3.
The chromatin around regulatory elements can be in one of five possible states. Inactive chromatin contains no histone modifications, and is thought to play little role in mediating gene regulation. Repressed chromatin contains the H3K27Me3/H3K9Me3 histone modifications and is associated with Polycomb complex recruitment that can play an active role in repressing gene expression. Active and poised chromatin contain active H3K4Me1/3, but while poised regions are unlikely playing a significant role in mediating gene expression, active regions that also would be marked by histone acetylation actively promote gene expression. Bivalent chromatin contains both active H3K4Me1/3 and repressive H3K27Me3/H3K9Me3, and as such likely don’t promote gene activation or repression, but are thought to be poised to do so.
1.7. Chromatin remodeling factors

Despite our incomplete understanding of the “Histone Code”, or even the full extent of possible modifications, our knowledge of the enzymes and complexes responsible for the deposition and removal of these modifications is relatively extensive. Many of these enzymes are maintained in complexes with scaffolding proteins and histone targeting proteins. These complexes range in activity from nucleosome rearrangement by the Brg1- or Brm-associated factors (BAF) SWI/tch/Sucrose Non-Fermentable (SWI/SNF) complex\textsuperscript{49}, to histone methylation by the Trithorax group (TrxG/Mll) complexes\textsuperscript{26,50}.

Many of these complexes can be traced all the way back to their yeast homologs, but have undergone duplication events and subsequent specialization in Drosophila and humans. The TrxG complexes are an excellent example of this. The main function of the TrxG complexes is the mono-, di, and trimethylation of histone 3 lysine 4. While in yeast all H3K4 methylation is carried out by the Set1-COMPASS complex\textsuperscript{26}, in Drosophila the functions of the Set1-COMPASS complex are divided between three TrxG complexes (dSet1,Trx,and Trr). These are further divided in mammals, which contain two complexes for each Drosophila complex for a total of six H3K4 methylase complexes (dSet1-Set1a/Set1b; Trx- Mll1/Mll2; Trr-Mll3/Mll4)(Fig.1.2)\textsuperscript{26,51}. The Set1a/Set1b, and the fly dSet1 complexes are associated with bulk deposition of H3K4me2 and H3K4me3 throughout the genome\textsuperscript{52,53}. Trx/Mll1/Mll2 however, are implemented in gene-specific H3K4 trimethylation of promoter elements, including those of the \textit{Hox} genes during development\textsuperscript{54}. The Trr/Mll3/Mll4 complexes, on the other hand, have been
associated with H3K4 methylation of specific genes in response to stimuli such as hormone-receptor signaling.$^{55-57}$

Figure 1.2. Evolution of Trithorax Group Complexes among yeast, drosophila, and mammals.
The TrxG complexes have diversified as they evolved from yeast through to mammals. While each complex contains unique methylases (orange) and specialized subunits (red, green, black and purple), all complexes share the same set of core subunits (blue).
While the TrxG complexes are required for the activation of regulatory elements, the polycomb group proteins (PcG) are involved in repression. The PcG assemble to form polycomb repressive complexes, which act together to facilitate epigenetic silencing of genes through chromatin remodeling\textsuperscript{34,65,68}. Polycomb repressive complex 2 (PRC2) contains the histone 3 lysine 27 methlyase subunit EZH2, which catalyzes the deposition of the repressive H3K27me3 mark\textsuperscript{65}. H3K27me3 then provides a binding surface for polycomb repressive complex 1 (PRC1), which comes in and facilitates oligomerization and condensation of the chromatin in order to maintain gene repression\textsuperscript{34,65,68}.

1.7.1. Mammalian TrxG complexes perform diverse functions through specific additional subunits

The TrxG complexes, and their mammalian homologs, contain a set of core subunits, found in all complexes, as well as a subset of class-specific subunits, which allow for functional diversity between the complexes. Wdr5, Dpy-30, Ash2l and Rbbp5 constitute the core subunits found in all complexes\textsuperscript{26,53}. The methylases Set1a, Set1b, Mll1, Mll2, Mll3, and Mll4 are the main distinguishing feature of TrxG complexes, and provide the complexes with the ability to mono-, di-, and/or tri- methylate H3K4\textsuperscript{26}. Additionally, Trr/Mll3/Mll4 complexes can also contain the histone demethylases Jmjd3 or Utx, which selectively remove the three methyl groups from the repressive H3K27me3\textsuperscript{26,58,59}. This alludes to a potential mechanism in which the Trr/Mll3/Mll4 complexes can be recruited to H3K27me3-marked repressed regulatory elements, remove the repressive H3K27me3 mark by way of Utx or Jmjd3, and add the active H3K4me1/3 marks, initiating gene transcription in response to particular stimuli\textsuperscript{26}. 
1.7.2. Long noncoding RNAs

Long non-coding RNAs (lncRNAs) have emerged in recent years as an addition to the transcriptomes of many species and have been found to play crucial roles in gene regulation and development. Specifically, lncRNAs have been tied into regulatory networks that control chromatin and cellular differentiation\textsuperscript{45}. One of the most famous lncRNAs is XIST that acts to initiate chromosome-wide H3K27me3 deposition in X inactivation\textsuperscript{46}. Additionally, the lncRNA NeST has been shown to regulate the expression of IFN\textgreek{g} and in doing so, alter susceptibility to pathogens\textsuperscript{47}. Furthermore, it has been reported that lncRNA expression maybe crucial for proper islet function\textsuperscript{59,98,99}, and that the expression profiles of lncRNAs in human pancreatic islets are altered by exposure to proinflammatory cytokines\textsuperscript{48}.

Additionally, lncRNAs have been shown to associate with chromatin and provide scaffolds for chromatin modifiers. This provides loci-specific recruitment of different chromatin modifiers, allowing for fine-tuning of gene expression\textsuperscript{93}. A well studied example of this is the lncRNA HOTAIR, a 2.2kb lncRNA located in the HOXC locus which is required for proper repression of the HOXD locus\textsuperscript{94}. HOTAIR acts as a scaffold and allows the recruitment and assembly of both the polycomb repressive complex 2 (PRC2) as well as, the LSD1/CoREST/Rest complex\textsuperscript{95}. In doing so, HOTAIR coordinates the repression of the HOXD locus as PRC2 is able to add the repressive H3K27me3 mark, while the LSD1 removes H3K4 methylation\textsuperscript{94,95}.
1.8. Thesis objective

The onset of T1D is characterized by immune infiltration and autoimmune assault of the pancreatic β-cells by cytokine-secreting immune cells. Although a great deal of research effort has been devoted to identifying the changes in gene expression patterns that take place during this process\textsuperscript{13-16}, the identification and characterization of the underlying mechanisms that cause the alterations to the gene expression profiles of the pancreatic β-cells remain to be elucidated. In other tissues, alterations to the epigenetic landscape have been shown to be the crucial mediators of gene expression changes in response to inflammatory cytokine exposure\textsuperscript{27,67}. For instance, chronic exposure to inflammatory cytokines has been shown to promote the removal of repressive histone modifications at regulatory elements that govern the expression of proinflammatory genes in smooth muscle cells in a mouse model of T2D\textsuperscript{27}, as well as in macrophages\textsuperscript{67}. As such, we hypothesized that IFNγ, IL-1β and TNFα exposure causes alterations to the chromatin state at regulatory elements throughout the β-cell genome, and that these changes in chromatin state are required to facilitate the corresponding IFNγ, IL-1β and TNFα induced gene expression changes. In line with this, the administration of histone deacetylase inhibitors (HDACi’s) has been shown to have a protective effect, and reduce IFNγ, IL-1β and TNFα induced β-cell dysfunction in both allogeneic and syngeneic mouse islet graft recipients\textsuperscript{60-63}. Accordingly, we further propose that prevention of IFNγ, IL-1β and TNFα induced alterations to the β-cell epigenome, by using small molecule inhibitors or shRNAs designed to inhibit chromatin modifier function, can potentially prevent alterations to the chromatin landscape and therefore, IFNγ, IL-1β and TNFα induced β-cell gene expression.
changes. This would identify drugs designed to target histone modifiers as potential avenues for future therapeutics for newly diagnosed T1D patients, as well as islet graft recipients.

In this study, we sought to map the localization of H3K4me1 in IFNγ, IL-1β and TNFα treated islets, as H3K4me1 has been shown to be an excellent way to identify active and poised regulatory elements, such as promoters and enhancers. We utilized chromatin immunoprecipitation coupled with next generation sequencing (ChIP-seq), as it has become an invaluable technique in the genome-wide identification of regulatory elements. By comparing this IFNγ, IL-1β and TNFα islet-treated H3K4me1 data with previously generated H3K4me1, H3K4me3, H3K9me3 and H3K27me3 data from untreated islets, we added greater and more dynamic detail to the picture of cytokine induced changes to the regulatory network of the β-cell, and successfully identified several chromatin modifiers as potential targets for future therapeutics.
Chapter 2  Materials and Methods

2.1 Chromatin Immunoprecipitation (ChIP)

For each islet ChIP experiment, islets from at least 10 adult (8-12 week old) CD-1 mice were isolated via collagenase digestion and purified by hand picking. Islets were allowed to recover overnight at 37˚C in Roswell Park Memorial Institute (RPMI) 1640 media (Life Technologies, Grand Island, NY, USA) supplemented with 10% Fetal Bovine Serum (Life Technologies), 1% penicillin/streptomycin (GE Healthcare Life Sciences, Logan, UT, USA) and 1% L-glutamine (GE Healthcare Life Sciences), hereafter referred to as complete RPMI. The following day the islets were transferred to complete RPMI (Control) or complete RPMI containing 0.273 ng/ml IL-1β (eBioscience, San Diego, CA, USA), 0.156 ng/ml TNFα (eBioscience) and 1.562 ng/ml IFNγ (eBioscience), and left to incubate at 37˚C. Following treatment, islets were dissociated using 0.05% trypsin (GE Healthcare Life Sciences) and gentle pipetting. Cells were then rinsed with PBS and fixed for 10 minutes with 1% formaldehyde (Fisher Scientific Company, Ottawa, ON, Canada). Fixation was stopped with 1M Glycine (Fisher Scientific Company), the cells were rinsed with PBS and then resuspended in 500ul of Cell Lysis Buffer (10mM Tris-HCl, pH 8(Fisher Scientific Company), 10mM NaCl (Fisher Scientific Company), 3mM MgCl2 (Fisher Scientific Company), 0.5% Igepal (Sigma-Aldrich, St Louis, MO, USA), Protease inhibitor cocktail (Thermo Fisher Scientific Inc., Waltham, MA, USA)) and dounce homogenized. Nuclei were isolated by centrifugation and resuspended in 500ul of Nuclear Lysis Buffer (NLB) (1% SDS (Fisher Scientific Company), 5mM EDTA (Thermo Fisher Scientific Inc), 50mM Tris HCl, pH8 and protease inhibitor cocktail). Samples
were split into two tubes containing 250ul each and sonicated on a Misonix Sonicator S-400 (Misonix Inc., Farmingdale, NY, USA) at 80% power for 12 min (30sec on, 30sec off) to achieve 200-500bp fragments. Samples were pooled back together and spun at max speed for 10min at 4°C. Supernatant was transferred to a new tube, 20ul was set as sample input. The rest of the sample was diluted with 800ul of ChIP Dilution Buffer (0.01% SDS, 1.1% Triton X-100 (Promega Corporation, Madison, WI, UAS), 150mM NaCl and 16.7mM Tris HCl pH 8) and 18ul 5M NaCl before being precleared with 20ul of protein A/G beads (Thermo Fisher Scientific Inc) for 1hr. After which the supernatant was removed from the beads and incubated with 5ug Rabbit anti-H3K4me1 (Abcam, Toronto, ON, Canada) or anti-H3K27me3 (Abcam) overnight at 4°C. At the same time 40ul of protein A/G beads were blocked with 1% FBS and 1% salmon sperm DNA. The Sample input was added to 180ul NLB and 5M NaCl was added to a final concentration of 0.192M and was incubated at 65°C overnight to reverse crosslinking. The following day the IP is added to the preblocked protein A/G beads and left to incubate for 4hrs at 4°C before being washed twice with low salt buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA 20mM Tris HCl pH 8 and 150mM NaCl), once in High salt buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris HCl pH 8 and 500mM NaCl) and twice in TE buffer (10mM EDTA and 10mM Tris HCl pH 8). The beads are then transferred into 150 ul Elution Buffer (EB) (1% SDS and 0.1M NaHCO₃) and left to incubate for 1hr at 50°C. The supernatant was transferred to a new tube and the beads rinsed with another 50ul EB, this was added to the new tube along with 5M NaCl to a final concentration of 0.192M and was incubated at 65°C overnight to reverse crosslinking. The sample input DNA was treated with 0.5ng RNase A and incubated at 68°C for 30 min and then 0.1 ng Proteinase K and incubated at 42°C for 30min. The DNA is then isolated via phenol chloroform extraction (Fisher Scientific Company) and ethanol precipitation. On day
3 the ChIPed DNA is treated with 0.5ng RNase A (Hoffmann-La Roche Limited, Mississauga, ON, Canada) and incubated at 68°C for 1hr before being isolated via phenol chloroform extraction and ethanol precipitation.

2.2 Mouse maintenance and Islet Isolation

CD-1 mice were maintained, according to the guidelines of the Canadian Council on Animal Care, in micro-isolator cages. The UBC Animal Care Committee approved all protocols. Islets were isolated from mixed sex, 8-12 week old mice via collagenase digestion. Male and female mice were anesthetized with isofluorane and euthanized by decapitation. The chest cavity was opened and the ampule was clamped with forceps. A syringe was inserted into the duct and 3ml of 1000U/ml collagenase XI (Sigma-Aldrich) in 1x Hank’s Buffered Salt Solution (HBSS) (Life Technologies) was perfused through the pancreas. The perfused pancreas was excised via careful dissection and placed in a 50ml tube containing an additional 2ml of 1000U/ml collagenase IX in HBSS. Pancreas were incubated for 15 min in a 37°C water bath before being shaken by hand for approximately 5min. 20ml of 1x HBSS supplemented with 1mM CaCl$_2$ (Fisher Scientific Company) was added to the tubes and they were spun at 1000g for 1min to pellet the islets. 20ml of supernatant was decanted off and the pellet was rinsed with another 20ml 1x HBSS supplemented with 1mM CaCl$_2$ and spun for 1min at 1000g. All supernatant was decanted off and the pellet was loosely resuspended in 25ml 1x HBSS supplemented with 1mM CaCl$_2$, passed through a 70µm filter, rinsed once with 25ml 1x HBSS supplemented with 1mM CaCl$_2$ and plated onto 10cm dishes in 10ml complete RPMI. Islets were subsequently hand picked and pooled.
2.3 Tissue Culture and Cell Lines

Mouse insulinoma 6 (MIN6) cells were cultured at 37°C in Dulbecco Modified Eagle Medium (DMEM) (Life Technologies) containing 4500 mg/L glucose and supplemented with 10% Fetal Bovine Serum 1% penicillin/streptomycin and 1% L-glutamine, hereafter referred to as complete DMEM. Cells were passaged using 0.25% Trypsin EDTA.

2.4 Library Construction and Sequencing

ChIP-seq library construction was performed on ChIP DNA from four cytokine treated H3K4me1 ChIP experiments pooled together, using the TruSeq ChIP Sample Preparation Kit (Illumina Inc., San Diego, CA, USA) as per the manufacturer’s instructions. RNA-seq was carried out on total RNA from islets from at least 4 adult CD1 mice, converted into a library of template cDNA using the TruSeq Sample Prep Kit (Illumina) as per the manufacturer’s protocol. Libraries were expanded using the Illumina Cluster generation protocol and sequenced on a HiSeq 2000 sequencer at Canada’s Michael Smith Genome Sciences Centre (Canada’s Michael Smith Genome Sciences Centre, Vancouver, BC, Canada). 75 bp single end sequencing was used for the ChIP-seq library and 75bp paired-end sequencing was used for the RNA-seq libraries.

2.5 Identification of H3K4me1 loci

75bp sequencing reads were received from the GSC and trimmed down to 36bp in order to be comparable to the previously sequenced data. The new 36bp reads were then mapped
to the mouse mm9 reference genome using Bowtie 2.2.1\textsuperscript{83}. The aligned reads were then filtered to remove reads with a minimum mapping quality less than 33 using samtools\textsuperscript{84}, duplicates were removed and the remaining reads were fed into ChromHMM\textsuperscript{44}, which utilizes a hidden Markov model-based approach to identify genomic regions that are enriched in the ChIP-seq data sets, over control genomic data, in 200bp bins throughout the genome. This produced 62171 cytokine H3K4me1 genomic regions and 52369 control H3K4me1 genomic regions. We filtered out all regions that were less than 200 bp as they would only contain a single nucleosome, as well as all regions with a reads/kilobase/million reads mapped (RPKM) less than 2. RPKM is a mathematical calculation used to normalize for total read length and the number of sequencing reads. This produced 43462 cytokine regions and 41518 control regions. We then merged both sets of regions in order to identify which regions were conserved or differentially enriched, resulting in 96722 total regions.

2.6 Characterization of H3K4me1 loci in control islets

Binding of Pdx1, Mafa, Neurod1, and Foxa2, as well as enrichment of H3K4me1, H3K4me3, H3K27me3, and H3K9me3, in untreated islets, at cytokine treated H3K4me1 loci was determined using previously obtained data\textsuperscript{38,39}, which we reanalyzed using the same thresholds and methods mentioned above. Detection of over-represented conserved transcription factor binding sites was carried out using Single Site Analysis by oPOSSUM\textsuperscript{85}.

2.7 Determination of gene expression and association with H3K4me1 state

Islets from at least four adult CD-1 mice were treated as described above. Total RNA from the islets was pooled and used for library generation. Libraries were generated using the TruSeq Sample Prep Kit (Illumina) as per the manufacturer’s protocol. 75bp paired-end sequencing reads were received from the GSC, and mapped to the mm9 genome and UCSC known transcripts using TopHat\textsuperscript{86}. Differential gene expression was determined using Cufflinks\textsuperscript{86}. Only transcripts with a fragments/kilobase/million reads mapped (FPKM) greater than 2 were considered expressed and used in subsequent analyses. The H3K4me1 enriched loci identified above were associated with the transcript with the closest start site within 100Kb. Gene ontology enrichment analyses were carried out using DAVID\textsuperscript{87}.

2.8 Identification of novel IncRNA transcripts in islets

Transcripts that did not map to known UCSC transcripts were identified using TopHat and Cufflinks. Potential IncRNA were identified as being greater than 100bp in length and having a maximum FPKM greater than 3 in either cytokine treated or untreated RNA-Seq data, resulting in 1182 putative IncRNAs. The sequences of these regions were used to create multiple species alignments (mm9, hg19, panTro2, gorGor1, ponAbe2, rn4, bosTau4, felCat3, and canFam2) in Galaxy\textsuperscript{88-90} (https://usegalaxy.org/). The alignments were fed through phyloCSF\textsuperscript{91} to assess their likelihood of being protein coding. All transcripts with a score less than 10 decibans were considered non-coding\textsuperscript{69}, resulting in the identification of 444 putative IncRNAs.
2.9 Quantitative PCR

Quantitative PCR (qPCR) was performed using the ViiA 7 Real-Time PCR System (Life Technologies) and Fast SYBR Green Master Mix (Life Technologies) or TaqMan Fast Advanced Master Mix (Life Technologies) and 0.25 µM forward and reverse primers. Cycling conditions were [40x 95°C for 15s, 60°C for 30s, 72°C for 1 min].

All samples and controls were assayed in triplicate. Obtained values were normalized to β-actin Ct values and the change in expression was calculated using the $2^{-\Delta\Delta Ct}$ method for gene expression. For ChIP-qPCR, the %recovery of each target site was calculated using the cycle difference between the input and ChIP DNA. All Primers were designed using Primer3 and are listed below.

2.10 Primer sequences

Table 2.1: Primer sequences

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<th>Target</th>
<th>Forward Primer Sequence</th>
<th>Reverse Primer Sequence</th>
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<td>AGG TCC CTG TCA TGC TTC TG</td>
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2.11 RNA extraction, reverse transcription and cDNA amplification

RNA was extracted using Trizol (Life Technologies) following the manufacturer’s instructions. RNA was then purified using the PureLink RNA kit (Life Technologies) as per the manufacturer’s instructions and RNA concentration was determined using the NanoDrop 2000 spectrophotometer (Thermo Scientific). Reverse transcription (RT) of RNA was carried out using the maximum amount of RNA available per replicate while keeping the total amount of RNA consistent in all samples. RNA was Dnase (Life Technologies) treated for 15min at room temperature and RT was done using 1x first strand buffer (Life Technologies), 0.5 µL DTT (Life Technologies), 1 µL (2U) Rnase OUT (Life Technologies) and 0.5 µL (10U) Superscript III (Life Technologies). RT was performed in a thermocycler under the following conditions [25˚C for 5min, 50˚C for 60min, 70˚C for 15min, ramp to 4˚C] and stored at -20˚C.

2.12 Adenoviral mediated RNAi knock-down and GSK-J4 inhibition

Three different pLKO vectors containing short hairpin constructs targeting Wdr5 under control of the hU6 promoter, and a scramble shRNA construct, were purchased from OpenBiosystems. These shWdr5 expressing vectors were screened for their ability to suppress Wdr5 in MIN6 cells. Subsequently, the sequence that suppressed Wdr5 by ~90%, and the scramble sequence, were inserted into pAdTrack using InFusion cloning (Clontech) and sequence verified. These were then used to make the pAdV-shWdr5 and pAdV-shScramble adenoviruses using the pAdeasy system as previously described92. Islets were
transduced with these adenoviruses at the indicated MOI’s for 3 hours and 48 hours later were treated with or without IFNγ, IL-1β and TNFα, as indicated, for a subsequent 3 hours. GSK-J4 (Sigma-Aldrich) or DMSO was used to treat islets at the concentrations indicated with or without IFNγ, IL-1β and TNFα for three hours.

2.13 Statistical analysis

Statistical analysis of the data was done using the paired, two-tailed student’s t-test by Prism5 (GraphPad Software). qPCR data was analyzed using the non-parametric Mann Whitney test to compare the ΔCt’s of the reference and the sample using Prism5. Data from at least three independent experiments are represented as mean +/- S.E.M. Statistical significance was accepted at P values <0.05.
Chapter 3  Results

3.1 IFNγ, IL-1β and TNFα induced alterations in H3K4me1 levels in mouse islets.

In this study, we sought to identify genome-wide changes in H3K4me1 levels in mouse pancreatic islets in response to exposure to the immune cell secreted cytokines IL-1β, TNFα, and IFNγ. These cytokines where chosen as they have been shown to be secreted by the immune system in the β-cell microenvironment during insulitis. For this, we performed ChIP-seq for H3K4me1 on islets treated with a cocktail of IL-1β, TNFα, and IFNγ for 96 hours. We then compared the IFNγ, IL-1β and TNFα treated ChIP-seq data to previously generated untreated islet ChIP-seq data and identified a total of 96,721 H3K4me1 enriched regions. Using a stringent cutoff of >3fold enrichment we identified 3,204 regions (3.3%) with increased H3K4me1 upon cytokine treatment, 84,573 regions (87.4%) that were unchanged, and 5,354 regions (5.5%) with decreased H3K4me1 enrichment after IFNγ, IL-1β and TNFα treatment (Fig 3.1a). We identified an additional 3,590 regions (3.7%) that were unmarked in control islets and acquired de novo H3K4me1 upon IFNγ, IL-1β and TNFα treatment, indicating that these represent de novo or latent cis-regulatory elements that emerge as a result of cytokine exposure.

Next we compared the distribution of H3K4me1 mapped reads for each of our classes to ensure that our classification of regions into de novo, increased, unchanged, and decreased accurately reflected the changes in H3K4me1 reads (Fig 3.1b). As anticipated, we see the greatest increase in associated H3K4me1 reads in the de novo regions upon IFNγ, IL-1β and
TNFα treatment. Keeping with our classification, we see an increase in the number of H3K4me1 reads after

When examining the location of these regions throughout the genome we found that Increased, Unchanged and Decreased regions tend were predominantly intronic (41.9%, 55.6% and 57.8% respectively), while De novo regions are more likely to be intergenic (47.1%)(Fig 3.2b). All region types had increased enrichment within 3kb of TSSs when compared to the genome (11.9% De novo, 14.9% Increased, 12.5% Unchanged, 9.4% Decreased and 6.6% Genome). This data implicates that IFNγ, IL-1β and TNFα treatment is causing alterations to H3K4me1 levels at enhancer elements.
Figure 3.1: Cytokine-induced alterations to H3K4me1 levels in Mouse Islets.
(A) Heatmaps of H3K4me1 read density in ±10Kb regions centered ChromHMM identified H3K4me1 enriched loci in Untreated and IFNγ, IL-1β and TNFα (Cyto) treated Islets. H3K4me1 read density is represented by the intensity of blue and red in the heatmaps: dark red indicates high read density while dark blue indicates low read density. 3590 regions contain de novo H3K4me1, 3204 regions show ≥3fold increase in H3K4me1, 84573 regions are unchanged and 5354 regions have ≥3fold decrease in the IFNγ, IL-1β, and TNFα (Cyto) treated islets vs Untreated islets. (B) Plots of average density of H3K4me1 reads ±10Kb around de novo, up, unchanged and down regions in the untreated (blue line) and IFNγ, IL-1β, and TNFα treated (red line) islets. (C) Violin plots of the number of H3K4me1 reads (RPKM) associated with the identified H3K4me1 enriched loci in each class (de novo, increased, unchanged, decreased) in untreated and IFNγ, IL-1β, and TNFα (Cyto) treated islets. The height indicates the range of RPKMs covered, while the width illustrates the abundance of genomic regions with that RPKM.
As cis-regulatory elements tend to regulate the nearest gene \(^{30,64}\), we associated each region with the nearest transcriptional start site and performed Gene Ontology analysis\(^{87}\) using the genes associated with unchanged regions as background (Fig 3.2a). Gene associated with \textit{de novo} regions were significantly enriched for GO terms related to many inflammatory processes such as B cell apoptosis, NF-κB binding and inflammatory response. Increased regions were associated with genes involved in similar processes; for instance, these regions correspond to the up regulation of macrophage differentiation, changes in interleukin-1β secretion, as well as an increase in cytokine secretion. Interestingly, the genes associated the Decreased regions were enriched for β-cell functional processes, including insulin receptor signaling, glucose transport and various cellular regulatory events that are typically triggered by insulin. As a whole, these data suggest that exposing islets to IFNγ, IL-1β and TNFα results in the \textit{de novo} generation and activation of cis-regulatory elements associated with genes involved in inflammation and in cytokine production, as well as the decreased activity of cis-regulatory elements associated with genes involved in β-cell function.
Figure 3.2: Association of identified H3K4me1-enriched regions with genes.
(A) Enrichment level (left graph) and p-values (right graph) of select gene ontology (GO) terms enriched using genes associated with the identified de novo, increased, and decreased loci. Genes associated with unchanged H3K4me1 were used as control. (B) Distribution of H3K4me1 reads into different genomic regions for the different H3K4me1 classes, compared to the percentage of the genome that falls into each category.
3.2 Characterization of H3K4me1 regions in untreated islets.

To examine whether islet-specific transcriptional regulators are associated with the above H3K4me1 marked regions, we analyzed transcription factor binding data for several transcription factors that regulate β-cell function: Pdx1, Foxa2, Neurod1 and Mafa, in untreated islets. These transcription factors are key regulators of many key β-cell processes\(^{38}\). As shown in Fig 3.3, all of these transcription factors were much more likely to be associated with Unchanged and Decreased H3K4me1 regions, individually or in combination. Remarkably, of these four transcription factors, Pdx1 was most commonly found to bind within H3K4me1 marked regions, with 1.11% *De novo*, 2.22% Increased, 10.30% Unchanged and 6.87% Decreased regions. These data indicate that β-cell genes, regulated by Pdx1, Foxa2, Mafa and Neurod1 are more likely to lose H3K4me1 upon IFNγ, IL-1β and TNFα treatment, and that increases in H3K4me1 are occurring at regions bound by alternative transcription factors.

![Heatmap of the fraction of de novo, increased, unaltered, and decreased loci bound by the indicated transcription factor combinations under untreated conditions. The darker the blue, the greater the percentage of regions that were bound by the given transcription factor or combination.](image)

**Figure 3.3: Fraction of identified H3K4me1-enriched regions bound by β-cell critical transcription factor in untreated islets.**

Heatmap of the fraction of *de novo*, increased, unaltered, and decreased loci bound by the indicated transcription factor combinations under untreated conditions. The darker the blue, the greater the percentage of regions that were bound by the given transcription factor or combination.
While H3K4me1 enrichment is strongly associated with active or poised cis-regulatory elements\textsuperscript{32,39}, it is only one part of a complex regulatory network. Therefore, we decided to analyze additional, previously generated\textsuperscript{38}, untreated islet ChIP-seq data sets to gain deeper insight into maintenance and function of the different classes of enhancers identified. To do so, we analyzed H3K4 trimethylation (H3K4me3), a mark enriched at active promoter elements\textsuperscript{29,35-37}, H3 lysine 9 trimethylation (H3K9me3), a mark enriched in heterochromatin\textsuperscript{24,32,65,66}, as well as tri-methylation of histone 3 lysine 27 (H3K27me3), a mark of active repression, associated with the polycomb repressive complex\textsuperscript{34,40,67}. Using a stringent cut-off of RPKM>2 to access the enrichment of H3K4me3, we found that Unchanged and Decreased H3K4me1 regions were much more likely to be co-enriched for H3K4me3 (21.99% and 19.39% respectively) than \textit{De novo} or Increased H3K4me1 regions (2.67% and 7.90% respectively) (Fig 3.4a,b). Next we evaluated the enrichment levels of H3K9me3 using the same stringent cut-off of RPKM>2. We found the opposite result of what was observed in H3K4me3, that is \textasciitilde2-2.5 fold more \textit{De novo} and Increased H3K4me1 regions marked by H3K9me3 than Unchanged and Decreased H3K4me1 regions (Fig 3.5a,b), although co-occupancy with H3K9me3 was low in all regions (0.1-0.4% of regions). This data implies that alterations in H3K4me1 are not occurring at H3K9me3, repressed regions of the genome.
Figure 3.4: Fraction of identified H3K4me1-enriched regions bound by H3K4me3 in untreated islets.

(A) Heatmaps of H3K4me3 read density in ±10-kb regions centred on the identified de novo, increased, unchanged, and decreased loci in untreated islets. H3K4me3 read density is represented by the intensity of blue and red in the heatmaps: dark red indicates high read density while dark blue indicates low read density. (B) Quantification of the percent of each region type that is marked by H3K4me3 (RPKM≥2) in untreated islets. The data indicates that unchanged and down regions are far more likely to be bound by H3K4me3 in an untreated state and therefore associated with active promoter elements.
Figure 3.5: Fraction of identified H3K4me1-enriched regions bound by H3K9me3 in untreated islets.
(A) Heatmaps of H3K9me3 read density in ±10-kb regions centred on the identified de novo, increased, unchanged, and decreased loci in untreated islets. H3K9me3 read density is represented by the intensity of blue and red in the heatmaps: dark red indicates high read density while dark blue indicates low read density. 
(B) Quantification of the percent of each region type that is marked by H3K9me3 (RPKM≥2) in untreated islets. The data indicates that de novo and up regions are more likely to be bound by H3K9me3 in an untreated state, but that very few regions of any type are associated with H3K9me3. Signifying that few changes are taking place in heterochromatin regions of the genome.
The acquisition and removal of H3K27me3 at regulatory elements plays a key role in gene regulation throughout cell development and fate commitment\textsuperscript{65,68}. As such, we sought to assess the enrichment level of H3K27me3 in our H3K4me1 regions using previously generated H3K27me3 data from untreated islets. We found that in untreated islets, regions with increased H3K4me1 upon IFN\textgreek{g}, IL-1\textgreek{b} and TNF\textgreek{a} treatment correlated more strongly with H3K27me3 than those that remained the same or with decreased H3K4me1 (Fig 3.6a). Increased H3K4me1 regions showed the highest enrichment for H3K27me3 (12.39\%), as compared to \textit{de novo} regions (6.35\%), Unchanged (3.75\%) and Decreased (1.20\%) (Fig 3.6b), suggesting that actively repressed cis-regulatory elements are gaining H3K4me1 upon IFN\textgreek{g}, IL-1\textgreek{b} and TNF\textgreek{a} exposure, and switching from a repressed state to an active/poised or bivalent state.
Figure 3.6: H3K27me3 enrichment in identified H3K4me1-enriched regions.
(A) Heatmaps of H3K27me3 read density in ±10-kb regions centred on the identified de novo, increased, unchanged, and decreased loci in untreated islets. H3K27me3 read density is represented by the intensity of blue and red in the heatmaps: dark red indicates high read density while dark blue indicates low read density (B) Quantification of the percent of each region type that is marked by H3K27me3 (RPKM≥2) in untreated islets. The data indicates that de novo and up regions are far more likely to be bound by H3K27me3 in an untreated state and in an actively repressed state.
3.3 Multiple repressed regions become H3K4me1 marked upon IFNγ, IL-1β and TNFα treatment

We next wanted to determine whether the de novo or increased H3K4me1 regions that were enriched for H3K27me3 in untreated islets were associated with any specific types of genes. To address this question, we associated H3K27me3/H3K4me1-marked regions with the nearest transcriptional start site and performed Gene Ontology analysis, using unchanged H3K4me1 regions as background (Fig 3.7a). We found that de novo H3K4me1 regions premarked by H3K27me3 were associated with genes linked to many inflammatory processes such as “stress response, inflammation and cytokines and Inflammatory Response”. The monocyte chemotactic protein Ccl2 is an example of a proinflammatory gene, premarked by H3K27me3, that acquires de novo H3K4me1 upon IFNγ, IL-1β and TNFα exposure (Fig 3.7b). Genes associated with Increased H3K4me1 regions premarked by H3K27me3 were enriched for apoptotic and developmental processes such as “Cell structure disassembly during apoptosis”, “Homeobox”, and “Regulation of cell development”. While genes associated with Decreased H3K4me1, premarked by H3K27me3 were linked to cell senescent activity.
Figure 3.7: H3K4me1 regions premarked by H3K27me3 are involved in inflammation. (A) Enrichment level (left graph) and p-values (right graph) of select gene ontology (GO) terms enriched using genes associated with the identified de novo and increased loci identified as enriched for H3K27me3 in untreated islets. (B) UCSC genome browser view of the genomic region around Ccl2. H3K4me1 enrichment data from IFNγ, IL-1β, and TNFα (Cyto) treated islets is shown in red, from untreated islets in blue, while H3K27me3 data from untreated islets is shown in black. All tracks are set to show a coverage depth range of 0 to 30. This data illustrates the de novo acquisition of H3K4me1 at previously H3K27me3 marked loci.
As less then 5% of de novo or increased H3K4me1 regions are bound by the key islet transcription factors Pdx1, Foxa2, Mafa and Neurod1 (Fig 3.3), we preformed motif analysis on de novo and increased H3K4me1 regions, both premarked by H3K27me3 or not, to detect over-represented conserved transcription factor binding sites (Fig 3.8). We identified conserved binding sites for Nfkb, Ar and Klf4 as being over-represented in de novo regions. Binding sites for Nfkb1 and Klf4 were highly over-represented in de novo regions premarked by H3K27me3. Noticeably, Nfkb, Rela and Mycn were among the most over-represented binding sites in increased H3K4me1 regions; whereas, Mycn, Klf4 and Nfkb1 were among the most over-represented in H3K27me3 premarked increased H3K4me1 regions. These results suggest that de novo or increased H3K4me1 is occurring at regulatory elements bound by known IFNγ, IL-1β and TNFα induced transcription factors, such as Nfkb.
3.4 **IFNγ, IL-1β and TNFα induced alterations to gene expression**

Next we sought to determine whether these changes in islet chromatin state corresponded with changes in transcription. To do this we performed RNA-sequencing on untreated and 96 hr IFNγ, IL-1β, and TNFα treated islets. We identified 27,877 total transcripts as being expressed in islets under each condition (Fig 3.9a). Performing differential gene expression analysis we identified 649 transcripts with increased expression and 481 transcripts with decreased expression. Based on gene ontology analysis of the differentially expressed transcripts that map to annotated genes, we found that many of the transcripts with increased expression upon IFNγ,
IL-1β and TNFα treatment are involved in inflammatory processes such as “CXC Chemokine” and “Inflammation” (Fig 3.9b). Interestingly, the decreased transcripts are associated with islet functional processes, including “Maturity Onset Diabetes of the Young” and “Response to Glucose Stimulus”. These correlate with other studies showing that IFNγ, IL-1β and TNFα exposure causes an up regulation of inflammatory processes and a down regulation of processes involved in β-cell function.
Figure 3.9: IFNγ, IL-1β and TNFα induced alterations to gene expression.
(A) Scatter plot of the FPKM of identified transcripts in untreated (x-axis) or IFNγ, IL-1β, and TNFα (Cytokine) treated islets (y-axis). The relative expression of transcripts in the IFNγ, IL-1β, and TNFα (Cytokine) treated islets as compared to in untreated islets is indicated by the colour of the dot, with darker blue indicating higher expression in IFNγ, IL-1β, and TNFα treated islets, and darker red indicating higher expression in untreated islets. Only transcripts with an FPKM of >2 in one of the samples are shown. (B) Enrichment level (left graph) and p-values (right graph) of select gene ontology (GO) terms enriched using genes identified as up regulated or down regulated by IFNγ, IL-1β, and TNFα exposure. Genes with unaltered expression were used as background control.
In addition to the transcripts mapping to annotated genes, we successfully identified 900 novel transcripts by performing *de novo* transcript assembly. Of the 900 novel transcripts, 444 are likely to be non-coding as they returned a score of <10 decibans using the Phylocsf algorithm\(^{91}\) indicating that the transcripts have no protein coding potential\(^{69}\). Examining the IFN\(\gamma\), IL-1\(\beta\) and TNF\(\alpha\) induced expression changes to these lncRNAs we discovered that while a similar proportion of lncRNAs and UCSC genes show increased expression upon treatment, a considerably higher percentage of lncRNAs exhibit decreased expression after IFN\(\gamma\), IL-1\(\beta\) and TNF\(\alpha\) treatment than USCS genes (Fig 3.10a). As many lncRNAs have been shown to exert their regulatory effects by acting as scaffolds to provide specificity to ubiquitously expressed chromatin modifiers by guiding these enzymes to specific genomic loci\(^{93,95}\), we then compared the lncRNA expression changes to the expression of the nearest gene. This revealed that almost 40% of lncRNAs that are down regulated by IFN\(\gamma\), IL-1\(\beta\) and TNF\(\alpha\) are associated with genes that are also down regulated (Fig 3.10b). For example, a lncRNA that lies ~70kb downstream of Ins1 shows a dramatic decrease in expression after IFN\(\gamma\), IL-1\(\beta\) and TNF\(\alpha\) exposure and coincides with a decrease in H3K4me1 near Ins1 (Fig 3.10c)\(^{70}\). Such transcriptional changes further support the notion that IFN\(\gamma\), IL-1\(\beta\) and TNF\(\alpha\) trigger up regulation of inflammatory processes and a down regulation of processes involved in \(\beta\)-cell function and that regulation of lncRNAs may play a pivotal role in the regulation of these activities.
Figure 3.10: IFNγ, IL-1β and TNFα induced alterations to lncRNA expression.
(A) Expression changes of lncRNAs compared to UCSC genes shows that more lncRNAs are down regulated by IFNγ, IL-1β and TNFα treatment. (B) Comparison of lncRNA expression changes to the expression of the nearest gene reveals that almost 40% of lncRNAs that are down regulated by treatment, are associated with genes that are also down regulated. (C) UCSC genome browser view of the genomic region around Ins1. H3K4me1 enrichment and RNA-seq data from IFNγ, IL-1β, and TNFα (Cytokine) treated islets is shown in red, from untreated islets in blue. All tracks are set to show a coverage depth range of 0 to 30. This is likely wrong for the RNA-seq data….. The lncRNA locus upstream of the Ins1 gene shows a decrease in expression and H3K4me1 levels upon IFNγ, IL-1β and TNFα treatment (red) corresponding with a decrease in Ins1 expression.
3.5 Increased H3K4me1 is associated with increased gene expression

To address whether the IFNγ, IL-1β and TNFα induced changes in chromatin state correlate with changes in gene expression, we associated each of the H3K4me1 regions with their nearest TSSs from our RNA-seq transcripts and looked at how the H3K4me1 enrichment levels change in relation to expression. In order to best match H3K4me1 enrichment with expression, we chose to focus on the H3K4me1 regions identified as likely promoters (within 2kb of transcriptional start site) or enhancers (2kb < 100kb) regions, and associated these with only transcripts that have an FPKM > 2 in at least one sample. As demonstrated in Fig 3.11a, almost 50% of transcripts with increased expression are associated with regulatory elements with increased or de novo H3K4me1. We also see a concomitant decrease in H3K4me1 enrichment with decreased expression. When we compared the RNA FPKMs associated with our H3K4me1 regions, we found significant increases in cytokine FPKMs for de novo and increased H3K4me1 regions (P-value= <0.0001 and 0.0096 respectively) (Fig 3.11b). As well as, a significant decrease in treated FPKMs in decreased H3K4me1 regions (P-value =0.0107). These data further support the idea that changes to the epigenome accompany changes in gene expression.

3.6 De novo enhancers at select chemokine genes are formed within 3hrs

Through analyzing our ChIP and RNA-seq data we identified many chemokine and cytokine genes that are in a repressed H3K27me3 marked state in untreated islets, but that gain
H3K4me1 enrichment and expression upon IFNγ, IL-1β and TNFα treatment. To determine how quickly this switch takes place, we performed an IFNγ, IL-1β, and TNFα treatment time course and determined the expression levels of a panel of 7 cytokine genes expressed in islets in response to IFNγ, IL-1β, and TNFα. Within one hour of treatment, the transcript levels of Cxcl10, Cxcl2 and Cxcl11 substantially increased, with all of seven genes reaching maximal expression within 6 hours in islets (Fig 3.12a). To ensure these expression changes were actually occurring in β-cells, and not in other cells within the islets, such as resident macrophages, we performed a similar time course using the mouse β-cell line, MIN6. A similar expression pattern was found, with all of these cytokines hitting maximal expression around 4hrs (Fig 3.12b). This supports the hypothesis that the gene expression changes we see in islets are principally the result of changes in gene expression in β-cells.
Figure 3.11: Increased H3K4me1 is associated with increased gene expression.

(A) The fraction of genes identified as upregulated (Up RNA), unchanged (Unchanged RNA), or downregulated (Down RNA) by exposure of islets to IFNγ, IL-1β, and TNFα that had associated H3K4me1-enriched regions with decreased, unchanged, increased or de novo H3K4me1 in response to IFNγ, IL-1β, and TNFα. (B) Violin plots of RNA-seq FPKMs associated with de novo, increased, unchanged and decreased regions in untreated or IFNγ, IL-1β, and TNFα (Cytokine) treated islets. The height indicates the range of FPKMs covered, while the width illustrates the abundance of genomic regions with that FPKM.
Next, in order to confirm that the induction of these genes by IFNγ, IL-1β, and TNFα is associated with corresponding chromatin state changes, we performed H3K4me1 and H3K27me3 ChIP-qPCR on MIN6 cells after 3 hours of IFNγ, IL-1β, and TNFα exposure. Ccl2 is blanketed by repressive H3K27me3 in untreated islets (black track), with low enrichment for H3K4me1 slightly upstream from the gene (Fig 3.13a). Upon 96hr IFNγ, IL-1β, and TNFα treatment, we see a drastic increase in H3K4me1 upstream and across the gene (red track). In MIN6 cells there is significant increase in H3K4me1 along with a concomitant significant decrease in H3K27me3 at both the promoter, and putative enhancer region after a 3hr treatment (black bar). We observed a similar significant effect at the promoter of Ccl20 (Fig 3.13b), where there is an increase in H3K4me1 and a decrease in H3K27me3 at the down stream putative enhancer. Similarly, Cxcl10 also shows a significant increased in H3K4me1 at the promoter and upstream putative enhancer, with a significant decrease in H3K27me3 at the promoter, following IFNγ, IL-1β, and TNFα treatment (Fig 3.13c). In short, these cytokine genes are not only becoming expressed rapidly, but are also undergoing a swift change in chromatin state, indicating that in as little as 3 hours, IFNγ, IL-1β, and TNFα can induce the activation of H3K27me3 repressed cis-regulatory loci and thereby significantly up-regulate the expression of various cytokine genes in β-cells.
Figure 3.12: Select chemokine genes are expressed within 3hrs of IFNγ, IL-1β and TNFα exposure.

(A) Expression time course of select cytokine genes upon IFNγ, IL-1β and TNFα treatment in islets, with most genes reaching maximal expression by 3 hours. Islets were incubated with IFNγ, IL-1β and TNFα for the given time period, RNA was then isolated and expression was measured via qPCR and compared to beta-actin (n = 3). (B) Expression time course of select cytokine genes upon IFNγ, IL-1β and TNFα treatment in min6, with most genes reaching maximal expression by 2 hours. Min6 cells were incubated with IFNγ, IL-1β and TNFα for the given time period, RNA was then isolated and expression was measured via qPCR and compared to beta-actin (n = 3).
Figure 3.13: De novo enhancers at select chemokine genes are formed within 3hrs.
UCSC genome browser view of genomic regions around (A) Ccl2, (B) Ccl20, and (C) Cxcl10. H3K4me1 enrichment data from IFNγ, IL-1β, and TNFα (Cytokine) treated islets is shown in red, from untreated islets in black, and H3K27me3 data from untreated islets is also shown in black. All tracks are set to show a coverage depth range of 0 to 30. Corresponding ChIP-qPCR for H3K4me1 and H3K27me3 levels (% recovery) around the promoter and enhancer regions of (A) Ccl2, (B) Ccl20, and (C) Cxcl10 from Min6 untreated or treated with IFNγ, IL-1β, and TNFα for 3 hours is shown below the UCSC browser tracks. Primer locations used in the ChIP-qPCR reactions are indicated by the red boundaries. * = p-value<0.05. All ChIP-qPCR were done in triplicate.
3.7 *De novo* enhancers at select chemokine genes do not sensitize these genes to restimulation

In select situations, stimuli can induce *de novo* enhancer formation with a concomitant induction in gene expression\textsuperscript{24-26}. In these situations, removal of the stimulus results in gene expression levels returning to normal, however the *de novo* enhancers can persist for some time\textsuperscript{24}. Upon restimulation, with the same or in some cases other stimuli, the genes respond faster and more strongly than they did initially\textsuperscript{24}. To address whether this was the case with the chemokine and cytokine genes in islets, we first performed a washout experiment to see if their expression would return to basal levels once our IFNγ, Il-1β, and TNFα cocktail was removed. As expected, most of the transcripts were reduced after 1hr without IFNγ, Il-1β, and TNFα, however the expression of *Cxcl10, Cxcl11, Il-15* and iNos remained elevated 48 hours after washout (Fig 3.14a). We next asked whether the initial IFNγ, Il-1β, and TNFα treatment primed the cells to restimulation. RT-qPCR of our proinflammatory cytokine panel revealed that an initial IFNγ, Il-1β, and TNFα treatment did not prime our cells to faster nor stronger expression upon restimulation (Fig 3.14b). Our data indicate that while *de novo* enhancer elements are being formed, and can persist after stimulus removal, they do not appear to sensitize the islets to future IFNγ, Il-1β, and TNFα exposure.
Figure. 3.14: **De novo enhancers at select chemokine genes do not sensitize these genes to restimulation**

(A) Time course of the relative expression of the indicated chemokines and cytokines in islets exposed to IFNγ, IL-1β, and TNFα for 3 hours then placed in media without IFNγ, IL-1β, and TNFα for the indicated time points (hours). Expression level was determined by qPCR and all samples were done in triplicate. (B) Following 3 hour IFNγ, IL-1β, and TNFα treatment and 48 hour washout, restimulation (green) of islets with IFNγ, IL-1β and TNFα does not result in faster, nor stronger cytokine gene induction than initial stimulation (black). All experiments were carried out in triplicate.
3.8 The Trithorax complexes are expressed in pancreatic islets

Now that we have established that IFNγ, IL-1β, and TNFα treatment results in both the addition of H3K4me1, as well as the removal of H3K27me3, we wanted to identify which chromatin modifiers are involved, as preventing their action may inhibit the IFNγ, IL-1β, and TNFα induced cytokine gene expression. The Trithorax complexes are a set of chromatin modifiers that can contain both an H3K4 methyltransferase, and a H3K27 demethylase\(^{26,53}\). First we checked to see if the TrxG genes are expressed in islets and whether their expression is altered in response to IFNγ, IL-1β, and TNFα via RT-qPCR. Indeed, all major subunits of these complexes are expressed in islets (Fig 3.15a) and their expression is not significantly altered by exposure to cytokine. This is further corroborated by our RNA-seq data (Fig 3.15b).
Figure. 3.15: The Trithorax complexes are expressed in pancreatic islets. (A) qPCR validation of the unaltered expression of the genes for the primary proteins in the TrxG complexes in IFNγ, IL-1β, and TNFα (cytokine) treated islets as compared to untreated islets. (B) Fold change (cytokine treated/untreated) of the expression level (FPKM) of the genes for the primary proteins in the TrxG complexes in untreated and IFNγ, IL-1β, and TNFα (cytokine) treated islets.
3.9 Adenoviral mediated RNAi knock-down of TrxG core subunit \( Wdr5 \) blunts chemokine and cytokine expression in dispersed Islets.

With the Trithorax group complexes identified as potential mediators of the chromatin state changes we see upon IFN\( \gamma \), IL-1\( \beta \), and TNF\( \alpha \) treatment, we sought to knock-down one of the core subunits, \( Wdr5 \), and assess the effect on pro-inflammatory gene expression. We chose to target \( Wdr5 \) as it is a core subunit of all TrxG complexes and is known to be essential for complex activity\(^{79-81} \). We began by creating an adenovirus expressing shRNA’s targeting \( Wdr5 \) and obtained >80% knock down of \( Wdr5 \) using our shWDR5 Adenovirus at an MOI of 30 on cells from ~100 dispersed islets (Fig 3.16a).

With approximately 80% depletion of \( Wdr5 \), we examined the effect it would have on IFN\( \gamma \), IL-1\( \beta \), and TNF\( \alpha \) induced proinflammatory gene expression. As compared to control shRNA and corresponding no-cytokine-treated condition, we observed a 3.6 fold reduction in \( Cxcl9 \) and \( Cxcl10 \) between shWdr5 and scramble transduced islets exposed to IFN\( \gamma \), IL-1\( \beta \), and TNF\( \alpha \) (Fig 3.16b, \( p<0.05 \)). We also see a modest but significant (\( p<0.05 \)) reductions in \( Ccl2 \), \( Ccl20 \) and \( \text{Il-15} \). We also examined the expression of a panel of key \( \beta \)-cell genes to evaluate whether suppression of \( Wdr5 \) has an effect on \( \beta \)-cell function. The results indicated no significant changes to the expression of these \( \beta \)-cell function genes. Taken together, these results indicate that suppression of \( Wdr5 \) does blunt the expression of proinflammatory cytokines, while having no significant effect on the expression of genes essential to \( \beta \)-cell function.
3.10 Inhibition of the TrxG demethylases Utx/Jmjd3 by the small molecule GSK-J4 dramatically inhibits IFNγ, IL-1β and TNFα induced proinflammatory gene induction

Having established that suppression of Wdr5 only modestly blunts the expression of proinflamatory cytokines, we applied an alternative approach to disrupt the functionality of the TrxG complexes. In particular, we utilized the small molecule Gsk-J4, a selective inhibitor of the H3K27 demethylases Utx and Jmjd396,97. Assessing the IFNγ, IL-1β, and TNFα induced expression level of Cxcl10 with varying concentrations of Gsk-J4, we identified 20µM as sufficient to prevent Cxcl10 gene expression (Fig 3.17a). Using this minimal but sufficient amount of inhibitor, we assessed the expression changes of the proinflamatory genes with and without IFNγ, IL-1β, and TNFα treatment. We found a significant reduction in all proinflamatory transcript levels in response to IFNγ, IL-1β, and TNFα, upon GSK-J4 treatment, including a 37.9 fold decrease in Cxcl11, a 33.8 fold decrease in iNos and a 22.3 fold decrease in Cxcl10 (Fig 3.17b, p<0.01). To assess effects on β-cell function, we monitored the expression of key β-cell genes and found no significant changes (Fig 3.17c). While we see no effect on the expression of β-cell function genes after 3 hours with GSK-J4, we do see a large increase in propidium iodide incorporation after 4 hours, and by 6 hours most of the islet cells appear to be apoptotic (Fig 3.18). Taken together; these results indicate that inhibition of H3K27 demethylases dramatically blunts the expression of proinflammatory cytokines and also has a minimal effect on the expression of β-cell function genes after 3 hours. However, extended exposure to GSK-J4 induces high levels of apoptosis by 6 hours, indicating that targeting the TrxG complex may yield beneficial approaches to prevent IFNγ, IL-1β and TNFα induced
proinflammatory chemokine and cytokine gene expression in β-cells; however, alternative mechanisms to inhibit the TrxG complex are required.
Figure 3.16: Adenoviral mediated RNAi knock-down of TrxG core subunit Wdr5 blunts proinflammatory cytokine expression in dispersed Islets.

(A) Relative expression of Wdr5 as determined by qPCR in islets treated with the indicated MOI of shWdr5 adenovirus. These results show that an MOI of 30 results in >80% suppression of the Wdr5 transcript. Relative expression as determined by qPCR of (B) select chemokine or cytokine genes or (C) select genes involved in regulating β-cell function in untreated or IFNγ, IL-1β and TNFα (cytokine) treated islets transduced with an shWdr5 expressing or a control adenovirus. * = p-value<0.05, ** = p-value<0.01.
Figure 3.17: Inhibition of the TrxG demethylases Utx/Jmd3 by the small molecule GSK-J4 dramatically blunts IFNγ, IL-1β and TNFα induced proinflammatory gene induction. (A) Dose response curve of Cxcl10 expression after 3 hours of IFNγ, IL-1β and TNFα treatment with increasing concentrations of GSK-J4 as determind by qPCR. Relative expression as determined by qPCR of (B) select chemokine or cytokine genes or (C) select genes involved in regulating β-cell function in untreated or IFNγ, IL-1β and TNFα (cytokine) treated islets incubated with 20 µM GSK-J4 or a DMSO control. * = p-value<0.05, ** = p-value<0.01.
Figure 3.18: GSK-J4 causes an increase in islet-cell death in 4-6 hours.
Representative images of propidium iodide incorporation at the indicated times in whole islets treated with 20µM GSK-J4. Propidium iodide (red) is only incorporated into dying cells, revealing a rise in islet-cell death after 4 hours of GSK-J4 incubation. Scale bar = 100µm.
Chapter 4  Discussion and Conclusion

The identification of cis-regulatory elements throughout the genome has become an invaluable tool to gain insight into the gene expression profiles of cells and the mechanisms that govern them. By comparing the enrichment profiles of several key histone modifications (for example, H3K4me1, H3K4me2, H3K4me3, H3K9ac and H3K27ac), it is possible to identify promoter and enhancer regions, and additionally classify them into chromatin states\textsuperscript{36,37,43,44}.

Previous work by our lab has shown H3K4me1 to be especially useful, as it is enriched at both promoter and enhancer elements that are in an active/poised or bivalent state in pancreatic islets\textsuperscript{38,39}. On the contrary, as H3K4me2 is typically found to co-occupy loci marked by H3K4me1 or H3K4me3, it is therefore, thought to be redundant for the identification of cis-regulatory elements. H3K4me3 on the other hand, is found primarily at promoter elements and strong enhancers, making it unable to identify the majority of enhancer elements. H3K9ac and H3K27ac mark active promoters and enhancers, but are lost when the elements are no longer in use, and thus are not suitable to identify bivalent regions\textsuperscript{29}. H3K4me1 however, is enriched at active/poised and bivalent enhancers and promoters; therefore it is the most robust modification for the identification of these regulatory elements\textsuperscript{26,35,36,39,43}. In line with this, many of genomic regions identified around genes with IFN\(\gamma\), IL-1\(\beta\) and TNF\(\alpha\) induced expression show concomitant increased H3K4me1 upon IFN\(\gamma\), IL-1\(\beta\) and TNF\(\alpha\) treatment in our data.
By the time a cell reaches terminal differentiation, it has passed through a variety of steps in its differentiation pathway, and at each of these steps along the way the epigenetic landscape is altered to adjust the expression of various transcripts required for the next phase of differentiation. A growing body of evidence shows that this process is not limited to differentiation; rather, the chromatin landscape is far more dynamic, responding to different stimuli and stresses. Here we show that not only is there a widespread change to the epigenetic landscape of pancreatic islets in response to a simulated autoimmune assault, but that some of these changes mark a shift from an H3K27me3 repressed state to an H3K4me1 actively marked state with concordant increases in gene expression, especially around key proinflammatory cytokine genes. This is in line with recent discovery of de novo enhancer elements being formed in mature cells in response to environmental stimuli, a process that has yet to be shown in islets. Utilizing our cytokine treated ChIP-Seq data, we were able to identify 3520 genomic regions that gain de novo H3K4me1 upon treatment with IFNγ, TNFα, and IL-1β. However, our initial work seems to show that these de novo enhancers do not prime the cells to further restimulation (Fig. 3.14b). Interestingly, the work done in macrophages indicates that de novo enhancers can be formed in a stimuli-specific manner, hinting that additional stimuli important to the pathological progression of diabetes, such as ER stress, gluco- and lipo-toxicity, may result in the unveiling of further de novo enhancers. As such, the full extent of β-cell de novo enhancers is yet to be fully determined.

As we further analyzed the ChIP-sequencing data, we found that a large number of proinflammatory cytokines, such as Cxcl9, Cxcl10, Cxcl11, IL-15, Ccl2 and Ccl20, become expressed by islets in response to IFNγ, TNFα, and IL-1β exposure. These genes are not only
associated with regulatory regions that gain *de novo* or increased H3K4me1, but are also marked by H3K27me3 in untreated islets. As H3K27me3 is associated with the polycomb complex group and is therefore found at repressed loci, this implies that these genes are maintained as an actively repressed state under normal conditions, and that this active repression must be removed in order to facilitate gene activation upon cytokine treatment.

While there is a clear trend toward increased gene expression coinciding with increased or *de novo* H3K4me1, the majority of increased and *de novo* H3K4me1 regions were not associated with increased gene expression. Similarly, many of the regions with reduced H3K4me1 upon cytokine exposure are not associated with genes down-regulated by IFNγ, IL-1β and TNFα exposure. We find this level of discrepancy to be expected for a number of reasons. First, we associated each H3K4me1 enriched region with the nearest transcriptional start site. While it is thought that this proximity-based association between regulatory elements and genes is true in most cases, over 66% \(^{23}\), it likely does not hold true for all cases. Secondly, gene regulation is a tremendously complex process, relying on the intricate communication of multiple regulatory elements for each gene. To this end, the expression of the vast majority of genes depend on numerous enhancer elements which may not all show the same trends in increased or decreased H3K4me1 upon IFNγ, IL-1β and TNFα stimulation. We may see an increase in H3K4me1 at some enhancers and a concomitant decrease at others, indicating that changes in H3K4me1 enrichment at a given enhancer may have a minimal impact on the overall expression levels of its target gene. Additionally, the heterogeneous make up of pancreatic islets, containing five endocrine cell types as well as other cell types, further muddles the results. That is, a small number of cells may be able to produce enough transcript to be noticeably increased in
our RNA-seq data, but due to their small number, do not contain enough DNA to be significantly enriched in ChIP-seq. Lastly, the stringent thresholds used throughout our ChIP-Seq and RNA-Seq analysis has likely led to many IFNγ, IL-1β and TNFα induced alterations in H3K4me1 enrichment, as well as transcript level being classified as unchanged. While these cutoffs may have removed some significant changes, by using more stringent thresholds we have greater confidence that the alterations that do fall into increase or decreased are likely to be biologically significant. In any case, it is evident that IFNγ, IL-1β and TNFα induced increases in transcript level were often associated with increased and/or de novo H3K4me1 enrichment.

The treatment of ex vivo islets with a cocktail of cytokines has become a widely used and accepted model of T1D \(^{14,48,74}\). While many studies choose to use a high dose of IFNγ, TNFα, and IL-1β for a short period of time, we elected to use a low dose (~1/64 of the typical dose) for a prolonged time frame (96 hours) in order to better replicate the initial onset and early pathogenesis of T1D. Surprisingly, even with this low dose of IFNγ, IL-1β and TNFα, we observed proinflammatory cytokine expression being induced within 1 hour and reaching maximal expression in 3-6 hours following treatment. Although our RNA-Seq data is from islets cultured in IFNγ, TNFα, and IL-1β for 96 hours, it is likely that up-regulation of many transcripts is also occurring within an hour of IFNγ, IL-1β and TNFα exposure. We were able to verify that the regulatory elements around Ccl2, Ccl20 and Cxcl10 exhibit increased H3K4me1 and concurrent decreased H3K27me3 enrichment within 3 hours of IFNγ, TNFα, and IL-1β stimulation. In addition, we show that once formed, these enhancers are capable of maintaining proinflammatory chemokine and cytokine gene expression for days, even after the removal of IFNγ, TNFα, and IL-1β. These results suggest that even a transient exposure to IFNγ, IL-1β and
TNFα from immune cells can have a dramatic and sustained effect on the production of proinflammatory cytokines and chemokines by β-cells, and likely drive the amplifying effect of β-cell autoimmune destruction.

The acquisition of active H3K4me1 marks and concomitant removal of H3K27me3 repressive marks of selected cytokine genes implicates the TrxG family of chromatin modifying complexes as likely mediators of this change. The TrxG complexes contain both the H3K4 methylases Mll1, Mll2, Mll3, Mll4, Set1a and Set1b, all of which are capable of adding H3K4me1 as well as the H3K27 demethylases Utx and Jmd3 responsible for removing H3K27me3. In addition to these histone-modifying subunits, all of the TrxG complexes contain the core proteins Wdr5, Dpy30, Ash2l, and Rbhp5 that are required for their function and proper complex formation. Here, we show that the components of the TrxG complexes are expressed in islets and that according to our RNA-seq data, their expression levels are not significantly reduced by cytokine exposure.

With TrxG complex expression confirmed in islets, we hypothesized that disruption of TrxG complexes should prevent IFNγ, IL-1β, and TNFα induced proinflammatory gene expression. In particular, RNAi mediated knock-down of Wdr5 is sufficient to blunt IFNγ, IL-1β, and TNFα induced gene expression changes of proinflammatory cytokines IL-15, Ccl20, Cxcl9 and Cxcl10, without greatly effect the expression of key β-cell functional genes.

Furthermore, inhibition of the H3K27 demethylase by the small molecule GSK-J4 results in an even more dramatic dampening of proinflammatory gene expression, whilst not affecting...
key β-cell functional gene expression. Notwithstanding these results, at the 20µM dose used in these studies, GSK-J4 caused nearly all islet cells to be apoptotic by 6 hours. This may be due to the islets cells dependence upon p16\(^{INK4A}\) expression, which requires the repression of Cdk4/Cdk6 by Jmjd3/Utx 82. Regardless, the ability of TrxG complex inhibition to prevent IFN\(\gamma\), Il-1\(\beta\), and TNF\(\alpha\) induced proinflammatory gene expression suggests that with additional refining, therapeutics targeting the TrxG complexes may be valuable adjuncts in treating early-diagnosed T1D, or in prolonging graft survival in islet graft recipients.

In conclusion, this work indicates that the underlying changes to the chromatin state around proinflammatory cytokine genes is occurring along side gene expression changes. These changes are rapid, taking place within 3 hours, and are taking place at previously repressed loci. By interrupting these changes to chromatin state we have managed to greatly reduce IFN\(\gamma\), Il-1\(\beta\), and TNF\(\alpha\) induced proinflammatory gene expression in mouse islets, highlighting chromatin modifiers as potential targets for novel therapeutics.

4.1 Conclusions

In summary, we conclude the following from the current study (also see Fig. 4.1):

- Exposure to the proinflammatory cytokines Tnf\(\alpha\), Il-1\(\beta\) and IFN\(\gamma\) caused alterations to H3K4me1 enrichment levels in islets.

- Upon IFN\(\gamma\), Il-1\(\beta\), and TNF\(\alpha\) stimulation, De novo enhancers are formed near previously H3K27me3 marked cytokine and chemokine regulatory elements.
• In response to IFNγ, IL-1β, and TNFα, these genes gain active expression and exhibit an increase in H3K4me1 and concomitant decrease in H3K27me3 at their regulatory elements within 3 hours.

• Despite formation, these De novo enhancers do not appear to sensitize the cells to future IFNγ, IL-1β, and TNFα exposure.

• shRNA mediated knock-down of core TrxG subunit Wdr5 reduces the activation of these cytokines.

• The small molecule inhibitor GSK-J4 is able to drastically reduce activation of these proinflammatory cytokines and chemokines.

Our data implies that alterations to the chromatin state around regulatory elements is required in order to facilitate IFNγ, IL-1β, and TNFα induced gene expression changes in mouse pancreatic islets. We have also demonstrated that chromatin modifiers are a prospective source of novel therapeutic targets and have the potential to help in the treatment and prevention of diabetes mellitus.
Figure 4.1. Working model proposed by this study.

Stimulation of pancreatic β-cells by the proinflammatory cytokines IFNγ, IL-1β, and TNFα caused the recruitment of the TrxG/MII complexes to the actively repressed regulatory elements of proinflammatory cytokines and chemokines. Once there, the TrxG/MII complexes deposit the active H3K4me1 histone modification while removing the repressive H3K27me3 modification. This in turn, allows for active expression of these genes and ultimately β-cell dysfunction and death. Interfering with the action of the TrxG/MII complexes by removing the core subunit Wdr5, or inhibiting the H3K27me3 demethylases prevents the activation of these proinflammatory cytokines and chemokines, prolonging β-cell survival.
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Appendix Figures: Bioinformatic workflows.

Figure A.1: ChIP-seq workflow

1. 75 bp Reads from Genome Science Centre
2. 75bp Trimmed to 36bp - SamTools
3. 36bp Reads mapped to mm9 genome – Bowtie 2.2.1
4. Filter out mapping quality < 33 - SamTools
5. Remove duplicates - SamTools
6. Identify enriched loci - ChromHmm
7. Filter out regions ≤ 200bp and RPKM < 2 - Excel
8. Merge / Intersect regions to identify sample specific Vs conserved regions - BedTools
9. Final ChIP-seq enriched regions
Figure A.2: RNA-seq workflow

1. **75bp Paired-end reads from Genome Science Centre**

2. **Mapped to mm9 and known UCSC genes - TopHat**

3. **Differential gene expression - Cufflinks**

4. **Remove transcripts with FPKM < 2 - Excel**

5. **Final RNA-seq transcript expression**
Figure A.3: IncRNA workflow

1. **Final RNA-seq transcripts not mapped to UCSC genes**
2. **Filter out transcripts < 100bp and FKPM < 3 - Excel**
3. **Multiple sequence alignments - Galaxy**
4. **Non-coding potential - phyloCSF**
5. **Remove transcripts with phyloCSF score > 10 decibands - Excel**
6. **Final RNA-seq transcript expression**