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Author
Zhou, Xi

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Epigenetic Studies Of Diabetes

A dissertation submitted in partial satisfaction of the

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by

Xi Zhou

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ABSTRACT OF THE DISSERTATION

Epigenetic Studies of Diabetes

by

Xi Zhou

Doctor of Philosophy in Molecular Biology

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Professor Anil Bhushan, Chair

Diabetes mellitus is growing at an alarming rate globally. My dissertation aims at understanding the epigenetic mechanisms of Diabetes pathogenesis and how we can utilize this knowledge to develop treatments for patients with this debilitating disease.

Diabetes is associated with loss of pancreatic beta cells and islet replacement therapy is limited by the source of donor islets. Two promising alternative approaches for beta cell replenishment are regeneration of endogenous beta cells and directed stem cell differentiation. In chapter 1, I used a transgenic mouse model that ectopically expresses Ezh2, to offset the loss of endogenous protein and uncovered new insights into plasticity of beta cell regeneration in adulthood. I show that reprogramming of the *Ink4a* chromatin by modulating the conserved Polycomb-Trithorax mechanism can rejuvenate the replication capacity of aged beta cells to provide a new strategy

for beta cell expansion. In chapter 2, I studied mouse embryonic stem (ES) cells to understand endodermal lineage specification, as current attempts to bring endoderm-derived stem cell therapy to a state of effectiveness have been hampered by the paucity of knowledge of the underlying mechanisms. I show that the pluripotency transcription factors and epigenetic modifications of the chromatin interact in order to implement control pathways that drive differentiation of stem cells towards the definitive endoderm lineage.

Type 1 Diabetes is caused by autoimmune assault of pathogenic T cells escaping the tolerance selection. In Chapter 3, I identified a novel interaction partner of the autoimmune regulator (Aire), JmjD6. My data suggest that JmjD6-mediated histone demethylation at Arginine 2 residues of target chromatin is essential to prime the chromatin of otherwise repressed peripheral tissue antigen (PTA), for subsequent Aire activation of the antigen peptides for tolerance selection.

Maternal obesity is concerning because of the deleterious effects in offspring of increased risks of metabolic syndromes including Type 2 diabetes and obesity. My current work is focused on whole genome methylation mapping of trans-generational epigenetic inheritance in a maternal high-fat diet mouse model. The single nucleotide resolution of this screening will reveal information on how inherited methylation markers contribute to the predisposition of metabolic diseases in progeny in the context of maternal obesity, which can be further utilized for intervention and disease prevention.

The dissertation of Xi Zhou is approved.

Guoping Fan

April D. Pyle

Christel H. Uittenbogaart

Antonio La Cava

Senta K. Georgia

Anil Bhushan, Committee Chair

University of California, Los Angeles

2013

To my parents, Caizong Zhou and Xiangling Gao

for their unconditional love

and support of my study abroad

To my brother, Sixiang Zhou

your accomplishment will be greater

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Part of Chapter 2 is adapted from a draft manuscript "Jmjd3 sequentially associates with two T-box factors; Tbx3 and Eomes to drive differentiation of endoderm", authored by Apriliana E. R. Kartikasari, Josie X. Zhou, Murtaza S. Kanji, David N. Chan, Arjun Sinha, Anne Grapin-Botton, Mark A. Magnuson, William E. Lowry and Anil Bhushan. The authors would like to acknowledge the support from the UCLA Broad Stem Cell Research Center High-Throughput Sequencing Core. I would like to thank Dr. Yuliang Tan for the ChIP-seq data analysis and the Pellegrini lab for assistance on bioinformatics.

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VITA

2007	Bachelor of Science, Molecular Cell Biology University of Science and Technology of China, Hefei, China
2008-2009	Teaching Assistant Department of Molecular Cellular and Developmental Biology, University of California, Los Angeles
2008-2012	Graduate Student Researcher Bhushan Lab Larry Hillblom Islet Research Center, Division of Endocrinology, Department of Medicine University of California, Los Angeles

PUBLICATIONS AND PRESENTATIONS

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INTRODUCTION

Diabetes

Diabetes is a chronic disease that affects 25.8 million children and adults-8.3% of the population- in the United States as of 2010. And it is expected that we will enter a Diabetes epidemic globally in the next decade(1). Diabetes is caused by inadequate insulin secretion/response to the metabolic demand, which results in high blood glucose. Insulin is a hormone synthesized and secreted by the pancreatic β cells into the blood stream to maintain glucose homeostasis, and therefore mediate energy consumption and storage for the body. While poorly controlled blood glucose level can cause discomfort and maybe life-challenging syndromes itself, prolonged exposure to hyperglycemia and hypoglycemia may result in severe complications that damage multiple organs and may be life threatening. There are three types of Diabetes, Type 1 Diabetes, Type 2 Diabetes and gestational Diabetes.

Type 1 Diabetes

Type 1 Diabetes is also known as juvenile Diabetes, as most of the Type 1 Diabetes is diagnosed in children and young adults. It accounts for 5-10% of Diabetes incidences, and is caused by autoimmune attack of the pancreatic β cells. To date, what is causing the onset of immune assault of β cells remains poorly understood, it is commonly believed that both genetic and environmental factors are involved. Type 1 Diabetes patients are treated with life-long insulin injections to supply the missing insulin and they must monitor their glucose levels on a regular basis.

Type 2 Diabetes

Type 2 Diabetes is the most common form, it accounts for 90% of the Diabetes cases and the incidence of Type 2 Diabetes increases with age. Type 2 Diabetes is generally characterized by insulin resistance and deficiency. Like in Type 1 Diabetes, our understanding of the pathogenesis of the disease has linked the onset of the disorder to a combination of lifestyle and genetic factors that is still largely under study. Type 2 Diabetes is managed by lifestyle interventions, combined with medications that help manage blood glucose homeostasis and increase insulin sensitivity, and insulin in some cases that fail to produce sufficient insulin.

Gestational Diabetes

Gestational Diabetes is diagnosed by high blood glucose during pregnancy in 3-10% of pregnancy. Hormonal changes during pregnancy can lead to insulin resistance in normal pregnancy, while a healthy pregnancy can compensate for the insulin resistance by expanding β cell mass and increasing insulin secretion, gestational diabetic patients fail to balance the insulin demand and this results in elevated blood glucose. Though for most Gestational Diabetes patients, their blood glucose levels return to normal after delivery, they are at increased risk of Type 2 Diabetes. Offspring born to Gestational Diabetes with uncontrolled blood glucose levels are also prone to develop metabolic diseases such as obesity and Diabetes later on in life. Gestational Diabetes is mostly treated with lifestyle interventions and glucose monitoring to maintain a normal blood glucose level. But in some severe cases, medications or insulin are needed.

Pancreatic β cells and Diabetes

In the pancreas, the β cells are organized into function units called islets of Langerhans, which

accounts for 1-2% of the pancreatic mass. However, these 1-2% of the cells are critical in determining the amount of insulin released to maintain normal range of blood glucose, and therefore have been under intensive studies for understanding the pathogenesis of Diabetes and devising new therapeutic approaches to diabetes as both Type 1 and Type 2 Diabetes are associated with loss of β cells (2-5). Currently, β cell replenishment strategies have been actively studied for the prospects of treating both Type 1 and Type 2 Diabetes; while islet transplant has shown promising results, this application is greatly restricted by the lack of sufficient donors (6-8). The success of islets transplantation has greatly supported the development of other β cell replenishment strategies that can potentially overcome the limit, of which two alternative approaches have shown great promise: stem cell or induced pluripotent stem cell based therapy and β cell regenerations (9-11). Both strategies require deeper understanding of mechanisms that regulate formation of pancreatic β cells, and capacity to establish, maintain and regenerate β cell mass under physiological and pathological conditions (12).

β cell development

The pancreas is differentiated from the definitive endoderm. During development, epiblast cells ingress through the anterior segment of the primitive streak where they undergo an epithelial-to-mesenchymal transition to give rise to mesoderm and definitive endoderm (13). Nascent definitive endoderm is marked by expression of transcription factors, *Sox17* and *Foxa2* (14-16). The anterior part of the definitive endoderm will become foregut, where the dorsal and ventral pancreatic buds will branch out under orchestrated patterning signaling (17). Pancreatic duodenal homeobox 1 (Pdx1) is expressed as early as embryonic day 8.5 (E8.5) in the foregut endoderm. Expression of Pdx1 characterizes the pancreatic progenitor cells that will eventually give rise to

all cell types in the pancreas, its expression goes down in the pancreas and reappear in pancreatic β cells selectively from embryonic day 11 through adulthood (18-20). Inactivation of Pdx1 in mouse has a apancreatic phenotype and further studies of the Pdx1 null mice have revealed its role as a transcription factor in regulating progenitor proliferations and lineage specifications of different endocrine cell types (21, 22). The Pdx1+ multipotent progenitors, under different signaling stimulates, can either proliferate to expand the progenitor pool or differentiate into more specialized endocrine progenitors that express Ngn3 (23). Expression of Ngn3 is transient, as they are specific to the progenitors but not in the differentiated endocrine cells. Upon expression of Ngn3, these progenitors are post-mitotic and pre-programmed to differentiate into endocrine lineages (24, 25). Generating β cells from ES cells is of great interest to replace the deficit of β cells in Diabetes, but it remains a significant challenge. Strategies developed to date to direct the differentiation process and induction of lineage specification at each stage has shown considerably decreased efficiency at each subsequent differentiation step. The lack of efficiency of directed pancreatic β cell differentiation implies an imperative need, to inspect both genetic and epigenetic molecular stimuli of development, and to understand the transcription networks that orchestrate each differentiation milestone.

Establishing β cell mass

Pancreatic β cell mass is established both prenatally and postnatally, but the source of new β cells is different in these two phases. Before birth, the majority of the β cells come from differentiation of the progenitors as data from rodent studies suggest that the pool of pancreatic progenitors persists through embryogenesis and the newly differentiated β cells are arrested in a mitotically quiescent state (26, 27). After birth, neonatal β cell mass undergos massive

expansion, and the new β cells come from self-replication of the existing β cells (28, 29). This progressive slow-down of replication rate occurs postnatally, and continues to decline through adulthood as replicative senescence takes place during aging (30). Although the bulk of the β cell mass is established during neonatal expansion, adult β cells can expand under physiological and pathological conditions such as obesity and pregnancy (31, 32).

β cell regeneration

β cell mass can regenerate after injury as well as expands in response to metabolic demands associated with growth, pregnancy and obesity in mice and humans (33, 34). The failure of β cells to expand and secrete adequate insulin to compensate for changes in insulin demand is thought to be a root cause for Type 2 Diabetes and gestational diabetes. However, the capacity of β cells to regenerate in mice and human is limited to young ages and this capacity declines rapidly with age (35-37). To restore functional β cells in Diabetic patients, this age-declined regenerative limit needs to be overruled, which would require better understanding of the underlying mechanisms that cause the replicative senescence. Studies suggest that the decline in β cell replication with age is due to increased transcription from the *Ink4a/Arf* locus (38). The *Ink4a/Arf* locus encodes $p16^{Ink4a}$ and $p19^{Arf}$; of which $p16^{Ink4a}$ is a negative regulator of CDK4 that curtails β cell replication with increased age (39). Germline deficiency of $p16^{Ink4a}$ leads to increased β cell proliferation (40). Genome wide association studies have linked the *Ink4a/Arf* locus to insulin insufficiency and risks of Type 2 Diabetes in humans, indicating a potential important role for $p16^{Ink4a}$-mediated control of β cell expansion in the pathogenesis of Type 2 Diabetes (41). Recent studies have shown that polycomb complexes that repress the *Ink4a/Arf* locus are dissociated in aged islets resulting in de-repression of the $p16^{Ink4a}$ and reduced β cell

replication (42, 43). These studies support the notion that epigenetic mechanisms play important roles in adult β cell self-renewal/regeneration, which can be further utilized to facilitate the development of regeneration strategies for treating diabetes.

Type 1 Diabetes and autoimmnunity

In Type 1 Diabetes, self-reactive T cells escape tolerance selections and attack β cells by reacting to pancreatic β cell-specific antigens (44). A series of islet anto-antigens have been identified, including insulin, islet cell autoantigen 69 (ICA69), islet antigen 2 (IA-2), glutamic acid decarboxylase 65 (GAD65), islet-specific glucose-6-phosphatase catalytic subunit related protein (G6PC2) and zinc transporter Znt8 located in the insulin secretory granule (45-48). Assays to detect auto-antibodies against these auto-antigens have been used clinically to predict Type 1 Diabetes in non-diabetic population. It is still unclear what triggered the antoimmune response to β cells; some studies have suggested it might be induced early in life (49). Notably, pancreatic β cells undergo a massive remodeling after wean in rodents, it has been proposed that the wave of neonatal β cell apoptosis that occurrs at this stage might expose antoantigens to initiate an autoimmune response in genetically predisposed subjects (50).

Establishing neonatal immune tolerance in the thymus

During T cell development, thymus stroma provides a distinct microenvironment for specific education and maturation of T cells (For review see (51). Thymus stroma contains two different regions, the cortex and medulla, consisting of distinctive types of epithelial cells. Thymocytes differentiate from double negative progenitors (CD4-CD8-) to double positive (CD4+CD8+) cells, and eventually mature into single positive cells (CD4+CD8- or CD4-CD8+) to be released

to the peripheral. T cells precursors first undergo T cell receptor (TCR) rearrangement to generate a T cell repertoire capable of different specificity in recognizing different antigens; a stochastic process essential to T cell mediated immunity. TCRs of mature T cells recognize foreign antigen peptides presented by self-major histocompatibility complexes (MHC) molecules displayed on antigen presenting cells (APCs) including macrophages, B cells, and dendritic cells (52). Developing T cell progenitors after TCR rearrangement then migrate to the cortex-medulla junction and undergo expansions and selections to develop functional competent and self-tolerant T cells (53). Only 2% of the thymocytes eventually migrate out of the thymus to the peripheral and the majority dies during either positive or negative selection processes in the thymus during T cell development. The positive selection ensures that T cells are functional in engaging self MHC-peptide complex; it takes place in the cortex(54). Self-peptides expressed by the cortical thymic epithelial cells (cTECs) are presented with MHC class I and class II molecules; T cells with appropriate affinity between TCR and MHC-peptide receive a survival signal, whereas T cells with too strong or too weak affinity with the self MHC-peptide complex will undergo activation induced apoptosis or death by negalect (For review see (55, 56). The restricted interaction between TCR and MHC molecules also determines the fate of thymocytes; recognition of MHC I molecules leads to development into single positive CD8+ cells and interaction with MHC II molecules signals maturation into CD4+ fate (57). Positively selected T cells then migrate to the medulla, where self-reactive thymocytes are negatively depleted by exposure to the self-peptide MHCs presented by medullary thymic epithelial cells (mTECs), a process called negative selection (For review see (56, 58, 59)). mTECs express a broad spectrum of peripheral tissue antigens for negative selections, many of which are regulated by the transcriptional regulator Aire (60). Single cell RT-PCR assay revealed heterogeneity in the

ectopic expression of peripheral tissue antigens in individual mTEC; the results show that Aire expression is associated with transcriptional up-regulation in a stochastic manner (61). Gene expression profiles from *Aire* null mTECs reveals that Aire is required to promote the expression of a diverse set of antigens in mTECs that are normally restricted to peripheral tissues. Of note, not all peripheral tissue antigens are regulated by Aire, such that the expression of GAD67 is not altered in *Aire* null mTECs (62, 63). Loss of functional Aire impairs clonal deletion of the self-reactive thymocytes, with the result that they escape into the peripheral tissues and attack various organs, leading to a severe autoimmune disease APECED (For review see (64)). These studies have suggested that Aire-mediated peripheral antigen tolerance is critical in establishing immune tolerance through adulthood. In particular, studies have suggested the neonatal window during which Aire mediates immune tolerance is of particular importance as escape of auto-reactive thymocytes after weaning is more tolerable and has less harmful consequences (65). The mechanism how Aire mediates the transcriptional activation of peripheral tissue antigens, especially in such a precise yet promiscuous manner, remains unclear. Recent structural evidence of Aire suggested epigenetic mechanisms could be critical in the target recognition and peripheral antigen trancriptional activation.

Notably, recent studies that depleted insulin in the Aire+ mTECs have induced autoimmune diabetes in mouse models, suggesting a pivotal role of central tolerance in establishing immune tolerance toward pancreatic β cells (66). The essential role of Aire induced tolerogenic mechanisms toward insulin support the notion that understanding the mechanism of Aire regulated peripheral antigen expression will contribute to developing therapeutic approaches for autoimmune diseases such as Type 1 Diabetes.

Maternal obesity and epigenetic inheritance

Pregnancy obesity is associated with increased risk of developing gestational diabetes, gestational hypertension and development of chronic metabolic defects in the progeny. According to a recent study, the risk of developing Gestational Diabetes is about 2.14, 3.56, and 8.56 times higher among women who are overweight, obese, and severely obese, respectively, compared with normal-weight pregnant women (67). Maternal obesity, like Gestational Diabetes, leaves a metabolic memory and predisposes the progeny to obesity, Diabetes, cardiovascular diseases and metabolic syndromes later on in life. With over 50% of the women of reproductive ages are overweight or obese; understanding the mechanism of the metabolic inheritance in the offspring has become an increasingly pressing issue for the coming obesity and diabetes epidemics. Studies of a maternal high-fat diet mouse model have revealed increased body length and insulin insensitivity in the second generation, and that the increased body size trait was preserved in the third generation via the paternal lineage (68, 69). Although heritable features affected by maternal high-fat diet have been recognized in several studies and an epigenetic mechanism is generally thought to contribute to the inheritability of dietary induced traits, how and through which epigenetic modification it is transmitted across generations has not been well characterized.

Epigenetics means “above the genome”, as it refers to mechanisms that regulate gene expression without altering the DNA sequence and therefore “above the genetic regulation”. Three major epigenetic modifications are identified to date, including DNA methylation, histone modifications and non-coding RNA. It is commonly accepted that epigenetic modifications

regulate gene expression by changing the local chromatin environment, chromatin structure and accessibility to transcription factors. Adapted epigenetic information can be propagated through mitotic cell divisions; however, they are not normally transmitted through meiosis to the future generation as the regulatory epigenetic marks are usually erased in zyogotes to ensure totipotency (70, 71). Few studies have provided evidence that paternal and/or maternal dietary condition induced epigenetic changes might be inherited transgenerationally, yet the mechanism remains to be elucidated systematically on a whole genome scale (72-75).

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CHAPTER 1: COMBINED MANIPULATION OF POLYCOMB AND TRITHORAX GENES CAN PROMOTE BETA CELL REGENERATION

Abstract

Reduced Ezh2 binding to the *Ink4a* locus facilitates the derepression of *Ink4a,* thus preventing beta cell replication. As *Ezh2* expression levels in the beta cell decline with age, we investigated whether replenishing *Ezh2* could reverse the age dependent repression of p16Ink4a. We generated an inducible pancreatic beta cell specific *Ezh2* transgenic mouse model and utilized it to show that transgene expression of *Ezh2* was sufficient to increase beta cell replication and regeneration in young adulthood. However, in aged mice, the transgene expression of *Ezh2* was insufficient to repress p16Ink4a due to enrichment of the trithorax group (TrxG) complex on the *Ink4a* locus. Knockdown of TrxG members, *MLL1* or *JmjD3* coupled with transgene expression of *Ezh2* enabled efficient repression of p16Ink4a and increased replication of aged beta cells. These results indicate that reprogramming of the *Ink4a* chromatin epigenome by modulating the conserved PcG-TrxG mechanism can rejuvenate the replication capacity of aged beta cells to provide a new strategy for beta cell expansion.

Introduction

Both type 1 and type 2 diabetes patients suffer from inadequate functional beta cell mass and regeneration of beta cell function is a critical goal (1, 2). Beta cell mass can regenerate after injury as well as expands in response to metabolic demands associated with pregnancy, obesity, insulin resistance, and growth in mice and humans (3-6). There is increasing evidence to suggests that replication of beta cells is the dominant means by which beta cells adapt to changing metabolic demands and during regeneration (7-10). The capacity of beta cells to regenerate or expand in mice and human is limited to young ages, suggesting that restoring functional beta mass in diabetic patients would require manipulating mechanisms that lead to the age-dependent decline in regenerative capacity (3, 11-13). Several studies indicate that this decline in beta cell replication with age is due to increased transcription from the *Ink4a/Arf* locus (14, 15). The *Ink4a/Arf* locus encodes p16Ink4a and p19Arf; of which p16Ink4a is a negative regulator of CDK4 that curtails beta cell replication (16, 17). Genome wide association studies have linked the *Ink4a/Arf* locus to insulin insufficiency and risks of Type 2 Diabetes (T2D) in humans, indicating a potential important role for p16Ink4a-mediated control of beta cell expansion in the pathogenesis of T2D (18).

Several studies have shown that members of the polycomb group (PcG) proteins regulate the *Ink4a/Arf* locus in islets (19, 20). PcG proteins are transcription repressors that mediate gene silencing by forming multiprotein complexes, called polycomb repressive complexes (PRC). Two distinct PRCs have been discovered to date, PRC1 and PRC2. Core components of PRC1 comprise Bmi-1 and ubiquitin ligase Ring1B, which catalyzes histone H2A monoubiquitination

at lysine 119 (21). Whereas the core PRC2 complex consists of EED, Suz12 and a histone methyltransferase--Enhancer of zeste homolog 2 (Ezh2), that has catalytic activity in methylating Histone H3 at lysine 27 (22, 23). PRC1 and PRC2 coordinate at a sequential manner to compact chromatin and mediate silencing of gene expression (24, 25). Following PRC2 recruitment and deposition of the repressive Histone H3K27me3 marks to the gene locus, PRC1 is enlisted and subsequently deposits Histone H2AK119ub to facilitate silencing of the locus (26-29). In islets from young mice, both PRCs are bound to the *Ink4a* locus to repress gene transcription (19, 20). Islets from aged mice show a decline in the levels and binding of PRCs to the *Ink4a* locus in aged islets that coincides with increase in binding of the Trithorax group (TrxG) protein complexes which results in enrichment of histone with methylated Histone H3 at lysine 4 (H3K4me3) resulting in transcriptional activation of the genes *Ink4a* (19). Increased levels of p16Ink4a results in inhibition of CDK4/6-CyclinD complex, which translate into reduced replication of beta cells (30).

The loss of *Ezh2* in beta cells of young mice results in premature induction p16Ink4a, which in turn blocks of beta cell replication and regeneration (20). These studies demonstrated the key role of *Ezh2* in maintaining repression of p16Ink4a in islets from young mice. Several studies have shown that the levels of Ezh2 declines with aging and islets from aged mice have reduced amounts of Ezh2 compared to islets from young mice (20, 31). This suggested that the limiting amounts of Ezh2 in aged islets could be insufficient in maintaining the repression of *Ink4a* resulting in de-repression of p16Ink4a expression. We hypothesized that replenishing *Ezh2* expression in islets of aged mice could reverse the de-repression of the *Ink4a* locus. Repression of p16Ink4a expression could then in turn rejuvenate the replicative capacity of beta cells and

could facilitate beta cell expansion of aged islet.

Here we report the generation and analysis of transgenic mice in which *Ezh2* can be conditional expressed in pancreatic beta cells. Conditional activation of *Ezh2* in young adult mice was sufficient to stimulate beta cell replication and regeneration through repression of p16Ink4a locus. However, induction of *Ezh2* in beta cells of aged mice failed to repress p16Ink4a and rejuvenate the capacity for replication and regeneration of beta cells. ChIP analysis indicated that despite increased expression of Ezh2, binding of Ezh2 was prevented due to the presence of TrxG complex along the *Ink4a* locus. Combining the knockdown of MLL1, a major enzymatic component of the TrxG complex with conditional activation of *Ezh2* was sufficient to repress p16Ink4a and promote increased beta cell replication. The elements of the PcG-TrxG-p16Ink4a pathway are highly conserved in human beta cells, indicating that reprogramming of the p16Ink4a chromatin epigenome by modulating PcG-TrxG could potentially be an effective strategy to replenish the deficit of beta cells in diabetic patients. Furthermore, this combined approach to knockdown *MLL1* with overexpression of *Ezh2* also resulted in increased proliferation of mouse embryonic fibroblasts (MEFs) suggesting that reversing PcG-TrxG dependent repression of p16Ink4a could be a general mechanism that can be utilized in regenerative therapy.

Results

Inducible beta cell specific *Ezh2* expression in vivo promotes age-dependent proliferation

Prior studies have shown that *Ezh2* mRNA and protein levels in islet beta cells decline with advancing age in mice and humans (20, 32). In aging mouse islets, decreased Ezh2 levels correlate with reduced association of Ezh2 and histone H3K27me3 levels at the *Ink4a* locus, accompanied by increased levels of H3K4me3 and p16Ink4a mRNA expression (19, 20). In islets from humans with advancing age, we observed similar changes (Supplementary Figure 1-S1A-C). Thus, evolutionarily conserved mechanisms linked to Ezh2 reduction may constrain beta-cell proliferation with age. To test the possibility that conditional *Ezh2* activation might be sufficient to rejuvenate islet beta cell proliferation, we constructed transgenic mouse lines permitting conditional *Ezh2* re-expression in aged mouse beta cells. First we created mice harboring a bi-directional tetracycline responsive element (TRE) that controlled expression of a transgene encoding myc-tagged *Ezh2* (*Myc-Ezh2TG*, abbreviated as EzTG) and beta-galactosidase (b-Gal; Figure 1-1A-B; see Methods). Intercrosses of EzTG mice with a mouse line expressing reverse tetracycline trans-activator (rtTA) in beta cells directed from rat insulin promoter elements (*RIP-rtTA*; (33), generated bi-transgenic progeny (*RIP-rtTA; EzTG*, abbreviated as bEzTG). Exposure of bEzTG mice to doxycycline (Dox) for one week induced expression of *EzTG* mRNA and protein (Figure 1-1C and J) and b-gal (Figure 1-1D) in pancreatic beta cells. By contrast, bEzTG mice without Dox exposure (-Dox control, Figure 1-1D, 1-1F, 1-2E), or single *RIP-rtTA* or *EzTG* transgenic mice exposed to Dox did not increase *EzTG* expression levels or b-Gal (data not shown). Thus, construction of bEzTG mice permitted studies of conditional *Ezh2* induction in islet beta cells.

To investigate whether *Ezh2* re-expression was sufficient to stimulate adult beta cell proliferation, we exposed 2-month-old bEzTG mice to Dox, when endogenous beta cell Ezh2

levels are nearly undetectable (20, 32). In bEzTG mice exposed to Dox for 1 week (Figure 1-1 E), we observed a 100% increase of the proliferation antigen Ki67 in islet Insulin+ cells by immunostaining, compared to littermate controls without Dox exposure (Figures 1-1F-G). Exposure to Dox for 2 weeks (see Methods) led to a 50% increase of beta cell mass as assessed by morphometry (Figure 1-1H). Immunohistochemistry revealed a striking reduction of p16Ink4a in islet beta cells from Dox-exposed bEzTg mice compared to controls (Figure 1-1I), a change confirmed by islet western blotting (Figure 1-1J). Collectively, these data suggest that re-expression of *Ezh2* in young adult beta cells is sufficient to reduce p16Ink4a and promote beta cell proliferation.

Diabetes incidence increases with aging (34) but the basis for this observation is unclear. Thus, we studied whether bEzTG induction led to similar beta cell phenotypes in mice with more advanced age. By 8 months of age, prior studies suggest that islets lose their capacity to proliferate beta cells to compensate for increased metabolic demands (13). In contrast to our findings with two-month-old bEzTG mice, exposure of 8 month-old bEzTG mice to Dox clearly induced *EzTG* mRNA and increased protein levels were evident, however, no detectably increase beta cell Ki67 staining or beta cell mass was observed (Figure 1-2A-C). Furthermore, p16Ink4a levels were unaltered (Figure 1-2D-E). Together, these data reveal that age-dependent mechanisms restrict the capacity of Ezh2 to repress beta cell p16Ink4a expression and induce proliferation. Increased Ezh2 levels have been associated with beta cell regeneration (20), suggesting *Ezh2* might play a key regulatory role in the beta cell regenerative response. We considered whether *EzTG* induction would promote beta cell regeneration in aged mice. To test whether age-dependent beta-cell regeneration reflects restrictions of metabolic need, we studied

bEzTG mice following beta cell injury from streptozotocin (STZ) injection. To test if *Ezh2* induction stimulated this regeneration, we injected 2 month-old bEzTG mice with STZ then exposed them to Dox to induce Ezh2 (Figure 1-2F). Quantification of the Ki67+ beta cells 7 days after STZ challenge accompanied by Dox revealed a 2-fold increase in proliferation compared to STZ-challenged bEzTG controls not exposed to Dox (Figure 1-2G-H). Thus, re-expression of *Ezh2* in young adult mice can stimulate regeneration after beta cell destruction. By contrast, a similar experimental strategy did not increase Ki67 labeling in 8 month-old bEzTG mice injected with STZ (Figure 1-2I-J). Together, these findings reveal a distinctive age-dependent beta cell response to *Ezh2* re-expression, and suggest that *Ezh2*-independent regulatory mechanisms accrue in aging to restrict the regenerative capacity of beta cells.

Ezh2 recruitment to the *Ink4a* locus in islets is age-dependent

Based on resistance of islet beta cell proliferation to *Ezh2* induction in aged bEzTG mice, we postulated that Ezh2 association at loci like *Ink4a* might be restricted by age. To test this possibility, we performed ChIP to assess the *Ink4a* locus in 2-month-old and 8-month-old bEzTG mice, respectively. As expected, ChIP revealed increased Ezh2 association at the *Ink4a* in islets from Dox-exposed 2-month-old bEzTG mice, compared to islets from control bEzTG mice not exposed to Dox (Fig 3A). Increased histone H3K27me3 levels at *Ink4a* accompanied this Ezh2 increase (Figure 1-3B). Consistent with prior studies, we also measured increased association of the PRC2 complex protein EED, the PRC1 protein Bmi-1, and ubiquitinated H2A at *Ink4a* locus in islets from Dox-exposed 2-month-old bEzTG mice (Supplementary Figure 1-S2A-C). By contrast, we failed to detect increased Ezh2, EED, Bmi-1 or H3K27me3 at *Ink4a* locus in islets

from Dox-exposed 8-month-old bEzTG mice (Figure 1-3F-G, Supplementary Figure 1-S2E-G), despite increased *Ezh2* transgene expression (Figure 1-2E). Thus, the *Ink4a* locus was resistant to Ezh2 association and *Ezh2*-dependent chromatin changes in aged islets.

The association of H3K4 methyltransferase MLL1 with the *Ink4a* locus increases in aged islets and could antagonizes association of Ezh2 (19). ChIP analysis revealed relatively low levels of MLL1 and H3K4me3 association at *Ink4a* locus in islets from 2-month mice and treatment of Dox did not alter this association. In contrast, 8-month-old control and bEzTG mice displayed binding of MLL1 and enrichment of H3K4me3 at the *Ink4a* locus. These observations led us to hypothesize that increased MLL1 and/or H3K4me3 levels at *Ink4a* locus in aged islet could impair association of Ezh2 with that locus and prevent repression of p16Ink4a.

MLL1 occupancy at the *Ink4a* locus restricts Ezh2 recruitment in aged islets

We postulated that disrupting MLL1 association with the *Ink4a* locus might permit Ezh2 recruitment to that locus in aging beta cells. To test this possibility, we used siRNA to knock down *MLL1* levels in cultured islets from 8-month-old bEzTG mice (see Methods). To enhance detection of beta cells in cultured islets, we used nuclear Pdx1 immunostaining to quantify beta cells that incorporated BrdU. Following exposure of islets to Dox, quantification of BrdU+ Pdx1+ cells revealed that, like in vivo, Ezh2 induction was insufficient to increase beta cell proliferation (Figure 1-4A-B). Knockdown of *MLL1* alone without *Ezh2* induction led to a modest reduction of p16Ink4a and increase in proliferation, but the combination of *Ezh2* induction and *MLL1* siRNA led to a 2.5 fold increase in BrdU incorporation (Figure 1-4A-B).

Consistent with these data, qRT-PCR and western blotting studies demonstrated that *Ink4a* mRNA and protein levels were clearly reduced by the combination of *Ezh2* induction and *MLL1* siRNA in 8 month-old bEzTG islets (Figure 1-4C-D). In these Dox-exposed bEzTG islets, ChIP analysis revealed that simultaneous *Ezh2* induction and *MLL1* knockdown led to increased Ezh2 association and H3K27m3 levels at *Ink4a* locus, accompanied by reduced MLL1 and H3K4me3 levels (Figure 1-4E-H). Together, these data support our view that the MLL1 protein complex restricts association of Ezh2 with *Ink4a* locus in aged islets.

Targeting JmjD3 enhances Ezh2-induced beta cell replication in aged islets

MLL proteins interact with histone H3K27 demethylases (35, 36), including UTX, UTY and JmjD3 (37). JmjD3 associated with established PcG targets, including *Ink4a*, and regulates H3K27me3 levels (38, 39). However, it remains unclear if MLL associates with JmjD3 in beta cells. We observed a modest increase in H3K27me3 at the *Ink4a* locus and reduced *Ink4a* mRNA levels in 8-month-old bEzTG islets with *MLL1* knockdown (Figure 1-4C, 1-4F), consistent with the possibility that H3K27 demethylase attenuation accompanying TrxG complex disruption in this setting might regulate p16Ink4a expression. To identify proteins associated with MLL1 in the TrxG complex, we performed MLL1 immunoprecipitation (IP) in mouse insulinoma Min6 cells. MLL1 IP revealed MLL1 association with JmjD3, along with another core component of the TrxG complex, RbBP5 (Figure 1-5A). Thus, we postulated that JmjD3 association with the TrxG complex associated with the *Ink4a* locus might increase with age. In human and mouse islets, we found that *JmjD3* mRNA levels increased with age (Figure 1-5C and data not shown). Consistent with this finding, ChIP analysis with JmjD3 antibody revealed increased association

with age of JmjD3 at the *Ink4a* locus of mouse and human islets (Figure 1-5B, 1-5D). Thus, increased JmjD3 demethylase association accompanies reduced H3K27me3 levels at the *Ink4a* locus in aging islets. To test the possibility that *JmjD3* might regulate induced Ezh2 activity in islets, we used siRNA targeting to knock down *JmjD3* in aged bEzTG islets. Following exposure of 8-month-old bEzTG islets to siRNA targeting *JmjD3* and Dox to induce *Ezh2*, immunostaining of Ki67 and Pdx1 demonstrated a 5-fold increase in beta cell proliferation (Figure 1-5E-F). This effect on beta cell proliferation is similar to that observed following simultaneous knockdown of *MLL1* and *Ezh2* induction in these islets. Similar to *MLL1* knockdown experiments, ChIP analysis revealed that simultaneous *Ezh2* induction and *JmjD3* knockdown led to increased Ezh2 association and H3K27m3 levels at *Ink4a*, accompanied by reduced MLL1 and H3K4me3 levels (Figure 1-4G-I, Supplementary Figure 1-S3A-B) Together, our findings suggest a 2-step mechanism of p16Ink4a-mediated aging that includes attenuation of PcG protein levels and accumulation of TrxG complexes including JmjD3 at the *Ink4a* chromatin in aging beta cells.

Dominant function of TrxG at the *Ink4a* locus is general to aging cells

We speculated that the regulation of *Ink4a* by PcG and TrxG proteins might be a general mechanism mediating p16Ink4a-based replicative senescence. To test this, we studied mouse embryonic fibroblasts (MEFS); like islet beta cells, aging MEFs, accumulate p16Ink4a (31). In serially-passaged C57BL/6 MEFs, levels of Ezh2 declined whereas *Ink4a* mRNA and protein levels increased, changes accompanied by reduced proliferation quantified by Ki67 immunodetection (Figure 1-S4 A-B). Like in pancreatic beta cells, Ezh2 and H3K27me3 levels

at the *Ink4a* locus decreased in MEFs from high passage, while MLL1 and H3K4me3 levels increased, confirming prior studies (Figure 1-S4 C-G; (31)). To test whether TrxG restricts Ezh2-dependent p16Ink4a repression in aged MEFs, we transfected MEFs at later passage with DNA vectors permitting myc-Ezh2 misexpression plasmid, MLL1 siRNA-mediated knockdown, or both. Consistent with the results in pancreatic beta cells, we observed that simultaneous *MLL1* knockdown and *Ezh2* induction increased fibroblast proliferation, accompanied by a 40% reduction of *Ink4a* mRNA levels. (Figure 1-6A-C). *MLL1* knockdown alone slightly enhanced BrdU incorporation, while *Ezh2* induction alone did not detectably alter BrdU labeling (Figure 1-6B). Taken together, these data support our findings and conclusions from studies of pancreatic beta cells and suggest that a sequence of TrxG and PcG protein regulation of the *Ink4a* to mediate proliferative decline is a general mechanism in senescing cells. Replicative rejuvenation may require a 2-step approach including dissociation of TrxG proteins and replenishment of the PcG proteins at the *Ink4a* chromatin.

Discussion

The mechanisms that regulate beta cell replication have attracted significant attention due to its importance in the restoration of beta cell mass in diabetic patients. The frequency of beta cell replication is high in the postnatal period in mice and humans but rapidly declines thereafter (11, 12). Previous work from our laboratories showed that during this period, polycomb genes bind and repress *Ink4a* to promote beta cell replication (19). Subsequent to the postnatal replicative period, polycomb genes dissociate from the *Ink4a* locus resulting in derepression of p16Ink4a and a decline in beta cell replication (13). The decline in the frequency of beta cell replication in adulthood overlapped with reduced expression levels of polycomb gene Ezh2 suggesting that

loss of Ezh2 expression could account for dissociation of Ezh2 from the *Ink4a* locus and the decline in beta cell replication.

We used bEzTG mice to offset the loss of endogenous Ezh2 levels and uncovered new insights into plasticity of beta cell regeneration in adulthood. In young adulthood, activation of Ezh2 expression in beta cells was sufficient to repress p16Ink4a and increase replication of beta cells. These results indicate that there is a period in young adulthood in which manipulation by PcG proteins can repress p16Ink4a resulting in beta cell replication. It is also worth noting that metabolic changes such as pregnancy and obesity within this period can result in adaptive expansion of beta cell mass to overcome insulin resistance (13, 40). Aged mice, on the other hand, were resistant to adaptive changes in beta cell mass and replenishing Ezh2 alone in aged mice was not sufficient to repress the *Ink4a* locus. Thus, although beta cell replication declines after the neonatal beta cell expansion, the cells retain the capacity for replication for a period before entering replicative sensecense. The fact that chromatin changes of the *Ink4a* locus accurately reflect the capacity of beta cell replication reaffirms the strategy of manipulating p16Ink4a expression for beta cell regeneration.

Recent work by Chen et al. (2012) has shown that signaling through the PDGF receptor pathway regulates the proliferation of beta cells with aging. During postnatal and early adult life, PDGF signaling functions to maintain high Ezh2 levels allowing for the expansion of beta cells. With aging, the levels of PDGF receptor in beta cells decline, resulting in a concomitant decrease of Ezh2 expression, which in turn leads to reduced proliferative capacity of beta cells (32). This

first phase of beta cell proliferation is thus sensitive to the levels of Ezh2 and signals that induce Ezh2 expression. However, later, as the levels of PDGF signaling and Ezh2 decline, there is a concomitant increase in the recruitment of MLL1-JmjD3 complex at the *Ink4a* locus. This is also accompanied by a build-up of JmjD3 levels in the beta cells. These events lead to decay of adaptive expansion of beta cells with aging. Our data from bEzTG mice shows that the p16Ink4a inducing activity of the MLL1-JmjD3 complex is dominant over the p16Ink4a repressive function of Ezh2. It is possible that decay of PDGF signaling with aging, or induction of other cellular signals in an age dependent fashion facilitates this phase of reduced beta cell proliferation governed by MLL1-JmjD3 complex. This phase is refractory to manipulations in Ezh2 levels, unless combined with loss of MLL1-JmjD3 complex. Identification of the signals that regulate this phase, will, therefore be a big step towards the development of possible therapeutic strategies towards expansion of beta cell mass.

METHODS

pBi-G-Myc-Ezh2 construct

pBI-G tet vector is used to express Ezh2 which contain a bidirectional tet-responsive promoter. The vector contain tet-responsive element (TRE) between 2 minimal CMV promoters that control the expression of b-galactosidase and the gene of interest. The plasmid is cloned as followed:

A mouse Ezh2 full length cDNA fragment was obtained by BssHII digestion, blunt-ended with T4 DNA Polymerase, followed by a NotI digestion from the parental plasmid from ATCC clone (Image clone ID: 6817366). This sequence was inserted into pcDNA3-Myc vector in frame with Myc-tag at N terminal, with the vector treated with EcoRI digestion, blunt-ended with T4 DNA Polymerase, then digested with NotI. PcDNA3-Myc vector was generated with Myc tag sequence being inserted into KpnI/BamHI sites. The myc-Ezh2 sequence was then isolated from pcDNA3-MycEzh2 by KpnI digestion, blunt-ended with T4 DNA Polymerase, digested with XhoI and then cloned into pBi-g Tet vector with the vector treated with PstI digestion, blunt-ended with T4 DNA Polymerase and followed with a SalI digestion.

Generating inducible bEzTG mice

A 9Kb DNA fragment that contains LacZ-TRE-MycEzh2 sequences was then obtained by AseI digestion of pBiG-MycEzh2 and injected into F2 donor embryos of C57BL/6 x DBA/2 and FVB/N to generate transgenic animals. Founder mice with the correct insert is then crossed into Rip-rtTA mice to generate inducible bEzTG mice with *Rip-rtTA; EzTG* double transgene.

Mouse husbandry and genotyping

All animal experiments were approved by the Animal Research Committee of the Office for the Protection of Research Subjects at UCLA. Animals were fed ad libitum and kept under a 12 hr light/dark circle. DNA extracted from the tails was used for PCR-based genotyping using standard methods. Primers used for genotyping bEzTG were as follows: Forward-5'-CACGCTGTTTTGACCTCCATAG-3' and Reverse-5'-TGGAAAATCCAAGTCACTGGTG-3'.

Doxycycline administration

Dox is administrated both in drinking water (2g/L) supplemented with sucrose (0.5%) shielded from light in red bottles, and food pellets (200mg/kg, Bio-Serv). Dox food and water are changed every 2-3 days. Gender matched littermates with *Rip-rtTA; EzTG* genotype on normal diet and drinking water are used as controls. For proliferation index analysis, pancreata are harvested after 2 weeks of Dox treatment. For beta cell mass analysis, pancreata are harvested after one month feeding procedure with Dox treatment on week 1 and 3, normal chow and water for week 2 and 4 (Figure 1-1A).

Regeneration studies

Single dose of 90mg/kg body weight streptozotocin (STZ; Sigma-Aldrich) was prepared fresh in 0.1 M citrate buffer (pH=4.5), injected intraperitoneally to the relative animals age groups. STZ

treated animals are exposed to Dox or control diet/water immediately after the injection for 1 week before the pancreata were harvest for proliferation index analysis.

Southern Blotting

Genomic DNA from control and transgenic mice were digested with PvuII, transferred to nylon membrane and incubate with Myc-Ezh2 full-length probe. PcDNA-Myc-Ezh2 is digested with KpnI/Xhol, 2.6kb length Myc-Ezh2 sequence is purified from the gel, used as a template to generate the probe. Probe preparation and detection is performed using DIG high primer DNA labeling and Detection Starter Kit (Roche), according to manufacture's instructions.

LacZ staining

LacZ staining of isolated islets was performed in islets from bEzTG and littermate control mice one week after Dox exposure. In brief, freshly isolated islets, were fixed at 4°C in 4% paraformaldehyde, 0.1% Triton X-100 in 100 mM PBS for 10 minutes, followed by three washes of cold PBS with 0.1% Triton X-100, and 2-hr incubation in PBS solution containing 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$, and 1 mg/ml X-gal at 4°C. After staining, images were collected with a dissecting microscope equipped with a Zeiss camera.

RNA isolation, RT-PCR and real-time qPCR

RNA from Islets is isolated with the RNeasy Mini/Micro kit (Qiagen) according to manufacturers' instructions. 200ng-1ug RNA is used for preparation of cDNA using Superscript III Reverse Transcriptase (Invitrogen) with the oligodT primers. The cDNA was then analyzed with regular PCR, or Real-time quantitative PCR (qPCR). Real-time qPCR was performed using ABI7900HT (Applied Biosystems) with initial denaturation at 95^0C for 20 seconds, followed by 50 cycles of 94^0C for 1 second and 60^0C for 20 seconds, then continued with a dissociation stage. The expression levels of each transcript were normalized to the housekeeping gene CyclophilinA (PPIA). Each real-time PCR experiment shown is a representative from at least three independent experiments; for each experiment, islets were pooled from three to four mice per specified group.

For lists of RT-PCR and real-time qRT-PCR primers used for mRNA expression analysis, see supplementary table 1 and 2.

Immunoprecipitation and Western blotting

2.5 mg. of anti-Mll1 antibody (or control IgG) was used for each immunoprecipitation. Antibodies were immobilized to Protein G Sepharose (Millipore) for 2-3 hours and washed with cold non-denaturing buffer (20 mM Tris-HCl pH=8, 137 mM NaCl, 10% glycerol, 1% NP-0, 2mM EDTA). The antibody-coated beads were then incubated with Min6 cell extracts prepared in non-denaturing buffer overnight at 4°C on a rotator. Beads were washed 3 times in ice-cold non-denaturing buffer and eluted in 1X SDS-PAGE gel loading buffer. The immunoprecipitates were analyzed by Western blotting with antibodies against JmjD3 (Millipore 07-1434), RbBP5 (Bethyl Laboratories A300-109A) and control Dnmt3a (Imgenex IMG-268A).

For western blotting, isolated Islets were lysed in cell dissociation buffer (Invitrogen) supplemented with protease inhibitors cocktail (Calbiochem). Protein concentration is measure with Dc protein essay (BIoRad) and 10ug-30ug of protein samples (or immunoprecipitates) are loaded to each lane of the SDS-PAGE gel as previously described (8). Target protein level is quantified against house keeping protein b-Tublin using imageJ. Each western shown is a representative from three independent experiments using different islet preparations.

Immunohistochemistry

Immnohistochemistry is performed as previously described (41). Pancreatic tissue was fixed in 4% formaldehyde, and processed for paraffin embedding. 5-μm Sections were deparaffinized and rehydrated, then subjected to antigen unmasking and permeabilization. The slides are then blocked with 3% BSA in TBST (0.1% Tween 20) and incubated with Primary antibody overnight at 4°C. Primary antibodies used for study included: guinea pig anti-insulin (1:400; Dako), mouse anti–Ki-67 (1:40; BD Biosciences), mouse anti-BrdU (1:2, GE Healthcare/Amersham RPN-202), mouse anti-p16 (diluted 1:250; Santa Cruz sc-1661). The slides are then washed and incubated with Donkey- and goat-derived secondary antibodies conjugated to FITC or Cy3 (1:200, Jackson ImmunoResearch Laboratories). Slides were mounted with Vectashield with DAPI (Vector Labs) prior to imaging using a Leica DM6000 microscope and Openlab software (Improvision). The immunofluorescence data presented are representatives of at least five animals per group in each group.

For analysis of the beta cell mass and proliferation index, 3-6 mice were analyzed in each group. For beta cell mass, pancreatic sections were stained for insulin and scanned using a Leica DM6000 microscope. Montage of the pancreatic area by DAPI and beta cell area by insulin staining were made with ImageJ. The cross-sectional areas of pancreata and beta cells were determined using an automated program on Openlab software. Proliferation index was calculated using Ki67 (or BrdU) staining as a marker for proliferation, insulin (or Pdx1) staining for beta cells.

Chromatic IP (ChIP) protocol

ChIP was performed as previously descrbied (42). In brief, isolated or cultured islets (150-200 islets per ChIP) were treated with 1% paraformaldehyde at room temperature for 10 minutes for crosslinking. The islets were washed and suspended in ChIP lysis buffer with protease inhibitors and sonicated with Biorupter (Diagenode). The chromatin was then pre-cleared with Protein A/G beads. Meanwhile, 10ul of Protein A or G beads were incubated with 2-5 mg of the antibody [anti-H3K27me3 (Diagenode), anti Ub-H2A (Millipore 05-678; the antibody is an IgM and therefore requires a bridging antibody, Millipore 12-488), anti-H3K4me3 (Diagenode), anti Bmi-1 (Millipore 05-637), anti Ezh2 (Clone AC22, Cell Signaling #3147), anti MLL1 (Millipore 05-765), anti JmjD3 (Millipore 07-1534) or normal mouse IgG as a control, at 4^{o} C for 2 hours on a rotator. The antibody-bound beads mix is then washed and incubated with pre-cleared Chromatin O/N at 4^{o} C on a rotator. After immunoprecipitation, the chromatin was eluted from the beads, subjected to reverse-crosslinking and the DNA was purified. The ChIPed DNA was quantified as percentage bound/input with real-time PCR, performed using ABI Master mix kit (Life Tech)

and the ABI equipment (Life Tech). Each ChIP experiment was performed 3-5 times, using independent pooled islet isolations from 4-8 mice.

Mouse islet isolation and Culture

Islets were isolated using the Liberase/DNase enzyme mix (Roche Diagnostics) as described (8). In brief, the pancreas is inflated with the enzyme mix through the common bile duct and digested at 37°C. Islets are enriched by gradient and hand picked. 100-200 islets are cultured in easy grip tissue culture dish (Falcon, 35mmX10mm) in Dulbecco's Modified Eagle's Medium (DMEM, Cellgro) supplemented with 10% fetal bovine serum (FBS). Islets cells were transfected after overnight resting in culture using Lipofectamine-2000 (Invitrogen), according to the manufacturer's instructions. The islets were harvested 48 hours after transfection and processed for immunostaining, protein extraction, RNA extraction and ChIP analysis. For proliferation analyses in islets, cultured islets were incubated with BrdU (1mg/ml) and/or Dox (2ug/ml) after transfection 48 hours before they are harvested. The islets are then fixed in 4% paraformaldehyde, embedded in HistoGel (Thermo scientific) and processed for paraffin embedded sections.

Human islets studies

Human islets were isolated as previously described (32). Equilibrated human islets from differente age groups ranging from 8 months to 70 years were processed to ChIP and Real-time qRT-PCR analysis similar to the mouse islets. ChIP results in human are normalized to GAPDH

locus. For JmjD3 mRNA and ChIP results (Figure 1-4C-D), samples were grouped into juvenile (<10ys) and adult (10 yrs+). ChIP results for Ezh2 and histones were shown with each age group. qRT-PCR restuls were normalized to b-actin. For Ezh2 mRNA, samples were grouped into young (<20 yrs) and old (20 yrs+).

MEFs culture

MEFs are isolated from E13.5 C57BL/6 embryos as previously described (ref). In brief, embryos were harvested in cold sterile PBS, embryo head and organs (everything inside body cavity) were carefully removed. The remainder tissue was minced and trypsinized to make single cell suspension. Trypsin was quenched by adding MEFs medium (DMEM with 10%FBS). The cells were centrifuged and cultured in dishes coated with gelatin, these cells are P0. Early passage MEFs were passaged every 2-3 days using trypsin, however, the proliferation slows down over a few passages and can be passed every 4-5 days or weekly towards the later passages.

Statistics

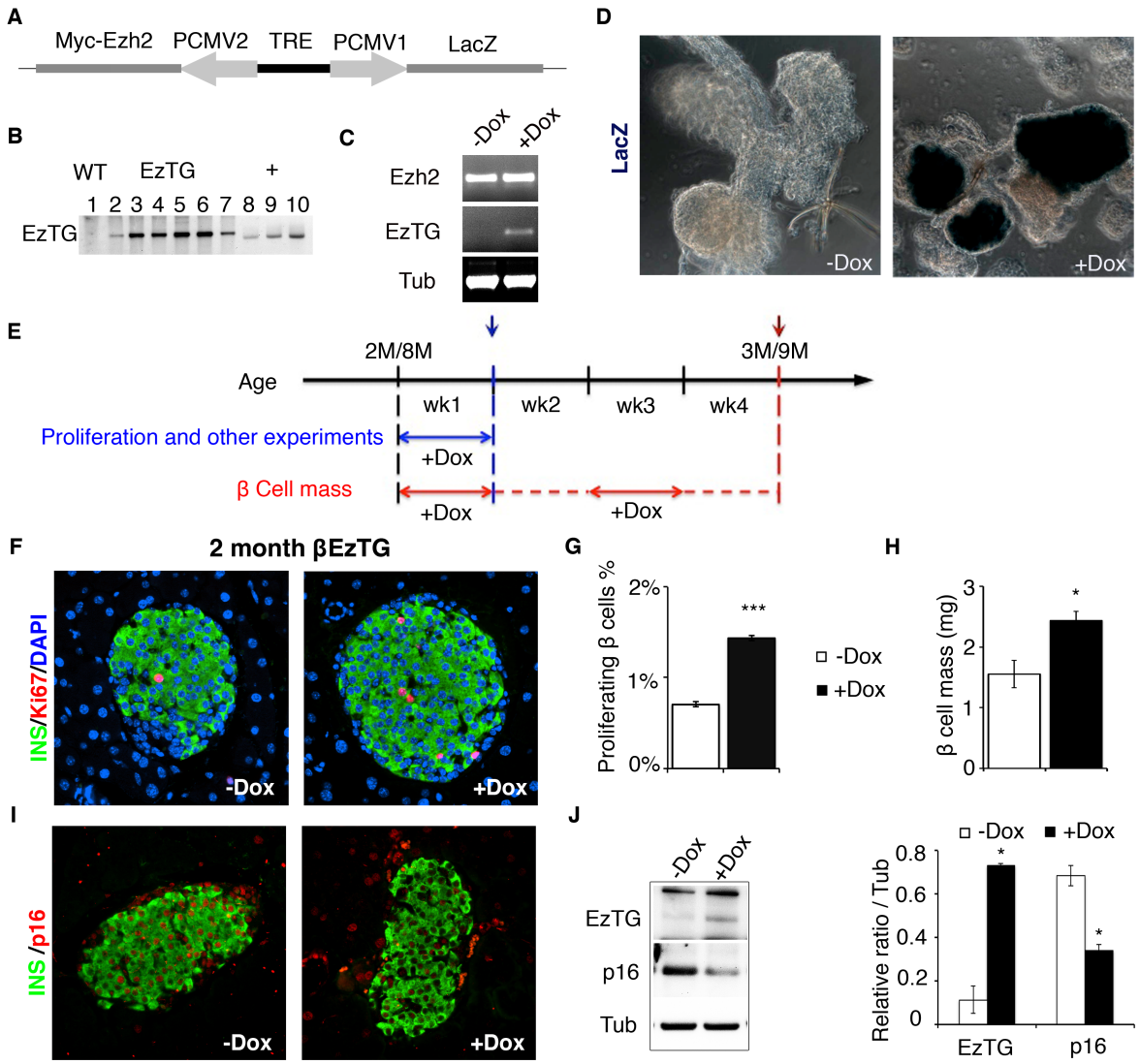
All data are expressed as means ± SEM. Mean and SEM values were calculated from at least triplicates of a representative experiment. Statistical significance was determined by an unpaired Student's *t* test. A p value <0.05 indicated statistical significance. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$.

Acknowledgements

This work was supported by grants from NIDDK, Juvenile Diabetes Research Foundation and the Helmsley Trust to AB. We thank Emily Snyder and Lendy Le for technical support.

Author contribution: JZ performed the experiments and analysis of bEZTG mouse and MEFs. SD performed experiments with JmjD3 studies in mouse. HF generated the EzTG construct and EzTG transgenic mouse. SK and SKK planned and performed human studies and critical suggestions to the manuscript. AB and JZ conceived and planned the experiments and interpreted data. AB and JZ wrote the manuscript.

Figure 1-1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Myc-Ezh2
PCMV2
TRE
PCMV1
LacZ
B
WT
EzTG
+
1 2 3 4 5 6 7 8 9 10
EzTG
C
-Dox
+Dox
Ezh2
EzTG
Tub
D
LacZ
-Dox
+Dox
E
2M/8M
3M/9M
Age
wk1
wk2
wk3
wk4
Proliferation and other experiments
+Dox
β Cell mass
+Dox
+Dox
F
2 month βEzTG
INS/Ki67/DAPI
-Dox
+Dox
G
Proliferating β cells %
2%
1%
0%
***
-Dox
+Dox
H
β cell mass (mg)
3
2
1
0
*
I
INS /p16
-Dox
+Dox
J
-Dox
+Dox
EzTG
p16
Tub
-Dox
+Dox
Relative ratio / Tub
0.8
0.6
0.4
0.2
0
*
*
EzTG
p16

Figure 1-1. Conditional mouse model of re-expressed Ezh2 in beta cells is sufficient to promote beta cell replication through repression of p16Ink4a

(**A**) Schematics of the targeting cassette containing LacZ-TRE-Myc-Ezh2TG. (**B**) Southern blotting detecting EzTG integration into the genome (Lane 2 to 7) as compared to the wt control (lane 1), EzTG vectors were used as a positive control (Lane 8 to 10). Expression level of EzTG mRNA (**C**) and b-galactosidase as shown by *LacZ* staining (**D**) in 1-week Dox treated bEzTG islets but not in littermate controls without Dox exposure. (**E**) Dox feeding schemes: bEzTG were treated with 1 week Dox food and drinking water before the pancreata or the islets are harvested for experiments. For measuring beta cell mass, bEzTG are treated with an extended Dox procedure with Dox treatment on week 1 and 3, pancreatic tissue are harvest after 1 month. (**F**, **I**) Pancreatic sections from the control (left panel) and Dox treated (right panel) 2-month bEzTG were immunostained with antibodies to insulin (green), Ki67 (red, **F**), p16 (red, **I**) and DAPI (blue). (**G**) Quantification of the percentages of Ki67 positive beta cells with or without Dox exposure. (**H**) Beta cell mass (mg) measurement. (**J**) Western blotting and quantification of p16Ink4a levels in isolated islets. n=5-6 animals per group. *$p < 0.05$, ***$p < 0.005$.

Figure 1-2

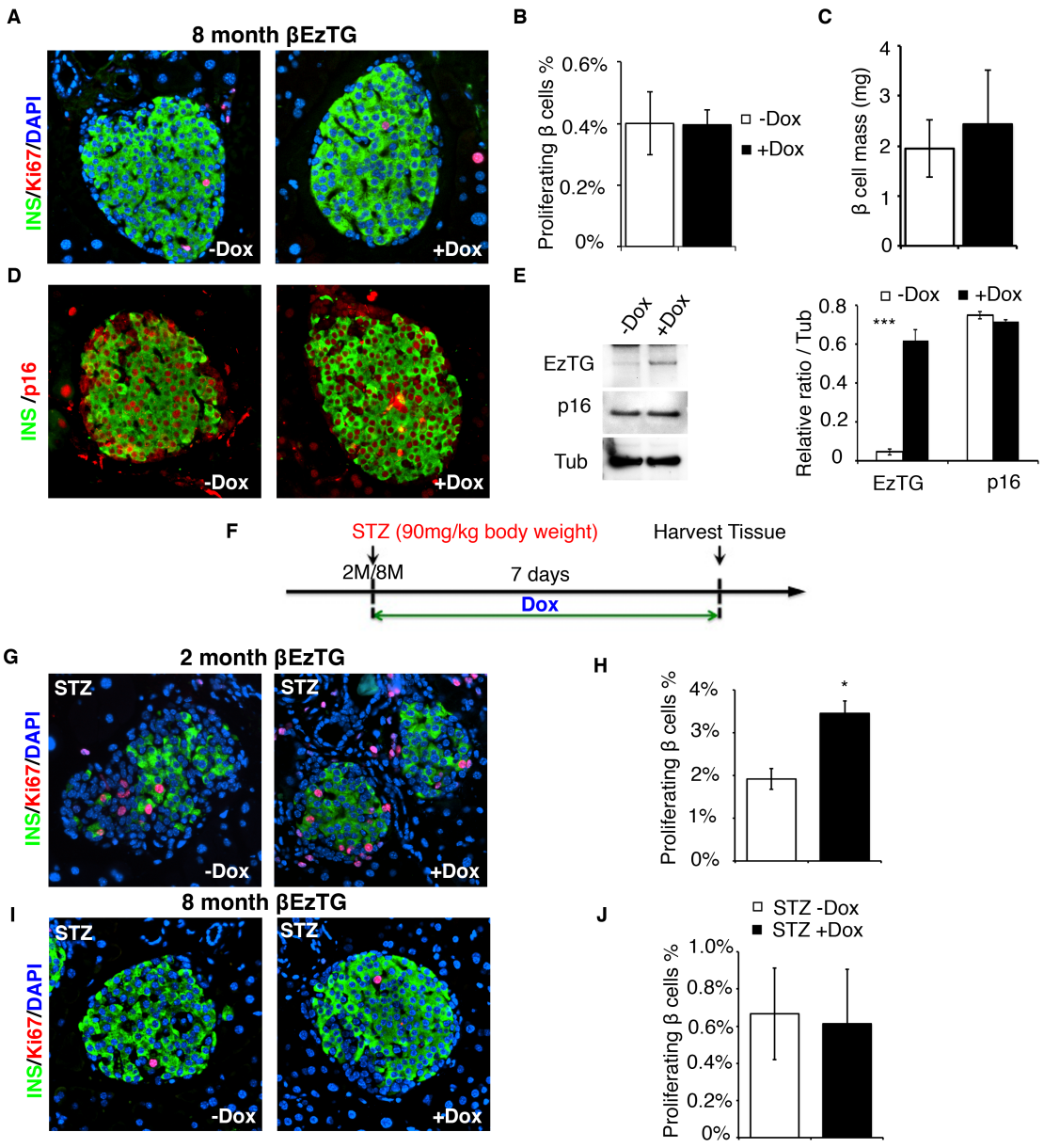

A
8 month βEzTG
INS/Ki67/DAPI
-Dox
+Dox
B
Proliferating β cells %
0.6%
0.4%
0.2%
0%
-Dox
+Dox
C
β cell mass (mg)
4
3
2
1
0
D
INS /p16
-Dox
+Dox
E
-Dox
+Dox
EzTG
p16
Tub
-Dox
+Dox
***
Relative ratio / Tub
0.8
0.6
0.4
0.2
0
EzTG
p16
F
STZ (90mg/kg body weight)
Harvest Tissue
2M/8M
7 days
Dox
G
2 month βEzTG
INS/Ki67/DAPI
STZ
-Dox
+Dox
H
Proliferating β cells %
4%
3%
2%
1%
0%
*
I
8 month βEzTG
INS/Ki67/DAPI
STZ
-Dox
+Dox
J
STZ -Dox
STZ +Dox
Proliferating β cells %
1.0%
0.8%
0.6%
0.4%
0.2%
0%

Figure 1-2. Re-expression of Ezh2 failed to promote beta cell replication or regeneration in 8-month bEzTG

(**A**, **D**) Pancreatic sections from the control (left panel) and Dox treated (right panel) 8-month bEzTG were immunostained with antibodies to insulin (green), Ki67 (red, **A**), p16 (red, **D**) and DAPI (blue). (**B**) Quantification of the percentages of Ki67 positive beta cells with or without Dox exposure. (**C**) Beta cell mass (mg) measurement. (**E**) Western blotting and quantification of p16Ink4a levels in isolated islets. (**F**) STZ and Dox treatment scheme: Dox is administrated after single does STZ (90mg/kg) injection; tissues were harvested 7 days after injection. Immunostaining of to insulin (green), Ki67 (red), DAPI (blue) in pancreatic sections from bEzTG animals 1 week after STZ injection at 2 month (**G**) and 8 month (**I**). Percentages of Ki67 positive beta cells with or without Dox exposure 1 week after STZ treatment are quantified in 2-month (**H**) and 8-month (**J**) age groups. n=3-6 animals per group. *$p < 0.05$, ***$p < 0.005$.

Figure 1-3

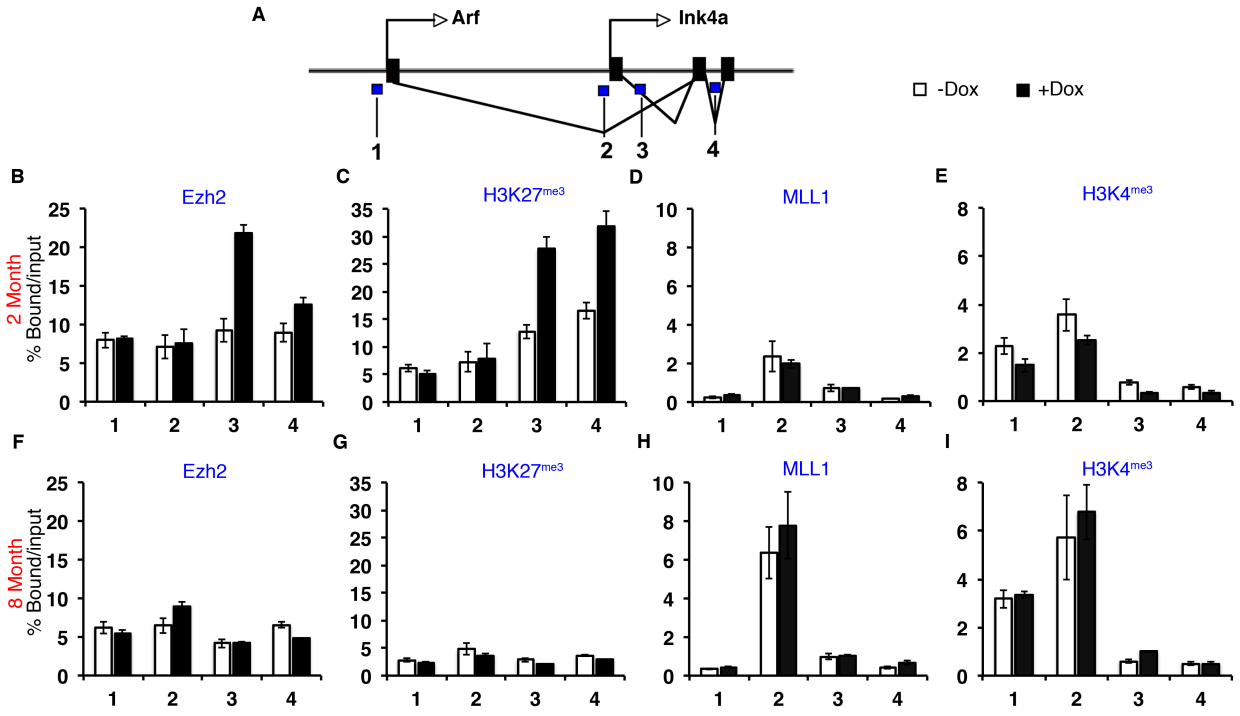


Figure 1-3. Ectopically expressed Ezh2 is recruited to *Ink4a* locus in 2-month bEzTG islets but not 8-month

(**A**) Schematic representation of the *Ink4a* locus, with blue regions marked 1-4 indicating the amplified regions in the ChIP studies. Representative ChIP analysis for the indicated antibody at the *Ink4a* locus in islets isolated from 2 month (**B-E**) and 8 month (**F-I**) old age groups.

Figure 1-4

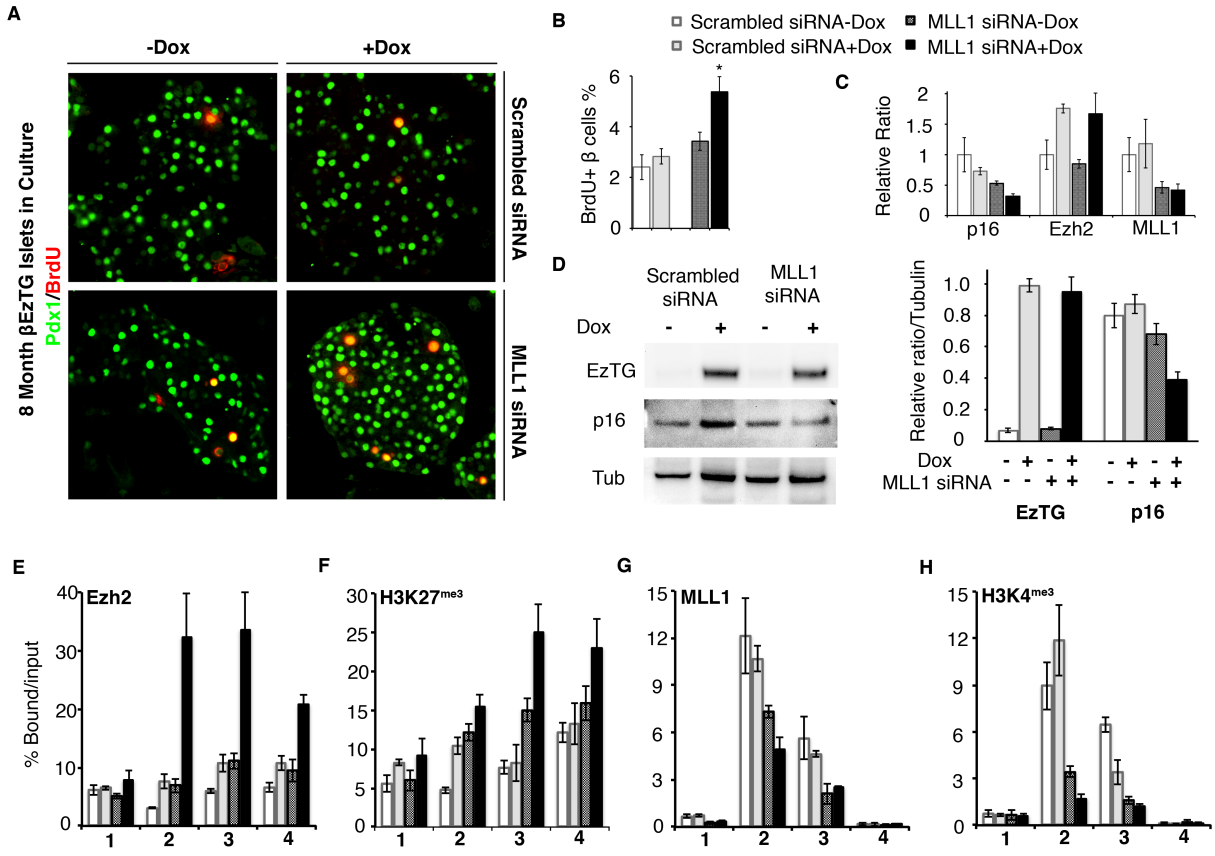
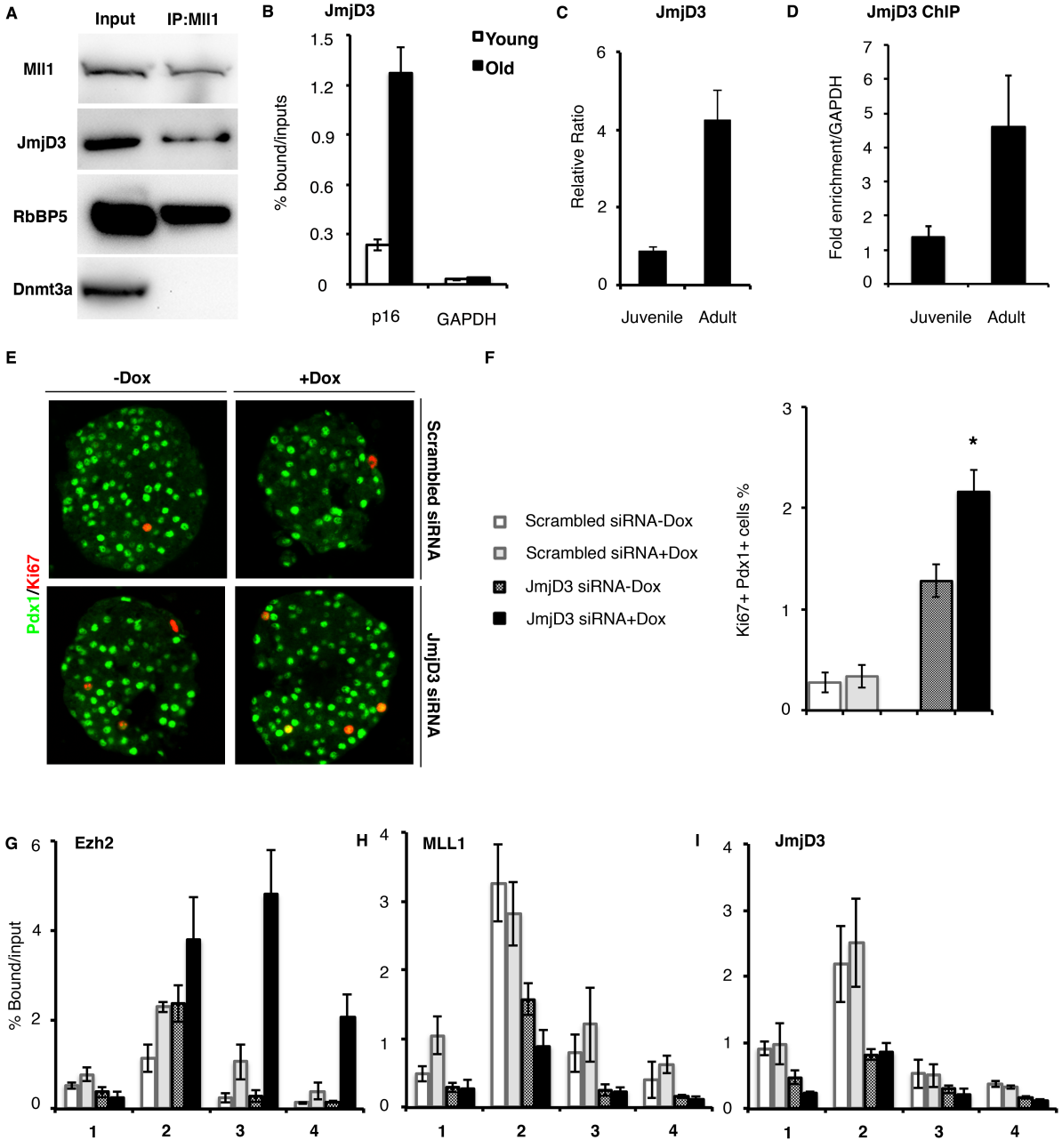

A
-Dox
+Dox
8 Month βEzTG Islets in Culture
Pdx1/BrdU
Scrambled siRNA
MLL1 siRNA
B
Scrambled siRNA-Dox
Scrambled siRNA+Dox
MLL1 siRNA-Dox
MLL1 siRNA+Dox
BrdU+ β cells %
C
Relative Ratio
p16
Ezh2
MLL1
D
Scrambled siRNA
MLL1 siRNA
Dox
EzTG
p16
Tub
Relative ratio/Tubulin
MLL1 siRNA
E
Ezh2
% Bound/input
F
H3K27me3
G
MLL1
H
H3K4me3

Figure 1-4. Disruption of MLL1 binding on the *Ink4a* locus permits Ezh2 recruitment in aged bEzTG islets

(**A**) 8-month-old bEzTG islets are treated with scrambled siRNA (Top left 100pM), Dox (Top right, 1ug/ml), MLL1 siRNA (Bottom left, 100pM) and both Dox and MLL1 siRNA (Bottom right) for 48hrs. BrdU is added in culture 48hr before harvest to measure proliferations. Islets sections are immunostained for BrdU (red) and Pdx1 (Green). (**B**) Percentage of BrdU positive beta cells in each experiment group. (**C**) Real time qRT-PCR of *p16Ink4a*, *Ezh2* and *MLL1* mRNA in each group. (**D**) Western blotting and quantification of EzTG (detected by Myc Ab), p16 and Tub protein levels. (**E-H**) Representative ChIP results for the indicated antibody at the *Ink4a* locus in islets treated with the same experiment conditions as in A. n=3 experiments.

Figure 1-5


A
Input
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Mll1
JmjD3
RbBP5
Dnmt3a
B
JmjD3
Young
Old
% bound/inputs
1.5
1.2
0.9
0.6
0.3
0
p16
GAPDH
C
JmjD3
Relative Ratio
6
4
2
0
Juvenile
Adult
D
JmjD3 ChIP
Fold enrichment/GAPDH
7
6
5
4
3
2
1
0
Juvenile
Adult
E
-Dox
+Dox
Scrambled siRNA
JmjD3 siRNA
Pdx1/Ki67
F
Scrambled siRNA-Dox
Scrambled siRNA+Dox
JmjD3 siRNA-Dox
JmjD3 siRNA+Dox
Ki67+ Pdx1+ cells %
3
2
1
0
*
G
Ezh2
% Bound/input
6
4
2
0
1
2
3
4
H
MLL1
4
3
2
1
0
1
2
3
4
I
JmjD3
4
3
2
1
0
1
2
3
4

Figure 1-5. Disruption of MLL1-JmjD3 complex with JmjD3 siRNA permits Ezh2 recruitment in aged bEzTG islets

(**A**) Cell extracts from Min6 cells were used for IP with anti-MLL1 antibody and analyzed by immunoblotting with MLL1, JmjD3, RbBP5 and DNMT3a (negative control) antibodies. (**B**) ChIP analysis of JmjD3 binding to the *Ink4a* locus in young and old mouse islets. (**C**) *JmjD3* mRNA levels in juvenile (<10 yrs) and adult (10yrs+) human islets as show by quantitative RT-PCR. (**D**) ChIP analysis showing JmjD3 binding to the *Ink4a* locus in human islets (**E**) 8-month-old bEzTG islets are treated with scrambled siRNA (Top left 100pM), Dox (Top right, 1ug/ml), JmjD3 siRNA (Bottom left, 100pM) and both Dox and JmjD3 siRNA (Bottom right) for 48hrs. Islets sections are immunostained for Ki67 (red) and Pdx1 (Green). (**F**) Percentage of Ki67 positive endocrine cells in each experiment group. (**G-I**) Representative ChIP results for the indicated antibody at the *Ink4a* locus in islets treated with the same experiment conditions as in A. n=3 experiments.

Figure 1-6

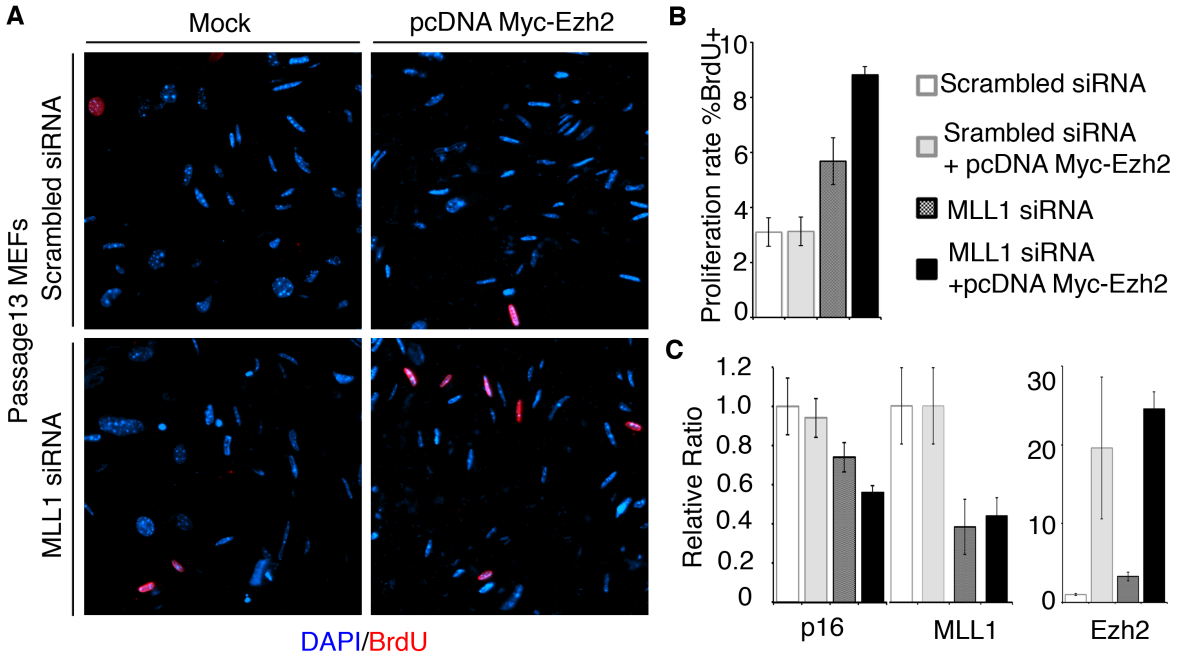

Figure 1-6. Disruption of MLL1 complex on the *Ink4a* locus permits Ezh2 recruitment in later passage MEFs

(**A**) Passage 13 MEFs were transfected with scrambled siRNA (Top left, 100pM), PcDNA Myc-Ezh2 (Top right, 2ug), MLL1 siRNA (Bottom left, 100pM) and both PcDNA Myc-Ezh2 and MLL1 siRNA (Bottom right) for 48hrs. BrdU is added in culture 48hr before immunostaining for BrdU (red) and DAPI (blue). (**B**) Percentage of BrdU positive MEFs in each experiment group. (**C**) Real time qRT-PCR of *p16Ink4a*, *Ezh2* and *MLL1* mRNA in each group. n=3 experiments.

Supplementary Figures

Figure 1-S1

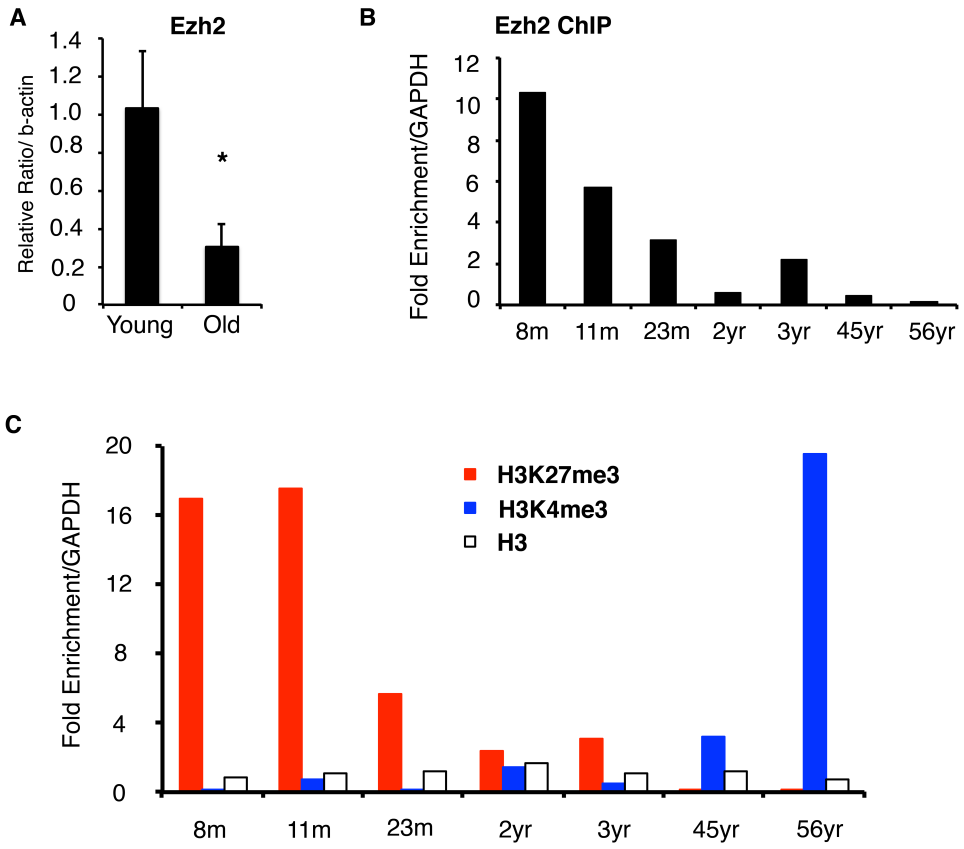

Figure 1-S1. Loss of Ezh2 in aging human islets

(**A**) Ezh2 mRNA level in young (<20 yrs) and old (20 yrs+) human islets. (**B**) ChIP analysis of Ezh2 binding to the *Ink4a* locus in human islets (8 months, 11 months, 23 months, 2 years, 3 years, 45 years and 56 years old). (**C**) ChIP analysis of histone modifications (H3K4me3 in green, H3K27me3 in red) in human islets as in (**B**). *$p < 0.05$

Figure 1-S2

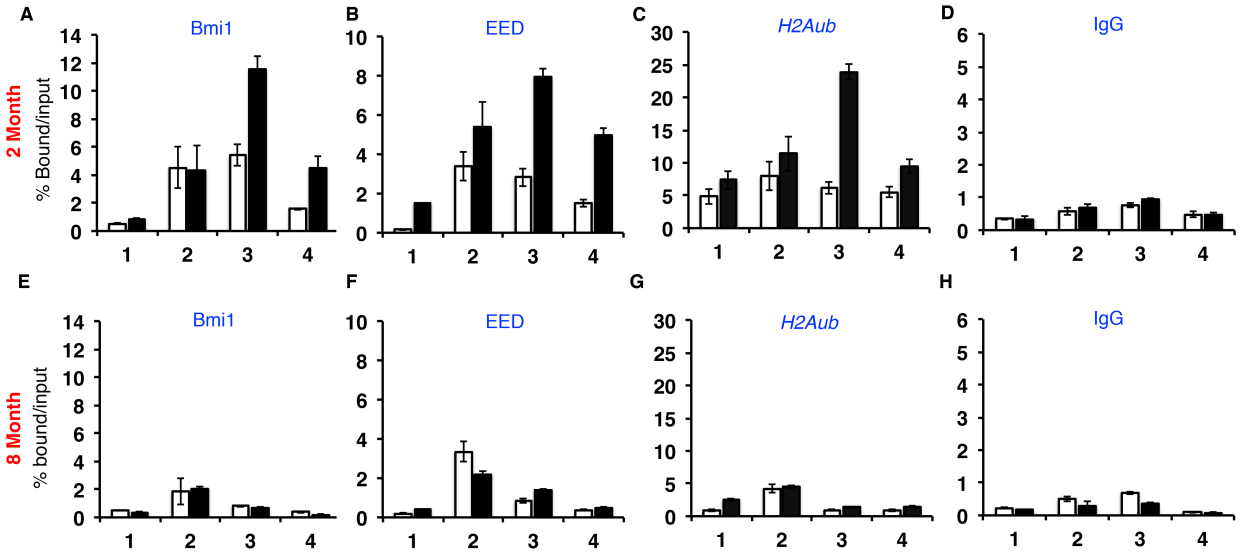

Figure 1-S2. Ectopically expressed Ezh2 recruited other PRC members in 2-month bEzTG islets but not 8-month

Representative ChIP analysis for the indicated antibody at the *Ink4a* locus in islets isolated from 2 month (**A-D**) and 8 month (**E-H**) old age groups.

Figure 1-S3

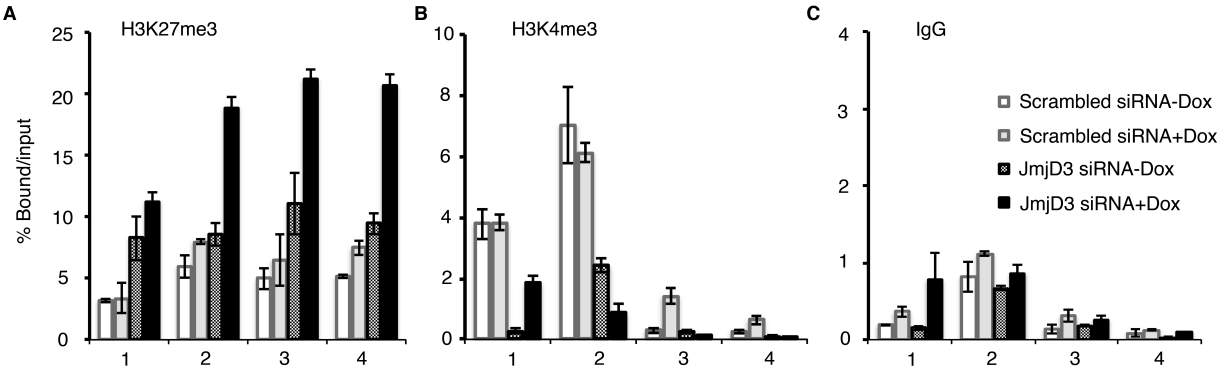

Figure 1-S3. Targeting JmjD3 siRNA altered histone codes at the *Ink4a* locus in aged bEzTG islets

(**A-C**) Representative ChIP results for the indicated antibody at the *Ink4a* locus in islets treated with the same experiment conditions as in A. n=3 experiments.

Figure 1-S4

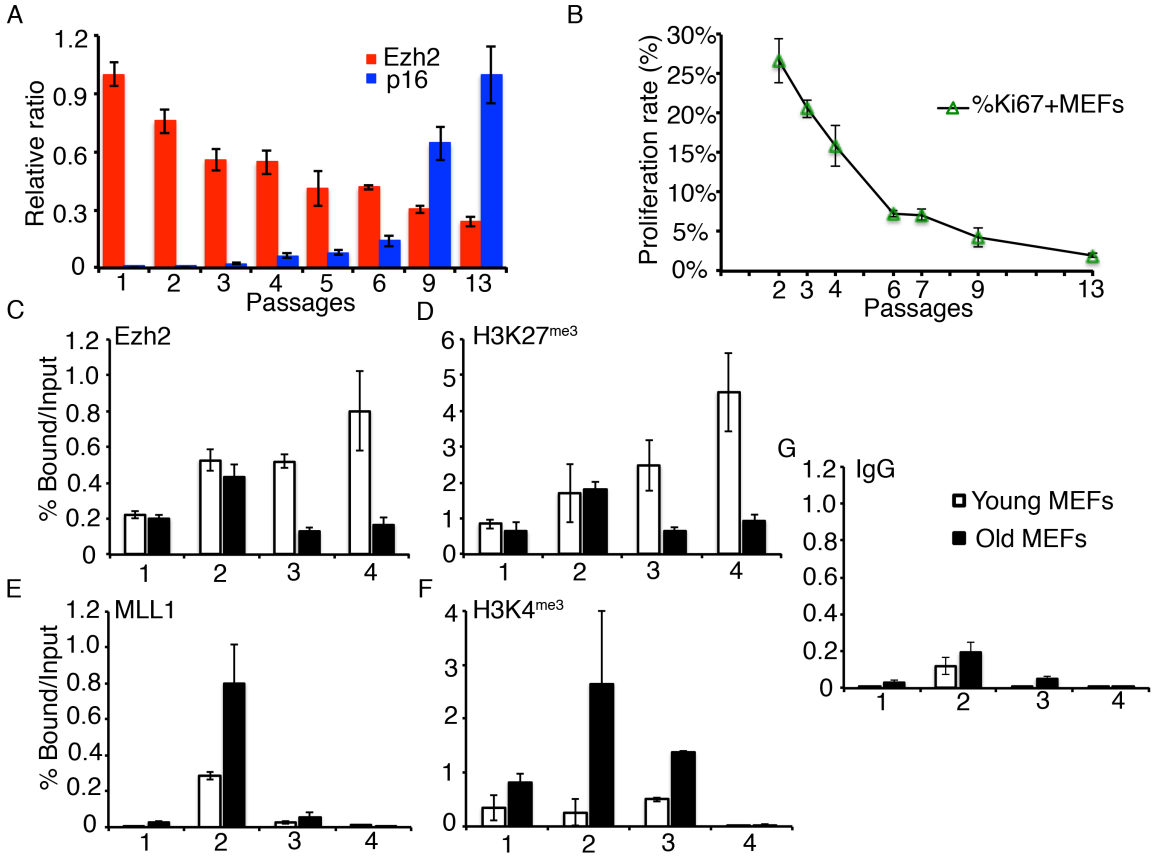

Figure 1-S4. Serial-passaged MEFs are associated with increased p16ink4a regulated by PcG-TrxG on the *Ink4a* locus

(**A**) Real time qRT-PCR showing p16ink4a increases with passages while Ezh2 declines. (**B**) Proliferations deceased as we passaged the MEFs. (**C-G**) ChIP analysis for the indicated antibody at the *Ink4a/Arf* locus in MEFs showing decreased Ezh2 and H3K27me3 and increased MLL1 and H3k4me3 in old MEFs. n=3 experiments.

Supplementary materials

Table 1-S1. RT-PCR primers

Gene	Forward	Reverse
Myc-Ezh2	5'-TTTCTGACGATTGGAACTAAACATAG-3'	5'-TTTCTGACGATTGGAACTAAACATAG-3'
Ezh-2	5'-TGTAGACAGGTGTATGAGTTTAGAG-3'	5'-GGTCAGCAGCTCCACACGTGAGAC-3'
Ink4a	5'-ATCTGGAGCAGCATGGAGTC-3'	5'-CTGAGGCGGATTTAGCTCTG-3'
Tubulin	5'-GTTGGCCAGGCTGGTGTCCAG-3'	5'-CTGTGATGAGCTGCTCAGGGTGG-3'

Table 1-S2. Real-time qRT-PCR primers

Gene	Forward	Reverse
MLL-1	5'- AGCAGCTCTCATTTGCAGGT -3'	5'- ACAACGGCATCATGGAGAAT -3'
Ezh-2	5'-TGGAAGCAGCGGAGGATA-3'	5'-GTCACTGGTGACTGAACACTCC-3'
Ink4a	5'- CCGTGTGCATGACGTGCGGG -3'	5'-GCTGCTACGTGAACGTTGCCC -3'
Cyclophilin	5'-GTTGGCCAGGCTGGTGTCCAG-3'	5'-CTGTGATGAGCTGCTCAGGGTGG-3'

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CHAPTER 2: PLURIPOTENCY FACTOR TBX3 RECRUITS JMJD3 TO DRIVE DIFFERENTIATION OF ENDODERM

Abstract

Stem cell differentiation depends on a complex network of transcriptional factors and chromatin organizations, yet the coordination of these events is poorly understood. Here we show the pluripotency factors team up with chromatin modifying enzymes to drive the expression of a key lineage regulator, *Eomes* during endodermal differentiation from embryonic stem (ES) cells. *Eomes* is maintained in a transcriptionally poised configuration in ES cells, altered binding patterns of ES cell factors Sox2, Nanog, Oct4 and Tbx3 associate with histone demethylase Jmjd3 at the enhancer element of the *Eomes* locus permits enhancer activation. Our results show that changes in pluripotency factors are the early determinants to drive stem cell differentiation towards specific lineages.

Introduction

A promising alternative source of pancreatic beta cells is differentiation from embryonic stem cells (1, 2). The attempts to bring endoderm-derived stem cell therapy to a state of effectiveness have been hampered by the paucity of knowledge of the underlying mechanisms, as each step in the step-wise lineage specification process is characterized by signaling pathways and downstream transcriptional networks regulated by epigenetic mechanisms (3). During development, epiblast cells ingress through the anterior segment of the primitive streak where they undergo an epithelial-to-mesenchymal transition to give rise to mesoderm and definitive endoderm (4). Genetic studies have shown that endoderm differentiation is regulated by lineage transcription factors including *Eomesodermin* (*Eomes*), *Sox17, Mixl1* and *Foxa2* (5-9). These genetic studies provided a dynamic picture of endoderm formation in the embryos and have guided studies of directed differentiation of ES cells.

ES cells maintain the ability to self-renew while retaining the ability to differentiate into any mature cell lineages. Differentiation of ES cells involves switching molecular circuits that maintain self-renewal and pluripotency to those that are associated with activation of lineage-specification and, ultimately maintenance of cell-type specific gene expression. There is growing recognition that genes that regulate ES cell pluripotency are also involved in germ layer lineage commitment (10-12). Over-expression of *Oct4* or *Nanog* individually mediates mesoendodermal lineage differentiation (13, 14); elevated Sox2 levels trigger differentiation towards a neural ectoderm lineage (15); induced Tbx3 expression induces an endoderm lineage (16). These findings suggest that pluripotency factors are not only involved in suppressing differentiation

when expressed at a level within a narrow window, but also in programming lineage commitment during early differentiation. Studies of over-expression and loss of function models suggested that each factor has the ability to affect cell fate, precisely how each lineage is orchestrated by the combined events of pluripotency factors is unclear.

Genome-wide studies have shown that the promoters of lineage regulators in ES cells are enriched for histone modifications associated both with gene activation such as trimethylation of histone3 lysine4 (H3K4me3) and with polycomb-mediated repression such as trimethylation of histone3 lysine27 (H3K27me3) (17-21). This bivalent chromatin profile keeps lineage regulators poised for rapid activation. In response to differentiation cues, the bivalent domains are resolved with the removal of H3K27me3 and activation of lineage-specific developmental genes. The Jumonji domain-containing proteins, Utx (Kdm6a) and Jmjd3 (Kdm6b), function as H3K27me3 demethylases (22-27). How the pluripotency factors act in concert with histone demethylases to remodel the chromatin and elicit lineage gene expression in driving fate decision remains an interesting question to be unraveled.

Here we used mESCs as an *in vitro* model to study the mechanism of pluripotency factors in regulating the switch from the pluripotency state to committed germ layers. Differentiation into definitive endoderm is of particular interest to us, we have identified a specific combination of pluripotency genes whose binding pattern is associated with endoderm and mesoderm lineage factors, characterized by decreased Nanog and Sox2, increased recruitment of Tbx3 and sustained Oct4 binding during embryonic body (EB) formation. Eomes is one of the lineage

specification factors, further investigation indicates *Eomes* is maintained in a transcriptionally poised configuration in ES cells. During early steps of differentiation, the T-box protein Tbx3 and the demethylase Jmjd3 bound to the *Eomes* enhancer to promote enhancer activation. Our results show the switch in binding patterns of pluripotency factors facilitates recruitment of an epigenetic modifier, Jmjd3, to drive stem cell differentiation towards definitive endoderm lineage.

Results

Genome-wide screen revealed changes in key pluripotency factor binding patterns during mES to EB formation were associated with endoderm differentiation

To investigate whether lineage commitment is determined by altering pluripotency gene binding patterns on a genome-wide scale, we performed chromatin immunoprecipitations followed by high throughput sequencing (ChIP-seq) of four key pluripotency factors namely: Oct4, Sox2, Nanog and Tbx3 in the mouse ES cell differentiation model. Leukemia inhibitory factor (LIF) was removed from culture medium to allow ES cells to aggregate and differentiate to form EBs (Figure 2-1A). EBs after 2 days of LIF withdrawal and ES cells were harvested and subjected to ChIP-Seq. ChIP-seq signals were analyzed and clustered based on the changes in binding to gene loci (See methods). We observed a sharp decrease in Sox2 binding, followed by Nanog, which is consistent with the previous finding that expression levels of Sox2 decrease strongly after day 1, Nanog after day 2 (Figure 2-1B) (14). Oct4 and Tbx3 bindings are sustained or moderately increased, consistent with the expression data that Oct4 and Tbx3 levels start to decrease after 3 days of LIF withdrawal (Figure 2-1B) (14, 16). Our results showed a slight increase in overall

binding of Oct4, possibly skewed due to the peak-calling threshold in different samples. Group 5 is of particular interest to us not only because of the striking changes in pluripotency factors, but also the strong acquisition of Tbx3 binding in differentiation. Tbx3 has been previously suggested to promote endoderm fate decision (16). Further investigation of group 5 genes revealed lineage factors involved in regulating early endoderm formation: *Eomes, Gsc and Gata6*.

To establish the functional correlation of the pluripotency factors and endoderm lineage commitment, we used a step-wise differentiation protocol to direct mES cells towards definitive endodermal fate (28, 29). Detailed expression analysis for transcription factors known to be involved in endodermal specification revealed that *Eomes* was induced in the early steps of differentiation and did not require Activin A for its induction (Figure 2-1C). The induction of other endodermal–specific transcription factors tested, including *Sox17, Gsc, Mixl1, Gata6 and Foxa2,* was not observed during early stages and Activin A treatment was required for transcriptional activation these genes (Figure 2-1C). The rapid induction of *Eomes* during the earliest steps of ES cell differentiation suggested that *Eomes* might be a direct target of pluripotency factor induced lineage transition.

Tbx3 is recruited to *Eomes* locus during differentiation

To identify the binding sites of pluripotency factors on the *Eomes* locus during differentiation, we mapped the extended *Eomes* locus in genome viewer. Previous studies showed that Nanog, Oct4 and Sox2 bound to the upstream *Eomes* enhancer region in human ES, and the binding of

Nanog and Oct4 persisted for at least 24 hr after differentiation (14). Our analysis confirmed that Sox2, Oct4 were bound to the *Eomes* promoter, Nanog bound to Eomes promoter and enhancer in mouse ES cells. In differentiated EBs, binding of Sox2 dissociated from the locus, binding of Oct4 persisted, while the binding of Nanog decreased (Figure 2-1A, 2-2A). Notably, we identified Tbx3 bound to the *Eomes* promoter and enhancer during differentiation (Figure 2-1A, 2-2A). ChIP analysis confirmed Tbx3 binding to the Eomes enhancer region in the differentiated cells, and decreased Nanog binding to the enhancer region during differentiation (Figure 2-2B). These data suggest that the acquisition of Tbx3 on *Eomes* might be an early signaling to endoderm fate and that Tbx3 might be involved in gene activation.

Tbx3 recruits Jmjd3 to activate *Eomes* transcription

To measure Tbx3 induced epigenetic changes at the Eomes enhancer region, we assessed changes in signature histone modifications during differentiation. Monomethylation of histone3 lysine 4 (H3K4me1), and acetylation of histone3 lysine 27 (H3K27ac) are typically present in developmental regulators that are inactive in ES cells but poised for activation during lineage specification (30-32). The marked change in H3K27ac on the *Eomes* enhancer during differentiation is followed by a significant decrease in the H3K27me3 mark (Figure 2-3A-C) consistent with activation of this enhancer element (30). We next investigated whether the enzymes responsible for removal of H3K27 methylation were recruited to the *Eomes* enhancer during differentiation. Transcription of *Jmjd3* was increased during differentiation (Figure 2-3F). ChIP analysis showed increased Jmjd3 binding at the *Eomes* enhancer in differentiated cells compared to ES cells (Figure 2-3D). These results indicated that increased occupancy of Jmjd3

corresponded with decrease in methylation of H3K27 at the *Eomes* enhancer. The recruitment of Jmjd3 and Tbx3 during the EB differentiation suggested the possibility that these factors were physically associated at the *Eomes* enhancer region. Coimmunoprecipitation of EB using Jmjd3 antibody subjected to western blotting using the Tbx3 antibody showed that Tbx3 co-precipitated with Jmjd3 (Figure 2-3E). These analyses suggested that Jmjd3 and Tbx3 were physically associated and co-occupied the *Eomes* enhancer during the early steps of differentiation.

Jmjd3 and Tbx3 are mutually required for induction of *Eomes*

To further investigate whether Jmjd3 and Tbx3 were mutually required for *Eomes* activation, we employed short hairpin RNA (shRNA)-mediated knockdown of *Jmjd3* and *Tbx3* (Supplementary Figure 2-4A-B). ChIP analysis on EBs formed from *Jmjd3*-knockdown ES cells showed that Tbx3 recruitment to the *Eomes* enhancer is prevented (Figure 2-4A). Conversely, Jmjd3 was not bound to the *Eomes* enhancer in EBs formed from *Tbx3*-knockdown ES cells (Figure 2-4B), reinforcing the idea that Tbx3 and Jmjd3 were associated. Unlike wildtype ES cells, *Jmjd3*-null ES cells failed to activate *Eomes* expression during differentiation (Figure 2-4C). Similarly, *Tbx3* knockdown prevented *Eomes* activation during differentiation (Figure 2-4D). These results suggest that both Jmjd3 and Tbx3 binding to the *Eomes* enhancer were required for *Eomes* transcriptional activation.

Discussion

Embryonic stem cells differentiate into a number of cell types, which makes them attractive for cell replacement strategies. How transcriptional factors direct chromatin dynamics to regulate cell fate decisions is often difficult to study in the embryo, and ES cells are a good model system to mimic early differentiation in the embryo. In this study we elucidate the molecular mechanism of pluripotency factors in altering their binding patterns and enlisting histone demethylase to direct stem cells to differentiate towards the definitive endoderm lineage. We have identified that the combination of decline in Nanog and Sox2, sustained Oct4 and increase Tbx3 regulations leads to endoderm lineage commitment. In particular, this pluripotency factor-induced early endoderm lineage specification process signals through activating *Eomes* enhancer. The shift to enrichment of pluripotency factor Tbx3 at the *Eomes* enhancer region during early differentiation facilitates the recruitment of Jmjd3, which then demethylates histone H3K27me3 and triggers a molecular switch from poised to active enhancer. Taken together, we have discovered a molecular mechanism by which the pluripotency factors direct the earliest lineage specification signal.

We show that the pluripotency transcription factors and epigenetic modifications of the chromatin interact in order to implement control pathways that drive differentiation of stem cells towards the definitive endoderm lineage. While we have focused on definitive endoderm specification in this study, other germ layer specification could utilize the pluripotency factor and epigenetic reorganization model. For instance, the *Brachyury* locus could be regulated in a similar fashion as *Eomes* during mesodermal specification. Our Chip-seq data show that early mesodermal factors Brachyury and Tbx6 are mediated by the same pluripotency factor patterns that directed *Eomes* activation during early differentiation, suggesting mesoderm and endoderm

lineages might share a permissive chromatin state before segregation. Analysis of mesoderm specification could shed light on whether the shared chromatin state exists, what factors are involved and at which step of differentiation did segregation of these germ layers happen.

Although modification of a single or combination of pluripotency factors can direct certain lineage commitment, the signals required to differentiate into an effective state is a dynamic and complex programming that call for comprehensive networking of epigenetic reorganization and transcription factors. Our ChIP-seq results confirmed previous results on the lineage specification effects of each factor and provide a dynamic and interconnected map of how these factors change during early differentiation. The binding or a pluripotency factor can be associated with gene silencing as well as gene activation; therefore, our ChIP-seq when combined with epigenome profiling will be more powerful in predicting the blueprints of lineage specification. Similar to the bivalent marks on gene promoters, H3K4me1 mark associates with poised enhancer and the acquisition of H3K27ac marks an active enhancer (32). Recent genome wide studies have suggested that RNA Polymerase (RNAP) II are paused at promoter proximal locations for rapid induction, phosphorylation at Ser 5 is associated with paused RNAP and phosporylation at Ser 2 releases the paused RNAP to elongation (33, 34). We speculate pluripotency factors, aside from the repressive functions in maintaining pluripotency, are also associated with prompting the poised to activated transition on gene loci of lineage factors. Understanding the pluripotency factors-induced lineage decisions in the context of epigenetic profiles on a whole genome scale is crucial to manipulate early lineage segregations.

Figures 2-1

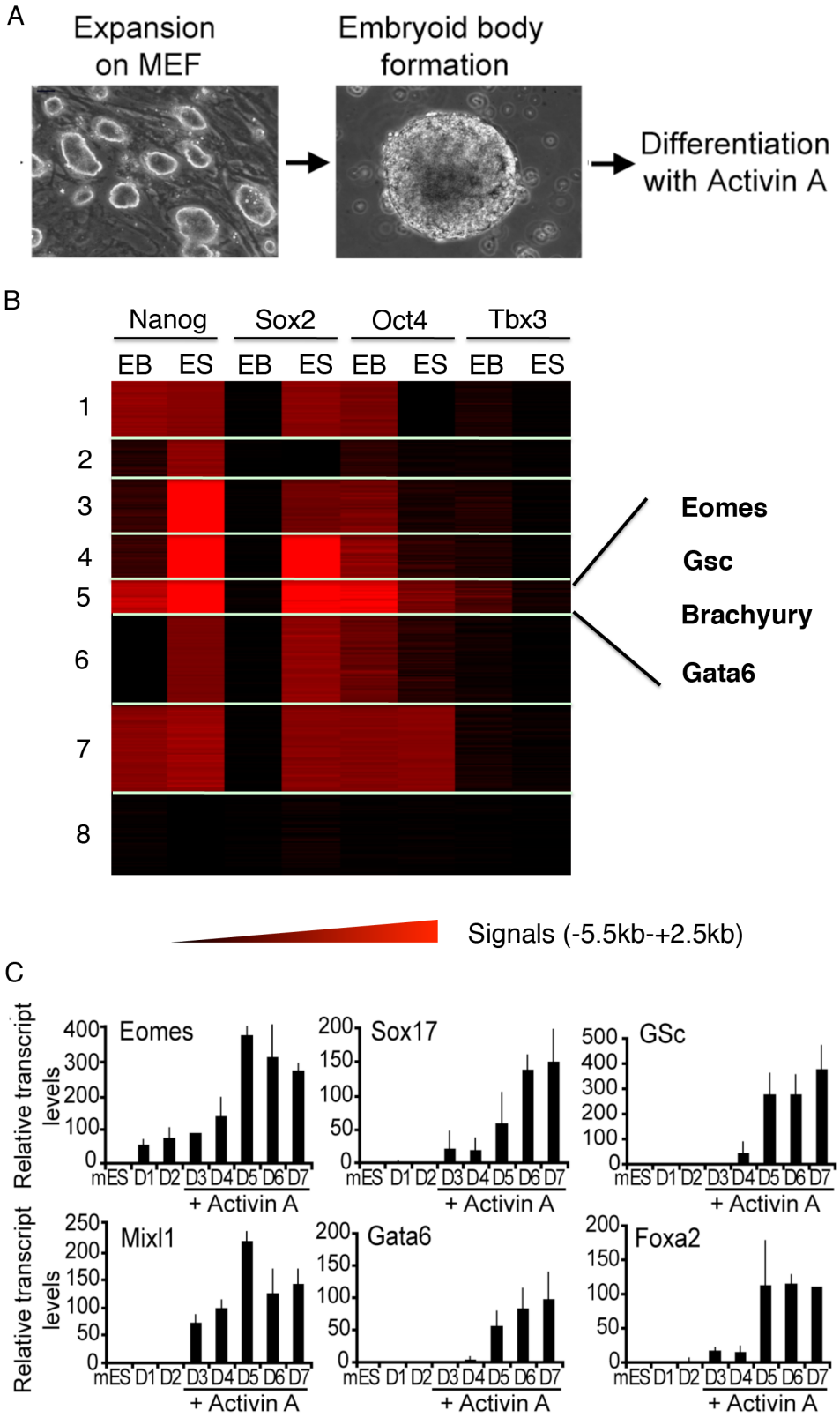
A
Expansion on MEF
Embryoid body formation
Differentiation with Activin A
B
Nanog
Sox2
Oct4
Tbx3
EB ES EB ES EB ES EB ES
1
2
3
4
5
6
7
8
Eomes
Gsc
Brachyury
Gata6
Signals (-5.5kb-+2.5kb)
C
Relative transcript levels
Eomes
Sox17
GSc
Mixl1
Gata6
Foxa2
mES D1 D2 D3 D4 D5 D6 D7
+ Activin A

Figure 2-1. Whole-genome screening of pluripotency factors in lineage determination

(**A**) Scheme of ES differentiation into EBs and endoderm with ActivinA. (**B**) Heatmap of pluripotency factor binding signals in ES cells and 2 day EBs. (**C**) Expression levels of the indicated genes during endoderm differentiation.

Figure 2-2

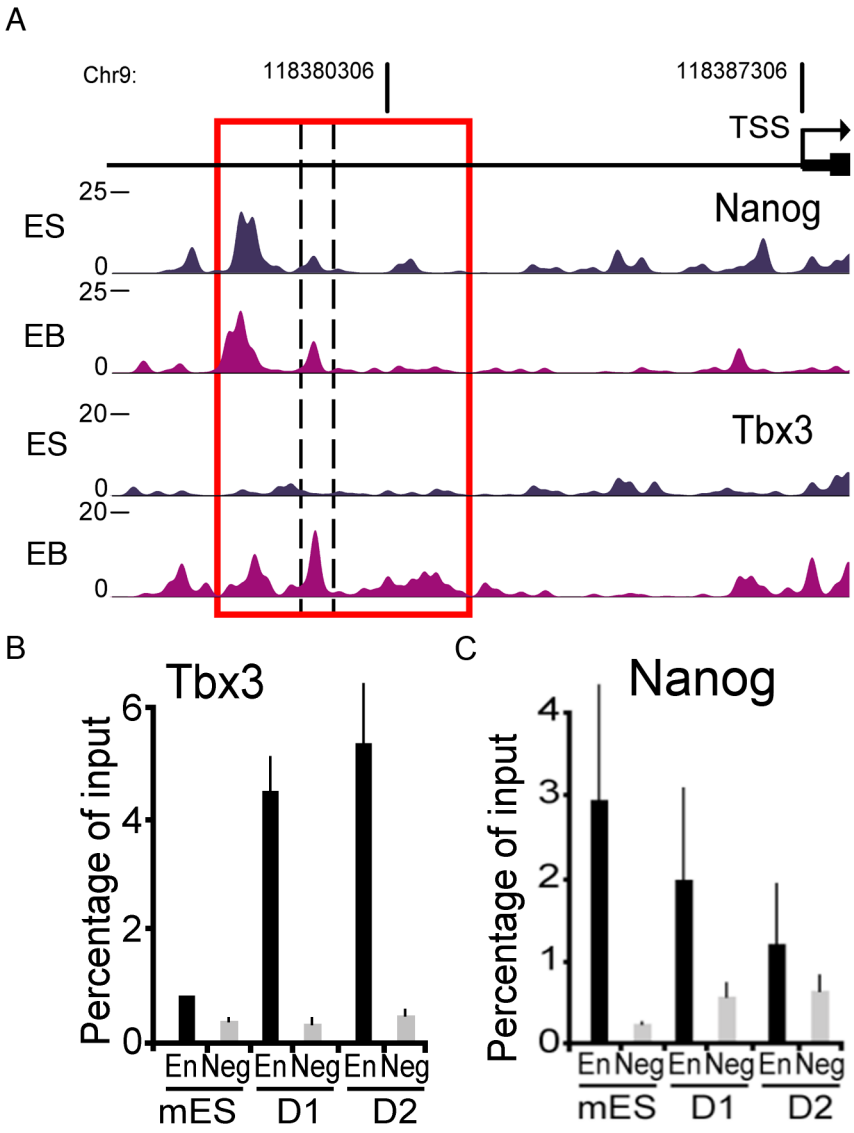

Figure 2-2. Tbx3 is recruited to *Eomes* enhancer region

(**A**) Genome map view of Nanog and Tbx3 binding patterns on *Eomes* locus. (**B**) Tbx3 ChIP in ES cells and EBs at day 1 and day 2 of differentiation. (**C**) Nanog ChIP in mES and EBs as in (**B**).

Figure 2-3

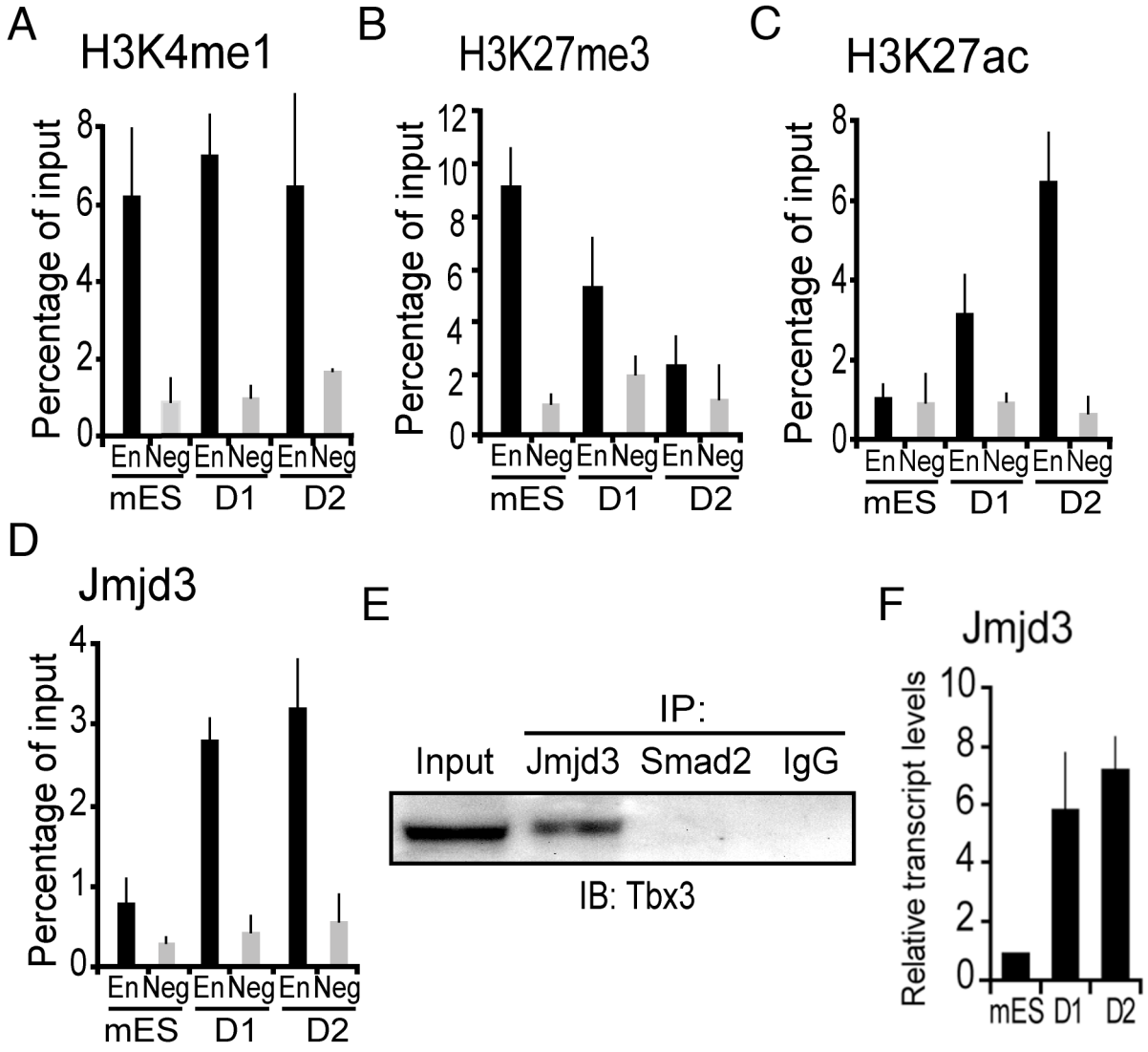

Figure 2-3. Tbx3 recruits Jmjd3 to activate *Eomes* enhancer

(**A-D**) ChIP analysis of indicated protein in ES cells and EBs at day 1 and day 2 of differentiation. (**E**) Jmjd3 IP immunoblot with Tbx3 antibody. (**F**) Jmjd3 mRNA levels during ES differentiation.

Figure 2-4

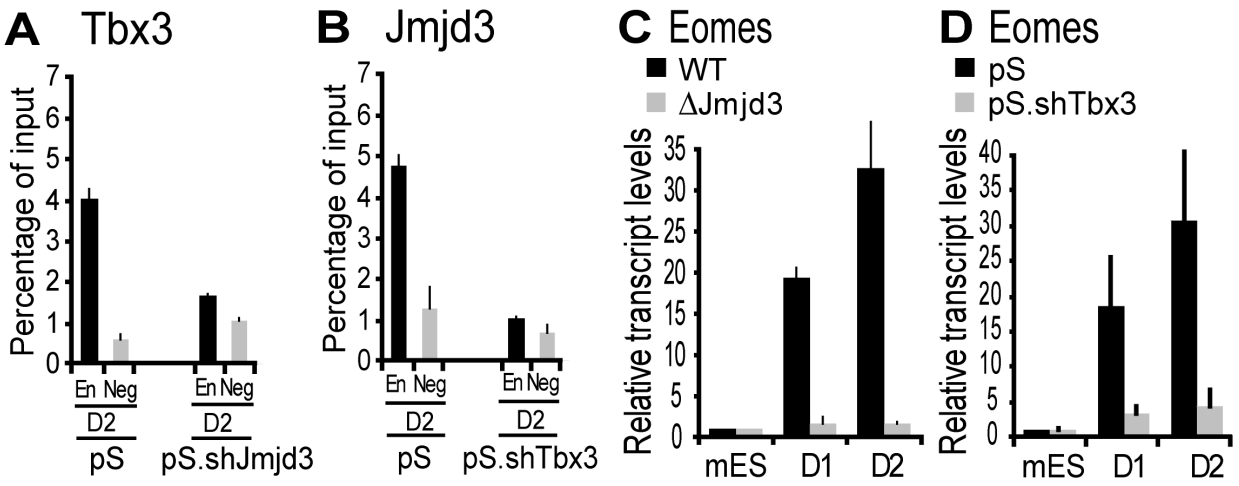

Figure 2-4. Tbx3 and Jmjd3 are mutually required for Eomes activation during differentiation

(**A**) Tbx3 ChIP in control (pS) and Jmjd3 knockdown (pS.shJmjd3) samples. (**B**) Jmjd3 ChIP in control (pS) and Tbx3 knockdown (pS.shTbx3) samples. (**C-D**) *Eomes* expression in Jmjd3 null ES cell line (**C**) and Tbx knockdown (**D**) during differentiation.

Methods and materials

Mouse and human ES cell culture, differentiation and RNA interference

All cells were cultured at 37^0C with 5% CO_2. Primary mouse embryonic fibroblasts (MEFs) were derived from E13.5 CF1 mouse embryos and mitotically inactivated using g-irradiation (UCLA). Sox17.GFP knock-in ES cells and Jmjd3-null ES cells (KO-2211 from Knock-out Mouse Project Repository (KOMP); this cell line carries deleted Kdm6b allele: $Kdm6b^{tm1(KOMP)Wtsi}$) were maintained on irradiated MEFs in mouse ES medium containing Knock-out Dulbecco's modified Eagle's medium (KO-DMEM; GIBCO), supplemented with 10% heat-inactivated fetal bovine serum (FBS, Hyclone), 0.055 mM β-mercaptoethanol (GIBCO), 2 mM L-glutamine (GIBCO), 0.1 mM nonessential amino acid (GIBCO), 5000 U/mL penicillin-streptomycin (GIBCO), 15mM HEPES (GIBCO) and 1000 U/mL LIF (Millipore/Chemicon). Jmjd3–null ES cells were selected for homozygous colonies in aforementioned medium using a highly concentrated G418 antibiotic (2.5 mg/mL) as previously described in (37). RNA interference was done as previously described (36) using puromycin (Sigma) selection at 1 μg/mL. All transfections were performed using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. shRNA-expressing pSuperpuro (Oligoengine) constructs were generated according to manufacturer's instructions. shRNA sequences are provided in Table 2-1. For differentiation, mES cells were initially cultured in non-adherent conditions at a density of 1 x 10^4 cells/mL for two days in supplemented KO-DMEM except in the absence of LIF. The formed embryoid bodies were then differentiated into definitive endoderm using 5 day treatment of 20 ng/mL Activin A (R&D) in 1:1 KO-DMEM/Neurobasal medium (GIBCO) supplemented with N2 (GIBCO), B27 (GIBCO), 0.055 mM β-mercaptoethanol, 2 mM L-glutamine, 0.1 mM nonessential amino acid, 5000 U/mL

penicillin-streptomycin, 15mM HEPES, and 20 ng/mL EGF (R&D) modified from (28, 29). The formation of GFP^+ cells was monitored using Leica DMLRE2 microscope. Human ES cells (HSF1) and human induced pluripotency stem cells (hiPS2) were maintained in human ES medium containing DMEM/F12 (GIBCO) supplemented with 2 mM L-glutamine, 0.1 mM nonessential amino acids, 5000 U/mL penicillin-streptomycin, 15 mM HEPES, 20% knockout serum replacement (Invitrogen), and 10 ng/mL basic FGF (R&D systems) as previously described (38). Hek293T cells were maintained in DMEM (GIBCO) supplemented with 10% heat-inactivated FBS (GIBCO), 2 mM L-glutamine and 5000 U/mL penicillin/streptomycin. HSF1 and hiPS2 were differentiated to endoderm using 100ng/mL Activin A for 5 days in DMEM/F12 with 2 mM L-glutamine, 15mM HEPES, 5000 U/mL penicillin-streptomycin and supplemented with FBS (0% from Day0-1, 1% from Day1-2, 2% from Day2-5) (39-41).

ChIP-seq

ChIPseq libraries were generated from the ChIP and input samples, using the Illumina **ChIP-Seq DNA Sample Prep Kit** according to manufacturer's instructions. These libraries were then subjected to sequencing using Illumina HiSeq2000. Base-calling and QC statistics were generated using Illumina software. All unique sequences were mapped by Bowtie (42) to the mouse mm9 genome. The number of unique reads was calculated in bins across the genome. Bins containing statistically significant Chip-seq enrichment were identified by comparison to a Poissonian background model. WIG files were generated for all ChIP-seq data sets. These files were subsequently used for visualization purposes and for obtaining average signal profiles.

Heatmap data were the log scale of the sequencing signal around -5.5kb to +2.5kb and clustered using cluster 3.0.

ChIP-qPCR

Chromatin immunoprecipitation (ChIP) was performed according to (43) with minor modifications. Briefly, single cell suspension of $1x10^5$ cells was cross-linked with 1% formaldehyde and incubated for 15 minutes at room temperature. Formaldehyde was deactivated by adding glycine to a final concentration of 125 mM. Cells were lysed using 100 μL lysis buffer consisted of 50 mM Tris-HCl pH8.0, 10 mM EDTA, 1% SDS, supplemented with 1x complete proteinase inhibitors (Calbiochem) and sonicated to yield DNA fragments with an average size of 500 bp using Bioruptor (Diagenode). A total of 1-2 μg of antibody was bound to 20 μL Protein-A/G Dynabeads (Invitrogen), depending on the antibody isotype, for 2 hours at 4^0C. One fourth fraction of the sonicated chromatin was incubated overnight at 4^0C with the antibody-bead complexes in total volume of 200 μL in RIPA buffer containing 10 mM Tris-HCl pH8.0, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS and 0.1% Na-deoxycholate supplemented with 1x complete proteinase inhibitors. After 4x washing with RIPA buffer and 1x with TE buffer, chromatin was eluted, followed by reverse-crosslinking at 68^0C for 4 hours with vigorous agitation in the presence of Proteinase K (Sigma). The DNA fragments were then purified using phenol-chloroform extraction and ethanol precipitation. ChIP-qPCR signals were calculated as percentage of input. Data are representative of two to three independent experiments, and error bars indicate standard error of the technical replicates. The primer sequences and primary and secondary antibodies used are in Table 2-2 and Table 2-3 respectively.

Coimmunoprecipitation and western blotting

For each coimmunoprecipitation, cells were lysed using 300 mL non-denaturing lysis buffer consisted of 50 mM Tris-HCl pH8.0, 150 mM NaCl, 2 mM EDTA, 1% NP40, supplemented with 1x complete proteinase inhibitors for 20 minutes at 4^0C. Coimmunoprecipitation was performed by incubation of the protein extract with 3-5 mg primary antibody at 4^0C overnight, followed by incubation with 60 mL Protein-A/G Dynabeads for 5 hours at 4^0C. Each coimmunoprecipitation was washed 3 times, each for 5 minutes, with the lysis buffer. The captured proteins were reduced in sample buffer (Invitrogen) and denatured by boiling for 10 minutes. Proteins were run on Nupage gels (Invitrogen). Gels were blotted onto PVDF membrane (Amersham), then stained with primary antibody as indicated, followed by secondary conjugated to horseradish peroxidase (HRP). The antibodies used are listed in Table 2-3. Membranes were developed using ECL western blotting detection system (Millipore) according to manufacturer's instructions.

RNA extraction, reverse-transcription and real-time quantitative PCR

Total RNAs were extracted using Absolute RNA Microprep Kit (Agilent Technology) or RNeasy Plus Mini Kit (Qiagen) and digested with RNase-free DNase (Fermentas) to eliminate DNA. The quality and quantity of the extracted RNA were assessed using Nanodrop (Spectrophotometers) and Bioanalyzer (Agilent Technologies). 0.5 μg of total RNA from each sample was denatured at 65^0C and then reverse transcribed using SuperscriptIII reverse transcriptase (Invitrogen) at 50^0C for 1 hour. Real-time quantitative PCR (qPCR) was performed

using ABI7900HT (Applied Biosystems) with initial denaturation at 95^0C for 20 seconds, followed by 50 cycles of 94^0C for 1 second and 60^0C for 20 seconds, then continued with a dissociation stage. Each qPCR reaction contained 1x FastSybrGreen Mix (Applied Biosystems), 500 nM of each primer and 100 ng cDNA. Relative expression of a certain target gene was determined using the comparative cycle threshold (Ct) method, where the amount of target cDNA was normalized to the internal control, cylophilin cDNA, and expressed as relative to the baseline normal control cDNA ($2^{-\Delta\Delta Ct}$). Data are representative of two to three independent experiments, and error bars indicate standard error of the replicates. Data are statistically analyzed using Student's t-test followed by Bonferroni correction for multiple comparisons. Primer sequences are included in Table 2-2.

Statistics

Bound sites from the ChIP-seq data were analyzed using Z-test FDR-adjusted *P*-values. A p-value cutoff of 10^{-6} was used to identify comparable numbers of bound sites. Other biological data are statistically analyzed using Student's t-test or z-test, followed by Bonferroni correction for multiple comparisons.

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Table 2-1. shRNA sequences used in this study

Targeted Gene	shRNA sequence (5' to 3')
Jmjd3	**CCTCTGTTCTTGAGGGACAA**TTCAAGAGA**TTGTCCCTCAA GAACAGAGG**
Tbx3 (44)	**GAATTGTACATAGCTGGAT**TTCAAGAGA**ATCCAGCTATGT ACAATTC**

Table 2-2. Probe and primers used in this study

Name	Distance from TSS	Sequence (5' to 3')
Eomes	-7kb	GCATTGCCTTCCGTCTACTCA
-Enhancer		TTAAGGCCCCATTTCCTGAA
Negative	Chr13	GCCACAGTTAGCCATCTGCA
		GCCCTGACCTCTGGACCCTA
Cyclophilin	cDNA	GTCTCCTTCGAGCTGTTTGC
		AGCCAAATCCTTTCTCTCCA
Sox17	cDNA	CACAACGCAGAGCTAAGCAA
		CGCTTCTCTGCCAAGGTC
Eomes	cDNA	ACCGGCACCAAACTGAGA
		AAGCTCAAGAAAGGAAACATGC
GSc	cDNA	GAGACGAAGTACCCAGACGTG
		GCGGTTCTTAAACCAGACCTC
Gata6	cDNA	GGTCTCTACAGCAAGATGAATGG
		TGGCACAGGACAGTCCAAG
Foxa2	cDNA	GAGCAGCAACATCACCACAG
		CGTAGGCCTTGAGGTCCAT
Mixl1	cDNA	CCATGTACCCAGACATCCACT
		CGGTTCTGGAACCACACCT
Jmjd3	cDNA	GTACAGACCCCCGGAACC
		TGGTGGAGAAAAGGCCTAAG

Table 2-3. Antibodies used in this study

Protein recognized	Raised in (isotype)	Company/Origin
H3K4me1	Rabbit (IgG)	Abcam
H3K27me3	Rabbit (IgG)	Millipore
H3K27Ac	Rabbit (IgG)	Abcam
H3	Rabbit (IgG)	Cell Signaling
Jmjd3	Rabbit (IgG)	Millipore
Nanog	Rabbit (IgG)	Bethyl Laboratories
Tbx3	Goat (IgG)	Santa Cruz
Oct4	Goat (IgG)	Santa Cruz
Sox2	Goat (IgG)	Santa Cruz
Mouse IgG	Rabbit (IgG)-HRP	Jackson Laboratories
Rabbit IgG	Donkey (IgG)-HRP	Jackson Laboratories

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CHAPTER 3: JMJD6 FACILITATES AIRE-MEDIATED TRANSCRIPTION OF PERIPHERAL TISSUE ANTIGENS IN THE THYMUS

Abstract

Understanding the molecular mechanism of Aire-mediated immune tolerance is of great interest to design treatment strategies for autoimmune diseases, including Type 1 Diabetes. Although consensus has been reached that Aire functions by activating transcription of the peripheral tissue antigens (PTAs) pool in the thymus medullar to be represented for negative selection, the precise mechanism is not well understood, partially due to the stochastic, widespread and promiscuous expression patterns induced by Aire. The assortment of Aire binding partners ranges from nuclear transporting, chromatin binding/structure, transcription and mRNA processing. Here we identified a novel Aire interacting partner, an arginine demethylase JmjD6. Aire-JmjD6 complex is enriched at the *INS2* promoter in sorted mTECs (Medullary Thymic Epithelial Cells) with non-methylated histone H3K4me0 and H3R2me0. These data suggest that JmjD6-mediated histone H3 demethylation at Arginine 2 residue on target chromatin is essential in priming the otherwise repressed PTA chromatin for subsequent Aire recruitment and transcriptional activation.

Introduction

Autoimmune diseases including Type 1 Diabetes are caused by immune attack of cells by self-reacting thymocytes that escape the tolerance selection. The origin of the pathologic T cells is not well understood for Type 1 Diabetes, however, the transcription levels of *INS* gene in the human thymus correlate with susceptibility to Type 1 Diabetes (1). Depletion of Insulin expression in the thymus also leads to rapid onset of autoimmune diabetes in mouse (2), suggesting a potential role of central tolerance in the pathogenesis of Type 1 Diabetes. During development, auto-reactive T cells are eliminated in the thymus stroma by exposure to peripheral tissue antigens in a process called negative selection (3). Negative selection requires the expression of a broad spectrum of genes that are normally expressed in the peripheral tissues (4). Peptides derived from these peripheral tissue antigens (PTAs) are then processed and loaded onto major histocompatibility complexe (MHC) molecules to be presented on the thymic epithelial cell (TEC) surface, making self-peptides visible for the differentiating thymocytes (5, 6). The maturing thymocytes migrating through the thymic stroma recognize these self-peptides and then undergo clonal deletion, therefore establishing immune tolerance to self antigens (7). This ectopic gene expression is carried out by a subset of specialized TECs in the medulla(mTECs) that expresses autoimmune regulator (Aire) (8).

Aire is a transcription factor that regulates the expression of PTAs reservoir, although the molecular mechanisms of how Aire directs the transcription of these PTA genes is only vaguely understood (9). Defects or mutations in Aire disrupt the transcription of PTAs and develop immune infiltrates into multiple organs, that manifest as APECED (Autoimmune

Polyendocrinopathy-Candidiasis-Ectodermal Dystrophy) in both humans and mice (9). The stochastic, cell type dependent expression manner, the broad range and magnitude of genes regulated by the Aire transcriptional footprint suggest that Aire, unlike classic transcriptional factors, activates transcription through mechanisms that do not involve recognition of a specific DNA binding motif (10-13). Recent studies reveal that Aire forms large multiprotein complexes, and is involved in releasing paused RNA-Polymerase II (RNAPII) from the promoter and promotes transcriptional elongation (14, 15). Identification of Aire partners has revealed a broad range of proteins spanning across nuclear transport, chromatin binding, transcription and pre-mRNA processing (16). Precisely how Aire functions within the different protein complexes is still unclear.

Recent structural studies have revealed more insights into the transcriptional function of Aire, and at the same time brought along some contradictory data. Aire contains multiple structural domains found in other transcription factors; including SAND domain (DNA binding), CARD (Caspase recruitment domain), four LXXLL motifs (protein-protein interactions) and 2 PHD (Plant homeodomain) finger domains. PHD1 specifically recognizes unmethylated histone H3 tails at the lysine 4 residue (H3K4me0) and Arginine 2 residue (H3R2me0) (17-19), while PHD2 domain acts as a docking site for transcription complexes and chromatin binding/structuring complexes to activate transcription (20). The structural data seem contradictory to the functional data (4, 8, 11, 21), as H3K4me0 is typically associated with gene repression. Sequence alignment of the PHD1 domain in Aire with other proteins that contain PHD domains have revealed structural properties in common with BHC80 and DNMT3L, both of which are correlated with gene silencing (22). The H3R2me0 recognition, however, is in support of Aire's role in

activating gene expression. The presence of a methylated R2 residue on the histone H3 tail, a mark usually associated with gene repressions abrogates AIRE binding to H3K4me0 promoters, suggesting H3R2me0 is the prerequisite recognition site over H3K4me0 (19). Regardless of the controversy in structural evidence, deletion or mutation of either the PHD1-histone H3 tail recognition domain, or PHD2-docking site for complex formation, results in inhibition of transcriptional up-regulation of previously reported Aire-dependent genes (20, 22). Examining the mechanistic roles of PHD domains in Aire-mediated transcription of PTAs, therefore, is critical to uncover the molecular mechanism of Aire-established central tolerance. In particular, understanding the mechanistic links and implications of Aire recognition of unmodified histone H3 tail with unique epigenetic markers—H3K4me0 and H3R2me0--is of great interest.

Recent studies have shown that H3R2 asymmetric methylation (H3R2me2a) catalyzed by arginine methyltranferase Prmt6 antagonizes H3K4 trimethylation (H3K4me3), mediated by MLL complex (23). Conversely, presence of the H3K4me3 marker abolishes H3R2 methylation (24). H3R2me2a can be actively removed by JmjD6—a histone arginine demethylase found to be strongly expressed in TECs (25, 26), implying a JmjD6 mediated epigenetic mechanism could facilitate Aire target recognition and subsequent function. Sequence alignments of homologous JmjD6 proteins from different species reveal that it contains a highly conserved JmjC domain and a conserved N terminal with no recognizable domain sequence, potentially functioning in protein interaction, as suggested by a surface model (27). Whether and how JmjD6 regulates Aire recognition self-antigens in the mTECs remains an interesting question. Here we hypothesize that Aire might function by forming a complex with JmjD6 to epigenetically modify normally repressed chromatin and up-regulate PTA expression.

Results

Aire forms a multi-protein complex with JmjD6 *in vitro*

To investigate whether Aire and JmjD6 function in the same protein complex, we transfected flag-tagged Aire into HEK 293T cells that express high levels of JmjD6. To identify binding partners of flag-tagged Aire, we performed co-immunoprecipitation (IP) with Flag and JmjD6 antibodies. Flag-tagged Aire was enriched in the IP samples as compared to the input and flow-through and JmjD6 was detected in Flag IP sample (Figure 3-1A). Similarly, a JmjD6 IP pulled down flag-tagged Aire (Figure 3-1B). Collectively, our data showed that Aire and JmjD6 can co-immunoprecipitate in HEK 293T cells, suggesting they might function in the same protein complex. Only a small fraction of the flag-tagged Aire was detected with JmjD6 IP, confirming previous data that Aire seems to form large protein complexes with a large set of proteins (16).

Aire co-localizes with JmjD6 on *INS2* promoter in enriched mTECs

To investigate whether an Aire-JmjD6 complex forms *in vivo*, we isolated mTECs from 4 weeks old C57BL/6 mice using flow cytometry (see methods, Figure 3-2A). To confirm the sorted cells represented an enriched mTEC population, mRNA was extracted from isolated mTECs and subjected to RT-PCR for expression of autoantigens and mTECs cells markers. Autoantigens including *insulin2, IA-2, Sox9* were expressed in the sorted mTECs, as well as markers like *CK5* and *Aire* that are specific to mTECs (Figure 3-2B). However, cTECs (cortical thymic epithelial cells) marker *CK8* was also detected in the sorted mTECs mRNA, possibly due to a slight

contamination of the cTECs in the flow cytometry. To test our Aire antibody, we immunostained embryonic day 18.5 (E18.5) thymus with Aire antibody, and we observed strong punctate nuclear Aire expression in a small subset of mTECs (Figure 3-S1A). To measure whether Aire and JmjD6 function in a complex to regulate PTA transcription, we performed ChIP analysis on the *INS2* locus-a putative Aire target, in the enriched mTECs. Aire has been previously reported to bind to gene promoters (28). We designed 3 sets of ChIP primers on the *INS2* locus, the 500 base pair upstream and 2 kb upstream primers were internal controls (Figure 3-3A). Both Aire and JmjD6 bound to the *INS2* promoter, but not the upstream regions (Figure 3-3B). *INS2* promoter was not methylated for H3R2 or H3K4, while the distant region was enriched on H3R2me2a (Figure 3-3B). To verify the specificity of Aire-JmjD6 function in the enriched mTECs, we performed similar ChIP in insulinoma cell line-Min6 cells transfected with Flag-Aire construct. In Min6 cells with ectopic Aire expression, we did not detect Aire binding to the *INS2* locus, while JmjD6 binding was observed throughout the locus and peaked at the promoter (Figure 3-S1B). Consistent with the highly active *insulin* transcription in Min6 cells, ChIP analysis showed a strong H3K4me3 at the *INS2* promoter and no H3R2me2a across the entire *INS2* locus (Figure 3-S1B). To conclude, Aire-JmjD6 complexes in regulating *INS2* expression is unique to the mTECs.

Discussion and future directions

The immune master regulator-Aire has been associated with the widespread transcriptional activation of PTAs in the mTECs. Recent structural discovery of the specific affinity to unmethylated histone H3 tails, in particular unmethylated lysine 4 and Arginine 2 residues by

PHD1 domain of Aire have implied epigenetic mechanisms are involved in the transcription activation of these weakly transcribed gene loci. Out data support the structural studies and identified a novel partner JmjD6 in priming the PTA promoters for Aire recruitment and function. We show that the Aire-JmjD6 complexes form both *in vitro* and *in vivo*, a partnership overlooked in the previous mass spectrometry, possibly due to the low amounts of the JmjD6 complex among the large set of other Aire complexes (16). We postulate that Aire-mediated PTA expression is a dynamic streamlined process involving multiple complexes at different stages of the transcription process including: nuclear transport, chromatin anchoring and structuring, transcription firing and elongation, pre-mRNA processing and packaging. Our data suggest that the Aire-JmjD6 complex is involved in the chromatin anchoring process; and erasure of the H3R2me2a by JmjD6 prepares the gene locus for Aire recognition and facilitates the recruitment of complexes for the next steps in the transcriptional activation streamline. A speculative illustration of JmjD6-Aire complex in priming the gene locus has been depicted in Figure 3-4.

The functional implication of how the specific recognition of unmethylated histone H3 tails by Aire modulates PTA chromatin and transcription in mTECs has not been studied. In this study, ChIP analysis of the *INS2* locus has revealed distinct transcription regulatory features of Aire complexes. Although H3K4me3 and H3R2me2a markers are mutually exclusive due to the antagonistic impacts on transcription (23), the unmethylated H3K4me0 and H3R2me0 can coexist, regardless of their contradictory transcription effects, and permit low to moderate transcription from the locus in the mTECs. Different from the fully activated *INS2* locus in Min6 cells, mTECs have low level of H3K4me3 and heavily methylated H3R2me2a in upstream 2 kb

region of *INS2* locus. The difference of *INS2* epigenetic profile in mTECs presumably reflects the unique functionality of Aire-induced *INS2* expression. A moderate expression of PTA is elicited by Aire to ensure adequate representation of INS2 peptides for negative selection, unlike the full-blown expression of *INS2* required to produce and secrete insulin in pancreatic beta cells to meet the metabolic demands. The over-expressed Aire is not recruited to the *INS2* promoter in Min6 cells, potentially restricted by the H3K4me3 modification and the MLL complex. The specificity to H3K4me0, therefore, could be a mechanism to reduce redundancy of Aire activation to ensure a broader representation of other weakly expressed PTAs.

Further, we hypothesize that Aire-JmD6 complex formation is a prerequisite in Aire recruitment to gene locus; defects in JmjD6 will potentially disrupts Aire recruitment to gene loci and abolish Aire mediated transcription activation. To test this hypothesis, I have utilized a conditional JmjD6 loss-of-function mouse model, generated by breeding JmjD6 floxed mouse into Aire-Cre transgenic mouse line. The breeding is currently ongoing and litters will be analyzed for defects in autoimmunity. JmjD6 was previously identified as a phosphatidylserine receptor (PSR) and it was misleadingly suggested to be involved in removal of apoptotic cells by phagocytes (29). JmjD6 -/- mice have since been generated and analyzed, although different groups have interpreted results differently and the role of JmjD6 has remained controversial (29-31). JmjD6 null mutation has perinatal lethality and defective developments of multiple organs has been reported, along with impaired thymic and thymocyte development(30). Examination of gene expression in heterozygous JmjD6 +/- has identified changes in the thymus gene expression profiles (data from the German mouse clinic). These data in combination with the high levels of JmjD6 observed in the thymus, suggest that JmjD6 plays an important role in thymocyte

development. Our data from the mouse model of *Aire-Cre, JmjD6 fl/fl* will add clarity to the role of JmjD6 in Aire+ mTECs, and test whether JmjD6 is involved in the negative selection process.

Figure 3-1

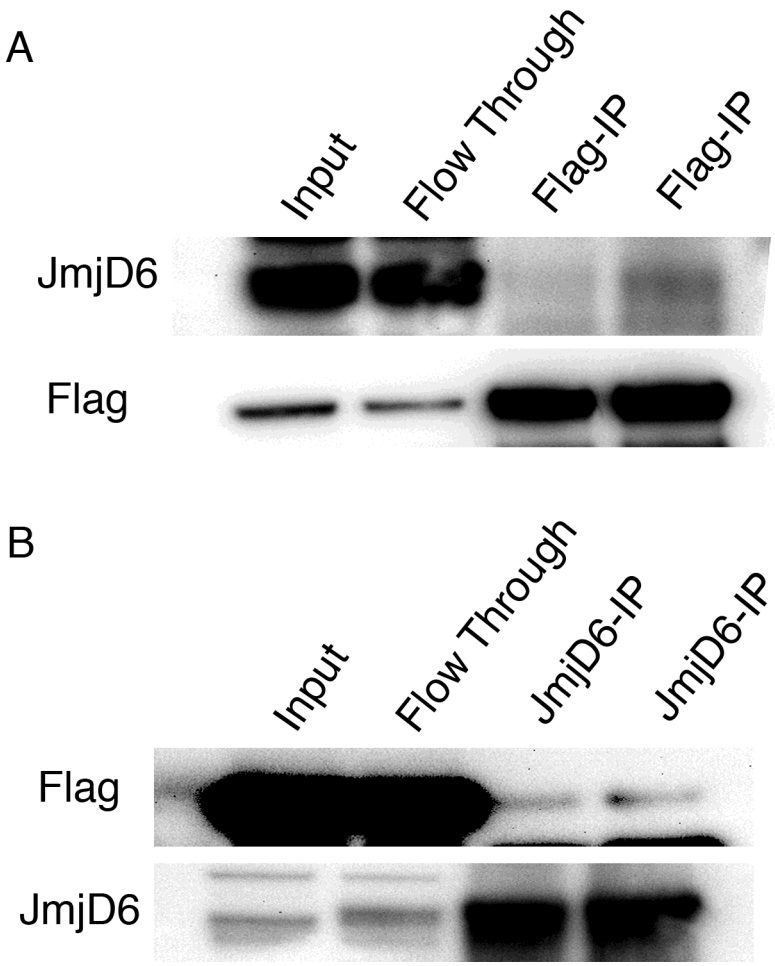

Figure 3-1. JmjD6 co-immunoprecipitates with Flag-Aire

(**A**) Input lysate, flow-through lysate, and two different amounts of Flag immunoprecipitates were immunoblotted with JmjD6 and Flag antibodies. (**B**) Input lysate, flow-through lysate, and two different amounts of JmjD6 immunoprecipitates were immunoblotted with Flag and JmjD6 antibodies.

Figure 3-2

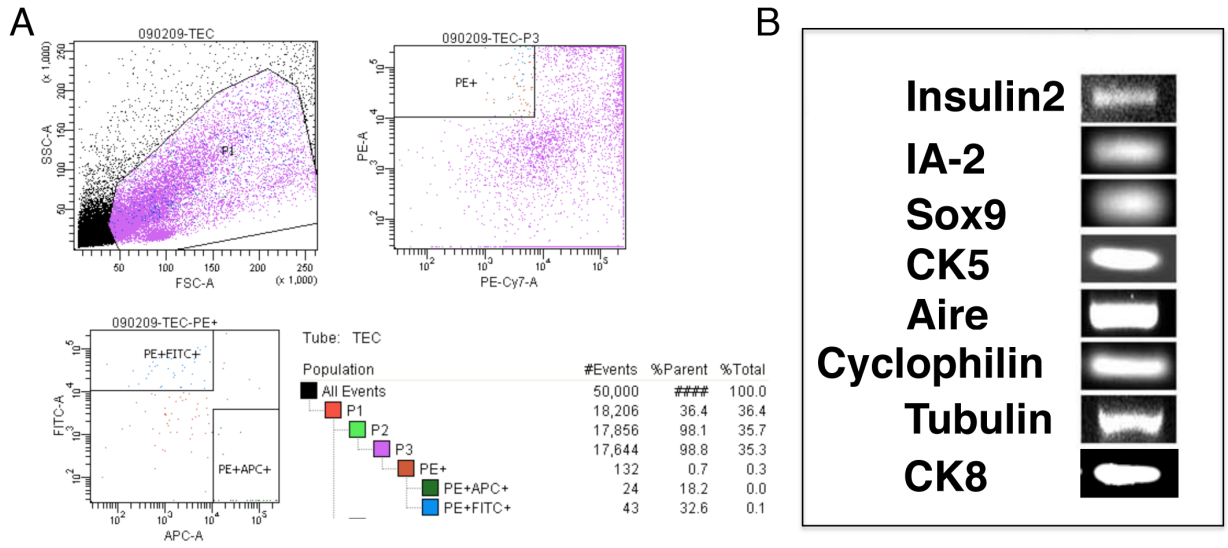

Figure 3-2. Isolating mTECs from the thymus

(**A**) Flow cytometry isolating PE-Epcam+, PE-Cy7-CD45-, FITC-UEAIhi mTEC population and PE-Epcam+, PE-Cy7-CD45-, APC+-Ly51+ cTEC population. (**B**) RT-PCR of mTEC mRNA with auto antigens *Insulin2, IA-2, Sox9*, and mTECs markers *Aire, CK5,* cTECs marker *CK8*, and house keeping genes *Cyclophilin* and *Tubulin*.

Figure 3-3

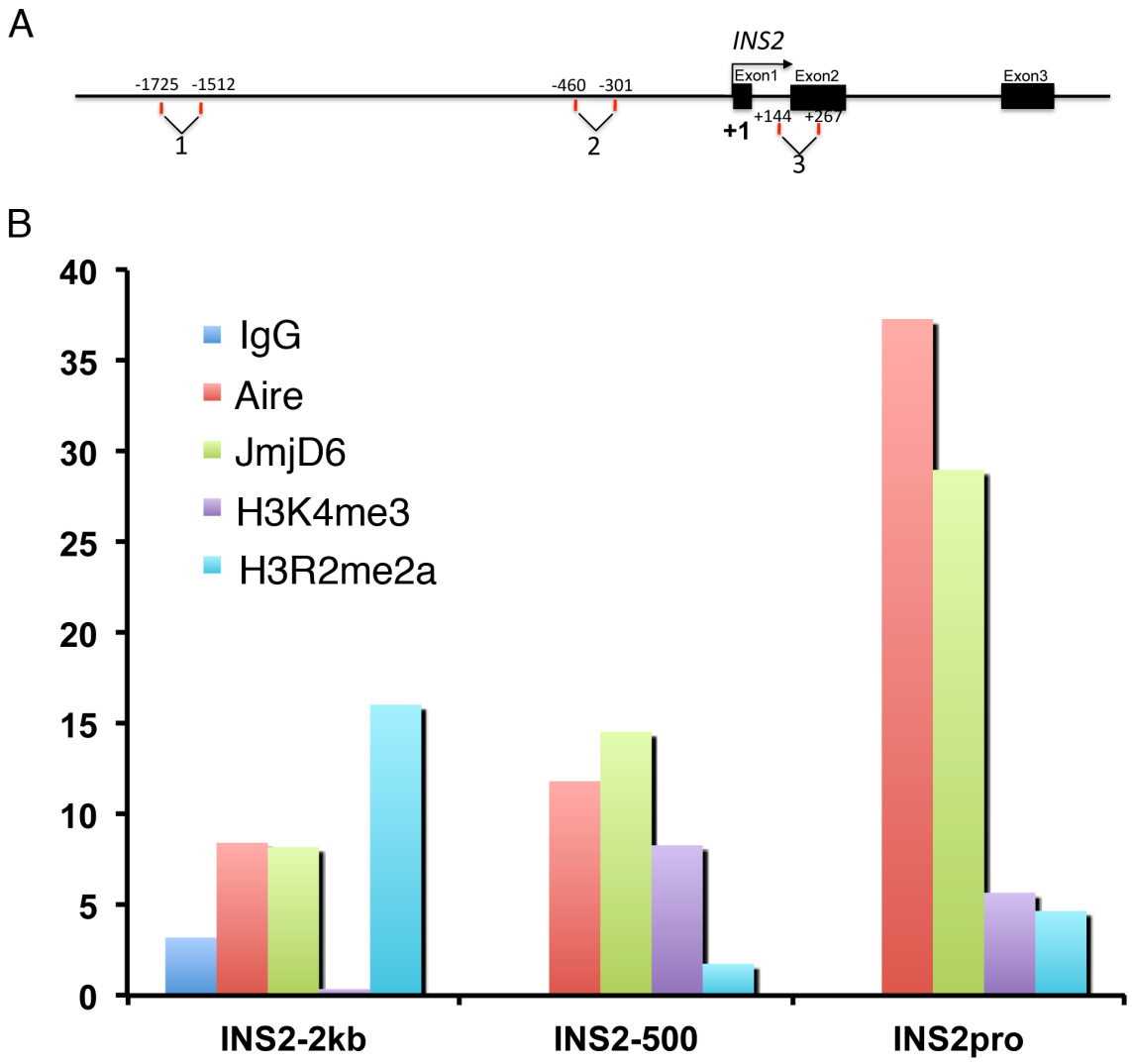

Figure 3-3. Aire-JmjD6 complex binds to *INS2* promoter to regulate insulin expression in mTECs

(**A**) Schematic of *INS2* locus and the ChIP primers. (**B**) ChIP analysis of the indicated antibodies on the *INS2* locus.

Figure 3-4

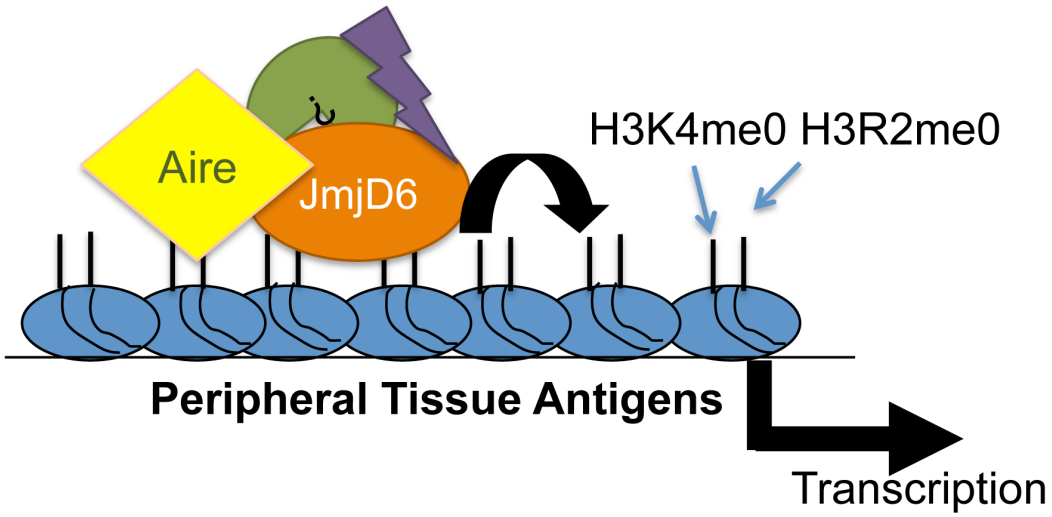

Figure3-4. Model of Aire-JmjD6 complex in regulating PTA transcription in the thymus

JmjD6 removes methylated H3R2me2a from inactive promoters and recruits Aire to gene loci with low transcriptional activity. Aire then further recruits other protein complexes to initiate transcription.

Supplementary Figures

Figure 3-S1

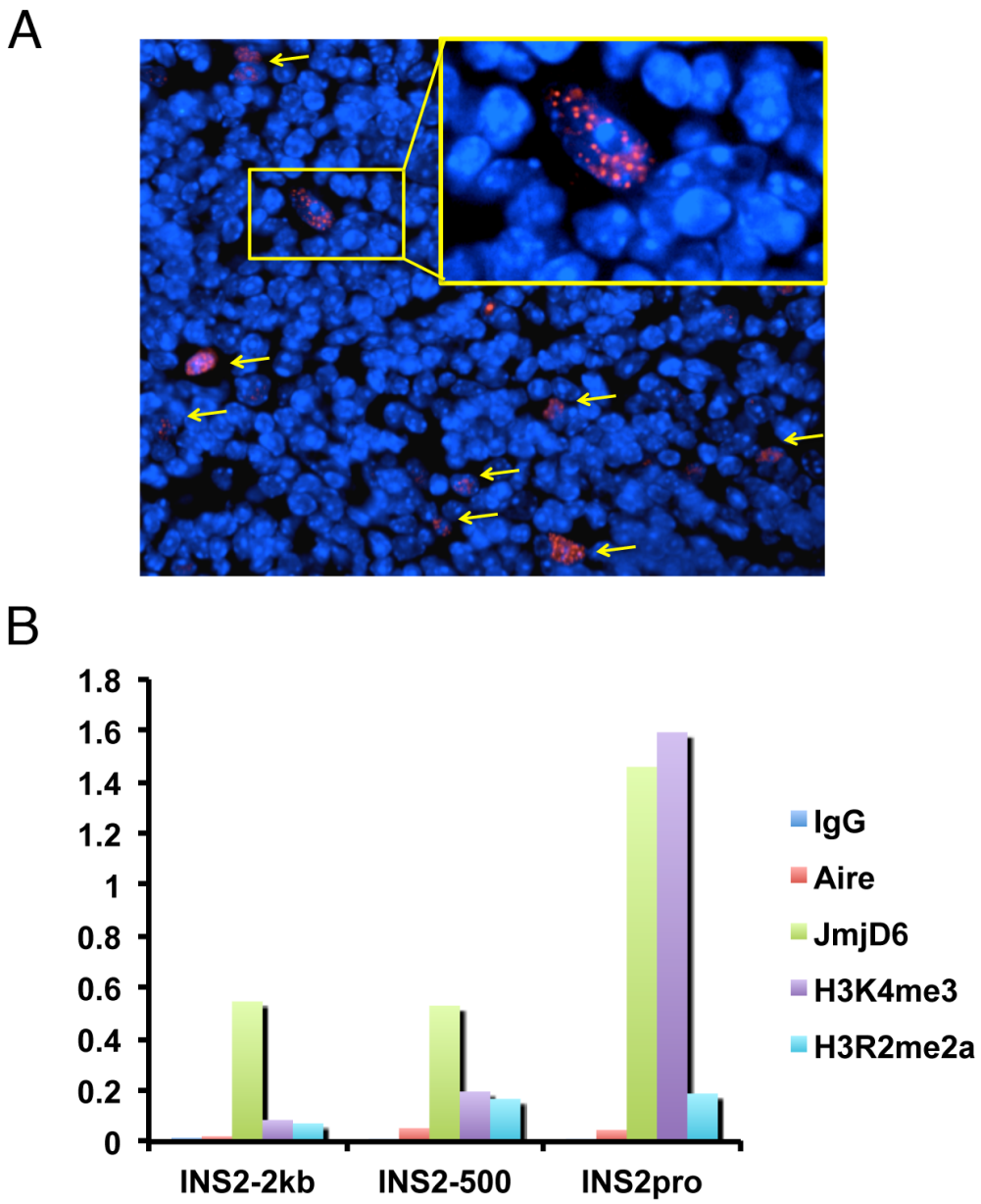

Figure 3-S1. Aire-JmjD6 complex did not form in Min6 cells with flagged Aire transfection

(**A**) Aire expression in the thymic medullar. (**B**) ChIP analysis of the indicated antibodies on the *INS2* locus.

Methods and Materials

Isolation of mTECs from mouse thymus

mTECs Isolation was performed as previously described (32). In brief, thymi from 4 weeks old C57B/6 mouse were harvested and digested in RPMI at 37 °C for 3-10 min digestions with Collagenase (0.125%)/DNase I (0.1%) (Roche) and a final digestion with 0.125% Collagenase/Dispase (Roche). Cells from all supernatant fractions were pooled and treated with RBC lysis buffer to remove erythrocytes. Pellet cells were resuspended in EDTA/FACS buffer and filtered through 70um nylon mesh. Single cell suspension was then blocked with BSA and immunostained with anti-CD16/32 purified (eBiosciences 14-0161) to block Fc receptors. TECs were incubated on ice for 30min with antibody cocktail containing anti-EpCAM-PE (eBiosciences 12-5791), Anti-CD45-PE-Cy7 (eBiosciences 25-0451) , UEA1-FITC (Vector FL-1061), biotin anti-CDR1 and SAV-APC (ebiosciences 13-5891 and 17-4317). After staining, samples were washed and sorted using the BD FACS Aria II flow cytomethric sorter (BD Biosciences).

ChIP analysis

Micro ChIP protocol (33) was used because of the low amount of sorted cell number. In brief, 2.4 ug of antibodies were bound to Dynabeads protein G. Cells were crosslinked with 1% of freshly prepared formaldehyde and lysed. Cell lysates were then sonicated with Bioruptor (Diagenode) and diluted in RIPA buffer. Antibody bound beads were then incubated with the chromatin for 2 hours at 4°C and washed with ice-cold RIPA buffer for three times before elution. The eluted DNA was then reverse cross-linked and digested with proteinase K overnight

and DNA was purified with phenol-chloroform and ethanol precipitation. Equal amount of DNA from the ChIP and Input samples were then subject to real time qPCR amplification (ABI) of gene loci of interest. The ChIP samples were quantified as percentage bound / input. Antibodies used in ChIP: mouse anti-Aire (gift from Matsumoto Lab), anti-JmjD6 (Abcam Ab10526), H3K4me3 (Diagenode Mab-152-050), H3R2me2a (Abcam Ab8046). Each ChIP experiment was performed 3-5 times. Primers used for *INS2* locus ChIP:

INS2 Pro: 5'-ACCCAGCCTATCTTCCAGGT-3' & 5'-CAAAGGTGCTGCTTGACAAA-3'

INS2 -500bp: 5'-GGCAGGACCATGATGAGACT-3' & 5'-CATTTCTCTCCCCCATGAGA-3'

INS2 -2kb: 5'-TGCCTGAATTCTGCTTTCCT-3' & 5'-GGCTGAGCATTTTTCCACAT-3'

RNA isolation and RT-PCR

RNA from sorted mTECs was isolated with the RNeasy Micro kit (Qiagen) according to manufacturers' instructions. 200ng RNA was used for preparation of cDNA using Superscript III Reverse Transcriptase (Invitrogen) with the oligodT primers. The cDNA was then analyzed with regular PCR and visualized on agarose gels.

Lists of RT-PCR primers used for mRNA expression analysis:

Aire: 5′–ACCATGGCAGCTTCTGTCCAG–3′ and 5′–GCAGCAGGAGCATCTCCAGAG–3′

IA-2: 5′–AGACAGGGCTCCAGATTTTGC–3′ and 5′-GGCATGGTCATAGGGTAGGAAG–3′

Insulin II: 5′–CAGCCCTTAGTGACCAGC–3′ and 5′–TCTCCAGCTGGTAGAGGG–3′

Sox9: 5'-CGAGCACTCTGGGCAATCT-3' and 5'-TGACGTCGAAGGTCTCAATG-3'

Co-IP and Western

2.5 ug of anti-Flag (Sigma F1804) or Anti-JmjD6 (Abcam Ab10526) antibodies were used for each immunoprecipitation. Antibodies were immobilized to Protein G Sepharose (Millipore) for 2-3 hours and washed with cold non-denaturing buffer (20 mM Tris-HCl pH=8, 137 mM NaCl, 10% glycerol, 1% NP-0, 2mM EDTA). The antibody-coated beads were then incubated with HEK 293T cell extracts prepared in non-denaturing buffer at 4°C on a rotator overnight. Beads were washed 3 times in ice-cold non-denaturing buffer and eluted in 1X SDS-PAGE gel loading buffer. The immunoprecipitates were loaded to each lane of SDS-PAGE gel, input and flow-through lysate samples were also loaded into separate lanes. The protein samples on the SDS-PAGE gel were transferred to nitrocellular membrane and blotted with antibodies against JmjD6 and Flag-M2. Each western shown is a representative from three independent experiments using different immunoprecipitates.

Immunohistochemistry

Immnohistochemistry was performed as previously described (34). Thymic tissue was fixed in 4% formaldehyde, and processed for paraffin embedding. 5-μm Sections were deparaffinized and rehydrated, then subjected to antigen unmasking and permeabilization. The slides were then blocked with 3% BSA in TBST (0.1% Tween 20) and incubated with Primary antibody (Mouse anti-Aire) at 4°C overnight. The slides were then washed and incubated with Donkey-derived

secondary antibodies conjugated Cy3 (1:200, Jackson ImmunoResearch Laboratories). Slides were mounted with Vectashield with DAPI (Vector Labs) prior to imaging using a Leica DM6000 microscope and Openlab software (Improvision).

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SUMMARY AND FUTURE DIRECTIONS

Last decade has seen a flourish of research in epigenetic mechanisms in tissue and organ development and various diseases. In particular, studies in cancer epigenetics have been advanced to clinical development stage of small molecule inhibitors of epigenetic events taking place during oncogenesis and malignancy. For instance, inhibitors targeting epigenetic regulators of DNA methylation enzymes (DNMTs) and histone deacetylases (HDACs), have been granted approval by the FDA for the treatment of malignant cancers (1). The success of epigenetic interventions in cancer malignancy leads a promising path to follow for other fields. When considering therapeutics that employ epigenetic inhibitors for chronic diseases such as diabetes, there is a clear need for such inhibitors to specifically target the tissue of interest as off target side-effects would be less desirable. The requirement of targeting accuracy in other disease fields, therefore, compels a comprehensive understanding of the underlying mechanism in developing therapies targeting epigenetic regulators. My work along with others in the field of epigenetics and diabetes has uncovered important roles of epigenetic modifications in the pathogenesis of Diabetes and developing treatments towards different aspects of diabetes. Here I summarize the impact of my dissertation work, highlight recent research in the fields of beta cell regenerative strategies including beta cell regeneration and stem cell derived beta cells as well as immune tolerance strategy for type 1 diabetes, and discuss future research directions on the roles of transgenerational epigenetic inheritance in gestational effects.

Beta cell regeneration

To achieve functional beta cell restoration in diabetic patients, the mechanisms regulating beta cell replication have been intensively investigated. Previous work from our lab and others have shown that genetic factors, in particular, cell cycle regulators such as cyclin D2, cdk4, p16, skp2 and p27 play pivotal roles in governing adult beta cell replication (2-8). Importantly, polycomb-p16 pathway has been reported to regulate the decline of beta cell replication with age, which also limits the regenerative capacity of aged beta cells (9-13). Epigenetic regulation of *p16* locus during aging, therefore, is a promising target for stimulating endogenous beta cell regeneration.

Using transgenic mice to offset the loss of endogenous Ezh2, we have advanced the concept of regenerative plasticity in adult beta cells. In young adulthood, activation of Ezh2 expression in beta cells was sufficient to repress *p16* and increase the replication of beta cells. This is consistent with previous observations that the beta cell mass in young adults can expand to adapt to metabolic demands accompanying pregnancy and obesity (11). Thus, results here and in prior work suggest that even in the absence of ongoing replication, beta cells in young adults retain the capacity for replication and expansion. By contrast, aged mice are resistant to adaptive changes in beta cell mass and replenishing Ezh2 alone in aged mice was not sufficient to repress the *P16* locus. Our finding that chromatin changes at the *p16* locus accurately reflect beta cell replicative capacity validates investigations of p16 expression to promote beta cell regeneration and, more generally, justifies studies of age-dependent chromatin changes in islet beta cells.

Our findings also suggest that efforts to regenerate beta cells will be advanced by identifying the signaling pathways that regulate expression and activity of PcG and TrxG factors in aging beta cells. Previously, Platelet-derived growth factor (PDGF) signaling has been shown to regulate beta cell Ezh2 levels and is an important conserved mechanism in sustaining beta cell replication during neonatal expansion of mouse and human islets (14). Coinciding with reduced levels of PDGF signaling and Ezh2 decline, here we observed increased recruitment of the Mll1-JmjD3 protein complex to the *p16* locus, accompanied by increased levels of JmjD3 in the beta cells. Moreover, findings with bEzTG mice here suggest that activation of *p16* expression by the Mll1-JmjD3 complex may prevent Ezh2-dependent repression of the *p16* locus in aged beta cells. Further studies are needed to determine if PDGF signaling or other pathways regulate the age-dependent changes of Mll1 and JmjD3 that limit beta cell replication.

The loss of H3K27me3 repressive marks at the *p16* locus could not solely be attributed to the decline in the binding of Ezh2 and our study highlights an active mechanism of removal of H3K27me3 marks involving JmjD3. We show that JmjD3 and Mll1 act together to modify two distinct lysines: JmjD3 demethylates H3K27, while Mll1 methylated H3K4 resulting in transcriptional activation of the *p16* locus. This mechanism of H3K27me3 removal appears to be very tightly regulated process, highlighted by the fact that while the levels of H3K27 methyltransferase Ezh2 decline with aging, there is a concomitant increase in the levels of H3K27 demethylase, JmjD3, in beta cells. The importance of JmjD3 is underscored by studies in MEFs, which show that ectopic expression of JmjD3 leads to increased expression of p16 and induction of replicative senescence (15). Our experiments with loss of JmjD3 in beta cells from

aged animals lead to repression of *p16* and induction of beta cell replications, suggesting a critical role for active removal of H3K27me3 marks in the regulation of *p16* locus.

Previous studies have demonstrated that *P16* repression by PcG proteins is a well-conserved mechanism across cell types (11), including during iPS cell generation (16)**.** Our experiments with MEFs show that re-expression of Ezh2 in late passage MEFs was insufficient to repress *P16* unless coupled with loss of the Mll1 complex, in a manner similar to pancreatic beta cells. Thus, TrxG-PcG mechanisms governing replicative senescence elucidated here may be generally relevant to creating regenerative strategies for multiple types of tissues and cells.

Small molecules targeting the TrxG-PcG pathways, therefore, can be harnessed to expand the adult beta cell pool in diabetic patients. However, given the fact that p16-mediated senescence is a general mechanism utilized by multiple tissues and cells and p16 is a tumor suppressor safeguarding normal cells from tumorigenesis, the TrxG-PcG modulation therapy need to be specifically delivered to the pancreatic beta cells in a moderate dose to minimize off target effects. Alternatively, modulation of TrxG-PcG can be used to expand the bank of donor islets prior to transplantation, overcoming the current limit in cadaveric cell source that restricts adoption of islet transplantation therapy.

Pancreatic endoderm derived from human ES cell differentiation

Human embryonic stem (ES) cells differentiate into a number of cell types, which makes them attractive for beta cell replacement strategies. The first successful differentiation protocol in producing hormone secreting endocrine cells (although not glucose responsive) by the Baetge group has stimulated interests of numerous groups to attempt modifying the step-wise differentiation protocols with the goal of making functional insulin producing cells *in vitro*. Following the guideline of developmental lineage factors identified during each step of pancreatic development, these step-wise differentiation protocols have very limited efficiency to yield Pdx1+ endocrine progenitor cells, or insulin secreting cells with minimal glucose response at best (17-22). Limited by the scant *in vitro* differentiation success, two groups have transplanted the progenitor cells into mouse models instead to permit maturation *in vivo*, islet like clusters and glucose-responsive insulin-producing cells are detected after engraftment, but part of these cells also mis-express other hormones such as glucagon (23, 24). In addition, teratomatous tissues and mesoderm structures are also detected in the grafts, making this an unsafe therapeutic approach (23). Moreover, the development of multi-hormone glucose-responsive insulin-producing cells cannot be replicated in nude rat with the same engraftment (25). The advancement to a stage of glucose-sensing insulin-secreting mono-hormonal cells that closely resemble functional beta cells in mouse models is exciting, but the low differentiation efficiency, formation of mesodermal structure and cyst, and the exist of multi-hormone cells indicate a long treacherous battle ahead to produce mature pancreatic beta cells from ES cells. The failure in producing mature beta cells efficiently with the Baetge protocol has also raised a question about the protocol itself: whether we are generating the wrong progenitors. Recent study using a modified version of Baetge protocol has more efficiently produced glucagon

producing alpha cells, casting doubt on the identity of the endoderm progenitor produced by the protocol (26). A revisit of the differentiation protocol and mechanism underlying the lineage decisions is required in lieu of the recent unsuccessful attempts.

ES cells have the unique property to self-renew and differentiate into cells of all lineages; and this ability is regulated by a group of transcription factors - pluripotency factors. My work in chapter 2 shows for the first time that aside from their roles in maintaining pluripotency, pluripotency factors are also involved in germ layer lineage specifications during differentiation. In particular, we elucidate the molecular mechanism that progressively restricts this differentiation potential and direct stem cells to differentiate towards definitive endoderm lineage. Our results lead us to suggest a factor relay model whereby an ES cell factor, Tbx3 acts on the enhancer elements of *Eomes* to establish a permissive chromatin states that restricts differentiation potential as well as primes the cells to respond to Activin A differentiation signal. Eomes also cooperates with Smad2/3 to act on bivalent domains within the promoters of core endodermal regulators to activate a transcriptional network leading to definitive endoderm specification (27).

We show that the sequence-specific transcription factors and epigenetic modifications of the chromatin interact in order to implement control pathways that drive differentiation of stem cells towards the definitive endoderm lineage. Both Tbx3 and Eomes are T-box transcription factors that act in a spatial and temporally distinct manner during differentiation. Tbx3 recruits the histone demethylase, Jmjd3 to influence the dynamics of chromatin structure. Jmjd3 is recruited

to the enhancer region of Eomes to resolve the bivalent state although the exact role of bivalent domains in regulating DNA-histone contacts and movement of RNA polymerase in gene transcription is not clear. Our results highlight how a few crucial factors can link changes in chromatin states to differentiation providing a simplification of the complex transcriptional circuits of lineage specification.

Jmjd3 is induced in response to different stimuli and subsequently contributes to the transcriptional activation of target genes. For example, Jmjd3 is induced by the transcription factor NF-kB in response to microbial stimuli to the control of gene expression in LPS-activated macrophages (28). Jmjd3 is essential in M2 macrophage development for anti-helmith responses (29). Jmjd3 is also induced upon activation of the RAS–RAF signaling pathway to activate the *INK4A–ARF* locus in response to oncogene- and stress-induced senescence (15). Jmjd3 is also induced by retinoic acid signaling in neural stem cells differentiation and interestingly, SMRT (also known as *NCoR2*, nuclear receptor co-repressor-2) is critical to repress Jmjd3 expression in the absence of retinoic acid stimulus (30). We show that Jmjd3 is induced during early differentiation to endoderm and whether SMRT is involved in repression of Jmjd3 in ES cells could be informative. Importantly, Jmjd3 binding on the endoderm lineage factor promoters correlates Smad2/3 binding and H3K27me3 reduction from the bivalent domains (31). Jmjd3 has also been shown to have a role in neural differentiation and immune cell development (30, 32-38). Mice with partial deletion of *Jmjd3* died perinataly due to premature defect in lung development (29), however the role of other H3K27 demethylase, Utx and Uty in compensating for Jmjd3 in endoderm formation was not evaluated in this study (39).

Analysis of histone demethyases in ES cells link the core transcription factor network to the regulation of chromatin status in pluripotent stem cell maintenance. Several ES cell core regulators target Jarid2 to maintain high levels of its expression in ES cells (40). Oct4 targets other histone demethylases, Jmjd1a and Jmjd2c, to positively regulate their expression (41). Jmjd1a demethylates K9H3me2 at the promoter regions of Tcl1, Tcfcp2l1, and Zfp57 to positively regulate the expression of these pluripotency-associated genes while Jmjd2c acts as a positive regulator for Nanog. Interestingly, Jmjd1a or Jmjd2c depletion leads to ES cell differentiation, which is accompanied by a reduction in the expression of ES cell-specific genes and an induction of lineage marker genes (41). These studies suggest that repression of Jmjd3 in ES cells could be integrated in the transcriptional factor regulatory network of ES cells and links core transcription factor network to the regulation of chromatin status during cellular differentiation. Overall our study provides a general framework for how ES factors regulate chromatin changes that provide a context for signal interpretation to drive differentiation of pluripotent stem cells into specific tissue lineages. Establishing the detailed principles on which cell lineages are defined and maintained is required for developing safe and successful cell therapies for a full potential of stem cell therapy.

Immune tolerance in Type 1 Diabetes

Type 1 Diabetes is recognized as a T cell mediated autoimmune disease, resulting from failure in both the central and peripheral tolerance mechanisms (42). Immune intervention strategies aiming to suppress immune response and induce/restore self-tolerance have been actively

assessed clinically for effects to halt or delay disease progression, as well as to reverse type 1 diabetes. Success from treating autoimmune diseases and transplantation has encouraged trials of immunosuppressants in recent onset type 1 diabetes, although restoration of beta cell functions is achieved with immunosuppressant administration including cyclosporine A, Rituximab and CTLA-Ig, their effects vanish after drug withdrawal (43-45). Insulitis precedes and accompanies the onset of type 1 diabetic, suggesting that inflammations are likely involved in the disease progression (46). Anti-inflammatory agents have been tested in treating type 1 diabetes: encouraging results have been reported from a pilot study with blockage of TNFa, trials with IL-1 antagonists are also currently being conducted (47, 48). The non-specific immune suppression experience proves the concept that it is possible to use immune intervention to halt type 1 diabetes progression and restore beta cell function, however, the requisite long term drug administrations has potential global side effects that are unacceptable. A more stringent and specific targeted immune therapy is therefore required to induce immune tolerance to beta cell autoantigens. Phase III trials delivering CD3 mocnoclonal antibody to induce immune tolerance has not been successful (49, 50). Antigen specific immunomodulation using beta cell antigens or peptides: insulin and GAD 65, has seen little efficacy (51, 52). Despite the unsuccessful attempts, regulatory T cells (Treg) have emerged as a very promising target for inducing specific immune tolerance locally in preventing systemic autoimmunity, and the potential tolerogenic effect of Treg cells is being actively sought after in type 1 diabetes (53). The experience gained from the various immune interventions to type 1 diabetes indicates that immunotherapy is possible to induce long-term remission of the disease, yet it requires more knowledge of the profound mechanisms causing immune attacks of beta cells.

The thymus provides an environment critical for the establishment of central tolerance. Thymic insulin expression as determined by the length of variable number of tandem nucleotide repeats (VNTR) at the proximal promoter is associated with susceptibility to type 1 diabetes, suggesting an important role of central tolerance in predisposition to type 1 diabetes (54-56). Of note, study of a mouse model lacking thymic insulin leads to a very rapid onset of autoimmune diabetes (57). The expression of thymic insulin is restricted to a small subset of the medullary TECs and regulated by the immune master regulator-Aire (58). The precise mechanism of Aire mediated peripheral tissue antigen expression is poorly understood. My study in chapter 3 identifies a novel partner of Aire in the mTECs-JmjD6, the association of the histone arginine demethylase at insulin promoter contributes to the priming of the locus for Aire induced transcription activation. Although we have focused on the *insulin* gene, other peripheral tissue antigens activated by Aire could also utilize the same epigenetic mechanism.

The discovery of a structure domain that reads a specific unmethylated histone H3K4 and H3R2 code suggestes Aire might employ epigenetic mechanisms to alter chromatin structures and activate transcription (59, 60). Our results indicate the recruitment of histone H3R2 demethylase by Aire potentially erases the H3R2 methylation marks associated with the otherwise repressed tissue antigens. Examining the changes in epigenetic modifications concomitant with Aire induced antigen expression could potentially uncover the mystery behind the promiscuous yet broad ranged PTA expression patterns elicited by Aire. The unconventional transcriptional programs induced by Aire in the mTECs also lead us to predict an unusual epigenetic activity involving atypical chromatin modifications and complex assemblies on gene loci normally restricted in other tissues.

Besides its primary expression in the mTECs, Aire is also found in peripheral lymphoid tissues (61, 62). Although the function of Aire in the periphery remains to be investigated, it is suggested to be involved in peripheral self-tolerance. A subpopulation of Aire expression cells in the peripheral is $CD11c^{int}B220^{+}$ dendritic cells; these cells are shown to be involved in peripheral education of tolerance to insulin (63). Aire expression in the thymus varies with age and the decline of *Aire* mRNA levels also impairs PTA expressions (64). It is therefore interesting to delineate signals upstream of Aire, and determine how transcription of *Aire* is regulated. Small molecule agonists of Aire might be useful to enhance/restore self-tolerance both in the central and the peripheral immune systems.

Previously identified as a phosphatidylserine receptor (PSR) and suggested in removal of apoptotic cells by phagocytes, JmjD6 is later discovered to be a histone arginine demethylase (65, 66). Recent study reveals a role for JmjD6 in lysine hydroxylation and indicates a potential role of JmjD6 in mRNA splicing, conflicting the study of JmjD6 mediated histone arginine demethylation (67). The controversy on the function of this protein, along with the conflict in nuclear and cytoplasmic expression patterns of JmjD6 in different cells raises questions about its role and function that need to be further clarified. Alternatively, based on current published conflicting results, JmjD6 might have multiple roles depending on where it locates and the specific cell type: cell surface association in immune cells might be associated with phagocytosis (68), cytosolic or nuclear localization might be associated with RNA splicing or histone modification(66, 67, 69). Regardless of what JmjD6 does in the cell, JmjD6 null mutation has

impaired thymic and thymocyte development, and defective developments of multiple organs resulting in perinatal lethality (70). Various groups interpret jmjD6-/- results differently and the role of JmjD6 has remained controversial (65, 69-71). The high levels of JmjD6 observed in the thymus stroma, suggest that JmjD6 plays an important role in thymocyte development. Studies to decode the role of JmjD6-Aire in mTECs, whether it be mRNA splicing, histone modification or transcriptional regulation would add significantly to 1) demystify the identity of JmjD6 in the thymus; 2) clarify the mechanism of Aire induced antigen expression in mTECs; 3) understand the mechanisms directing central tolerance.

Gestational diabetes and trans-generational epigenetic inheritance

Pregnancy obesity is concerning not only because of the adverse maternal health effects of having gestational diabetes, hypertension etc. in the pregnant women, but also of the potential deleterious effects of predisposing the developing child to increased risk of developing chronic diseases such as diabetes, obesity, cardiovascular diseases and metabolic syndromes in later life (72, 73). With the rate of obesity increasing at an alarming rate globally, understanding the mechanism of how maternal obesity and high-fat diet affect offspring is of particular interest to the field of diabetes prevention. Animal models of maternal high-fat have characterized trans-generational traits that persisted across two generations to the maternal high-fat diet, suggesting a heritable programming is initiated by the maternal nutrition condition (74, 75). Although epigenetic inheritance is often proposed to contribute to the observed trans-generational heritability of traits through maternal diet, few studies have been directed to address this question.

Epigenetic inheritance, as commonly proposed to act as an adaptive mechanism to pass acquired environmental information to the next generation, has not been well understood to date. It has been hypothesized to contribute to the epidemic breakout of obesity and Diabetes: from the "hunt and gather" era, humans have accumulated traits that can preserve and store energy; these accumulated epigenetic mechanisms continue to work in an environment with abundant food and energy supply, which eventually exacerbates the growing events of obesity and diabetes worldwide. Early evidence of epigenetic inheritance in mammals came from nuclear transplant of pronuclei at one-cell stage to eggs of different genotype, the nucleo-cytoplasmic hybrids displayed different gene expression patterns that were transmitted to the offspring (76, 77). However, what mechanism carries the epigenetic inheritance to the next generation is unclear. Studies of the trans-generational effects of parental diet have attributed the heritable traits to DNA methylations with little evidence. Increased DNA methylation on the *Pdx1* proximal promoter has been associated with intrauterine growth retardation (IUGR) mouse model that predispose fetus to the onset of diseases including T2D in a recent study (78). However, whether the hypermethylated *Pdx1* promoter is an epigenetic mark inherited from the dam or acquired during development with the IUGR condition is unclear. Until recently, Carone et al. 2010 and Ng et al. 2010 have reported paternal diet induced reprogramming of metabolic signaling through differential cytosine methylation profiles, providing evidence of heritable epigenome by DNA methylation (79, 80). These studies, however, did not address where these methylation marks come from and whether they were inherited from epigenetic events of paternal somatic/germ cells in response to diet, as they did not compare the offspring with the paternal epigenome. The question remains whether the maternal effect of diet is transmitted through DNA

methylation or other form of epigenome programming. It is therefore of great interest to characterize the mechanisms that carry the maternal information to the future generations.

DNA methylation refers to the addition of methyl group to cytosine in the context of CpG dinucleotide, and this epigenetic mark is usually associated with gene silencing (81, 82). Methylation marks, established and maintained by DNA methyltransferases (DNMTs), play critical roles in tissue specific gene regulation, X chromosome inactivation, gene imprinting and genome stability (83, 84). Cells of different tissue origin and at different development stages have differential methylation patterns (85). DNA methylations have long been thought to be relatively stable marks, recent studies of 5-hydroxymethylcytosine (5hmC) and the enzyme family - ten-eleven translocation (TET) that mediates the conversion of 5-methylcytocine (5mC) to 5hmC have drastically challenged our view of this epigenetic mark (86). Whether DNA demethylation occurs actively or passively is still under intensive debate, it is suggested that 5hmC is a transition state that facilitates the subsequent DNA demethylation (87-89). During development, the primordial germ cells undergo a wave of chromatin reprogramming between E11.5 and E12.5 including global demethylation to establish totipotency (90). Recent studies have revealed high levels of TET proteins and 5hmC accompanying the demethylation process, suggesting a possible redundant regulatory role of 5hmC in the systematic erasure of DNA methylation (91). Notably, the genome-wide methylation and hydroxy-methylation analysis has identified rare promoters and regulatory elements that evade the comprehensive DNA demethylation in primordial germ cell formation, providing potential mechanistic avenues to transmit epigenome information across multiple generations (91). Investigating whether trans-

generational inheritance of maternal high-fat diet is functionally associated with DNA methylation memory is of particular interest.

My current work in the lab aims at dissecting the potential mechanistic links of trans-generational inheritance of maternal diet induced changes and DNA methylation in offspring of a maternal high-fat diet model. We established a maternal high-fat mouse model and harvest liver tissues from both the maternal and the offspring groups to conduct whole genome screening of methylation profiles. Previous study of maternal high-fat diet identified increased hepatic insulin like growth fater-2 (Igf-2) and peroxisome proliferator activated receptor-alpha (Ppara) to be involved in fetal growth and fat metabolism (92), suggesting that developmental and metabolic programming is possibly induced by maternal high-fat diet. Notably, Zhang et al. 2009 also identified a group of reduced mi-RNAs that could potentially target methyl-CpG binding protein 2 (MeCP2), providing links to DNA methylation in the transmission of metabolic memory. Our RRBS results, when analyzed, will provide evidence of epigenetic inheritance in maternal high-fat diet induced effects in offspring.

Maternal effect is complicated because trans-generational inherited effects cannot be separated from in-utero exposure since the F1 embryos as well as the primordial germ cells of F2 are both exposed to the same in-utero environment. Although in epidemiological studies of trans-generational inherited traits, like our maternal high-fat obesity pregnancy model did not exclude in-utero effects. Our model, therefore, mimics an obesity pregnancy case as these moms are likely exposed to high-fat food during pregnancy, and thus provides an opportunity to investigate

the mechanistic basis of the predisposition to metabolic disease of the progeny. Further studies to eliminate in-utero effects will be conducted in F3 to identify if the inherited epigenetic changes identified in F1 are heritable along multiple generations.

While the current interests of epigenetic trans-generational inheritance have focused on DNA methylation, it is highly likely that the other epigenetic mechanisms or combination of the epigenetic events participate in the heritable epigenome. For example, noncoding RNAs including siRNAs, piRNAs are involved in epigenetic inheritance (93-96), it is also likely the nc RNA mediated changes in chromatin states to establish the inheritance. Due to the unconventional mode of inheritance, evidence of trans-generational epigenetic inheritance has been actively sought after in different animal model in recent years, the reasons why epigenetic inheritance exist deserves more thoughts. It is clear that epigenetic inheritance is not wide spreading, and in gametes development most of the epigenetic marks are erased to ensure a fresh start-totipotency. So why do we need to inherit epigenetic information if we have DNA, the genetic material carrier doing the major work of inheritance? Does it have evolutionary function? One would argue that if it exists in multiple organisms, nature has selected it for a reason that we do not fully understood yet. Epigenetic modifications occurring in cells can be divided into two major categories: 1) Programmed epigenetic modifications by the genetic information, those that occur during development in mediating cell lineages would fall into this category. 2) Fluctuated epigenetic modifications influenced by the environment, those events in response to diet, stress, viral infections, etc. would fall into this category. The trans-generational epigenetic inheritance we refer here is inheritance of the epigenetic information accumulated due to the second category. It is possible that these environmental signals carried through via epigenetic memory confer

biodiversity, and those that provides evolutionary advantage will eventually be captured in the genome. Many questions remain to be answered, including what signals regulate the decision of which the epigenetic modifications are maintained versus erased, what determine the duration to carry on modifications at a locus, can these marks be reset and how. Knowledge of the epigenetic mechanisms taking place during maternal obesity as well as its inheritance will help answer these questions as well as how the epigenetic factors are contributing to the negative gestational effects and predisposition to diabetes.

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