IDENTIFICATION AND CHARACTERIZATION OF MAFA COREGULATORS: MLL3/4 AND ITS ROLE IN MOUSE AND HUMAN ISLET β-CELLS

By

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INTRODUCTION

Diabetes Mellitus and the Pancreas

Pancreatic Cell types and functions

The pancreas is a multifunctional organ in mammals, located between the stomach, duodenum, and spleen. It possesses two important roles: an exocrine function through secretion of digestive enzymes from acinar cells, and an endocrine function through hormone secretion from Islets of Langerhans. The digestive enzymes produced in acinar cells are secreted through the pancreatic ducts into the duodenum, where they aid in the breakdown of carbohydrates, proteins, and lipids. The Islets of Langerhans (referred to hereafter as islets) contain several different hormone-producing cell types, and secrete these hormones into the circulating bloodstream.

Islets contain 5 distinct cell types: α-cells, β-cells, δ-cells, ε-cells, and pancreatic polypeptide (PP)-cells (**Figure 1**). These cells each produce and secrete a different hormone involved in the regulation of metabolism and glucose uptake, with α-cells producing glucagon, β-cells producing insulin, δ-cells producing somatostatin, ε-cells producing ghrelin, and PP-cells producing pancreatic polypeptide. While the β-cell is the most predominant cell type in the islet, the proportion of cells in the islet is partly dependent on the organism: mouse islets contain ~74% β-cells, 19% α-cells, and 6% δ-cells, while human islets contain ~54% β-cells, 34% α-cells, and 10% δ-cells.[1] Additionally, there is significant variability in the proportion of β-, α-, and δ-cells in the human, with β-cells ranging from 28% to 75% of the islet.[1] In combination with the data showing that proportions of islets cell types change dependent on the age of the

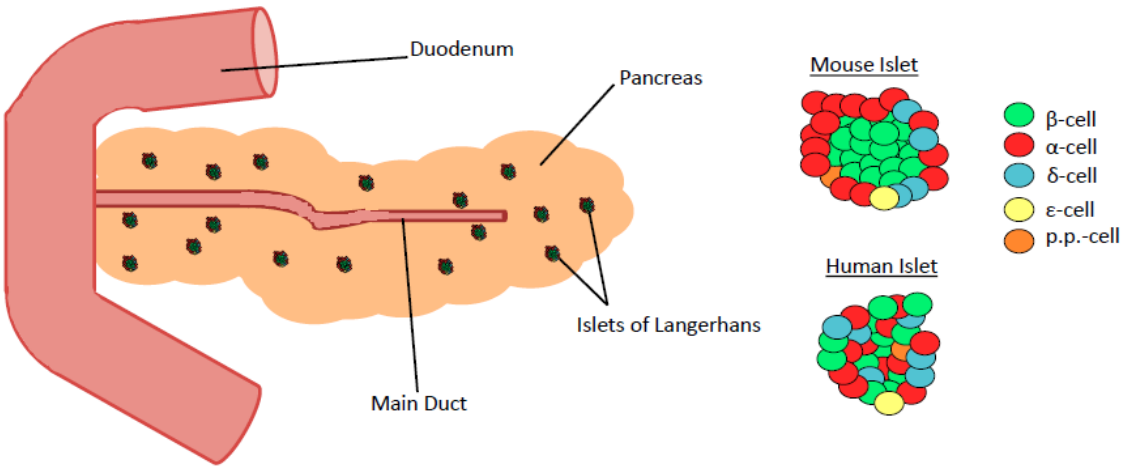


Figure 1. The Pancreas and the Islets of Langerhans. Schematic of the pancreas, and of the islets of Langerhans. Mouse islets and human islets differ both in their composition and architecture.

individual[2], this suggests that islet proportions vary from person to person, as well as within the same pancreas.

The predominant cells responsible for regulating glycemia are the α- and β-cells. β-cells regulate blood glucose levels by secreting insulin, which stimulates peripheral tissues such as the liver, fat, and skeletal muscle to take up and store glucose from the blood, thus preventing hyperglycemia. The counter-regulatory hormone glucagon stimulates glugoneogenesis and glycogen breakdown in the liver, causing a rise in blood glucose levels.[3] In addition, these hormones display paracrine activity within the islet, with insulin secreted from β-cells regulating the secretion of glucagon from α-cells, and vice versa.[4] Excess blood glucose results in hyperglycemia, while insufficient blood glucose will result in hypoglycemia.

Role of the β-cell in Diabetes Mellitus

The term Diabetes Mellitus refers to metabolic disorders characterized by chronic hyperglycemia (≥ 7mM fasting blood glucose), associated with either β-cell destruction or defective β-cell function and insufficiency.[5] Type 1 Diabetes (T1D) results from a near total autoimmune destruction of the β-cells, while Type 2 Diabetes (T2D) results from peripheral insulin resistance and β-cell dysfunction. This disease highlights the central role of the β-cell in regulating glycemia. In addition to β-cell dysfunction, α-cells have also been shown to display impaired function in rats with chemically ablated β-cells, a model of T1D.[6] Under normal conditions, α-cells secrete glucagon at low glucose to stimulate the release of stored glucose from the liver. In these defective α-cells, glucagon is secreted at high glucose concentration, counter to their normal function and further contributing to hyperglycemia. Without treatment, chronic hyperglycemia can result in blindness, nephropathy, neuropathy, and lead to increased risk for other

problems such as cardiovascular disease. Acute hyperglycemia can result in stupor, coma, or even death.

In addition to T1D and T2D, monogenic forms of the disease also exist, frequently referred to as Maturation Onset Diabetes of the Young (MODY). MODY can be caused by mutations of several genes, including genes associated with β-cell function such as Glucokinase (*GCK*), subunits of the K_{ATP} channel *ABCC9* and *KCNJ11*, and insulin itself (*INS*). However, a majority of the MODY genes identified are transcription factors, including *PDX-1*, *HNF1α*, *HNF1β*, *HNF4α*, *NEUROD1*, *KLF11*, and *PAX4*. Transcription factors regulate genes that direct the differentiation of β-cells, as well as genes that directly function in glucose-stimulated insulin secretion.

When faced with persistent hyperglycemia, the normal response of the pancreas is to increase insulin release, resulting in hyperinsulinemia and possibly increased β-cell mass (**Figure 2**).[7] In T2D, β-cells fail to compensate and chronic hyperglycemia leads to β-cell dysfunction, and decreased β-cell mass.[8,9] Interestingly, some transcription factors have also been shown be affected in T2D. For instance, levels of PDX-1, NKX6.1, MAFA, and MAFB have been shown to be reduced in islets from T2D patients in comparison to healthy individuals.[10] In mouse models of T2D, loss of MafA and Nkx6.1 transcription factors is preceded by the cytoplasmic to nuclear translocation of the transcription factor FoxO1, an indication of oxidative stress.[10] When oxidative stress is reversed by β-cell-specific overexpression of the antioxidant enzyme glutathione peroxidase-1 (Gpx-1), MafA expression is restored.[11]

Several mouse models of T2D exist, including the leptin-receptor deficient mouse (db/db), a Glut4-Cre Insulin Receptor Knockout (GIRKO) mouse where the insulin receptor is deleted in peripheral tissue , and a β-cell specific overexpression of ATP-insensitive Kir6.2 channel (K_{ATP}-GOF) mouse.[12,13(p4),14] In these models, there is a

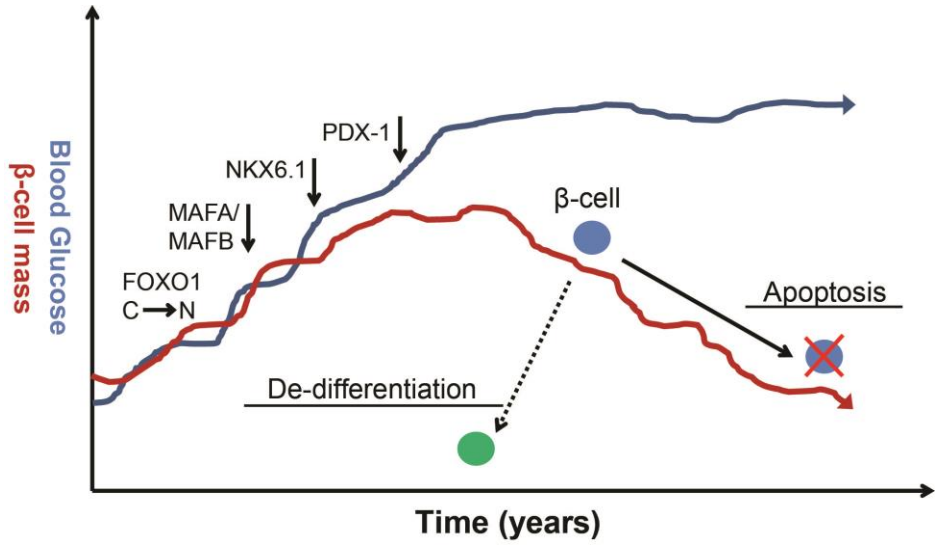


Figure 2. Development of Type 2 Diabetes over time. Red line denotes predicted changes in β-cell mass, the blue line denotes average blood glucose levels. FOXO1 cytoplasmic to nuclear translocation is proposed to precede sequential loss of MAFA, MAFB, NKX6.1, and PDX-1 to in the development of Type 2 Diabetes.

significant loss of β-cells in response to chronic hyperglycemia, yet there is very little concomitant increase in β-cell apoptosis. Instead, loss of β-cell mass appears to be predominantly driven by β-cell dedifferentiation, as measured by re-expression of developmental markers such as Ngn3.[15,14] In GIRKO mice, lineage tracing of β-cells revealed that previously insulin$^+$ cells had lost expression of maturity markers such as MafA and Pdx-1, and re-expressed the developmental transcription factors such as Ngn3, Oct4, and Nanog. It remains unclear whether this process also occurs in humans. Some groups have reported increased protein expression of Ngn3 in islets of T2D patients,[16] while research from our lab reported that there is no change in mRNA expression of *Ngn3* in T2D patients, although there were changes in *Oct4* expression.[10] This indicates that transcription factors not only play a critical role in guiding β-cell development, but that their loss can be a signature of dysfunction and disease.

Current Limitations in Treatment

Current therapies for diabetes depend on the type and severity of the disease. Treatments for T2D generally seek to address the underlying cause of peripheral insulin resistance, obesity, while simultaneously lowering blood sugar.[17] The most broadly used pharmacological agent in treating T2D is metformin, which acts in the liver to inhibit gluconeogenesis. In addition, patients are counseled towards lifestyle changes that would lead to weight loss and increased exercise. However, many patients still become increasingly hyperglycemic, and some will require exogenous insulin treatment. The International Diabetes Federation predicted that the number of patients living with Diabetes would increase from 366 million in 2010 to 552 million by 2030. This highlights the importance of developing a better understanding of the factors that govern β-cell function, in order to develop novel treatments for diabetes.

T1D patients are treated with exogenous insulin, and must regularly monitor their blood glucose multiple times per day. Interestingly, transplanted islets from cadaveric donors have been successfully used to treat T1D, with patients able to maintain euglycemia for at least a year following transplantation.[18,19] Unfortunately, this treatment also requires long term use of immunosuppressive drugs in order to prevent both alloimmune and autoimmune rejection. Additionally, there are simply not enough donated islets of sufficient quality available for the number of T1D patients.[20] A long-term goal of researchers has therefore been to generate islets from precursor cells such as stem cells.[20] This would eliminate the need for cadaveric donors, and ensure that quality and quantity could be carefully controlled. The advent of induced pluripotent stem cells (iPSC) has raised the possibility that a T1D patient may be able to provide the necessary precursor cells, thus preventing the need for long-term immunosuppressive therapy.[21]

Significant progress has been made in establishing a differentiation protocol for the production of functional β-cells. Initial protocols were able to differentiate hESCs into insulin$^+$ cells, but these cells were frequently polyhormonal and failed to properly secrete insulin in response to glucose.[22,23,24] These cells also failed to express MAFA, a key marker of β-cell maturity in both humans and mice. Within the past year, protocols have been developed which produce insulin$^+$ cells that do not co-express other islet hormones, that express MAFA and that release insulin in a glucose-dependent manner.[21] Furthermore, upon transplantation these cells are able to ameliorate the hyperglycemia of T2D mouse models, which is the ultimate goal of these strategies. However, while significant progress has been made in establishing differentiation protocols to produce functional β-cell cells, many challenges remain.[25] A better understanding of β-cell development and the factors required for cell maturity and function remains necessary to continue the improvement of these protocols, to adapt

them to other sources of cells such as iPSC cells or transdifferentiation of other mature cell types, and to ensure that transplanted cells stay committed to their β-cell lineage.

Islet-Enriched Transcription Factors

A vast body of literature has arisen over the past two decades utilizing the elegant genetics of the mouse model system. These studies have highlighted the importance of transcription factors in guiding the differentiation of the pancreas, through a step-like progression, to produce mature endocrine cells including the β-cell.[26] However, transcription factors are not only critical in guiding cell development, but also in controlling function. This section will describe a subset of those transcription factors critical for β-cell development and function.

The Maf family of transcription factors

The founding member of the Maf family of proteins was identified as viral Musculoaponeurotic Fibrosarcoma (v-maf, homologous to c-Maf in rodents), an oncogene in avian virus AS42.[27] Subsequent studies identified other highly related proteins, including Neural Retina Leucine zipper (NRL), MafB, and L-Maf/MafA, all of which possess a conserved leucine zipper and basic DNA binding domain (see **Figure 3**). NRL was first identified in a cDNA screen of human retina and retinoblastoma cell lines.[28] MafB was initially identified as a cDNA affected by the mouse Kreisler mutation, causing abnormalities in hindbrain and inner ear development.[29] L-Maf (homologous to MafA in rodents) was identified initially as a novel Maf member expressed in the quail neuroretina and chicken lens.[30,31]

To date, there are 11 known Maf family members, which are further divided into two subgroups: large Maf proteins and small Maf proteins. Small Maf proteins (MafK, MafF, MafG, MafT, and Maf-S) lack a transactivation domain and are thought to repress

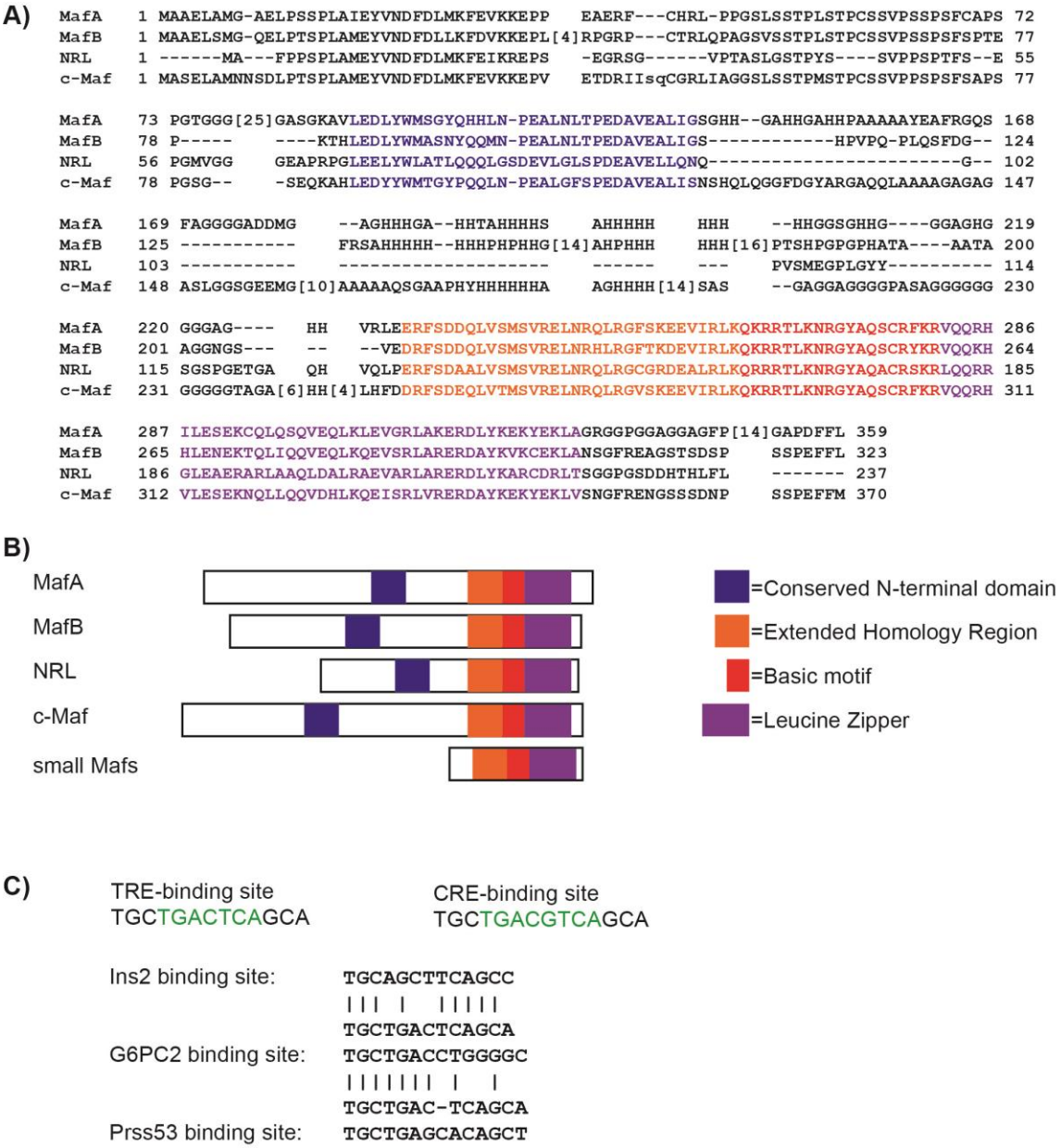


Figure 3. The Maf family of transcription factors. A) Sequence alignment shows the similarity between the 4 large Maf proteins. Blue represents a conserved N-terminal domain, orange the extended homology region, red the basic motif, and purple the leucine zipper. **B)** Diagrammatic representation of the large Mafs and a representative small Maf. **C)** Tre- and Cre-type MARE binding sites. These binding sites are often different from the consensus MARE bindng sites, as shown by genomic binding sequences for Ins2, G6PC2, and Prss53 bound by MafA in β-cells (top) compared to a consensus MARE sequence (bottom).

transcription through heterodimerization with other proteins, including members of the Cap n' Collar (CNC) transcription factor family.[32,33] Recently, expression of the small Mafs has been reported in the islet β-cell, although the extent to which they compete with large Maf proteins for binding and their function in the islet remains unclear.[34] Transgenic overexpression of MafK has been shown to inhibit β-cell activity, while overexpression of a dominant-negative MafK improves β-cell function by increasing insulin transcription and secretion.[35,34] This suggests that small Mafs may play an inducible role in order to repress the activity of their Large Maf counterparts. Large Maf proteins (c-Maf, MafA, MafB, and NRL) contain a transactivation domain which shares significant homology among these proteins.[36,37]

The conserved DNA-binding domain of the Maf proteins shows weak similarity to the DNA-binding domains of AP-1 superfamily members.[38] The AP-1 superfamily consists of Fos and Jun transcription factors, which are well known for their ability to bind the phorbol-12-O-tetradecanoate-13-acetate (TPA)-responsive element (TRE; TGACTCA) and the cyclic AMP-responsive element (CRE; TGACGTCA). Interestingly, the consensus Maf recognition element (MARE) contains a TRE element, with Maf proteins also able to bind a similar sequence containing the Cre element (see **Figure 3** and 39). This consensus site was generated from a screen of random 20 base pair nucleotides using the v-Maf DNA binding and dimerization domains as bait.[39] However, when examining ChIP-seq data generated using a MafA antibody in adult mouse islets,[40] this binding site appears to be highly variable (**Figure 3C**).

Another characteristic of the AP-1 family is its members' ability to heterodimerize with each other. For example, Fos and Jun proteins were shown *in vitro* to heterodimerize for increased transcriptional activation.[41] Indeed heterodimerization can affect DNA binding ability, as Fos was unable to bind the TRE binding site by itself, but when heterodimerized with Jun DNA binding was observed.[42] Indeed, some of the Maf

proteins are also able to heterodimerize with these AP-1 family members, such as c-Maf and NRL heterodimerizing with Fos and c-Jun if expressed in the same cell.[38,43,44] Of the large Maf family of proteins, only MafA and MafB have been shown to be expressed in the β-cell, as NRL appears to be restricted to the nervous system,[45] and c-Maf expression has only been reported in the α-cell.[46]

MafA

MafA is expressed in the developing nervous system, lens, and pancreas in birds, but has so far only been detected in the lens and pancreas of mammals.[47] MafA was first identified in the pancreas as the transcription factor capable of binding the C1-RIPE3b1 element of the rat *insulin II* enhancer.[48,49,50] The insulin II enhancer has previously been shown as a well characterized region, bound by several other islet-enriched transcription factors, and the C1-RIPE3b1 element conferred β-cell specific activity.[51] The identification of MafA as the C1-RIPE3b1 binding protein therefore implicated it as an important driver of β-cell specific transcriptional activity.

Further characterization of MafA's role in the β-cell revealed that MafA is first expressed in the mouse at E13.5 exclusively in insulin$^+$ cells within the pancreas, where it remains expressed throughout life.[52] Pancreatic endocrine cell development is generally broken into two phases: an early primary transition, where an epithelial outgrowth of foregut endoderm produces the first few insulin$^+$ and glucagon$^+$ cells, and a second transition, where there is a massive expansion in endocrine cell numbers as they divide and terminally differentiate. Endocrine cells produced during the primary transition are not thought to populate the adult islet.[53,54] The expression of MafA at 13.5 places it in the secondary transition, which is later than many other previously identified islet-enriched transcription factors (i.e. Pdx-1, NeuroD1, Ngn3, Pax6, *etc*). This suggested that MafA may play a role in β-cell terminal differentiation and function, and not

development. This hypothesis was confirmed by investigation of global MafA knockout and a pancreas specific-knockout, which lacked a developmental phenotype.[55,56] Interestingly, in humans MAFA is nearly undetectable in the early post-natal period, but is only detected at higher levels in adulthood (Dr. Holly Cyphert, personal communications).[57,58,10]

Functional characterization of MafA further supported a role in directing β-cell-specific function. *In ovo* electroporation of chick embryos to overexpress MafA leads to the generation of insulin^{+} cells.[59] Likewise, in a mouse pancreas-specific knockout of MafA (*MafA*$^{\Delta panc}$), several β-cell enriched genes (including *insulin2, Slc2a2, and Slc30a8*) are downregulated.[56] This translates into an impairment in glucose stimulated insulin secretion (GSIS), confirmed by both intraperitoneal glucose tolerance test and perifusion analysis of isolated islets. Additionally, perifusion analysis of islets from *MafA*$^{\Delta panc}$ mice indicated that impaired GSIS was due to a defect in first phase insulin secretion, and second phase insulin secretion was trending towards impairment (although not statistically significant).[56] In first phase insulin secretion, insulin granules that are already docked at the cell membrane are released, while second phase insulin secretion is driven by mobilization of cytoplasmic granules to the membrane. Electron microscopy analysis of *MafA*$^{\Delta panc}$ β-cells revealed that there were fewer insulin granules docked at the membrane, as well as fewer present in the cell. This suggests that MafA is critical for proper β-cell maturation and function.[56]

MafA activity in the β-cell is controlled in part through phosphorylation. MafA is heavily phosphorylated, and phosphorylation impacts MafA dimerization, DNA binding, and degradation.[60,61] Furthermore, phosphorylation in the N-terminal region of MafA (which contains the conserved Maf transactivation domain) is required for dimerization and DNA binding of the C-terminal portions of MafA.[60]. Phosphorylation of the N-terminal domain of MafA is driven in part through Glycogen Synthase Kinase-3β (GSK-3β).[61] This

phosphorylation subsequently recruits the histone acetyltransferase p300/CBP-associated factor (p/CAF), which aids in MafA target gene transactivation as well as protects MafA from degradation.[62]

MafA activity is only required postnatally in β-cell maturation, and lacks a defined developmental function, a role that is played by MafB. Analyses of both global MafA knockouts (*MafA*$^{-/-}$) and pancreas-specific knockouts (*MafA*$^{\Delta panc}$) have shown that MafA is required for proper islet architecture, β-cell proliferation and mass, regulation of several important genes, and GSIS.[55,56] MafA regulates a large number of genes, as identified via microarray analysis of isolated islets from *MafA*$^{\Delta panc}$ mice. Many of these genes have known roles in β-cell function, such as the glucose transporter *Slc2a2*, the Zinc transporter *Slc30a8*, *glucokinase*, *insulin*, and many others.

In humans, little is known about the function of MAFA, and much of what is known is correlative. For instance, early differentiation protocols for directing human Embryonic Stem Cells (hESCs) to insulin-producing cells resulted in the expression of MAFB, but not MAFA.[22] These cells were frequently polyhormonal (usually expressing both glucagon and insulin), and displayed impaired GSIS; thus supporting a role for MAFA in β-cell maturation in humans similar to its role in mice. Interestingly, recent advances and refinements in hESC differentiation protocols have led to the production of monohormonal insulin^{+} cells which are much more glucose-responsive.[21] Correspondingly, these cells express MAFA. Further supporting a role for MAFA in human β-cell maturation is data from a newly generated human β-cell line, EndoC-βH2.[63] These cells were derived from human fetal pancreas tissue, and contain excisable LoxP sites flanking the immortalizing *hTERT* and *Large T-antigen* genes. When these genes are excised upon exposure to Cre recombinase, the cells no longer proliferate and become more glucose-responsive. This also correlates with an ~8-fold increase in expression of MAFA, whereas other transcription factors (for instance, those

associated with MODY such as PDX-1 and NEUROD1) show no increase or only a minimal increase. Taken together, this data indicates that MAFA is likely playing a similar role in β-cell maturation and function in humans as in mice.

MafB

The expression pattern of MafB is distinct from that of MafA in mice and humans. In mice, MafB is expressed first starting at around embryonic day 10.5 (E10.5) in $Ngn3^+$ endocrine cells in mice, and is subsequently expressed in $insulin^+$ and $glucagon^+$ cells throughout development, before becoming restricted to α-cells around 2-3 weeks after birth (**Figure 4**).[64] Interestingly, MafA and MafB do not appear to be functionally redundant. *In ovo* overexpression of MafB by electroporation in chick embryos leads to $glucagon^+$ cells, not the $insulin^+$ cells seen with MafA. Likewise, in pancreas-specific knockout of MafA, MafB is upregulated in a subset of β-cells, yet these islets remain dysfunctional.[56] This suggests that, despite their high sequence homology and overlapping target genes, MafB plays a distinct role in β-cell development, but may be dispensable for postnatal function. This has been confirmed in mouse models of pancreas-specific MafB knockout, where β-cells do exhibit a developmental delay, but are able to function normally by at least 3-weeks of age (Elizabeth Conrad, personal communications).

The expression pattern of MAFB in humans is different from that of mice. While MAFB is expressed developmentally in both α-cells and β-cells, MAFB does not become restricted to α-cells postnatally, but maintains its expression in both α- and β-cells in the adult.[58] Adult human β-cells therefore co-express both MAFA and MAFB. This is particularly interesting as MAFA and MAFB are thought to heterodimerize, yet in mice appear to have some non-overlapping roles. This could indicate that MAFA and MAFB are able to form both homodimers and heterodimers in adult humans, with distinct roles

for each. In mice, co-expression of MafA and MafB occurs during late embryonic development in β-cells, and it remains unclear how Maf regulation occurs during this time, and whether MafA and MafB regulate these genes in distinct homodimeric complexes or as a heterodimer. In addition, as mentioned above, MafA is controlled post-translationally through phosphorylation. Although MafB is phosphorylated on several amino acids, phosphatase treatment does not inhibit MafB dimerization or DNA binding.[60] As of yet, phosphorylation of MafB does not play any known roles, although it remains possible that phosphorylation regulates the ability of MafB to interact with other proteins, such as coregulators.

The function of MafB in the β-cell has been difficult to analyze, as global knockout of MafB is perinatal lethal due to central apnea or renal failure.[65,66] Developmentally, however, these mice display reduced numbers of insulin$^+$ and glucagon$^+$ cells, although the total number of endocrine positive cells remains the same, as measured by Isl1 expression.[67] This suggests that MafB is required for proper β-cell specification. Indeed, MafB is found to regulate several genes critical for β-cell function (such as *G6pc2*, *Slc30a8*, *Slc2a2*, etc.), which are subsequently regulated by MafA postnatally. However, perinatal lethality of global MafB knockouts has prevented the postnatal analysis of MafB and its possible roles in β-cell maturation.

Interestingly, our lab has recently generated a pancreas-specific knockout of MafB, which does seem to have similar developmental defects to what has previously been shown.[64] However, these mice do express MafA in β-cells postnatally, and lack a detectable phenotype after 3-weeks of age (Elizabeth Conrad, personal communications). Combined pancreas-specific deletion of both MafA and MafB leads to death shortly after birth, with pups showing severe hyperglycemia (Elizabeth Conrad,

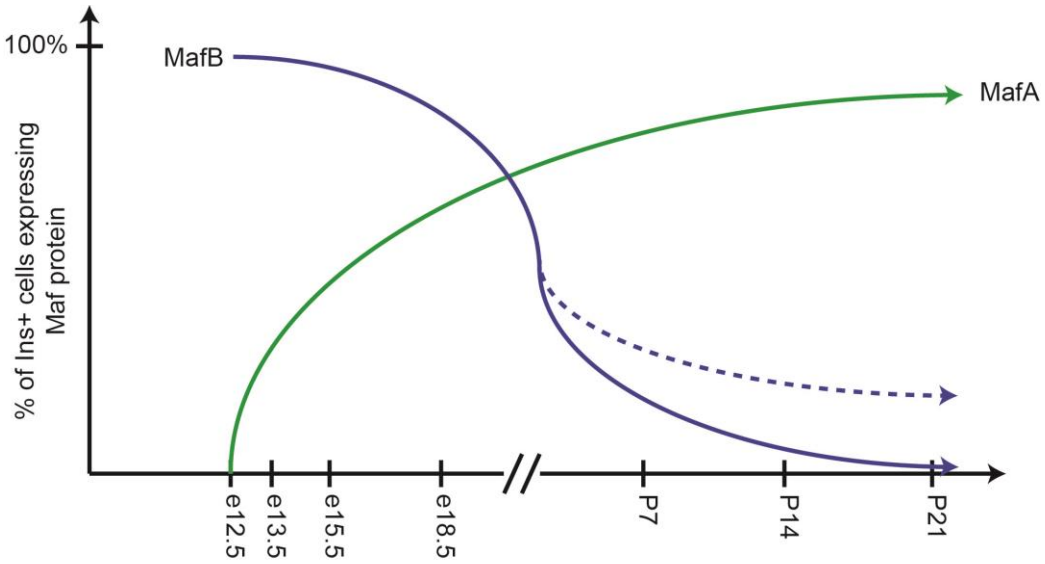


Figure 4. Mouse β-cells transition from MafB expression to MafA after birth. The green line represents MafA expression in Ins+ cells, the purple line represents MafB expression in Ins+ cells. The dashed purple line indicates MafB expression is retained in a portion of Ins+ cells in *MafA*$^{\Delta panc}$ mice.

personal communications). This indicates that while MafA and MafB have many distinct functions, together they play overlapping roles essential to the β-cell.

Pdx-1

Pancreatic and duodenal homeobox gene-1 (Pdx-1, or IPF-1 in humans), is a homeodomain-containing transcription factor. Early studies in mice showed that a global null allele prevented pancreas formation and led to death shortly after birth.[68,69,70] Pdx-1 is first expressed at e8.5 in the dorsal endoderm, prior to budding of the pancreas.[71] Lineage tracing experiments have subsequently shown that Pdx-1$^+$ progenitor cells give rise to all pancreatic cell types.[72] In the adult, Pdx-1 expression is maintained at high levels in the β-cell, where it plays important roles in regulating genes such as *Ins1*, *Slc2a2*, and *MafA*, as well as maintains β-cell identity by preventing expression of other proteins such as the α-cell marker glucagon.[73,74] Thus Pdx-1 plays multiple roles in regulating not only pancreatic development, but also mature β-cell function and identity.

Interestingly, in humans heterozygous mutations in *PDX-1* lead to MODY4,[75] whereas homozygous deletion leads to pancreatic agenesis, similar to the rodent.[70] In islets from T2D humans, PDX-1 is also one of 4 islet-enriched transcription factors downregulated as a result of oxidative stress.[10] Together these studies indicate that PDX-1 plays a critical role in the human disease. Pdx-1 expression also drives the endocrine lineage towards the β-cell when misexpressed during development.[76] However, Pdx-1 expression alone is incapable of reprogramming postnatal α-cells or exocrine cells to insulin$^+$ cells, indicating that it is necessary but not sufficient to drive β-cell transdifferentiation.[76,77] Correspondingly, early attempts to generate functional β-cells from human ESCs *in vitro* revealed that while PDX-1 expression is induced, very few of these cells become monohormonal β-cells and lack normal glucose-responsiveness.[22] More recent work on hESC differentiation has produced

monohormonal insulin$^+$ cells that are glucose response.[24,21] These cells also express PDX-1, indicating that as in rodent models, PDX-1 is necessary but not sufficient to drive functional human β-cell formation.

Pdx-1 is also one of the best-studied pancreas transcription factors for its role in coregulator recruitment. It mediates glucose-regulated insulin transcription through recruitment of the acetyltransferase p300 to the insulin promoter.[78] Pdx-1 also recruits the histone deacetylase proteins HDAC1 and HDAC2 to the *insulin2* promoter under low glucose conditions, thus removing histone acetylation and reducing *insulin2* transcription.[79] However, in addition to controlling histone acetylation, Pdx-1 has recently been shown to recruit the ATP-dependent chromatin remodeler Brg1 of the SWI/SNF complex to not only *insulin2* regulatory elements, but also Pdx-1 binding sites near *MafA* and *Pax4*.[80] This interaction also appeared to be regulated by glucose, with fewer Pdx-1/Brg1 interactions in low glucose conditions. This suggests that Pdx-1 is playing multiple roles in coordinating both histone acetylation and chromatin architecture, and that this recruitment can be carefully controlled by physiological conditions. Furthermore, PDX-1/BRG1 interaction appears to be downregulated in islets from T2D patients. This indicates that not only is there a reduction in the expression levels of Pdx-1 under pathophysiological conditions, but that chronic hyperglycemia may also affect transcription factor/coregulator interactions.

Ngn3

Neurogenin 3 (Ngn3) is a basic helix-loop-helix transcription factor, one of three closely related members of the neurogenin transcription factor family.[81] Ngn1, Ngn2, and Ngn3 are expressed in the developing nervous system, yet only Ngn3 is expressed in the developing pancreas.[81,72] Within the pancreas, Ngn3 is expressed in endocrine progenitor cells prior to hormone expression, and mice lacking *Ngn3* fail to develop

endocrine cells, and are extremely hyperglycemic only two days after birth.[72] Ngn3 expression is also maintained at low levels in a subset of endocrine cells after birth, and plays a role in maintaining β-cell function.[82] In contrast to Pdx-1, there is currently very little known about Ngn3 coregulators.

Interestingly, recent reports have shown Ngn3 to be an important factor in reprogramming other cell types to β-cells, in combination with MafA and Pdx-1.[77,83] Other studies have demonstrated that β-cells seem to de-differentiate in mouse models of FoxO1-ablation or models of T2D such as leptin receptor deficiency (db/db). , Evidence for β-cell de-differentiation includes increased expression of Ngn3 as well as other markers of progenitor cells such as Oct4 and L-myc.[15] This data is particularly of interest when considering that, despite a significant loss of insulin+ cells in patients with T2D, there is relatively little β-cell apoptosis observed in these islets.[16] Increases in NGN3 immunolabeling have also been reported in the pancreas of T2D humans.[16,14] However, our laboratory did not observe increases in *NGN3* mRNA in islets isolated from T2D patients, although we did detect increased levels of *OCT4* mRNA.[10] Ngn3 is therefore a critical transcription factor not only in the differentiation of the endocrine lineage, but may also be involved in de-differentiation in cases of extreme β-cell stress. The precise mechanisms through which it exercises this transcriptional control remain unknown.

Other Islet-enriched transcription factors

As mentioned previously, a majority of the identified MODY genes encode transcription factors (*PDX-1*, *HNF1α*, *HNF1β*, *HNF4α*, *NEUROD1*, *KLF11*, and *PAX4)*. Most of these transcription factors have been characterized *in vivo* for their roles in the development of the mouse islet, and specifically the β-cell. Indeed, pancreatic β-cell development has been shown to progress through a step-wise expression of various transcription factors, leading from definitive endoderm to mature β-cell (**Figure 5**).[26]

Many of the transcription factors expressed during mouse β-cell development are MODY genes in humans, and heterozygous loss of gene function in humans (or homozygous loss in mice) leads to β-cell defects and severe hyperglycemia. For instance, *NeuroD1*$^{-/-}$ mice have a severe reduction in the number of β-cells at birth, and die shortly after birth with hyperglycemia.[84] Heterozygous mutations affecting either NEUROD1 DNA binding or transactivation are associated with development of diabetes in humans.[85] This demonstrates the utility of mouse models in examining the function of transcription factors in β-cell development.

However, while all of these transcription factors produce a pancreatic phenotype in mice, nearly all are more severe than deletion of MafA, which lacks a known mutation related to human disease. Several of these transcription factors have multiple roles in the pancreas, including cell specification, proliferation, and subsequent function. One example of a transcription factor with multiple roles is Pdx-1, where mice lacking Pdx-1 exhibit pancreatic agenesis, and deletion at 1-month of age in β-cells reveals additional roles in maintaining β-cell identity after birth.[73] By comparison, MafA is expressed much later than most other islet enriched transcription factors, is specific to the β-cell, and only affects adult β-cell function, not development.[56] In addition, in humans MAFA is one of the only islet-enriched transcription factors whose expression correlated with β-cell maturity in hESC differentiation protocols,[22,21] and MAFA induction has been shown to correlate with GSIS in human cell line models.[63] We have therefore focused our research on the role of MafA, as it plays a critical role in maturation and function of the mouse β-cell, and MAFA appears to play a similar role in the human β-cell.[56]

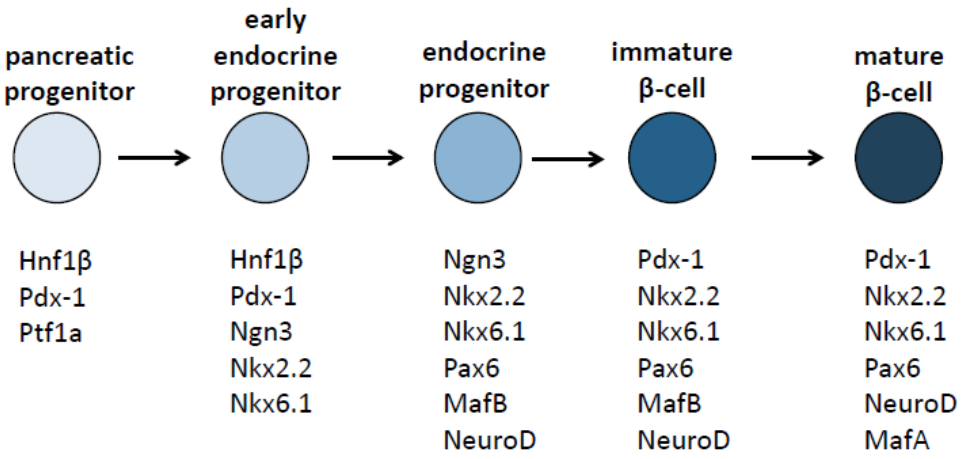


Figure 5. Transcription factors involved in β-cell differentiation. An abbreviated list of the transcription factors involved in each step of β-cell development. Adapted from Ref 26.

Coregulators and Transcription

History and Function

DNA, the fundamental blueprint for life, is present in every living organism. In eukaryotes, DNA is rarely found unbound by protein, but is instead tightly wrapped around octamers of histone protein. Each octamer contains two subunits each of histone 2A, 2B, 3, and 4.[86] One hundred and forty-six base pairs (bp) of DNA wrap around a single histone octamer, forming a nucleosome. All of these histones contain an N-terminal tail that extends away from the octamer, and can undergo many different post-translational modifications, including acetylation, ubiquitination, methylation, phosphorylation, and others. Through these modifications, nucleosomes can thus function not only in the compaction of DNA, but in allowing structural control of DNA transcription without affecting the DNA sequence itself. Additionally, distal DNA regulatory elements can be brought into close proximity via chromatin looping, allowing for local control over DNA architecture through distal control elements.[87] A combination of histone modifications and transcription factors bound to DNA sequence-specific enhancers can either encourage the recruitment of the transcriptional machinery (see **Figure 6**), including RNA polymerase II, or inhibit it.

Histone modifications (marks) on specific residues play an important role in controlling gene activation or repression. This specificity is important, as the specific histone amino acid being modified will determine whether the mark is activating or repressing. For instance, Histone 3 Lysine 4 trimethylation (H3K4me3) is an activating mark frequently found near actively transcribed promoters, whereas Histone 3 Lysine 27 trimethylation (H3K27me3) is found near actively repressed promoters.[88] Histone acetylation is generally an activating mark, and can occur in enhancers such as with Histone 3 Lysine 27 acetylation (H3K27ac),[89] or at promoters such as Histone 3 Lysine 9

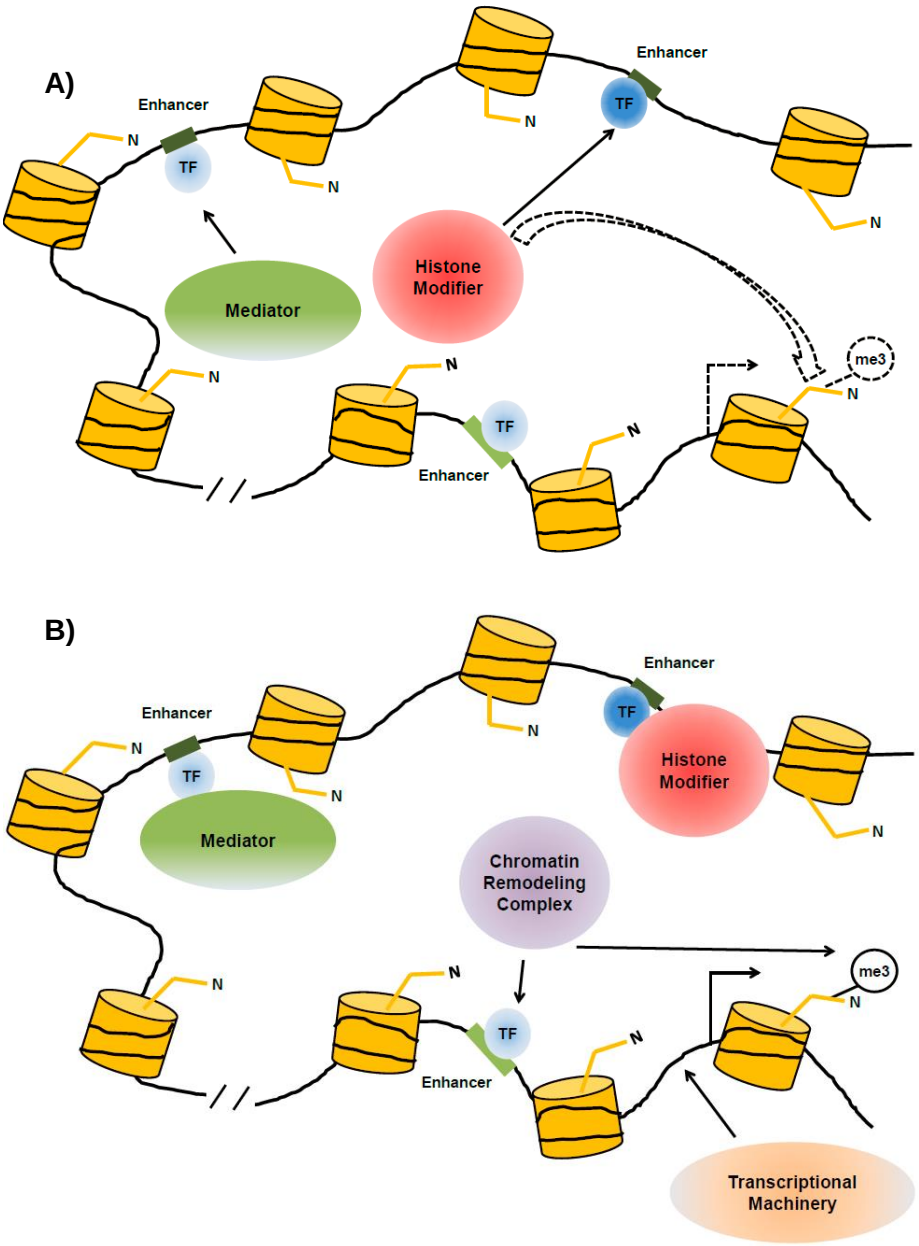


Figure 6. Model of Chromatin Architecture and Transcription. A) Coregulators such as the Mediator complex can be recruited to control chromatin looping, while histone modifiers can add post-translational modifications to either neighboring histones, or distal histones via chromatin looping. **B)** Histone modifications can act as binding sites for histone modifiers and chromatin remodeling complexes, and allow for recruitment of the transcriptional machinery and ultimately RNA Polymerase II. TF = Transcription Factors.

acetylation (H3K9ac).[90] Indeed, both activating and repressive histone modifications can be present at the same loci. This largely occurs during development, when the same gene is marked by both activating H3K4me3 and repressive H3K27me3, generating a bivalent state.[88] These opposing marks can prime a gene for subsequent activation or repression during later stages of development.

Control of nucleosome architecture and transcription is primarily mediated through proteins known as coregulators. The Nuclear Receptor Signaling Atlas (www.nursa.org) currently lists ~320 known coregulators, with diverse functions including histone modification, DNA methylation, and chromatin rearrangement. DNA methylation is primarily thought to repress transcription, while ATP-dependent chromatin remodeling can have both activating and repressive effects on transcription.[91] Chromatin modifiers make up a large fraction of the known coregulators, and are responsible for a variety of post-translational modifications as well as amino acid specificity in their marks. For instance, proteins of the yeast Complex Proteins Associated with Set1 (COMPASS) are responsible for mono-, di-, and trimethylation of Histone 3 Lysine 4 (H3K4). This is accomplished through a conserved SET domain, which gets its name from the Su(var)3-9, Enhancer of Zeste (EZ), and trithorax (Trx), proteins which all share a conserved methyltransferase domain. These marks can occur on enhancers (as is the case of mono methylation), or near transcriptional start sites (as is the case of trimethylation), but are always activating. The COMPASS proteins do not methylate lysine residues 9 or 27 of Histone 3.[92] Thus each coregulator is associated not only with a specific post-translational mark, but frequently a specific histone and amino acid for its target.

These histone modifications have a significant impact on transcription. In addition to interacting with transcription factors, coregulators preferentially interact with specific modifications and DNA itself. For instance, several coregulators contain a Plant homedomain (PHD) finger, which binds the methylated lysines of histones. One example

of this is the BPTF subunit of the nucleosome remodeling factor (NURF) complex, which binds to H3K4me3 through its PHD domain.[93] In the absence of H3K4me3, the BPTF subunit is no longer able to bind to the nucleosome, and subsequent regulation of transcription by the NURF complex is compromised. This requirement that histones are marked by H3K4me3 to provide a binding site for BPTF and the NURF complex demonstrates that histone modifications provide an additional level of spatial coordination, along with the sequence-specific binding of transcription factors, for controlling recruitment of coregulators and subsequent regulation of chromatin architecture and RNA Polymerase II recruitment.

Transcription factors and Coregulators

While coregulators are capable of directing transcription, they lack the ability to bind DNA in a sequence specific manner. Instead, they rely on transcription factors for this function. Transcription factors are capable of binding DNA in a sequence specific manner (see **Figure 3**) for the purpose of recruiting coregulators to specific DNA regulatory elements. Coregulators are then able to either activate or repress transcription by controlling DNA accessibility or RNA polymerase II recruitment.

There are many known examples of transcription factors recruiting coregulators for gene regulation. One example is the Farnesoid X Receptor (FXR), a nuclear receptor that binds DNA and recruits the MLL3/4 complexes to its target regulatory sequences when stimulated by its ligand chenodeoxycholic acid (CDCA) in human liver HepG2 cells.[94] The MLL3/4 complexes are members of the COMPASS family of H3K4 methyltransferases, which direct gene activation. Knockdown in HepG2 cells revealed that, in the absence of MLL3 and MLL4 (or their required subunit, NCOA6), FXR target genes were not H3K4 trimethylated. Furthermore, this led to a loss of expression of these target genes. This study highlighted three important functions of coregulators: that

they can control gene activation (or repression) through their role in regulation of histone modifications, that they display specificity in their recruitment by selective interaction with transcription factors, and their recruitment can be temporally controlled by post-translational processes such as ligand binding (in the case of nuclear receptors) or post-translational modifications such as phosphorylation.

While transcription factors are frequently studied individually for their ability to control transcription, many transcription factors bind near to one another, suggesting coordinated action. For instance, characterization of *insulin* enhancers in rat and human have revealed that multiple transcription factors bind neighboring regulatory sequences.[51] These transcription factors include, among others, Pdx-1, NeuroD1, MafA and Glis3.[95] Indeed, these transcription factors seem to play synergistic roles in gene activation, as reporter assays utilizing the rat insulin enhancer have shown that combinations of these 4 factors leads to greater stimulation than would be predicted by their individual binding.[95,96] Previous work has shown that Pdx-1 and NeuroD1 cooperate to recruit the acetyltransferase p300 to the insulin enhancer,[97] and a more recent study has suggested that Glis3 may also contribute to the recruitment of the acetyltransferase CBP.[98] In addition, p/CAF has been shown to localize to and acetylate the mouse *Insulin2* promoter, although whether this is mediated through MafA or another transcription factor remains unclear.[99] Regulation of the insulin promoter by multiple transcription factors and coregulators suggests that it is the coordinated actions of transcription factors and their recruited coregulators that guide transcription of target genes.

Coregulators in the Pancreas

While there is much known about the coordinating roles of transcription factors and coregulators in several tissue types, the identity of these complexes in the pancreas

is less well understood. While there are hundreds of known coregulators, relatively few have a known interacting transcription factor in the pancreas (**Table 1**). The most well described example is Pdx-1, which interacts with p300[97], Set7/9[100] , Hdac1/2[79] , Pcif1[101], and Bridge-1[102]. More recently, Pdx-1 was found to also interact with the SWI/SNF complex.[80] In contrast to Pdx-1, MafA has only been linked to p/CAF.[61] p/CAF global null mice lack overt defects, indicating that it plays a minor or redundant role as a coregulator.[103]

While few coregulators have been directly linked to islet-enriched transcription factors, there are coregulators that have a known role in the pancreas, but for which the corresponding transcription factor is unknown. One example of this is the HDAC family of proteins. HDACs have several roles not only in normal development, but are frequently the target of drugs to fight diseases such as cancer.[104] In mice, many HDACs are expressed in the developing and adult pancreas such as Hdac1-7.[105] Broad inhibition of HDACs through small molecule inhibitors leads to a change in lineage allocation, with more ductal cells and $Ngn3^{+}$ proendocrine cells. Follow up studies using global knockout mice for *Hdac4*, *Hdac5*, and *Hdac9* were examined for pancreatic phenotypes.[106] These studies revealed that removal of *Hdac4* or *Hdac5* led to increased δ-cell number, while removal of *Hdac5* or *Hdac9* resulted in an increase in β-cell number. However, these studies mainly rely on analyzing changes in cell number, and do not provide mechanistic insight into the changes in gene expression. Interestingly, Hdac1 and Hdac2 interact with Pdx-1 under low glucose conditions, a time when the histones neighboring the insulin promoter are de-acetylated.[79] This interaction appeared to be linked to dephosphorylation of Pdx-1, as treatment with okadaic acid (a phosphatase inhibitor) prevented the interaction of Pdx-1 and Hdac2 under low glucose conditions. However, direct recruitment by Pdx-1 of Hdac1 or 2 to the insulin promoter was not

Table 1. Known coregulators of islet-enriched transcription factors

Protein	Coregulators	Additional Coregulators identified in our lab
MafA	p/CAF	MLL3/4 complex
Pdx-1	Bridge1, CBP/p300, Pcif, Hdac1/2, Set7/9	SWI/SNF complex, NuRD complex, MCM complex
NeuroD1	Bridge1, CBP/p300	
Nkx2.2	Grg3, Dnmt3a, Hdac1	
Pax6	p300	

shown, and many questions remain about the mechanisms involved.

Dnmt1 is another coregulator with a known role in the pancreas. Dnmt1, along with Dnmt3a and Dnmt3b, are DNA methyltransferase proteins responsible for methylation of the 5' position of a cytosine-guanine dinucleotide (CpG).[107] This DNA methylation mark, when present near the transcriptional start site (TSS) or within the first exon of a gene, is associated with repression of that gene. Dnmt1 maintains the DNA methylation status during cell replication, and Dnmt3a/3b are responsible for *de novo* methylation of DNA.[107] Interestingly, β-cell specific loss of *Dnmt1* led to conversion of β-cells to α-cells, in part due to the de-repression of the gene encoding the α-cell specific Arx transcription factor.[108] Furthermore, de-repression was linked to the activity of the histone methyltransferase Prmt6, which methylates histone 3 on arginine 2. This histone methylation has been shown to be antagonistic to H3K4 trimethylation, specifically through inhibition of Wdr5 binding and disruption of the MLL family of proteins. Further work has suggested that DNA methylation is the result of recruitment of Dnmt3a by Nkx2.2 to the *Arx* promoter.[109] Indeed, mice with a β-cell specific deletion of *Dnmt3a* phenocopied mice lacking the Nkx2.2 repressive domain; both mutant mice displayed an increase in α-cell number, a concomitant decrease in β-cell number, and evidence of β-to-α-cell transdifferentiation.[109] However, the precise roles of Nkx2.2 and Dnmt3a in gene repression, and the mechanisms by which disruption leads to β- to α-cell conversion, remain unknown. In order to expand upon our understanding of transcription factor/coregulator activity in the pancreas, we identified previously unknown coregulators of MafA in Chapter II. We then further characterized the role of the MLL3/4 complexes in the pancreatic β-cell.

The MLL3/4 Complex

The Mixed-Lineage Leukemia (MLL) proteins are conserved histone methyltransferase proteins that form the core catalytic component of at least 6 complexes in mammals.[92] These complexes are grouped into three sets: The SET1A/SET1B complexes, the MLL1/2 complexes, and the MLL3/4 complexes (see **Figure 7**). Each set shares common subunits with the other complexes (Rbbp5, Ash2l, Wdr5, dpy30), yet also contains unique subunits of their own.[110] For instance, MLL1 and MLL2 interact with the protein Menin, while the remaining MLL family members do not. The MLL family of proteins all appear to be broadly expressed, with the core MLL proteins and their subunits present in almost every tissue tested. Unlike the MLL1 and MLL2 complexes, which have been shown to have distinct roles,[111] the MLL3 and MLL4 complexes have been shown to be largely overlapping and capable of compensating for one another.[110] As an example, both MLL3 and MLL4 bind the *ABCA1* gene, where they regulate H3K4 trimethylation.[112] However, siRNA-mediated knockdown of either *MLL3* or *MLL4* alone in human liver HepG2 cells does not affect target gene expression, while simultaneous knockdown of both *MLL3* and *MLL4* results in downregulation of ABCA1. Therefore for simplicity the MLL3 and MLL4 complexes will jointly be referred to as the MLL3/4 complexes, or MLL3/4.

The MLL complexes all share the ability to methylate histone 3 lysine 4 (H3K4), mediated through a conserved S-methyltransferase SET domain (**Figure 7**).[113] However, individual MLL complexes have been suggested to preferentially mono-, di-, or trimethylate H3K4. For instance, the homolog of mammalian Set1A and Set1B in *Drosophila melanogaster*, dSet1, appears to be responsible for a majority of the global di- and trimethylation of H3K4.[114,115] MLL1 appears much more targeted in its activity, only recruited to less than 5% of H3K4 trimethylated promoters in mouse embryonic

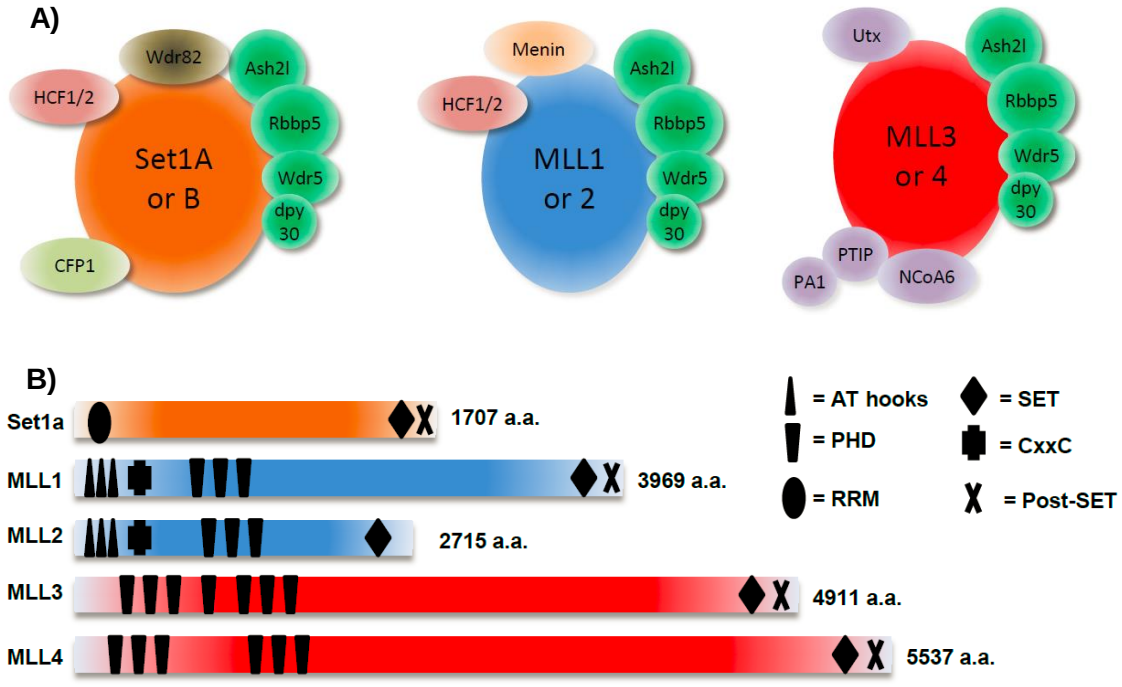


Figure 7. The MLL family of proteins. A) Schematic of the MLL family of proteins and their respective subunits, both shared and unique. Shared components are green. **B)** Schematic of the domains found in the MLL proteins. Set1B is not shown, but is similar to Set1A. All are characterized by a conserved SET methyltransferase domain.

fibroblasts (MEFs).[116] The MLL3/4 complexes, however, are proposed to have two different roles. One laboratory has reported that deletion of either the single *Drosophila* homolog to *MLL3/4*, *Trr*, or the simultaneous knockdown of MLL3 and MLL4 in a mammalian cell line produces global decreases in H3K4 monomethylation, with limited effect on trimethylation.[117,118] However, only global alterations in H3K4 monomethylated protein levels were examined, either by immunofluorescence or western blot. Alternatively, a different laboratory found via ChIP assays that the MLL3/4 complexes are specifically recruited to sites of H3K4 trimethylation, and that knockdown of both MLL3 and MLL4 results in a loss of H3K4 trimethylation.[94,119] Therefore whether the MLL3/4 complexes are primarily responsible for H3K4 mono- or trimethylation, and whether this occurs at promoters or enhancers, remains largely controversial.

However, while its specific histone modification remains unclear, the role of the MLL3/4 complexes as a coactivator is clear. The unique subunits of the MLL3/4 complexes have also been shown to play critical roles in the activity of the complexes. Nuclear Co-Activator 6 (NCoA6), one of the best-characterized subunits, plays a critical role in recruitment of the complex to target promoters or enhancers based on its ability to directly interact with both nuclear receptors and transcription factors. In the absence of NCOA6, the MLL3/4 complexes are no longer recruited and subsequent H3K4 methylation and gene activation are lost.[120] NCOA6 was first identified to interact with Retinoid X Receptor (RXR), Retinoic Acid Receptor (RAR), and Estrogen Receptor α (ERα) in yeast two-hybrid screens in a ligand dependent manner.[121] NCOA6 binds the Farnesoid X Receptor (FXR) in human liver HepG2 cells, also in a ligand-dependent manner.[94] NCOA6 mediates many of these interaction through two LXXLL motifs, which have been well characterized for their ability to bind nuclear receptors.[122] Non-nuclear receptor transcription factors are also able to interact with NCoA6, as demonstrated by NCoA6 interaction with p53 through the p53 interacting partner p53bp1 in multiple cell

types including mouse embryonic fibroblasts.[119] However, it is currently unclear which region of NCoA6 is responsible for mediating many of its other interactions, including its interaction with p53bp1.

Interestingly, NCOA6 also interacts with the Hepatic Nuclear Factor 4 alpha (HNF4α) transcription factor in HepG2 cells, and with the Constitutive Androstane Receptor (CAR) controls expression of the *CYP2C9* gene, which plays a critical role in drug metabolism.[123] HNF4α also plays a role in the pancreatic β-cell, where it is required for GSIS.[124,125,126] β-cell specific deletion of *Hnf4a* results in glucose intolerance at 10-weeks in females, and 24-weeks in males.[126] While the exact target genes that regulate this effect are not yet known, it is important to note that several MafA target genes (*Ins2*, *Slc2a2*, *Gck*, *G6pc2*) are unaffected in *Hnf4* α mutant mice, indicating likely non-overlapping roles for these two transcription factors. Additionally, whether HNF4α is able to interact with the Mll3/4 complexes in the β-cell remains unclear.

Another unique subunit of the MLL3/4 complexes is Ubiquitously Transcribed tetratricopeptide repeat protein X-linked (UTX), also known as KDM6A. Unlike the other unique subunits of the MLL complexes, UTX has a catalytic role as a histone demethylase. Specifically, it is able to remove histone 3 lysine 27 (H3K27) trimethylation, a repressive histone mark.[127] As mentioned previously, H3K4 trimethylation and H3K27 trimethylation are frequently found at the promoters of either active or repressed genes, respectively. When they are both present on the same gene, the gene is considered "poised", and awaits either activation or repression by resolving this bivalent state to only H3K4me3 or only H3K27me3.[88] Taken together, this would implicate the MLL3/4 complexes as prime candidates for resolving the bivalency of genes during development, as they possess the ability to place activating H3K4 trimethylation marks and remove repressive H3K27 trimethylation marks.

Both the MLL1/2 complexes and the MLL3/4 complexes have known roles in the pancreas. The Mll1 protein has been detected on the *Ink4a* promoter in isolated islets, where it is responsible for H3K4 trimethylation and prevents the recruitment of the PRC2 repressive complex.[108,128] Ink4a is an inhibitor of Cdk4 and hence β-cell replication, and increased *Ink4a* expression in aging mice is associated with a decrease in proliferation.[129] This correlates well with data on mutations in *MENIN* (a unique subunit of the MLL1 and MLL2 complexes), which have been associated with the development in humans of β-cell-derived tumors, called insulinomas.[130] Furthermore, 75% of these mutations are inactivating,[131] suggesting that MLL1 and MLL2 complexes may primarily be involved in controlling cell proliferation.

Interestingly, the mouse Mll3/4 complexes have previously been suggested to have a role in islet β-cell function. Mice which are globally heterozygous for *NCoA6* (*NCoA6*$^{+/-}$), a unique subunit of the Mll3/4 complexes, develop glucose intolerance and have reduced β-cell mass.[132] In agreement with these findings, human mutations in NCOA6 have been linked to decreased β-cell function via Homeostasis model assessment (HOMA), but were not linked to insulin resistance.[133] However, while Mll3/4 is known to direct H3K4 methylation, the effects of decreased NCoA6 expression on histone marks in the β-cell were not examined. Therefore it is possible that the Mll3/4 complexes may drive both H3K4 methylation as well as the removal of H3K27 trimethylation (via the unique subunit Utx) in order to regulate β-cell gene transcription.

In the developing β-cell, it is not yet clear when H3K4/H3K27 bivalency is resolved. Xie *et al*[134] used a combination of stem cell differentiation and *in vivo* maturation to look at histone modification changes during human endocrine development. Stem cells were differentiated into pancreatic endoderm, expressing both PDX-1 and NKX6.1, prior to transplantation into mice. After 20-weeks, these cells matured into functional endocrine cells, including β-like cells capable of secreting insulin

in a glucose-regulated manner. Interestingly, it was during this final *in vivo* maturation that these cells experienced the greatest loss of H3K4/H3K27 bivalency, which correlated with the onset of MAFA expression in these cells.[134] This provides preliminary evidence that resolution of H3K4/H3K27 bivalency is important in β-cell maturation, and suggests that MAFA could play a role in its resolution.

Overview and Aims of the Dissertation

MafA has previously been studied in our laboratory for its ability to bind the *insulin2* promoter, for its unique β-cell specific expression pattern, and for its role in β-cell maturation and function in mice.[50,135,56] Additionally, MAFA correlates with human β-cell maturation and function in multiple studies: its expression is lacking in immature β-cells,[21] it is expressed in functionally mature β-cells,[22] it correlates with increased insulin responsiveness in human cell lines,[63] and it is downregulated in instances of T2D.[10] Microarray analysis of mouse islets from a pancreas-specific deletion of *MafA* generated a large list of genes related to β-cell activity which were downregulated in the absence of MafA.[56] Furthermore, ChIP-seq analysis of MafA in adult mouse islets implicated it in direct control of several of these genes, such as *Ins2*, *Slc2a2*, *Slc30a8*, *Sytl4*, and others.[40] However, as with most islet-enriched transcription factors, mechanisms of MafA-directed transcriptional regulation are poorly understood. Indeed, the only known coregulator for MafA prior to these studies was the acetyltransferase p/CAF, which has previously been associated with the regulation of *insulin2* in mice.[61,99] However, p/CAF mutant mice lack a β-cell phenotype,[103] and while there are hundreds of previously identified coregulators, few have been shown to interact with transcription factors in the β-cell.

In order to further characterize mechanisms by which MafA exerts transcriptional control, my thesis work aimed to identify other coregulators that interact with MafA in the

β-cell. Chapter II describes the results of our immunoprecipitation/mass spectrometry approach, in which several potential coregulators were identified. The Mll3/4 complexes were chosen as a candidate for further study for their known role in gene activation,[136] the description of a pancreatic phenotype in global heterozygous NCoA6 mice,[132] and the absence of a known interaction between the Mll3/4 complexes and other known islet-enriched transcription factors. The function of these complexes was further characterized and compared/contrasted to that of MafA, both *in vitro* and *in vivo*. Interestingly, during the course of this work a human β-cell line was generated by collaborators and utilized here to examine the effects not only on MAFA-mediated transcription, but MAFB-mediated transcription as well. Chapter III concludes with additional preliminary data and possible future directions for the research described in this dissertation.

Chapter II

MLL3 AND MLL4 METHYTRANSFERASES INTERACT WITH TO THE MAFA AND MAFB TRANSCRIPTION FACTORS TO REGULATE ISLET β-CELL FUNCTION

Introduction

As discussed in Chapter I, transcription factors primarily regulate gene activation or repression by recruitment of coregulators, which often influence expression by directly binding to the basal transcriptional machinery and/or through remodeling of the chromatin structure. Coregulator recruitment is, in turn, controlled by the spatial and temporal expression patterns and posttranslational modifications of the transcription factor and/or coregulator. Unfortunately, little is known about the coregulators recruited by islet-enriched transcription factors. Although there are hundreds of known coregulators (*http://www.nursa.org/*), such knowledge is limited to candidate studies linking, for example, Pdx-1 to p300[97], Set7/9[100], Hdac1/2[79], Pcif1[101], and Bridge-1[102]. In contrast, MafA has only been linked to p/CAF.[61]

In this chapter, our goal was to identify previously unknown interacting partners of MafA, particularly coregulators. To accomplish this goal, we considered several biochemical approaches for purification and identification of proteins. MafA is a heavily phosphorylated protein, and these phosphorylation events can regulate coactivator recruitment (such as p/CAF),[61] or play critical roles in MafA dimerization and DNA binding.[60] Therefore techniques which rely on overexpression of epitope tagged constructs, such as Tandem Affinity Purification (TAP-tagging)[137], were not considered, as overexpression may lead to an abundance of hypophosphorylated MafA. We instead utilized the 'in cell' Reversible Cross-link Immunoprecipitation (Re-CLIP) and mass spectrometry (MS) approach to isolate coregulators of MafA from mouse β-cells. Re-

CLIP was initially developed in the Reynolds laboratory at Vanderbilt, and utilized successfully to identify binding partners of p120-catenin.[138] This unbiased proteomics approach detects interactions via immunoprecipitation of endogenous protein.

We performed Re-CLIP for MafA using the mouse βTC-3 cell line. This cell line was derived from transgenic mice expressing the SV40 T Antigen from the rat insulin promoter.[139] βTC-3 cells divide relatively quickly (every ~36 hours), and maintain a stable cell identity for at least 50 passages. Additionally, βTC-3 cells appear to represent a mature mouse β-cell, as they express MafA but do not express MafB (data not shown). Re-CLIP and MS analysis allowed us to identify several potential interacting partners for MafA. Interestingly, all nine subunits of the Mll3 and Mll4 Histone 3 Lysine 4 (H3K4) methyltransferase complexes were identified in the MafA immunoprecipitates, while none of the unique subunits of the other mammalian MLL complexes were detected (e.g. Menin, Mll1, Mll2 of Mll1/2 complexes; Set1A, Set1B, Wdr82 in Set1A/B complexes). (Mll3 and Mll4 will be referred to as Mll3/4 for simplicity.) These methyltransferases were also found to interact with MAFB, a closely related transcription factor co-expressed with MAFA in adult human islets.[64,58] Notably, mouse islet β-cell function is compromised in a heterozygous null mutant of *NCoA6*, a key subunit of Mll3/4.[132] Decreased NCOA6 levels were found to reduce expression of a subset of MAFA and MAFB regulated genes in human and rodent β-cell lines, with evidence provided that this results from limiting gene transcriptional start site (TSS) H3K4 trimethylation. Interestingly, there is essentially complete overlap of MafA and Mll3/4 transcriptional control in islets isolated after embryonic β-cell-specific removal of *NCoA6* or *MafA in vivo*. These results suggest that MLL3/4 coactivator recruitment by MAFA and MAFB is important to both the formation and adult function of islet β-cells.

Materials and Methods

Cell Culture and Immunoblotting Analysis

Mouse βTC-3 cells were maintained in DMEM containing 25 mM glucose, 10% fetal bovine serum, 100 U/mL penicillin/streptomycin[140]. Human EndoC-βH1[141] cells were grown in DMEM containing 5.6 mM glucose, 2% bovine serum albumin, 50 μM 2-mercaptoethanol, 10mM nicotinamide, 5.5 μg/mL transferrin, 6.7 ng/mL selenite, 100 U/mL penicillin, and 100 U/mL streptomycin. βTC-3 cells were infected with an adenovirus overexpressing GFP alone or a Flag-tagged MafA (kindly provided by Dr. Qiao Zhou, Harvard University) at a multiplicity of infection (MOI) of 200 under conditions described previously [142]. Nuclear extract was prepared 48 hours post-infection as described below. For immunoblotting [60], primary antibodies are listed in **Table 2**. HRP-conjugated α-rabbit, α-mouse, or α-goat secondary antibody was used at 1:2000 (Promega). Immunoblots were quantitated with NIH ImageJ software.

Reversible Cross-linking Immunoprecipitation (Re-CLIP) and Immunoprecipitation assays

The Re-CLIP protocol was adapted from Smith *et al.*[138] Briefly, ~10^9 βTC-3 cells were cross-linked using 0.1 mM Dithiobis[succinimidyl propionate] (DSP) in PBS for 30 minutes at 37°C. Cells were then harvested, and nuclear extract prepared by high salt 400 mM NaCl) extraction.[60] Magnetic protein G beads (Life Technologies) were covalently cross-linked with MafA antibody (20 μg), and either pre-incubated with a MafA blocking peptide (^{32}KKEPPEAERFC^{42}, 100-fold excess antibody) or with PBS alone. Extract and beads were incubated for 3 hours at 4°C, washed with RIPA buffer (10 mM Tris pH=8.0, 140 mM NaCl, 0.5% Triton X-100, 1% Sodium Deoxycholate, 0.1% SDS), and eluted in RIPA supplemented with 200 mM Dithiothreitol (DTT). The eluted proteins

Table 2. Antibodies

Immunoprecipitation

Target	Host Species	Company and Catalog #
IgG	Rabbit	Bethyl #P120-101
MafA (mouse and human)	Rabbit	Bethyl #A300-BL1225
Flag	Mouse	Sigma #F1804
NCoA6	Rabbit	Bethyl #A300-410A
MafB	Rabbit	Bethyl #A300-BL658

Western Blot

Target	Host Species	Concentration	Company and Catalog #
MafA	Rabbit	1:1000	Bethyl #A300-BL1225
PCAF	Mouse	1:1000	Santa Cruz #13124
Pdx-1	Rabbit	1:10000	Provided by Dr. Chris Wright
Rbbp5	Mouse	1:2000	Santa Cruz #271072
NCoA6	Rabbit	1:1000	Bethyl #A300-410A
Wdr5	Rabbit	1:1000	Santa Cruz #135245
UTX	Rabbit	1:1000	Bethyl #A302-374A
Nkx6.1	Mouse	1:1000	Developmental Studies Hybridoma Bank
HNF1α	Goat	1:1000	Santa Cruz #6548
Nkx2.2	Goat	1:1000	Santa Cruz #15015
Pax6	Rabbit	1:1000	Covance #PRB-278P
Ash2l	Mouse	1:1000	Abcam #ab50699
MafB	Rabbit	1:1000	Bethyl #A300-BL658

Immunofluorescence

Target	Host Species	Concentration	Company and Catalog #
Insulin	Guinea Pig	1:1000	Dako #A0564
MafA	Rabbit	1:500	Novus #NBP1-00121
NCoA6	Rabbit	1:1000	Sigma #HPA004198
Glucagon	Mouse	1:1000	Sigma #G2654

EMSA

Target	Host Species	Company and Catalog #
MafA (mouse)	Rabbit	Bethyl A300-BL1225
MAFA (human)	Rabbit	Novus #NBP1-00121
MAFB (human)	Rabbit	Bethyl A300-BL658

were visualized by PAGE/silver staining and protein identification determined by liquid chromatography/MS/MS (LC/MS/MS) or MudPIT analysis in the Vanderbilt University Proteomics Core. Immunoprecipitation experiments were performed with the antibodies listed in **Table 2** at 10 µg each as described above for Re-CLIP, omitting the DSP step and substituting PBS for RIPA.

Sucrose gradient ultracentrifugation and Electrophoretic Mobility Shift Assay (EMSA)

Sucrose gradients were performed as described earlier.[60] Briefly, βTC-3 nuclear extract (600-1000 µg) was collected by high salt extraction[60] and separated over a 5-35% sucrose gradient (4.5 mL total volume). Fractions (300 µL each, excluding the first 500 µL) were analyzed by immunoblotting, rat *insulin II* C1 element gel shift[60], and immunoprecipitation analysis. HeLa cells were transfected with expression plasmids encoding human MAFA and/or MAFB, and nuclear extract utilized for rat *insulin II* C1 element gel shift.[60] Antibodies used for gel shift are listed in **Table 2**.

siRNA treatment of β-cell lines

Knockdown in βTC-3 and EndoC-βH1 cells was achieved using ON-TARGETplus siRNAs of mouse *MafA* (#J-041353-09), mouse *NCoA6* (#J-041129-6), human *MAFA* (#L-027343-01), human *MAFB* (#L-009018-00), and human *NCOA6* (#L-019107-00). The targeting siRNA (0.5 nanomoles) or a non-targeting control (#D001810) (GE Dharmacon) was introduced into βTC-3 cells ($4x10^6$) using Buffer V (Lonza #VVCA-1003) with an Amaxa Nucleofector 2 (Program G-016; Lonza, Walkersville, MD). EndoC-βH1 cells ($2x10^6$) were transfected with siRNAs (50 picomoles) using the Dharmafect #1 reagent (GE Dharmacon #T-2001) following the manufacturer's protocol. Nuclear extract, RNA, and chromatin were collected 72 hours post transfection.

Quantitative PCR (qPCR) and Qiagen RT^2 Profiler PCR array analysis

RNA was collected from βTC-3 cells using the RNeasy kit (Qiagen), whereas the Trizol reagent (Life technologies) and the DNA-Free RNA Kit (Zymo Research) were employed for EndoC-βH1 cells and mouse islets. The iScript cDNA synthesis kit (Biorad) was used for cDNA synthesis. The qPCR reactions were performed with the gene primers listed in **Table 3** on a LightCycler 480 II (Roche), and analyzed by the ΔΔCT method [143]. The Qiagen RT^2 Profiler PCR array for human diabetes genes (Cat. No. 330231 PAHS-023ZA) was screened with 1 µg of cDNA from EndoC-βH1 cells following manufacturer guidelines.

Mouse lines, Intraperitoneal Glucose Tolerance Test (IPGTT), and Islet Isolation

Floxed (fl) *MafA* [64] or *NCoA6* [144] mice were crossed with rat *insulin* II promoter-driven Cre (RIP-Cre) [145] transgenic mice to delete *MafA* (termed *MafA*$^{\Delta\beta}$) or *NCoA6* (*NCoA6*$^{\Delta\beta}$) specifically in β-cells. All the mice were maintained on a mixed genetic background (C57BL/6J, I129, Balb/c, 129S6/SvEvTac, FVB/N), although *MafA*$^{\Delta\beta}$ mice were mostly C57BL/6J. *MafA*$^{\Delta\beta}$, *NCoA6*$^{\Delta\beta}$, *MafA*$^{fl/fl}$ and/or *NCoA6*$^{fl/fl}$ mice were fasted for 6 hours in the morning before the IPGTT. Blood glucose levels were measured prior to (time 0), and then 15, 30, 60, and 120 minutes after an intraperitoneal injection of 2mg/g body weight glucose prepared in sterile PBS (20% w/v). Islets from 8-week old mice were isolated using collagenase P (Roche) digestion and hand-picking [146]. The Vanderbilt University Institutional Care and Use Committee approved all of these studies.

Tissue collection and Immunofluorescence

Pancreata were dissected and fixed in 4% paraformaldehyde in PBS for 4 hours

Table 3. Primers used in qPCR experiments.

Gene	Species	Forward	Reverse
MafA	Mus musculus	CCTGTAGAGGAAGCCGAGGAA	CCTCCCCCAGTCGAGTATAGC
NCoA6	Mus musculus	TTGCAGGAGGCCCAAATAACATGC	TCCAGGTTTGGGTCCTGTCATCAT
G6PC2	Mus musculus	ACCTGGTCCTTTCTGTGGAGTGTT	TTCAAAGGCCTCGGCTACTAGCAT
preIns2	Mus musculus	GGGGAGCGTGGCTTCTTCTA	GGGGACAGAATTCAGTGGCA
Ins2	Mus musculus	GGCTTCTTCTACACACCCAT	CCAAGGTCTGAAGGTCACCT
Slc2a2	Mus musculus	GTTAATGGCAGCTTTCCGGTC	CAGTTCGGCTATGACATCGGT
Slc30a8	Mus musculus	AGCTTCCTGTGTTTCCTAGGCCAT	AATCTATTCCGACGGCTGCCTCAT
Lifr	Mus musculus	GCTCGCAGGCTCAAACATGACAAT	GAAGGGTATTGCCGATCTGTCCTGAA
Sytl4	Mus musculus	TGAAGTTGCACCGCCAGAAACT	AAGGCAATCTTGCCCGTCACA
Atp2a2	Mus musculus	AGCCCAGAGAGATGCCTGCTTAAA	AGAGCAGGAGCATCATTCACACCA
Prss53	Mus musculus	ACACTGGGTCCAGGTTGGAATCAT	CCAAGAAAGCTGCCTCGTGAACAT
Rab6	Mus musculus	AGGCAAGTGTCAATTGAGGAGGGA	TTCTCTGCTTCTGTCCTGTGTGCT
CCND2	Mus musculus	GAGTGGGAACTGGTAGTGTTG	CGCACAGAGCGATGAAGGT
PRKAA1	Mus musculus	CATGTGGCTGGGTGTGTAAAG	CAGAGGGAGGAGTTAAGGAGAAG
G6PD	Mus musculus	CCACTGCCACCACCAGTAAA	GAAAGATGGGTGGCTCAGATAGG
AGT	Mus musculus	GGCGCTGAAGGATACACAGAA	CCAGGTCAAGATGCAGAAGATG
MAFA	Homo sapien	TGAGCGGAGAACGGTGATTTCTAAGG	GGAACGGAGAACCACGTTCAACGTA
MAFB	Homo sapien	ACCTTGGCTAAGGCGAGAGTAG	CTTCAGCCTGGAGAGAAGTTACTC
NCOA6	Homo sapien	TCCTCTCTGGGCTCCATATAC	GCTGGGTTCATTTGTCTGTTC
G6PC2	Homo sapien	GCAGGGCTTTATGGGCTATT	AGTTCATTTCCTCCAAGGTCAG
INS	Homo sapien	AGAGGCCATCAAGCAGATCACTGT	AGGTGTTGGTTCACAAAGGCTG
ROBO2	Homo sapien	AAGCAACATGTCTTTCCACATT	TTGGATGCTGCTTGGTGACT
SLC2A1	Homo sapien	GGACAGGCTCAAAGAGGTTATG	AGGAGGTGGGTGGAGTTAAT
SLC2A2	Homo sapien	CTAGGCAGAGCTGCGAATAAA	CTAGTTGGGAGTCCTGTCAATTC
PRKAA1	Homo sapien	CAGGTCATCAGTACACCATCTGA	TCATCCAGCCTTCCATTCTTACAG
G6PD	Homo sapien	GTAGGCAGCCTCTCTGCTATAA	TGGGCTGTTTGCGGATTTAA
AGT	Homo sapien	AAAGGCCAGCAGCAGATAACAA	CAGCACACTTAGACCAAGGAGAAA

followed by paraffin embedding [50]. The entire e15.5 pancreas was serial sectioned (6 µm per section) and every 10th section analyzed by immunofluorescence. Three sections at least 120µm apart were examined at 8-weeks for changes in islet architecture. The primary antibody list is provided in **Table 2**. Species matched secondary antibodies were used for immune detection at 1:1000 (Jackson ImmunoResearch Laboratories). Slides were mounted with Dapi Fluoromount-G (SouthernBiotech #0100-20) and images acquired on a Zeiss Axio Imager M2 widefield microscope with Apotome.

Glucose Stimulated Insulin Secretion (GSIS)

EndoC-βH1 cells ($2x10^6$) were placed in medium containing low glucose (1.1 mM) for 12-14 hours. Low or high glucose (15.5 mM) medium was then added for 1 hour. Medium and cell lysates (lysis buffer: 20 mM Tris, 1% Triton X-100, 10% glycerol, 137 mM NaCl, 2 mM EGTA, 1X protease inhibitor tablet [Roche Diagnostics]) were collected and analyzed for insulin content by the Vanderbilt Hormone Assay Core. GSIS was performed with 8-week old islets in the Vanderbilt Islet Procurement and Analysis Core. Briefly, islets were size matched, incubated overnight in 5.6mM glucose, and then treated with 5.6 mM or 16.7 mM glucose for 1 hour. The insulin content in the medium and cell lysate was analyzed by radioimmunoassay. Insulin secretion capacity was calculated as media insulin over cell lysate insulin content. Glucose stimulated levels are presented as the 15.5 mM/1.1 mM values for EndoC-βH1 cells, and 16.7 mM glucose/5.6 mM glucose for isolated islets.

Statistics

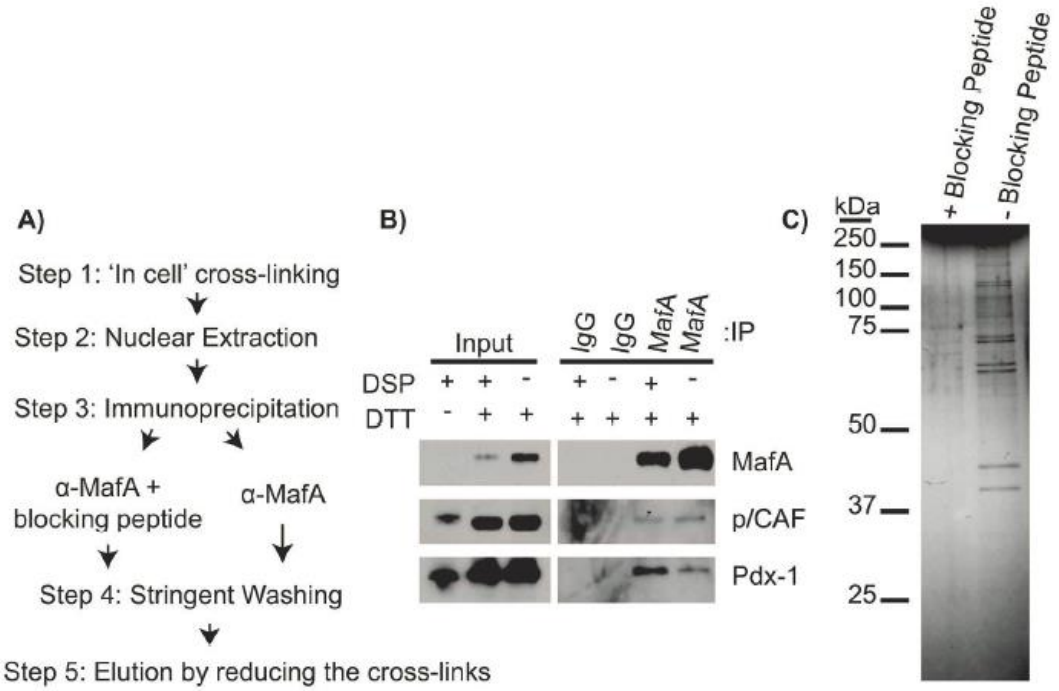
Mean differences were tested for significance using a Student two-tailed t-test. The minimal level of statistical significance is listed in the figure legend.

Results

Identification of MafA interacting proteins using Re-CLIP

MafA interacting proteins were isolated from mouse βTC-3 cells using a combined Re-CLIP/MS strategy (**Figure 8A**).[138] Amine-reactive DSP was used to covalently cross-link MafA to neighboring proteins, and MafA antibody to precipitate bound proteins. The signal-to-noise of the procedure was improved by utilizing the MafA antibody blocked by MafA peptide in the control immunoprecipitation (as opposed to non-immune IgG), and by washing precipitated proteins with RIPA buffer. Bound proteins were eluted from MafA by addition of DTT to cleave the disulfide bond in the cross-linker. As expected, Pdx-1[140] and p/CAF[61], known MafA interacting proteins, were bound to MafA following Re-CLIP (**Figure 8B**). Moreover, many proteins were selectively bound to MafA in the immunoprecipitation conducted in the absence of blocking peptide (**Figure 8C**).

Many of the proteins that immunoprecipitated with MafA and were subsequently identified by MS analysis have known roles as coregulators (**Table 4**). It is notable that no other islet-enriched transcription factors (e.g. Pdx-1) or the p/CAF coactivator were identified by MS, presumably reflecting their relatively low abundance. This indicates that the results in **Table 4** represent only a portion of MafA interacting proteins. Our analysis focused on determining the significance of Mll3/4 coactivator binding to MafA, since the evolutionarily conserved COMPASS family of methyltransferases is strongly associated with gene activation due to their ability to mono-, di-, and tri-methylate H3K4 within the enhancer and/or promoter region.[147] All nine proteins of the ~1.5 MDa Mll4 complex were found by MS (**Figure 8D**), and Mll3 was additionally detected via the more sensitive MudPIT analysis (**Table 4**). However, none of the unique COMPASS subunits of mammalian Mll1/2 (i.e. Mll1, Mll2, menin, Hcfc1/2, Psip1/2) or Set1A/B (i.e. Set1A,



D)

Protein	% Coverage	#Peptides from Control IP	# Peptides from MafA IP
Rbbp5	34	4	13
Ash2l	30	0	9
Wdr5	39	4	12
Dpy30	55	2	4
Utx	30	3	27
PTIP	10	0	6
NCoA6	2	1	3
PA1	15	0	3
MLL4	4	1	3

Figure 8. MafA interacting proteins in β-cells were identified by Re-CLIP/MS. A) Schematic of the Re-CLIP protocol used for isolating MafA interacting proteins from mouse βTC-3 cells. DSP was the cross-linker used in Step 1 and DTT was used to elute MafA cross-linked proteins in Step 5. **B)** Immunoprecipitations performed with βTC-3 cells using control IgG or MafA antibody in the absence (-) or presence (+) of DSP treatment. The precipitate was then immunoblotted with MafA, p/CAF, and Pdx-1 antibodies. The input lanes contain ~1% of the nuclear extract, treated with (+) or without (-) DTT to show the absence of MafA at its normal molecular weight when cross-linked. **C)** A representative image of proteins precipitated by MafA antibody pre-incubated with a blocking or scrambled control peptide after SDS-PAGE and silver staining. **D)** A representative MS result from Re-CLIP for the MLL3/4 complex proteins.

Set1B, Cfp1, Wdr82, Bod1, Bod1l) were detected. The ability of MafA to bind to Mll3/4 was also independently demonstrated in βTC-3 cells expressing an adenovirus-driven Flag-tagged MafA (**Figure 9**).

MafA co-migrates with the high-molecular weight Mll3/4 complexes upon sucrose density gradient separation

βTC-3 nuclear proteins were size separated in a 5-35% sucrose gradient to determine if the mobility of the ~90kDa MafA dimer was impacted by interaction with Mll3/4. MafA was found in two distinct gradient size fractions. The faster mobility MafA containing fractions 6-8 co-migrated with islet-enriched transcription factors of relatively low molecular weight (**Figure 10A**; Pdx-1, 31 kDa, Fractions 3-5; Nkx6.1, 38kDa, Fractions 3-5; Hnf1α, 67kDa, Fractions 6-8; Nkx2.2, 30kDa, Fractions 3-5; Pax6, 50kDa, Fractions 6-8), while the high molecular weight fraction 14 co-migrated with the Rbbp5 (59kDa), NCoA6 (220 kDa), Wdr5 (36kDa), and Utx (154 kDa) subunits of the Mll3/4 complex. In contrast, few other islet-enriched transcription factors were found in this high molecular fraction. Antibody super-shift and competitor analysis illustrated that the binding properties of MafA in Fractions 7 and 14 were identical (**Figure 10B**). In addition, immunoprecipitation experiments conducted with antibodies to NCoA6 or MafA demonstrated that MafA interacted with several Mll3/4 components in Fraction 14 (**Figure 10C**). These results strongly indicate that a significant fraction of MafA is associated with the Mll3/4 complexes in β-cells.

Table 4. MudPIT results from Re-CLIP. The proteins detected in both MudPIT analyses are in green, and those found only in one are depicted in red. The proteins in bold are in the MLL3/4 complex. All proteins listed had greater than two-fold peptide enrichment in the MafA Re-CLIP compared to control.

Transcription factors	Co-regulators		Other proteins of various functions	
Cux1	**Ash2l**	**Dpy30**	Otud4	MCM7
Zbtb20	**MLL4**	HDAC6	Baiap2	NONO
Trps1	**MLL3**	Uty	Cabin1	SFPQ
Zfhx3	**Utx**	Wiz	Cpsf1	Matrin-3
Dach2	**Rbbp5**	HDAC2	RPA1	Ddx5
Dach1	**NCoA6**	Chd4	SSBP1	Gm5580
Myt1	**PTIP**	Rbbp7	Kiaa1522	Ddx3x
Myt3	Fmr1	Cdyl	Snrnp200	MCM6
Myt1l	Ehmt2	Rcor3	Mios	Actn4
Hmbox1	Gm10093	Rcor1	Fip1l1	Actn1
znf516	Ehmt1	LSD1	Myef2	Ina
Zfp644	**PA1**	Smarca4	Rps3a2	Neurotrimin
Trp53	**Wdr5**	Smarce1	Elmsan1	Sorbs2
Zhx2	Ogt	Smarca5	Nvl	Ctnnb1
Srfbp1	Chd7	Smarcc2	Pgam5	Ctnna1
Nfic	Sin3a	Taf5l	Wdr33	Plekha6
Baz2b	Hcfc1	Ctbp1	Vrk3	Ubap2
GATAD2A	Trim28	Arid1a	Evi5l	Azi1
Cdc5l	Smarcb1		Usp7	Lmo7
Mox2			Spats2	Dhx9
Dido1			Kpna2	
Ubn1				
HIRA				
Tjp2				
Tjp1				
COE2				
Gtf2i				
Cnot1				
Trim56				

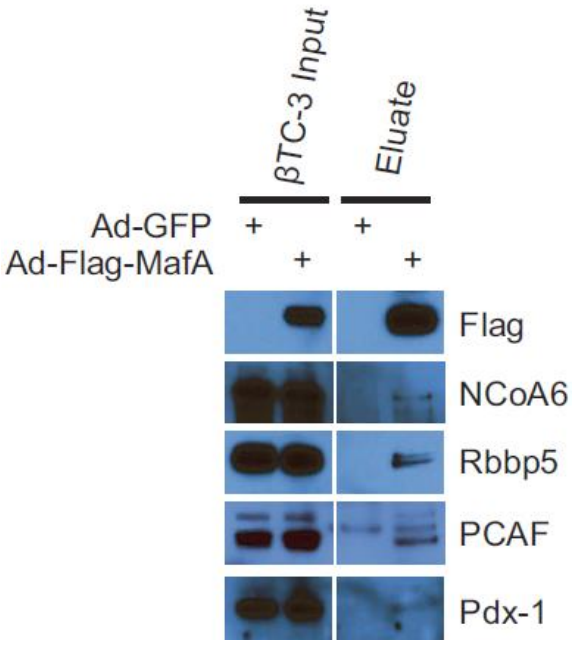


Figure 9. Flag-tagged MafA interacts with the MLL3/4 complex. βTC-3 cells were infected with an adenovirus expressing either GFP or a Flag-tagged MafA construct. After 48 hours, nuclear extract was collected and immunoprecipitated with an anti-flag antibody. The precipitated protein was probed with the antibodies listed. A representative western blot is shown, N=3.

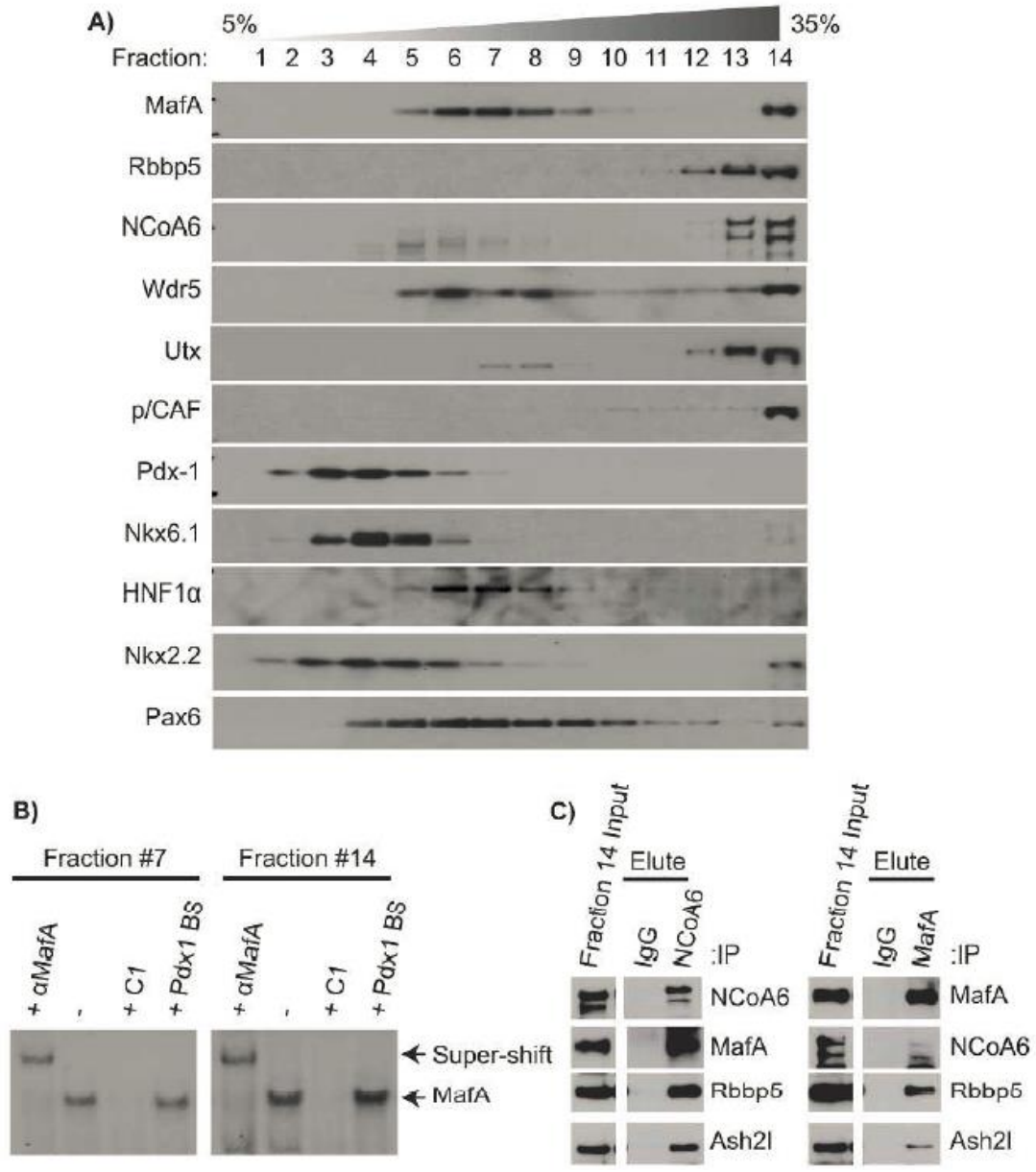


Figure 10. Sucrose gradient sedimentation reveals that MafA comigrates with the MLL3/4 complex. A) βTC-3 nuclear proteins were separated in a 5-35% sucrose gradient, and fractions were screened for MafA, MLL3/4 (i.e. Rbbp5, NCoA6, WDR, and UTX), p/CAF, Pdx-1, Nkx6.1, HNF1α, Nkx2.2, and Pax6 by immunoblotting. A significant portion of MafA was present in Fraction 14, which contains the MLL3/4 complex. **B)** MafA homodimer in Fractions 7 and 14 binds specifically to the rat *insulin II* C1 enhancer element. MafA in these fractions was super-shifted by α-MafA antibody, while only unlabeled C1 and not an *insulin* Pdx-1 binding element (Pdx-1 BS) competed for binding. **C)** Immunoprecipitations performed on Fraction 14 with antibody to NCoA6 (left) and MafA (right), which were then probed with MafA, NCoA6, Rbbp5, or Ash2l antibodies. The input lane contained ~5% of Fraction 14.

A subset of MafA regulated genes is activated by the Mll3/4 complexes in mouse βTC-3 cells

We next analyzed how Mll3/4 affected MafA activity by depleting *MafA* or *NCoA6* from βTC-3 cells by siRNA-directed knockdown. The NCoA6 subunit lacks a DNA binding domain, and is involved in recruiting Mll3/4 to a variety of transcription factors and nuclear receptors.[94,112,148,120] Furthermore, Mll3 and Mll4 have redundant roles in liver, and NCoA6 is required for activity of both methyltransferase complexes.[112] Notably, mouse *NCoA6*$^{+/-}$ mutants have impaired glucose clearance,[132] while overexpression of NCoA6 in rat INS-1 β-cells increases cell function.[133] In addition, polymorphisms in *NCOA6* are associated with decreased human β-cell function.[133] Our objective was to determine the interdependence of Mll3/4 activity and MafA-mediated transcriptional control.

In order to determine the overlap between MafA and NCoA6-directed control, a list of MafA target genes was generated by comparing genes bound by MafA in adult islets via ChIP-seq[40] with a list of genes downregulated in *MafA*$^{\Delta panc}$ islets[56] (**Table 5**). These genes will subsequently be referred to as MafA target genes. The expression of these MafA target genes was then examined after depletion of either MafA or NCoA6. Both MafA and NCoA6 protein levels in βTC-3 cells were effectively (>80%) depleted by siRNA treatment (**Figure 11A**). The effect of MafA and NCoA6 knockdown on MafA target genes was determined via RT-qPCR (**Figure 11B**). As expected, the expression of most of these genes was decreased upon depletion of MafA (**Figure 11B**; *Slc2a2*, *Ins2*, *Lifr*, *Prss53*, *Atp2a2*), while depletion of MafA had no impact on *NCoA6* levels. In contrast, only a small subset of these MafA target genes was affected in the *NCoA6* knockdown (i.e. *Slc2a2, Prss53*). *Slc2a2* is a β-cell-specific glucose transporter, and *Prss53* is a serine protease of unknown function.[149] This relatively small overlap between

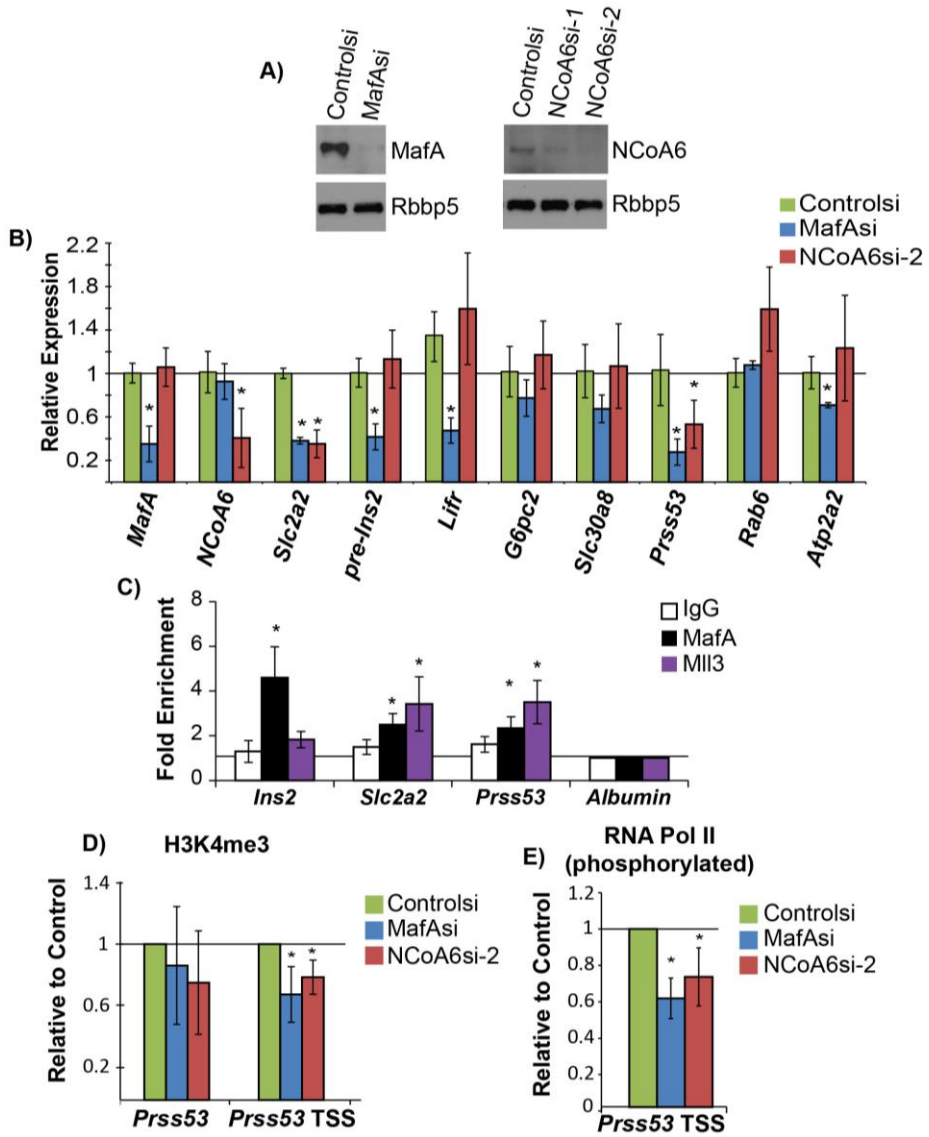


Figure 11. Knockdown of NCoA6 reduces gene expression of a subset of MafA targets in βTC-3 cells. Control siRNA (Controlsi) or siRNA specific to *MafA* (MafAsi) or *NCoA6* (NCoA6si-1 and -2) were introduced into βTC-3 cells. **A)** MafA and NCoA6 were immunoblotted after corresponding depletion, with NCoA6si-2 most effective at reducing protein levels and used in all subsequent experiments. Rbbp5 levels were unaffected by siRNA treatment and served as a loading control. Densitometric analysis indicated effective knockdown of MafA (MafAsi = 0.04* ± 0.02 standard deviation) and NCoA6 (NCoA6si-2 = 0.16* ± 0.12). *p<0.05, N=3. **B)** NCoA6 affects expression levels of a subset of MafA-regulated genes in βTC-3 cells. mRNA expression was determined by qRT-PCR, normalized to *Gapdh* expression. N=3-6. **C)** ChIP analysis reveals that MafA and Mll3 are bound to the same region of *Slc2a2* and *Prss53*. Fold enrichment was calculated using percent input and normalized to background binding at the *Albumin* promoter, N=4. **D)** H3K4 trimethylation levels were reduced near the TSS of *Prss53* in MafAsi and NCoA6si treated βTC-3 cells in ChIP analysis. Percent input was calculated, and normalized to Controlsi treated cells. N=3. **E)** *MafA* or *NCoA6* knockdown in βTC-3 cells decreased phosphorylated RNA polymerase II binding to the TSS region of *Prss53* in ChIP assays, N=3. All error bars indicate standard deviation; *p<0.05.

Table 5. Identification of direct targets of MafA. This list was compiled upon comparing the *MafA*$^{\Delta panc}$ microarray data to mouse islet MafA ChIP-Seq results. Shown is the fold expression change seen in the original microarray data, as well as the nearest ChIP-Seq peaks for MafA and the height (fold enrichment over input) of the peak.

Name	Gene Name	Microarray Fold Change	ChIP-seq Peak height	Distance of Peak from TSS
G6pc2	NM_021331	-5.7	18	-257
Ins2	NM_008387	-2.7	12	-92
Slc2a2	NM_031197	-4.1	15	-1586
Slc30a8	NM_172816	-2.6	10	-72
Lifr	NM_001113386	-3.3	11	-306
Sytl4	NM_013757	-8.5	6	12086
Atp2a2	NM_001110140	-3.2	7	959
Prss53	NM_001081268	-6.8	8	969
Rab6	NM_024287	-2.2	9	-7767
			13	117
CCND2	NM_009829	-2	7	-68388

MafA and NCoA6 was considered likely as a number of other potential MafA coregulators were identified here and previously (e.g. p/CAF[61], **Table 4**), although it is possible that some fraction of Mll3/4 activity is independent of NCoA6. In addition, ChIP analysis showed that Mll3 and MafA bound within the same control regions of the *Slc2a2* and *Prss53* genes (**Figure 11C**). Moreover, H3K4 trimethylation and levels of elongating, carboxy terminal tail-phosphorylated RNA polymerase II were significantly reduced within the TSS region of *Prss53* upon knockdown of *MafA* or *NCoA6*, the regulatory pattern expected for MafA-recruited Mll3/4 activity (**Figure 11D, E**). While only a 30-40% reduction in H3K4me3 and phosphorylated RNA polymerase II was observed, this effect is likely biologically relevant, as we saw a 40-60% reduction in gene expression. This confirms what has been found in other studies examining knockdown of MLL3/4, in which a ~40-50% reduction in H3K4me3 and RNA Polymerase II at the promoter of *HOXC10* led to a ~70% decrease in *HOXC10* expression.[150]

Human MAFA and MAFB interact with the MLL3/4 complexes

Our next objective was to determine if human MAFA and closely related MAFB bind MLL3/4. For these experiments, we utilized the EndoC-βH1 cell line, one of only two widely available human β-cell lines.[141] EndoC-βH1 cells were generated from human fetal pancreas, using a lentiviral vector expressing SV40 large T antigen from a rat insulin promoter. This cell line is glucose responsive and exhibits a similar pattern of transcription factor expression to that of primary human β-cells. Unlike primary human islets, which display a significant deal of variability depending on the age of the individual, the cause of death, and the isolation procedure itself,[151] EndoC-βH1 cells provide a consistent and reproducible source of cells for our experiments. Additionally, they allow for certain manipulations such as siRNA-mediated knockdown, which would be difficult in primary human β-cells.

As mentioned, these cells display a pattern of transcription factor expression similar to primary human β-cells, which is not entirely conserved between mouse and human. Specifically, MafB is only produced in insulin$^+$ cells during mouse embryogenesis, and plays an important role in β-cell formation.[64] In contrast, human MAFB is expressed in developing and postnatal β-cells, being coexpressed with MAFA in adult islet β-cells.[58] These basic leucine-zipper proteins bind to MARE sequences as homo- or hetero-dimers[50,43], as also observed in MAFA and MAFB transfected HeLa cell extracts (**Figure 12A**). Gel shift analysis indicates that both MAFA:MAFB and MAFB:MAFB complexes are also formed in nuclear extracts prepared from the human EndoC-βH1 β-cell line (**Figure 12B**), although they were difficult to distinguish due to their similar size (MAFA at ~45 versus MAFB at ~42 kDa). Super-shift analysis with MAFA and MAFB antibodies provided further support for these protein heterodimerization (**Figure 12A**: lane 3-5; **Figure 12B**: lane 3). Significantly, immunoprecipitation experiments demonstrated that both MAFA and MAFB were able to associate with multiple proteins of the MLL3/4 complexes in EndoC-βH1 cells (**Figure 12C;** NCOA6, UTX, RBBP5), although whether this results from binding MAFB:MAFB and/or MAFA:MAFB is unclear. In addition, MAFA and MAFB were able to interact with the islet-enriched PAX6 and ISL1 transcription factors as well as P/CAF; however, these likely represent relatively weak or indirect binding due to their absence from the Re-CLIP/MS analysis performed in mouse βTC-3 cells (**Table 4**).

MAFA, MAFB, and the MLL3/4 complexes are necessary for glucose-responsive insulin secretion in EndoC-βH1 cells

MAFA, *MAFB*, or *NCOA6* were siRNA depleted from EndoC-βH1 cells to analyze their significance to human β-cell gene expression and function. Protein levels

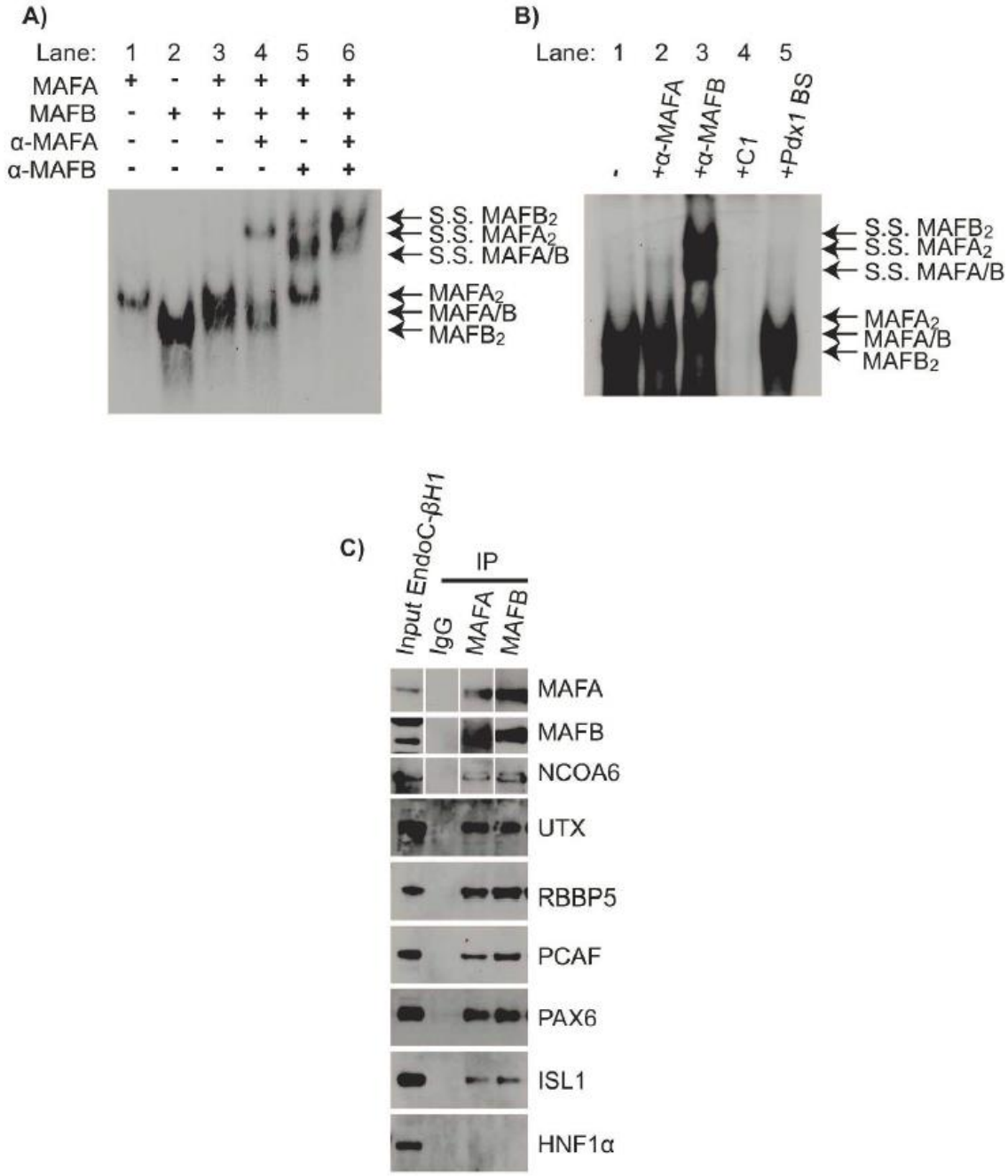


Figure 12. MAFA and MAFB associate with MLL3/4 in human EndoC-βH1 cells. A) MAFA and MAFB produced in HeLa cells form both homodimers and a heterodimer. Super-shift (S.S.) reveals three distinct complexes, with α-MAFA and α-MAFB altering homo- and heterodimer formation. **B)** MAFB:MAFB and MAFA:MAFB appear to be the predominant complexes formed in gel shift analysis EndoC-βH1 nuclear extracts. Note that complex formation is profoundly impacted by addition of α-MAFB. The rat insulin II C1 and Pdx-1 binding element competitions illustrate the specificity of complex binding. **C)** Proteins in MLL3/4 were precipitated from EndoC-βH1 nuclear extracts with antibodies to MAFA and MAFB. Immunoblotting was performed for MLL3/4 (NCOA6, RBBP5, UTX), islet-enriched transcription factors (PAX6, ISL1, HNF1α) and the mouse MafA-associated P/CAF coactivator.[18] N=3, Representative blot shown.

were reduced by ~50-70% under these conditions (**Figure 13A**). The impact on transcriptional control was determined by examining known genes regulated by mouse MafA and a candidate set available on a commercial array composed of 84 genes associated with T2DM onset, development, and progression. *INSULIN, G6PC2*, *PRSS53* and *SLC2A1* were significantly affected by *MAFA* or *MAFB* depletion in EndoC-βH1 cells (**Figure 13B**). However, their expression was unchanged by *NCOA6* depletion, presumably due to differences in human MAFA/MAFB:MLL3/4 control in relation to rodents and/or the β-cell lines used in our analysis. Notably, the levels of three genes linked to T2DM and important to GSIS were reduced upon *NCOA6* depletion (**Figure 13C**), specifically AMP-kinase subunit *PRKAA1*[152], Glucose-6-Phosphate Dehydrogenase (*G6PD*)[153], and Angiotensin (*AGT*)[154]. In addition, expression of each was compromised in *MAFB* siRNA treated EndoC-βH1 cells, while *MAFA* depletion only affected *AGT*. Moreover, MAFA, MAFB, and MLL3 were all co-recruited to the same endogenous 5'-flanking control region of the *AGT* gene (**Figure 13D**). Furthermore, GSIS in EndoC-βH1 cells was compromised upon knockdown of *MAFA*, *MAFB*, or *NCOA6* (**Figure 13E**). Collectively, this data supports an important role for MLL3/4 in MAFA and MAFB activity in human β-cells.

Conditional knockout of MafA or NCoA6 in islet β-cells in vivo decreases MafA target gene expression and glucose responsive insulin secretion

To evaluate the influence of MafA:Mll3/4 on islet β-cells in mice, we compared the phenotypes of β-cell-specific loss of either *NCoA6* or *MafA*. Rat *insulin II*-driven Cre transgenic mice[145] were bred with $MafA^{fl/fl}$[64] and $NCoA6^{fl/fl}$[155] mice to generate β-cell-specific removal of *MafA* ($MafA^{\Delta\beta}$) and *NCoA6* ($NCoA6^{\Delta\beta}$) during development (**Figure 14A**). Roughly 70% of mutant insulin$^+$ cells lacked the MafA or NCoA6 proteins by e15.5 (**Figure 14B**), a time at which MafB is still expressed in developing β-cells.[156]

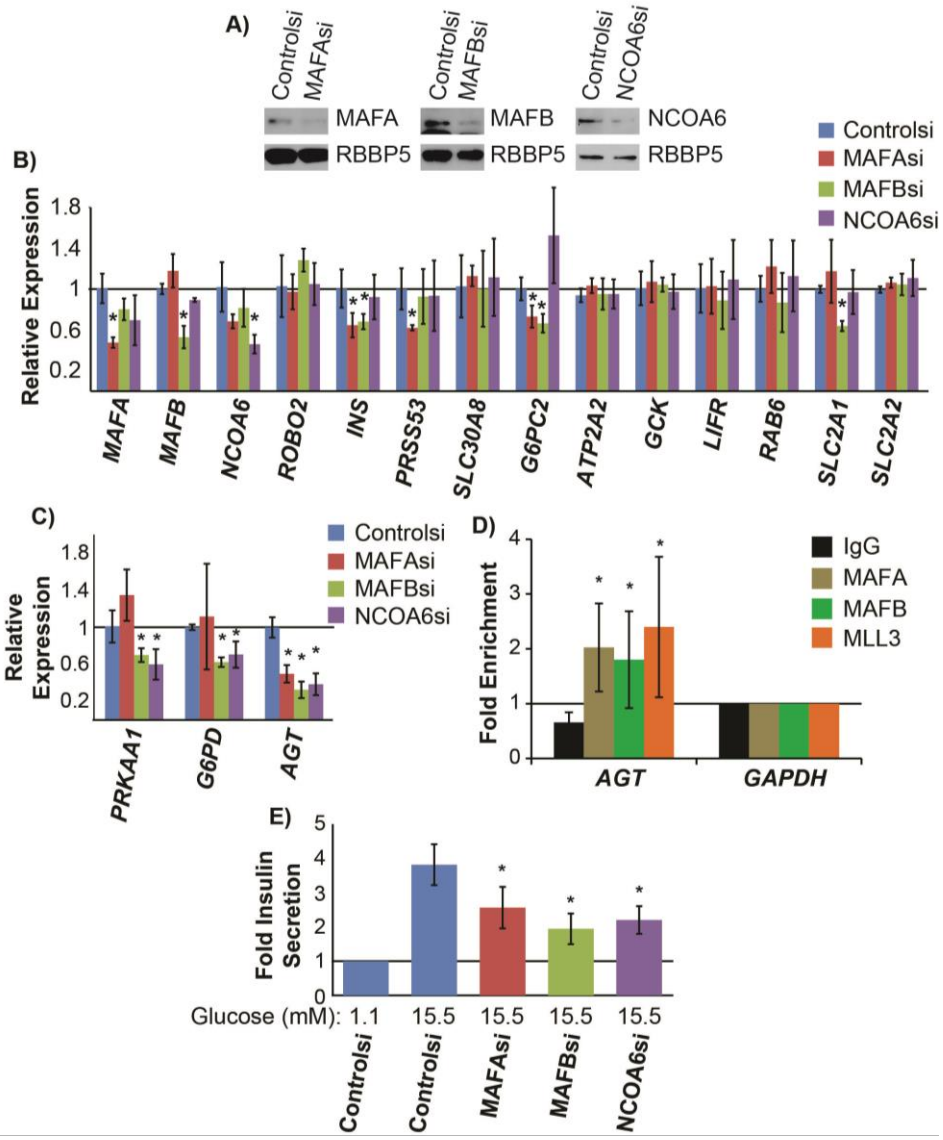


Figure 13. MAFA, MAFB, and MLL3/4 are required for glucose stimulated insulin secretion in EndoC-βH1 cells. A) Protein levels of MAFA, MAFB, and NCOA6 were reduced upon targeted siRNA treatment. Densitometric analysis indicated effective knockdown of *MAFA* (0.44*±.18), *MAFB* (0.53*±.29), and *NCOA6* (0.26*±0.05). *p<0.05, N=3. **B)** The impact of knockdown on β-cell mRNA levels was determined using *bona fide* MafA-activated genes of mouse β-cells and by **C)** candidate gene screening using the Qiagen human diabetes RT^2 profiler array. qPCR results were normalized to *GAPDH* expression. Error bars depict standard deviation, N=4. **D)** MAFA, MAFB, and MLL3 bind within the same region of *AGT*. Fold enrichment was calculated from the percent input normalized to *GAPDH*, N=4. **E)** Glucose stimulated insulin secretion was measured one hour after glucose stimulation in cells treated with siRNA for 72 hours. Results are presented as fold stimulation at 15.5 mM versus 1.1 mM glucose; all measurements were normalized for insulin content. Error bars indicate standard deviation. N=4, *p<0.05.

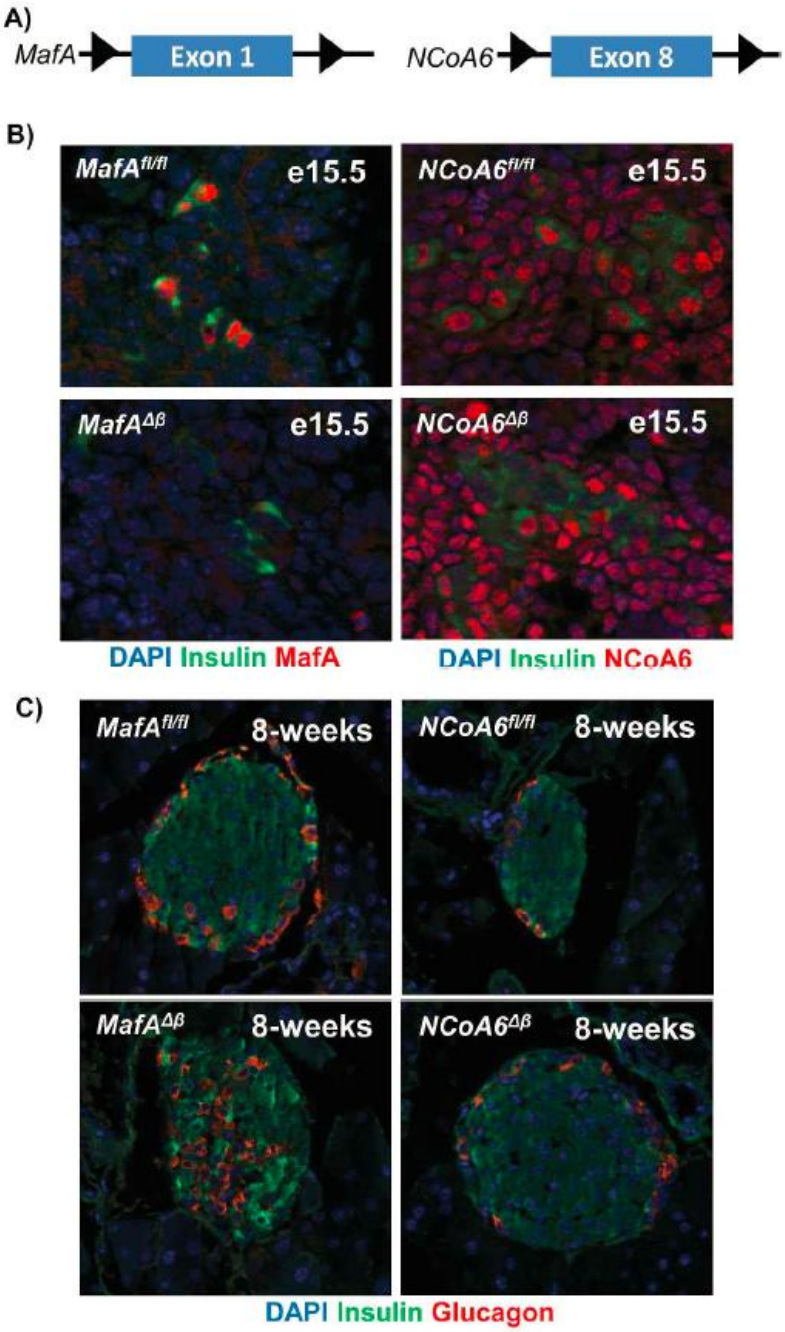


Figure 14. Islet architecture is unchanged in *NCoA6$^{\Delta\beta}$* mice. A) *MafA$^{\Delta\beta}$* and *NCoA6$^{\Delta\beta}$* mice were generated by breeding *MafA$^{fl/fl}$* and *NCoA6$^{fl/fl}$* mice with rat *insulin II* enhancer/promoter (RIP)-driven Cre transgenic mice. MafA protein coding sequences are only present within Exon 1, while deletion of Exon 8 causes a frame-shift that prevents the production of NCoA6. **B)** Representative images illustrating the effectiveness of MafA and NCoA6 removal from insulin^{+} cells at e15.5; ~72% of insulin^{+} cells lack MafA or NCoA6. N=3. **C)** Islet architecture is only distorted in *MafA$^{\Delta\beta}$* mice at 8-weeks.

Similar to the pancreas-specific deletion of MafA (*MafA*$^{\Delta panc}$)[56], *MafA*$^{\Delta\beta}$ mice were euglycemic according to their fed blood glucose levels (data not shown), and displayed impaired islet architecture, where glucagon^{+} α-cells were no longer restricted to the outer mantle of the islet (**Figure 14C**). Adult *NCoA6*$^{\Delta\beta}$ mice were also euglycemic, but their islet architecture was unchanged. In addition, only *MafA*$^{\Delta\beta}$ mice displayed the impaired whole body glucose clearance observed in *MafA*$^{\Delta panc}$ mice ([56], **Figure 15**). Controls with RIP-Cre alone did not show impaired whole body glucose clearance, likely due to the mixed genetic background of the mice (data not shown).

Strikingly, most of the genes downregulated in 8-week old *MafA*$^{\Delta\beta}$ mouse islets were also significantly compromised in 8-week old *NCoA6*$^{\Delta\beta}$ islets (**Figure 16A**). This downregulation of target mRNA led to decreased levels of target proteins, such as Slc2a2 and Slc30a8 (**Figure 17**). Furthermore, this significant overlap in gene regulation between *MafA*$^{\Delta\beta}$ and *NCoA6*$^{\Delta\beta}$ indicates that the Mll3/4 complexes are critical coregulators of MafA. Differences in the extent of MafA and Mll3/4 gene regulation overlap between mice and cell lines could be due in part to the presence of MafB during development, which could play a role in directing the recruitment of the Mll3/4 complexes to these same target genes (see Chapter 3, **Figure 18**). Additionally, GSIS was impaired in islets isolated from both *MafA*$^{\Delta\beta}$ and *NCoA6*$^{\Delta\beta}$ mice (**Figure 16C**), as found upon *MAFA*, *MAFB* or *NCOA6* depletion in human EndoC-βH1 cells (**Figure 13D**). However, while islets from *MafA*$^{\Delta\beta}$ mice showed significant loss of GSIS, in *NCoA6*$^{\Delta\beta}$ islets the basal insulin secretion level was unusually high in relation to islets from wild type controls and *MafA*$^{\Delta\beta}$ mice(**Figure 16C**). This is a sign of β-cell immaturity,[157] but is also a phenotype seen in islets from mice with a pancreas-specific deletion of MafB at 2-weeks of age (Elizabeth Conrad, personal communications). However, in the pancreas-specific deletion of MafB, islets from 8-week old mice do not display impaired basal insulin

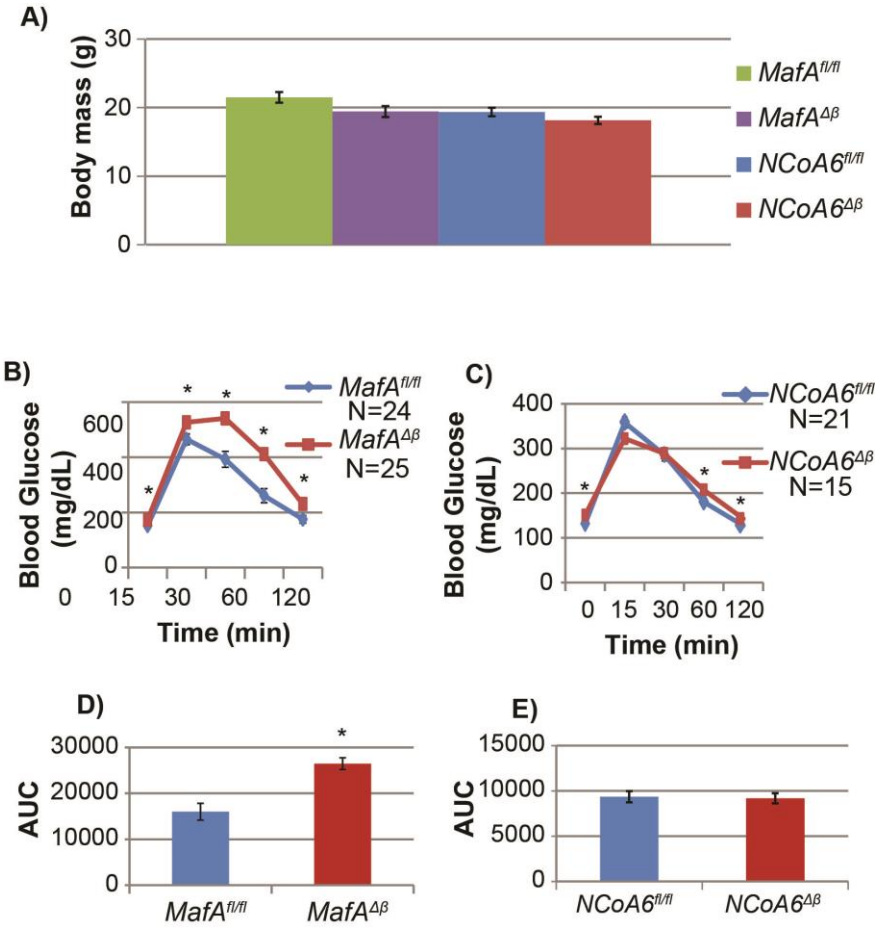


Figure 15. *NCoA6^{Δβ}* mice do not develop glucose intolerance. A) Body mass is not significantly different between controls and *MafA^{Δβ}* and *NcoA6^{Δβ}* at 8-weeks old. (**B** and **C**) Intraperitoneal glucose tolerance tests were performed with 8-week old *MafA^{Δβ}, NCoA6^{Δβ}*, and control littermates. Only *MafA^{Δβ}* mice displayed a significant defect in their ability to clear blood glucose. Numbers of each genotype used are indicated. (**D** and **E**) Area under the curve results from *MafA^{Δβ}* (*p<0.05) and *NCoA6^{Δβ}* (p=0.84) mice.

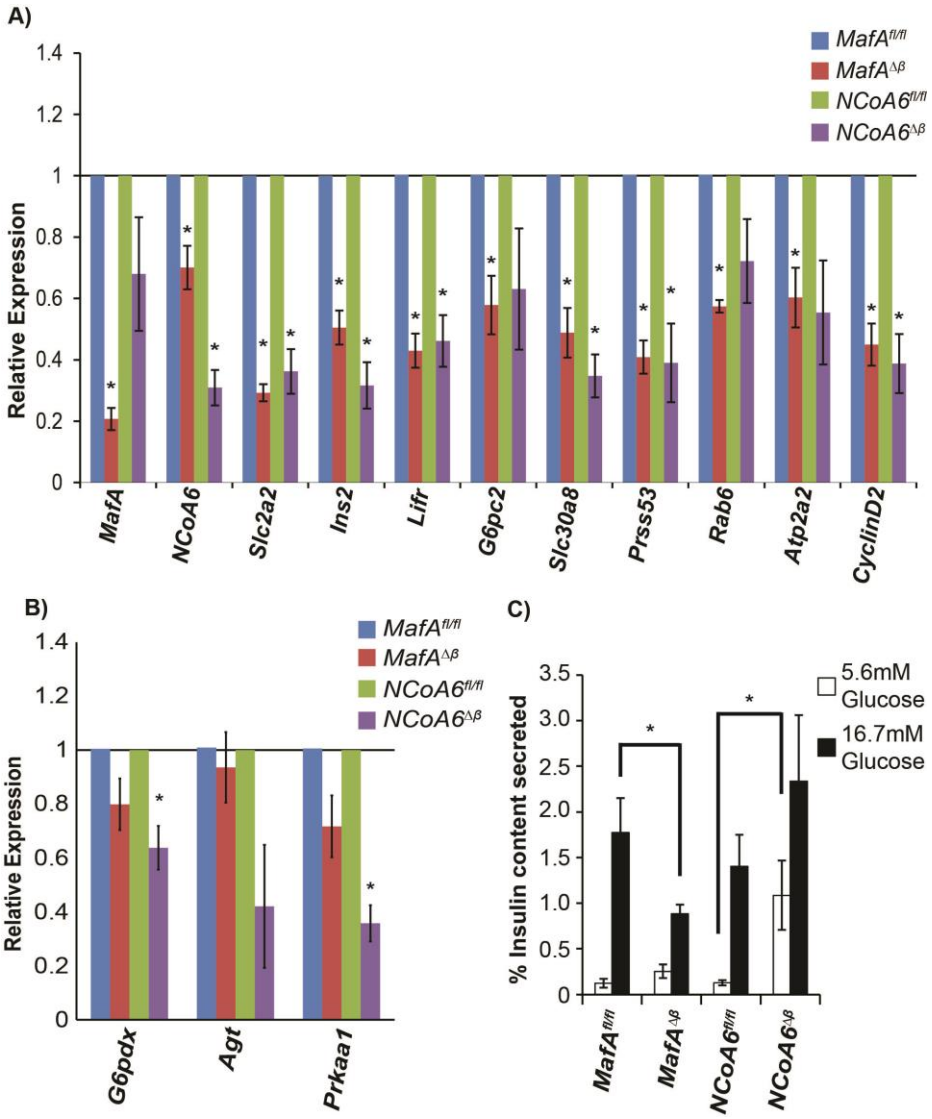


Figure 16. Glucose stimulated insulin secretion is impaired in *MafA*$^{\Delta\beta}$ and *NCoA6*$^{\Delta\beta}$ islets. A) Expression of all the direct targets of mouse MafA were decreased in 8-week old *MafA*$^{\Delta\beta}$ islets, but not **B)** those identified as human MAFA- and/or MAFB-regulated genes by Qiagen human diabetes RT^2 profiler screening (see **Figure 7C**). Moreover, essentially all of these genes were also compromised in *NCoA6*$^{\Delta\beta}$ islets. Error bars indicate SEM, N=3-5. Fold expression was normalized to control littermates. **C)** Isolated islets were incubated overnight in low glucose (5.6mM), and then treated with 5.6mM or stimulating 16.7mM glucose for 1 hour. The data is presented as percent (%) of total insulin content secreted. Error bars represent SEM, * $p<0.05$, N for *MafA*$^{fl/fl}$=3, *MafA*$^{\Delta\beta}$=4, *NCoA6*$^{fl/fl}$=3, *NCoA6*$^{\Delta\beta}$=3.

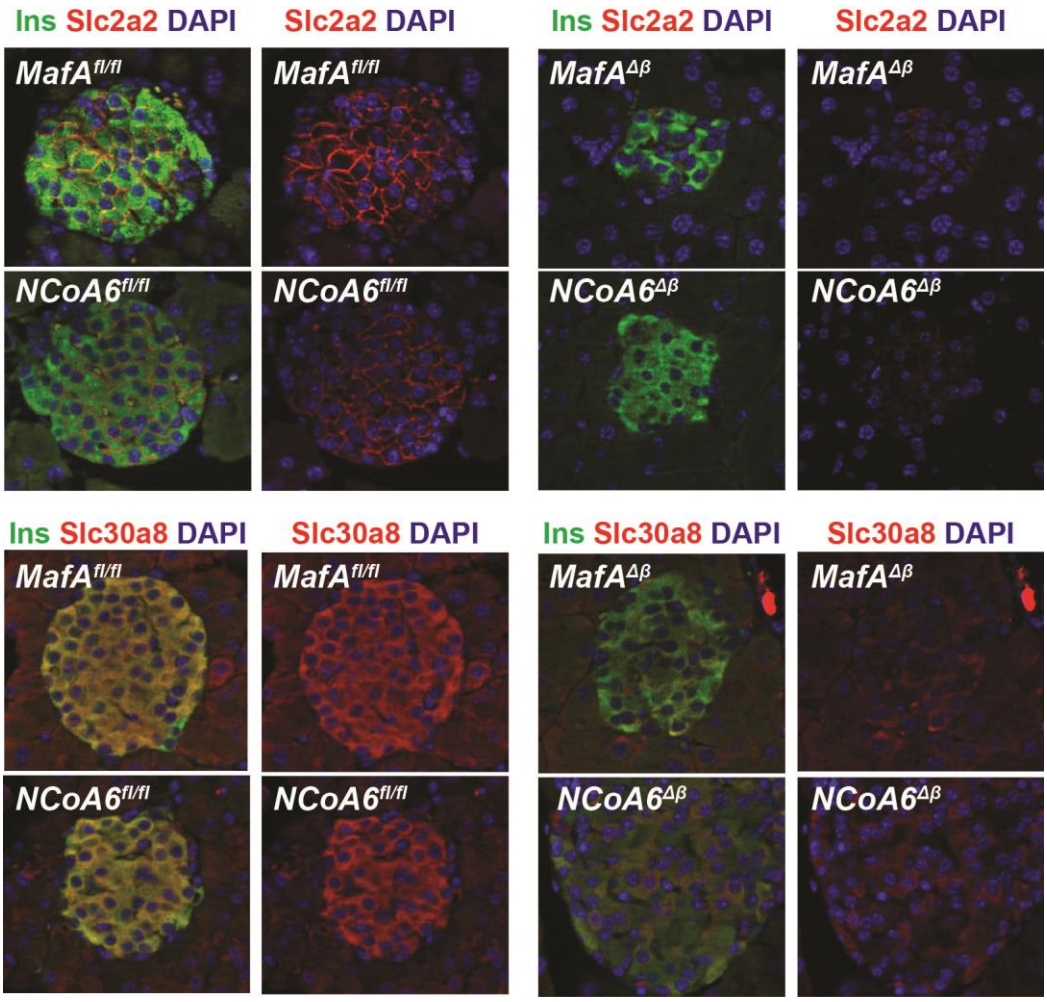


Figure 17. Slc2a2 and Slc30a8 protein levels are reduced in *MafA*$^{\Delta\beta}$ and *NCoA6*$^{\Delta\beta}$ mice. Immunofluorescence analysis of *MafA*$^{\Delta\beta}$, *NCoA6*$^{\Delta\beta}$, and control littermates at 8-weeks. All images are representative.

secretion, likely due to eventual compensation by MafA. This further supports a model by which the Mll3/4 complexes are recruited during development to more MafA:MafB target genes, and that after development in a mature MafA-only β-cell, this complex is only recruited to a subset of MafA-regulated genes.

Discussion

Gene induction involves the recruitment of coregulators by enhancer-bound transcription factors like MAFA and MAFB. These transcription factor-bound coregulators ultimately influence recruitment of the RNA polymerase II transcriptional machinery. We used an unbiased 'in cell' chemical cross-linking, immunoprecipitation, and MS strategy to identify coregulators of the MafA transcription factor, an essential regulator of postnatal rodent islet β-cell proliferation and activity. Many distinct coregulator candidates were identified in our biochemical screen, and we focused on the Mll3/4 methyltransferase complex because of the established role of this coactivator in gene transcription. Our studies demonstrate that the recruitment of MLL3/4 by MAFA and MAFB is essential to islet β-cells.

MLL3 and MLL4 are members of the evolutionary conserved COMPASS family of methyltransferases that are strongly associated with gene activation due to their ability to methylate H3K4 within enhancer and/or promoter regions. Strikingly, all of the proteins in the Mll3/4 complexes were identified in the MafA antibody precipitates by MS, and not the other mammalian MLL complexes (e.g. Mll1/2 complex or Set1A/B complex). MLL3/4 binding to MAFA was also confirmed by immunoprecipitation analysis in mouse and human β-cell lines (**Figures 7**, **9**). Moreover, a significant fraction of MafA was bound to the ~1.5 MDa Mll3/4 complexes by sucrose gradient centrifugation (**Figure 10**). Interestingly, the islet-enriched Nkx2.2 and Nkx6.1 transcription factors were also found in the same high molecular weight fraction. Although the identity of all components of

these islet-enriched complexes is unknown, a very high molecular weight complex would be predicted from the association in β-cells of Nkx2.2 and Nkx6.1 with the Histone deacetylase-1 corepressor, the Groucho-3 transcription factor repressor, and the DNA (cytosine-5)-methyltransferase 3A corepressor to prevent expression of the α-cell specification factor Arx.[109] Likewise, Pax6 co-migration with MafA:Mll3/4 could reflect binding to high molecular proteins like p300.[158] However, neither Nkx2.2, Nkx6.1, Pax6, nor any other islet-enriched transcription factors were detected by MafA antibody in Re-CLIP experiments (**Table 4**), strongly suggesting that these proteins are not directly, and certainly not abundantly, associated with MafA:Mll3/4. At least some interaction is likely with PAX6 and ISL1 in human β-cells based upon the MAFA and MAFB antibody precipitation results in human EndoC-βH1 cells (**Figure 12C**). Collectively, these data strongly implied that MafA recruitment of Mll3/4 was highly specific and important to β-cell activity.

Immunoprecipitation experiments conducted with the EndoC-βH1 cell line showed that both human MAFA and MAFB are capable of binding and recruiting the MLL3/4 complexes. However, reducing the protein levels of these transcription factors or the core NCOA6 subunit of the MLL3/4 complexes in human (**Figure 13**) and mouse (**Figure 11**) β-cell lines disclosed that the coactivator only influenced some MAFA/MAFB activated genes. For example, only the *Slc2a2* and *Prss53* genes were bound and coregulated by MafA and Mll3/4 amongst a variety of tested MafA-regulated genes in βTC-3 cells (**Figure 11**). Importantly, and as expected, recruitment of Mll3/4 by MafA regulated H3K4 trimethylation levels of the *Prss53* gene as well as RNA polymerase II engagement.

In contrast to what was found upon reduction of *NCoA6* levels in β-cell lines, nearly all of the islet MafA target genes were dependent upon NCoA6 in 8-week old *NCoA6*$^{\Delta\beta}$ islets (**Figure 16A**). It is possible that this is due to NCoA6 interaction with

other transcription factors in the islet. However, as noted previously, *NCoA6*$^{\Delta\beta}$ mice do not display as severe a defect as seen with deletion of other islet-enriched transcription factors (for example, Pdx1, Ngn3, NeuroD1, *etc*). Because of the early action of Cre-recombinase in developing MafA^{+}/MafB^{+} β-cells, the broader regulation of MafA target genes observed in *NCoA6*$^{\Delta\beta}$ mouse islets is instead likely due to the activity of Mll3/4 on MafB-bound enhancers during development. Notably, this supposition is consistent with analysis of the *MafB*$^{-/-}$[64] and *MafA*$^{\Delta panc}$[56] mutants, which established that many genes first regulated by MafB developmentally were subsequently regulated by MafA after birth.[56] In *NCoA6*$^{\Delta\beta}$ mice, the loss of Mll3/4 recruitment to MafB-bound enhancers during development may explain why *NCoA6*$^{\Delta\beta}$ islets, exhibited impaired basal insulin secretion similar to islets from mice with a pancreas-specific deletion of MafB (**Figure 16C** and Elizabeth Conrad, personal communications). The results also indicate that Mll3/4 is involved in some, but not all, MafA- and MafB-regulated gene expression. Thus, while MLL3/4 was necessary for GSIS in both human and mouse β-cells, it did not have the same influential effect on islet cell architecture as MafA (**Figure 14C**).

Our understanding of the genes regulating GSIS in human β-cells is very limited. The results in EndoC-βH1 cells suggest that regulation by MAFA/B:MLL3/4 of AMP-kinase subunit *PRKAA1*[152], Glucose-6-Phosphate Dehydrogenase (*G6PD*)[153], and Angiotensin (*AGT*)[154] expression may be important (**Figure 13C**). For example, Angiotensin appears to have a direct role in regulating β-cell activity[159]. However, this candidate list certainly represents only a fraction of the gene products involved in β-cell function and future efforts will be aimed at identifying more using ChIP-Seq and RNA-Seq. It is also important to note how similar the phenotypes of the MafA, MafB and NCoA6 mouse models were, and yet different from the mouse models of other islet-enriched transcription factors present in high molecular weight complexes (**Figure 10**). Thus, islet β-cell-specific deletion of *Pax6*, *Nkx2.2*, or *Nkx6.1* produces a much more

severe effect on cell identify and causes lethality soon after birth due to hyperglycemia.[52,53,54]

In conclusion, our combined proteomic, cell line and mouse strategies have led to the identification and characterization of MLL3/4 as a novel coactivator of the MAFA and MAFB transcription factors in islet β-cells. Future efforts will be aimed at determining how other isolated candidate coregulators, such as corepressor complexes Sin3, NuRD, and CoREST, impact MAFA and MAFB control. These ideas will be discussed further in Chapter 3. It will also be of interest to determine if coregulator recruitment is influenced by homodimeric (i.e. MAFA:MAFA, MAFB:MAFB) or heteromeric (MAFA:MAFB) transcription factor composition and the physiological or pathophysiological state of the β-cell.

Chapter III

SUMMARY AND FUTURE DIRECTIONS

Thesis Summary and Significance

Previous attempts to determine the genes that regulate pancreatic development have mainly focused on the identification of transcription factors.[26] While this has greatly expanded our knowledge of the factors involved in directing differentiation, and even identified specific transcription factors capable of transdifferentiation of other cell types into β-cells, our mechanistic understanding of these cell fate decisions is still lacking. Specifically, it remains unclear which coregulatory interactions are necessary to direct the histone modifications, chromatin remodeling, and RNA Polymerase II recruitment necessary for transcription of important β-cell genes. Recent work by Bramswig *et al* has highlighted the important roles that histone marks plays in directing differentiation and cell identity.[163] They demonstrated that the H3K4 and H3K27 methylation states of α- and β-cells correlated well with the mRNA expression levels of the affected genes, with H3K4me3 being activating and H3K27me3 being repressive, and bivalently marked genes having low levels of expression. They also showed that treatment with a methyltransferase inhibitor led to partial endocrine cell fate conversion (α- to β-cell), indicating that methyltransferases play an important role not only in establishing cell identity, but in maintaining it. However, most coregulators lack a DNA binding domain, and rely on transcription factors for their recruitment to specific loci. It is therefore critical to understand not only which transcription factors guide β-cell development, but how these transcription factors are guiding coregulator recruitment to affect transcription, as transcription factor expression itself may not be sufficient to direct β-cell differentiation.

Within the pancreas, MafA is a β-cell specific transcription factor involved in cell maturation and function in mice.[56] Here, we sought to more completely define our understanding of how MafA drives transcriptional regulation by identifying, in an unbiased manner, its interacting partners. My thesis has predominantly characterized the MLL3/4 complexes, as these were the most abundant known coregulators detected in our Re-CLIP experiments. Additionally, the MLL3/4 complexes have a known role in catalyzing H3K4 methylation, one of the best-studied histone modifications for gene activation. Surprisingly, our Re-CLIP experiments did not identify the acetyltransferase p/CAF, or other islet-enriched transcription factors present in our immunoprecipitations and known to interact with MafA (**Figure 8, Table 4**). This likely represents an issue of relative abundance, and highlights the importance of an unbiased approach for identifying coregulatory interactions, as interactions identified via candidate screens may overestimate the abundance of those interactions, and their importance to the transcription factors function. However, relative abundance also does not always correlate with importance of the interaction, and does not preclude a role for other coregulatory complexes and transcription factors not identified here in MafA-directed transcriptional control, as context-dependent recruitment likely also plays an important role. As seen in data from **Figure 16**, there seems to be a significant overlap of target genes between MafA and the Mll3/4 complexes *in vivo*. When compared to the data from our mouse cell lines, this indicates that Mll3/4 is an essential coregulator of MafA and likely MafB *in vivo*, and is recruited more broadly in a developmental context as opposed to post-maturation (**Figure 18**). We also confirmed the existence of this interaction, and its importance to β-cell function, in a human β-cell line. Lastly, we show that for at least the target gene *Prss53* in mice, MafA appears to direct Mll3/4 recruitment for the purpose of H3K4 trimethylation of the promoter, and RNA polymerase

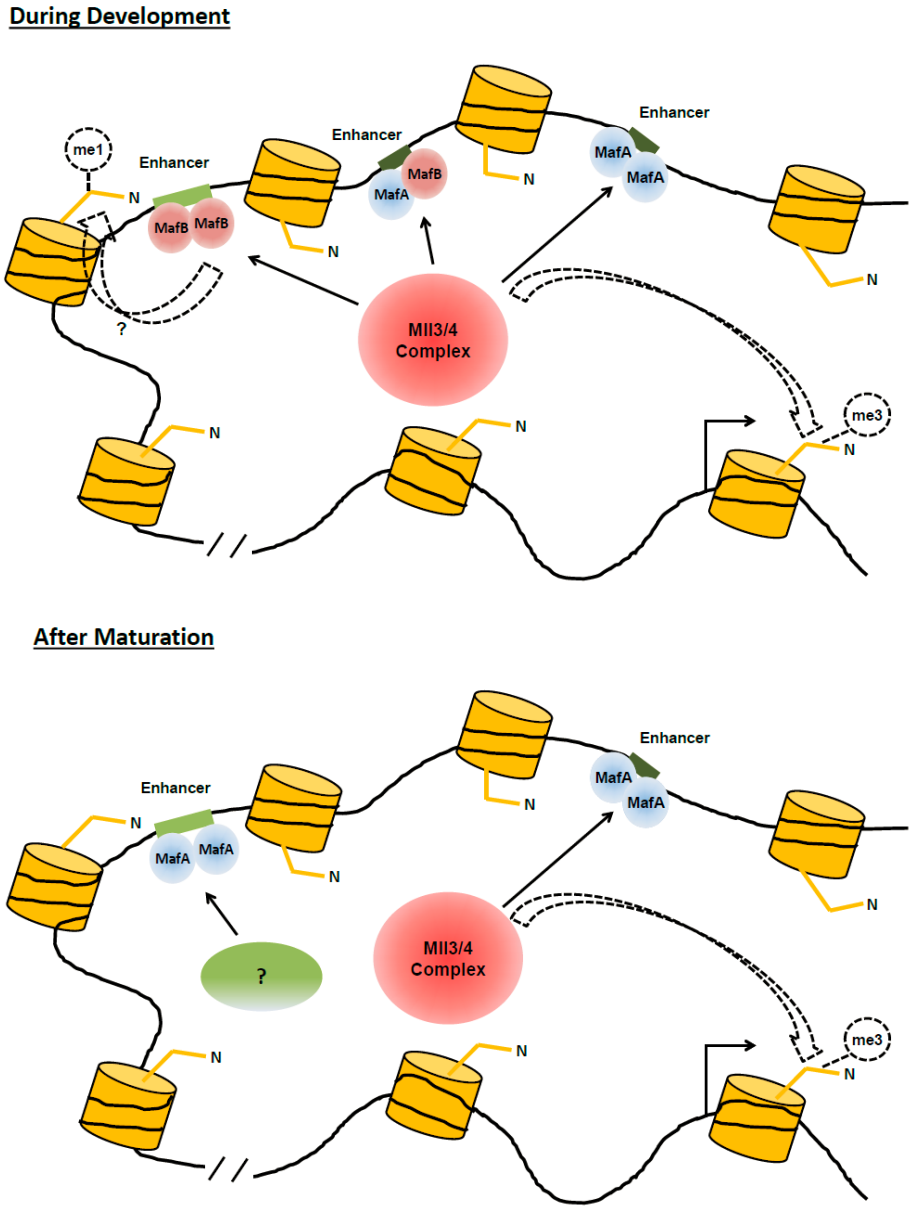


Figure 18. Model of MafA recruitment of the MLL3/4 complex. During β-cell development, MafA and MafB recruit the Mll3/4 complexes to enhancers of Maf target genes for the purpose of H3K4 methylation (either me1 or me3) and subsequent activation. After maturation MafB is silenced, and MafA only recruits the Mll3/4 complexes to a subset of MafA target genes for the purpose of H3K4me3 near the TSS.

II phosphorylation. Taken together, this data supports a model that MafA, and likely MafB, are recruiting the Mll3/4 complexes to regulate histone methylation and gene activation of Maf target genes.

Perspectives and Future Studies

Determine whether mouse MafB also interacts with the MLL3/4 complex

As seen in **Figure 12**, human MAFB does interact with the MLL3/4 complex, although whether as a homodimer or as a heterodimer with MAFA remains unclear. In contrast to humans, in mice, MafB is silenced in β-cells shortly after birth but maintained in α-cells. This has precluded the study of MafB:Mll3/4 interaction in our mouse cell lines and in isolated islets from adults. However, when comparing the phenotype of the mouse models (**Figure 16**), which represent effects during both development and maturation, with that of the mouse βTC-3 cell line (**Figure 11**), which is thought to represent a mature β-cell, it appears that MafB plays at least a partial role in directing Mll3/4 recruitment during β-cell development. It remains to be determined when this MafB:Mll3/4 interaction occurs, what effect MafA:MafB heterodimerization has, and what regulates MafA:MafB:Mll3/4 interactions.

To determine whether mouse MafB interacts with the Mll3/4 complexes, mouse MafB was overexpressed in the mouse βTC-3 cell line by infection with an adenovirus expressing MafB. This is not the ideal condition to study this interaction, as MafB expression is driven by the CMV promoter and therefore is vastly overexpressed compared to what may be observed under developmental conditions. As MafB, like MafA, is a heavily phosphorylated protein, overexpression could lead to problems such as hypophosphorylation, which may have a direct effect on coregulatory interactions. Additionally, the βTC-3 cell line appears to represent a terminally differentiated β-cell, as

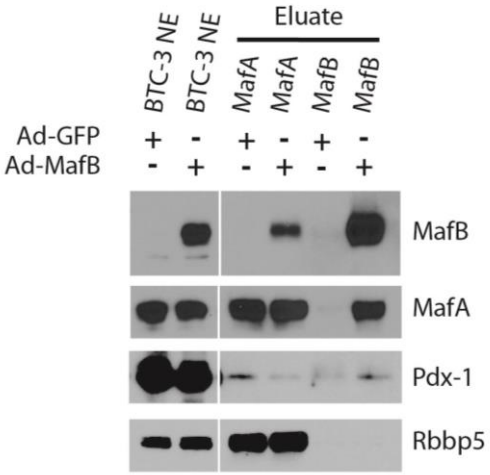


Figure 19. Mouse MafB does not interact with the MLL3/4 complex. βTC-3 cells were infected with adenovirus overexpressing either GFP or MafB, as indicated. MafA and MafB immunoprecipitation revealed that MafA and MafB heterodimerize, and interact with Pdx-1 and p/CAF. MafB does not interact with Rbbp5.

these cells only express MafA and do not express MafB. *In vivo* MafB is transiently expressed during β-cell development, and it is possible that certain activating signals such as phosphorylation are present during this time window, which are absent in a mature primary β-cells and our βTC-3 cells.

Nevertheless, I was able to overexpress MafB, which heterodimerized with MafA in immunoprecipitation experiments (**Figure 19**). Interestingly, MafB also interacted with Pdx-1, a known interacting partner of MafA and proposed to be interacting partner of MafB. This could indicate that any hypophosphorylation of MafB due to overexpression does not interfere with protein-protein interactions. However, Rbbp5 (a representative member of the Mll3/4 complex) was not detected in these immunoprecipitation experiments (**Figure 19**), whereas Rbbp5 did interact with adenovirally overexpressed MafA (**Figure 9**). This could indicate that the Mll3/4 complexes do not, in fact, interact with mouse MafB. Alternatively mouse MafB may only be able to interact with Mll3/4 during developmental timepoints. In the context of a mature β-cell, it is possible that mouse MafB is unable to interact with Mll3/4, which would make it evolutionarily advantageous to silence MafB after birth, so as not to act as a dominant negative to MafA function.

In order to further examine the role of MafA:MafB:Mll3/4 during development, MafA and MafB conditional mutant mice were generated using transgenic mice carrying an *Ngn3* promoter-driven Cre (Ngn3-Cre).[164] *Ngn3*-Cre is expressed developmentally in all endocrine cells prior to hormone expression. Simultaneous inactivation of both *MafA* and *MafB* using *Ngn3*-Cre (termed $MafA^{\Delta endo}MafB^{\Delta endo}$) produces mice that die within the first few days of life of apparent hyperglycemia **(Table 6** and data not shown), indicating the activity of these large Mafs are required for production of functional pancreatic islets. This was also true of mice with pancreas-specific inactivation of *MafA* and *MafB* using

Table 6. *MafAB$^{\Delta endo}$* animals die within 3 weeks of birth. Expected and actual genotypes obtained from the *MafA$^{fl/fl}$MafB$^{fl/fl}$* x *MafA$^{\Delta endo/+}$MafB$^{\Delta endo}$* mating. * p = 0.000171.

MafA$^{fl/fl}$MafB$^{fl/fl}$ x MafA$^{\Delta endo/+}$MafB$^{\Delta endo}$

Potential Genotypes	Mendelian Ratio	# pups expected out of 59	Actual # pups	Actual ratio
MafAB$^{\Delta endo}$	0.25	14.75	0*	0
MafA$^{\Delta endo/+}$MafB$^{\Delta endo}$	0.25	14.75	21	0.36
MafA$^{fl/+}$B$^{fl/fl}$	0.25	14.75	18	0.31
MafA$^{fl/fl}$B$^{fl/fl}$	0.25	14.75	20	0.34
Total			59	

Pdx-1-Cre (Elizabeth Conrad, personal communications). *Ngn3*-Cre-mediated inactivation of *NCoA6* (termed *NCoA6*$^{\Delta endo}$), however, is not lethal, and the mice appear to develop normally, with the production of β-cells and normal glucose tolerance (data not shown). Indeed, *NCoA6*$^{\Delta endo}$ mice appear to phenocopy the *NCoA6*$^{\Delta \beta}$ mice described earlier, thus confirming that while the activity of the Mll3/4 complexes displays significant overlap with the activity of MafA and MafB, there are also key differences that allow for the development of functional β-cells in *NCoA6*$^{\Delta endo}$ mice but not *MafA*$^{\Delta endo}$*MafB*$^{\Delta endo}$ mice.

However, some differences do exist between the *NCoA6*$^{\Delta \beta}$ and *NCoA6*$^{\Delta endo}$ mouse models. *Slc2a2*, *Prss53*, and *Slc30a8* were all downregulated in islets from 8-week old *NCoA6*$^{\Delta \beta}$ mice (**Figure 16A**), yet none of these genes are significantly downregulated in islets from 8-week old *NCoA6*$^{\Delta endo}$ mice (**Figure 20**). This could simply be the result of an insufficient number of animals analyzed (as N=3 for these experiments). Strikingly, however, both *MafB* and *glucagon* are upregulated in *NCoA6*$^{\Delta endo}$ islets by ~2.5 fold, while *MafA* expression is unchanged. *MafB* expression was unchanged in islets from 8-week old *NCoA6*$^{\Delta \beta}$ mice (data not shown). *MafB* upregulation could indicate an attempt to compensate for loss of MafA:MafB-driven Mll3/4 activity in *NCoA6*$^{\Delta endo}$ islets. Indeed, the absence of significant downregulation of *Slc2a2*, *Prss53*, and *Slc30a8* in *NCoA6*$^{\Delta endo}$ islets could indicate that MafB is able to drive expression of these genes in an Mll3/4-independent manner, likely through recruitment of other coactivators. Thus, understanding the complete network of MafA:MafB-coregulator interactions, as well as when these interactions occur, will be critical in understanding the mechanisms behind MafA/MafB-driven gene regulation.

One way in which these interactions could be detected would be using a Proximity Ligation Assay (PLA).[165] PLA experiments are performed similarly to

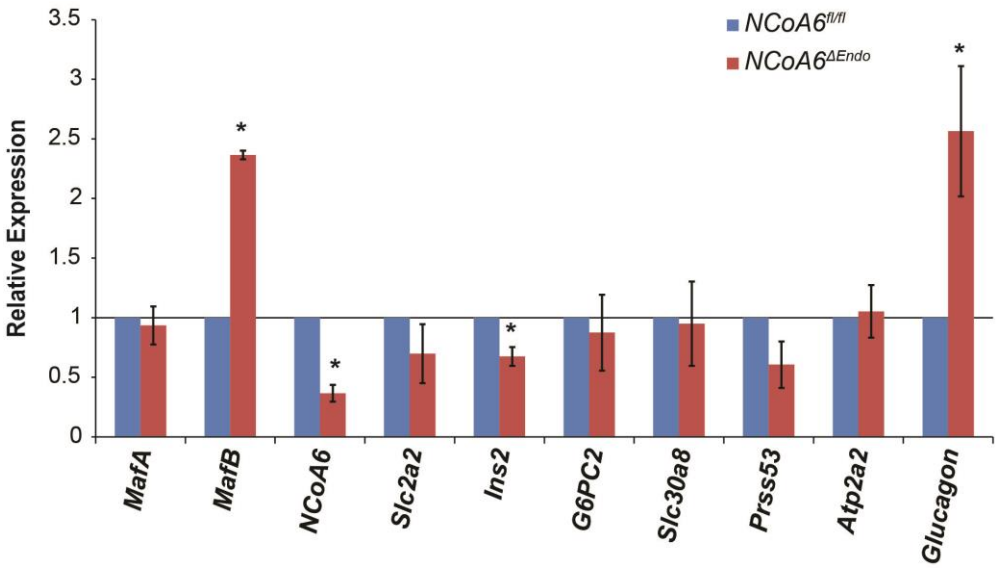


Figure 20. *NCoA6$^{\Delta endo}$* islets display compromised gene expression. mRNA expression levels were measured in 8-week old isolated islets. N for *NCoA6$^{fl/fl}$* = 3, for *NCoA6$^{\Delta endo}$* = 3, * indicates $p<0.05$.

immunofluorescence experiments, but instead of using fluorescently-labeled secondary antibodies, the secondary antibodies are instead conjugated to complementary oligonucleotides. When proteins and their primary antibodies are within 40nm, the oligonucleotides will be close enough to anneal to one another, and rolling-circle amplification can be used to produce a fluorescent signal. Therefore individual protein-protein interactions can be visualized and quantitated at a single-cell level. This technique has a distinct benefit over traditional immunoprecipitation in that embryonic mouse tissue can be utilized where MafB is still expressed in the β-cell.

However, this technique has several limitations. First, it requires very high quality and high specificity antibodies. In addition, these antibodies need to recognize target antigens in the presence of the target protein's binding partners. If the epitope on the target protein is blocked by the presence of another protein, this would produce a false-negative. Second, PLA only detects the presence of protein-protein interactions, and does not provide information about the context of this interaction. For instance, while a PLA signal may detect the interaction of a transcription factor with a coregulator, it does not provide information about where this interaction is occurring (ie on which gene). Finally, this technique cannot distinguish direct from indirect interactions. This means that, while PLA can be a powerful technique for suggesting protein-protein interactions, the absence of a signal cannot be used as evidence of a lack of interaction. We have attempted PLA with both MafA and MafB antibodies in mouse and human tissue samples, yet none of the antibodies tested have yielded a consistently positive result. This is true for PLA with either MafA or MafB and members of the Mll3/4 complexes, as well as PLA with either MafA or MafB and the known interacting protein Pdx-1. Thus, as we currently lack suitable antibodies for MafB PLA in tissue, a better technique to determine whether MafB is able to recruit the Mll3/4 complexes would be re-ChIP, where two sequential ChIPs are performed to determine whether two proteins are bound to the

same loci. Unfortunately, a significant amount of starting material would be required for re-ChIP experiments, and obtaining this much material from embryonic material would not be feasible.

Recently, transgenic mice that misexpress MafB in the β-cell under the control of a MafA promoter were generated by a previous graduate student in the Stein lab. These mice, termed MafBTg, continue to express MafB in the β-cell into adulthood (Dr. Holly Cyphert, personal communications). By combining these mice with our *MafA*$^{\Delta\beta}$ system, isolated mouse islets could be used to detect MafB:Mll3/4 complexes via traditional immunoprecipitation. Unfortunately, immunoprecipitation would require a large amount of starting material (islets from at least 20 mice per IP), and a MafB:Mll3/4 interaction in β-cells may be masked by the presence of α-cells which also express MafB. Instead, it may be possible to use isolated islets for ChIP and Re-ChIP experiments to examine occupancy on β-cell specific genes, as these genes are likely not bound by both MafB and Mll3/4 in the α-cells. This should allow us to determine whether MafB is bound to the same loci as Mll3/4, in the absence of MafA. Additional experiments could also be performed with MafBTg animals on a wild type background, where both MafA and MafB are co-expressed in the adult β-cells and are capable of heterodimerizing (Dr. Holly Cyphert, personal communications). This would allow us to begin to distinguish whether the MafA and MafB homodimers or the MafA:MafB heterodimer have distinct coregulator recruitment properties. Unfortunately, re-ChIP from adult islets still requires a large amount of starting material.

Therefore the best way to detect an interaction of mouse MafB and the Mll3/4 complexes remains PLA. In addition to determining whether and when mouse MafB interacts with Mll3/4, tissues from diabetic and pre-diabetic mice and humans could be examined, to determine whether acute or chronic diseases affect the interactions of MafA:MafB and the MLL3/4 complex. However, as mentioned above, preliminary

experiments with both MafA and MafB were unable to detect endogenous interactions. This is likely due to the quality of the antibodies being unsuitable for this assay. It is also possible that due to the large size of the Mll3/4 complexes (at least ~1.5 MDa), the assay is sensitive to which Mll3/4 complex subunit is examined, as some complex subunits are not at an optimal distance from MafA for PLA. We are still attempting to address these issues.

Determining whether and when mouse MafB is able to interact with the Mll3/4 complexes would be of great interest, as it could help explain the differences in MAFB expression between mouse and human. In our human EndoC-βH1 cell line, MAFB is able to interact with the MLL3/4 complex, although possibly through a heterodimer with MAFA. In mice, preliminary data in **Figure 19** indicates that MafB may not interact with this complex after maturation, even though MafB is capable of heterodimerizing with MafA. Therefore it is possible that in mice, silencing of MafB is critical to allow for the recruitment of the MLL3/4 complexes to shared MafA:MafB target genes. However, if this were true then one would predict that MafB expression in adult mice would have an inhibitory effect on gene expression, by acting as a dominant negative through heterodimerization with MafA and/or directly binding to these genes without the ability to recruit the Mll3/4 coactivor. In the MafBTg mice mentioned above that co-express MafB with MafA in adult β-cells, loss of MafA target gene expression was not observed, indicating that MafB is able to interact with the Mll3/4 complex, or that lack of interaction does not affect MafA target gene activation (Dr. Holly Cyphert, personal communications). This would also be consistent with the conservation of the MAFB protein between mouse and human, as they share 97% identity. Therefore a significant amount of work remains to resolve these discrepancies.

Determine the extent of overlap between the Mll3/4 complexes and MafA/MafB

As shown in **Figure 16A**, MafA and the Mll3/4 complexes share a large overlap *in vivo* in regulation of the MafA target genes previously identified (**Table 5**). This large overlap was initially unexpected for two reasons: There are relatively few genes affected by depletion of NCOA6 in mouse and human β-cell lines (**Figures 8 and 10**), and there were several other coregulatory proteins identified in **Table 4** that would appear to act independently of the Mll3/4 complexes. However, as previously reported by our lab, MafA and MafB share a large number of overlapping target genes during development, with control transitioning from MafB during development to MafA postnatally.[64] This indicates that both MafA and MafB are likely directing recruitment of the Mll3/4 complexes during development, and that Mll3/4 recruitment is restricted to a subset of MafA targets after maturation (**Figure 18**). In support of a role for MafB-driven Mll3/4 recruitment developmentally, the high levels of basal insulin secretion observed in islets from *NCoA6*$^{\Delta\beta}$ mice are similar to what was observed in islets from 2-week old mice with a pancreas-specific deletion of MafB (Elizabeth Conrad, personal communications), and is a characteristic of immature islets[157] (**Figure 16C**). However, the extent to which Mll3/4 and MafA/MafB target genes overlap has yet to be determined.

In order to determine the gene regulatory overlap between MafA and the Mll3/4 complex, the *MafA*$^{\Delta\beta}$ and *NCoA6*$^{\Delta\beta}$ mice generated previously may be used for RNA-Seq analysis. This should allow us to determine the extent of overlap between MafA regulated genes and Mll3/4 regulated genes, as well as identify genes that are regulated only by MafA or Mll3/4. However, utilizing isolated mouse islets for these experiments may not be sufficient. Current islet isolation protocols involve the collagenase-driven digestion the pancreas, in order to purify the islets from the exocrine tissue by hand-picking, although a small amount of acinar tissue usually remains. However, several different cell types are present within islets, which may generate significant background

in RNA-Seq. This can be seen in microarray analysis of islets from *MafA*$^{\Delta panc}$ mice.[56] One of the largest changes in gene expression in this analysis was the gene *amylase*, which is expressed in pancreatic acinar cells but not islets. In order to generate a better signal-to-noise and limit contaminating tissue, β-cells would need to be sorted via Fluorescently Activated Cell Sorting (FACS). Thankfully, a transgenic mouse line exists which expresses GFP from the mouse insulin promoter (MIP-GFP), and is readily available for use.[166]

We performed preliminary experiments to sort β-cells from *MIP-GFP;NCoA6*$^{\Delta\beta}$ mice, *MIP-GFP;MafA*$^{\Delta\beta}$ mice, and their respective control littermates. The conditions were optimized to find the appropriate buffer with minimal effect on viability, the appropriate conditions for digestion to a single cell suspension, and a quenching step was added following the digestion according to the recommendations of Dr. Guoqiang Gu. With this protocol, a single 8-week old mouse could yield between 2,000 to 5,000 FAC-sorted β-cells. A representative FACS plot from this isolation is shown in **Figure 21**, showing separation based on GFP fluorescence and viability using the 7-aminoactinomycin D (7-AAD) viability dye. It is recommended that at least 10,000 cells are used for isolation of RNA for RNA-Seq, indicating 2-4 mice would be needed for each sample. RNA-Seq would be preferable to microarray, as the sensitivity is greatly increased, and relative amounts of alternatively spliced genes could be determined.[167]

Once this analysis is performed on *MafA*$^{\Delta\beta}$ and *NCoA6*$^{\Delta\beta}$ mice, the affected genes could be compared between the two. This analysis would determine not only genes that are regulated by both MafA and the Mll3/4 complexes, but also gene regulation that is distinct. If a large majority of MafA-regulated genes are also regulated by the Mll3/4 complexes (similar to the results seen in **Figure 16A**), this would further support our hypothesis that the Mll3/4 complexes are essential for MafA:MafB activity.

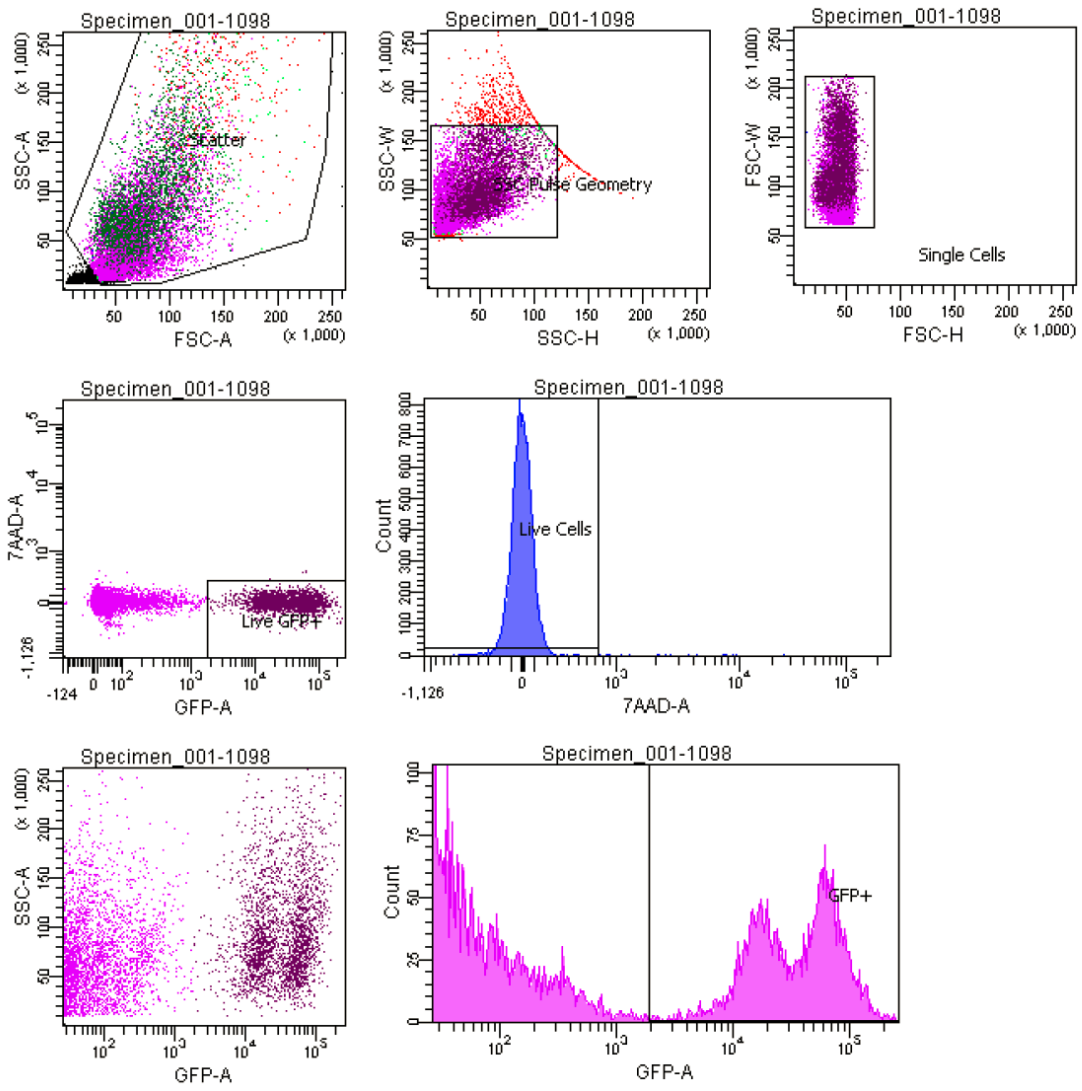


Figure 21. FACS of β-cells from *NCoA6^{Δβ}* mouse. Isolated islets were digested to single cells, and then submitted for FACS using the signal from GFP to mark insulin^{+} cells, and 7-AAD as a viability dye. Of the 10,153 single cells sorted, 2,644 were both 7-AAD-negative (i.e. viable) and GFP-positive (i.e. insulin^{+}).

ChIP-Seq of isolated embryonic tissue or adult islets for both MafA and the Mll3/4 complexes would further verify how much of this regulation may be direct. Finally, identifying MafA-regulated genes that are not regulated by the Mll3/4 may indicate roles for other coregulators identified in our Re-CLIP/MS analysis (**Table 4**).

As mentioned, our data indicates that in *NCoA6*$^{\Delta\beta}$ mice the Mll3/4 complexes are regulating a subset of MafA:MafB target genes during development and postnatally (**Figure 18**). In order to distinguish developmental and post-maturation gene regulation, an inducible Cre system could be utilized, such as the mouse insulin promoter-CreER (MIP-CreER) mouse,[168] to drive excision of exon 8 of *NCoA6* postnatally in mature β-cells. We would predict that post-β-cell-maturation loss of NcoA6 would result downregulation of a subset of MafA target genes, similar to what was seen with knockdown of *NCoA6* in the mouse βTC-3 cell line (**Figure 11**). Histone modification patterns are established during development, and it is likely that they may not be actively reversed post-maturation. Additionally, recruitment of Mll3/4 by MafA/MafA homodimer may be distinct from recruitment by MafB:MafB homodimer or MafA:MafB heterodimer. Finally, the necessary signaling events to drive MafA recruitment of Mll3/4 may be lacking after maturation. However, removal of *NCoA6* in mature β-cells and comparison to removal of *MafA* in mature β-cells may address some of the unanswered questions remaining from our cell line analysis.

Alternatively, it would be interesting to determine which genes are regulated by the MLL3/4 complexes independent of MafA. As mentioned previously, the MLL3/4 complexes have multiple roles in other tissues, such as in adipogenesis,[148] in mediating nuclear receptor signaling,[136,169] and in the regulation of genes associated with other pathways such as cholesterol efflux.[170] However, as many of these genes are driven by transcription factors not expressed in the β-cell, it is unlikely that the MLL3/4 complexes are recruited for their activation in the β-cell. As noted in **Figure 10**, the MLL3/4

complexes appear to have some specificity for MafA in comparison to other islet enriched transcription factors, and Re-CLIP analysis of Pdx-1 did not identify the MLL3/4 complexes ([80] and Brian McKenna, personal communications). Additionally, mouse models for many of these transcription factors display severe pancreatic phenotypes, which frequently cause death due to hyperglycemia shortly after birth, in stark contrast to what was observed with *NCoA6*$^{\Delta\beta}$ mice. It is therefore likely that the MLL3/4 complexes predominately regulate MafA target genes via MafA recruitment; however, we cannot rule out a role for other islet-enriched transcription factors in recruitment of Mll3/4 complexes. RNA-Seq of the *NCoA6*$^{\Delta\beta}$ mice, as described above, could help clarify this. Additionally, this could suggest roles for other coregulators, including other members of the COMPASS family of proteins, in driving islet-enriched transcription factor function.

Further characterize the other transcription factors and coregulators that interact with MafA

As seen in **Table 4**, several coregulators were identified in our Re-CLIP experiments but were not further characterized. This includes the histone deacetylase Hdac2, which is capable of histone deacetylation and gene repression. Interestingly, Hdac2 is part of three distinct corepressor complexes: the Sin3 complex, the Nucleosome Remodeling and Deacetylation (NuRD) complex, and the CoRepressor for Element-1-Silencing Transcription factor (CoREST) repressor complex.[171] Interestingly, members of each of these three complexes were identified in the ReCLIP/MS analysis of MafA: Sin3A for the Sin3 complex, Chd4 from the NuRD complex, and Rcor1, Rcor3, Lsd1, and Hmg20b of the CoREST repressive complex (**Figure 22**). Rbbp7 was also observed in our analysis, and is present in both the Sin3 complex and NuRD complex. These corepressor complexes are thought to be largely non-redundant, as mouse

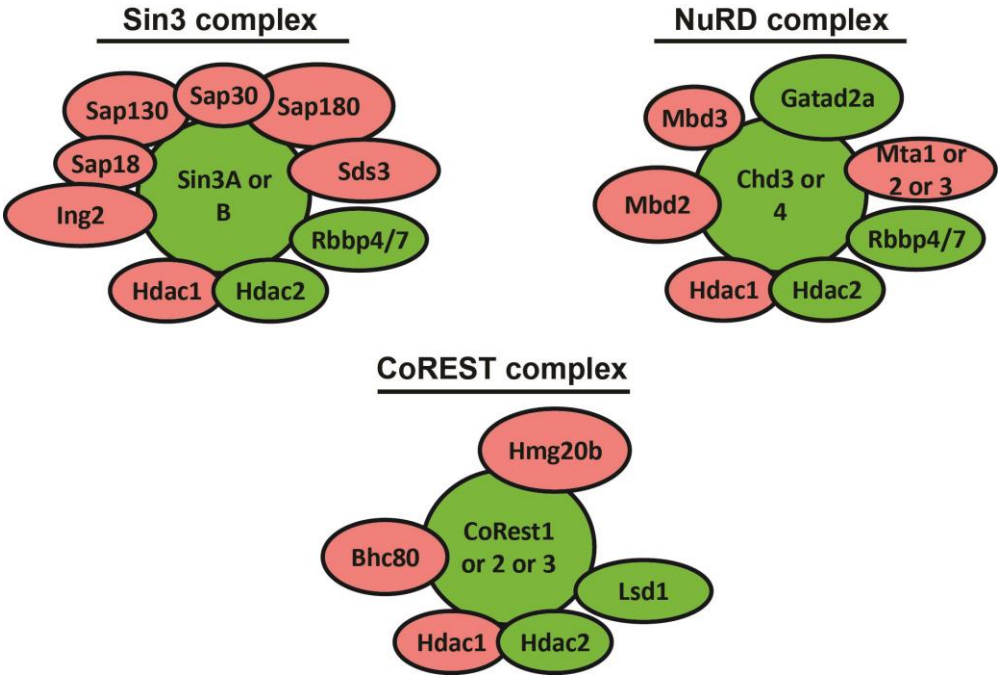


Figure 22. The Hdac2-containing complexes. The proteins labeled in red were not present in Re-CLIP/MS analysis to identify MafA interacting partners (Table 4), while the proteins labeled in green were present.

knockouts of individual complex subunits (Sin3A[172], MBD2/3[173], Lsd1[174]) lead to embryonic lethality. Thus, these complexes represent several distinct pathways through which MafA could be directing gene repression. Unfortunately, relatively little is known about these complexes in the pancreas, with a few studies looking only at Hdac1 but not other members of these complexes. For instance, Nkx2.2 was shown to recruit Hdac1 to the promoter of the *Arx* gene to mediate its repression in β-cells, yet further analysis was not performed to determine whether the Sin3A, NuRD, or CoREST complexes were also present.[109] Additionally, in humans INSM1 was shown to co-immunoprecipitate with HDAC1, yet no additional examination was performed to identify sites of co-recruitment or its role.[175] This leaves a significant amount of work left to determine the role of these complexes, and their relationship to MafA in the β-cell.

In addition to coregulators, several transcription factors were identified to interact with MafA that have known roles in the islet, such as the three members of the Myt family: Myt1, Myt1l, and Myt3.[176,177,178,179] Myt1 is expressed in the developing endocrine cells and adult islet β-cell, yet deletion has minimal effect on the pancreatic β-cell, likely due to compensation by other Myt family members.[177] More recently, Myt3 has been shown to be expressed in the mature β-cell, but is not expressed developmentally, and is involved in regulation of β-cell survival and in regulation of the expression of some islet-enriched transcription factors.[179] As discussed in Chapter I, transcription factors are capable of playing coordinating roles in coregulator recruitment and gene activation, as is the case with Pdx-1, NeuroD1, MafA, and Glis3.[98] It is therefore possible that Myt family members, and other transcription factors identified here, play similar roles in synergistic activation of at least some MafA target genes.

In order to further study potential coregulators of MafA, the primary goal should be to find antibodies suitable for chromatin immunoprecipitation (ChIP). As the ChIP-Seq analysis of MafA has already been performed by others,[40] there is a wealth of

information about MafA binding sites available. Once binding targets for these coregulators are established, Re-ChIP could then be performed. Re-ChIP is a performed similar to ChIP experiments, except that two different antibodies are used sequentially, allowing for the detection of co-occupancy of two different proteins on the same piece of chromatin. Assuming co-occupancy, ChIP experiments in the presence or absence of MafA (via knockdown in cell lines, or from isolated islets of *MafA*$^{\Delta\beta}$ mice) would allow for the detection of direct recruitment by MafA. This could further be analyzed via examination of histone modifications in the presence or absence of MafA and its coregulator. However, Xu *et al*[180] recently found that histone modifications are frequently less dynamic in cell lines than *in vivo*, indicating that coregulators may only be required during specific time windows during development. This idea was reinforced by our data in the βTC-3 cell line compared to our *NCoA6*$^{\Delta\beta}$ mouse model. We demonstrated in the mouse βTC-3 cell line that the Mll3/4 complexes are required for activation of only a subset of MafA target genes, whereas we saw a near complete overlap *in vivo* of MafA target gene regulation between *MafA*$^{\Delta\beta}$ and *NCoA6*$^{\Delta\beta}$ mice.

Ultimately, studies involving new coregulators should be extended *in vivo*, using mouse models or human samples when available. A starting point for this analysis could be the use of PLA to determine whether these interactions occur *in vivo*, and at what timepoints. Unfortunately, as mentioned above, this technique remains technically difficult for MafA and MafB. Secondly, a mouse model of β-cell specific removal of these coregulators could provide insight into the level of overlap between the coregulators and the MafA, as seen in **Figure 16**. Finally, human analysis would be essential, in order to determine whether these mechanisms and interactions are conserved. For this analysis, human islets could be utilized for ChIP or Re-ChIP experiments, as well as for examination of chromatin marks surrounding MAFA/MAFB binding sites. Additionally, it may be possible to utilize other techniques such as siRNA-mediated knockdown in

primary human islets; however, the efficiency and reproducibility of such techniques in human islets is not yet clear. Therefore, additional manipulations are possible using the EndoC-βH1 cell, as seen in **Figure 13**. Although this cell line was generated from human fetal pancreas, these cells express significant levels of MAFA similar to adult humans, whereas islets from pre-pubescent humans express nearly undetectable levels of MAFA (Dr. Holly Cyphert, personal communications). By utilizing the EndoC-βH1 cells, it is not only possible to gain insight into the roles of MAFA coregulatory complexes in humans, but also gain some insight into the function of MAFA/MAFB coexpression, and how this might direct mouse and human β-cell development.

Identification and characterization of MAFA and MAFB coregulators in human β-cell lines

As noted in Chapter I, the ultimate goal of this research is to better understand the mechanisms by which MAFA regulates β-cell maturation in humans, using the mouse as a model organism. However, as we have found in Chapter II, there are differences between genes regulated by MafA and/or MafB in mice and genes regulated by MAFA or MAFB in humans. Therefore, in order to translate this research to humans, it will first be necessary to understand which genes are regulated by MAFA and MAFB in the human. We have identified a small candidate list of genes regulated by MAFA and MafB in **Figure 13**, but a more systematic approach would better establish the breadth of MAFA and MAFB regulation, as well as whether MAFA and MAFB may have independent roles. A future direction would therefore be to knockdown or knockout *MAFA* and *MAFB* independently, as well as together, and perform RNA-Seq on these cells in order to generate a list of target genes. In addition, ChIP-Seq using non-crossreactive MAFA and MAFB antibodies would provide insight into which MAFA/MAFB regulated genes are direct target genes of MAFA and MAFB, and which may be targets of a homodimer versus a heterodimer of both.

One possible approach to this question would be to knockout *MAFA*, *MAFB*, or both genes in the EndoC-βH1 cell line using the Clustered, Regularly Interspaced, Short Palindromic Repeat (CRISPR)-Cas9 system.[181] CRISPR-Cas9 utilizes the nuclease activity of the Cas9 protein to induce double-stranded DNA breaks at specific genomic loci chosen by the researcher. When these double stranded DNA breaks are repaired, random insertions/deletions can occur that may disrupt nearby gene function. Individual cells are sub-cloned and screened to identify the specific mutation that occurred. Using this technique, it is theoretically possible to knockout any gene not directly necessary for cell survival or proliferation from the genome.

Unfortunately, one caveat to this technique is that, following CRISPR-Cas9, single cell colonies must be grown and characterized in order to ensure successful mutation, and to check for possible off-target effects. This makes the utilization of this technique in EndoC-βH1 (or EndoC-βH2) cells difficult, as these cells proliferate at a rate of ~1 division per week. To generate a subcloned population of ~1 million cells would take approximately 5 months, assuming optimal growth conditions. For this reason, it may be beneficial to consider alternative approaches.

In Chapter II, we utilized siRNA-mediated knockdown via traditional lipid-based transfection reagents in EndoC-βH1 cells. Unfortunately, this technique proved to be low efficiency (~50%, **Figure 13**), and simultaneous knockdown of both *MAFA* and *MAFB* was even less efficient (~25-30%, data not shown). Such inefficient knockdown may be insufficient to identify many of the genes regulated by MAFA and MAFB in the human β-cell lines. One additional approach currently under consideration is nucleofection-mediated siRNA knockdown, a technique utilized in our mouse β-cell lines in **Figure 11**. In preliminary experiments, this technique dramatically improves the efficiency of siRNA-mediated knockdown to ~65-80% (data not shown). However, nucleofection is a form of electroporation that is extremely harsh on the cells, routinely causing death in roughly

half of the treated cells. Therefore we are currently in the process of testing the effects of nucleofection alone, to ensure that this approach does not impair the ability of the EndoC-βH1 cells to grow and respond to glucose. Another approach currently under consideration is the use of lentivirus to deliver shRNA targeting MAFA and/or MAFB. This technique has been used successfully by the Ferrer laboratory in EndoC-βH1 cells to knockdown MAFB, although their efficiency was only slightly better than our lipid based (~60% vs ~50%).[182] However, lentivirus infection allows for the utilization of positive-selection markers, which may further increase this efficiency by selecting for cells with stable integration of the virus.

Once efficient knockdown conditions have been established, we should be able to identify more target genes of MAFA and/or MAFB using a combination of RNA-Seq and ChIP-Seq. Of interest, we have recently obtained the EndoC-βH2 cell line mentioned briefly in Chapter I. This human β-cell line is different from the EndoC-βH1 cells in that the human telomerase reverse transcriptase (*hTERT*) and large T antigen of simian virus 40 (*SV40LT*) genes used for immortalization are flanked by loxP sites. This allows for excision of *HTERT* and *SV40LT* upon expression of Cre, after which the EndoC-βH2 cells no longer proliferate, and both *MAFA* and *INSULIN* are upregulated. This appears to mimic in some ways the maturation of β-cells in the rodent. In rodents, decreased proliferation, increased expression of *MafA*, and decreased basal insulin secretion (as a % of insulin content) are all characteristics of a mature β-cell.[63] Therefore, in future studies the EndoC-βH2 cells may prove to be a good model for human β-cell maturation, allowing for manipulations that would not be feasible with primary human β-cells.

In addition to identifying further targets of MAFA and MAFB in human β-cells, it would also be possible to pursue the identification of coregulators for both MAFA and MAFB in the EndoC-βH1 or EndoC-βH2 cells. An approach such as Re-CLIP/MS we

outlined in Chapter II could identify coregulators of MAFA and MAFB in human cells. Currently, this strategy is being utilized in our lab to identify coregulators of MAFA and MAFB in humans, as well as coregulators unique to either MAFA or MAFB. As expected, the MLL3/4 complexes were present in the MS analysis of both MAFA and MAFB experiments (Dr. Holly Cyphert, personal communications), consistent with our observations in **Figure 12**. Once new binding partners are identified, it would be of interest to determine whether the interaction is specific to MAFA or MAFB. While knockdown experiments similar to those utilized in Chapter II could help determine a role for these coregulators in MAFA:MAFB-mediated gene activation, the ultimate goal would be ChIP-Seq and Re-ChIP experiments, in order to determine whether these factors are directly recruited by the MAFA/MAFB transcription factors. Here, the EndoC-βH2 would provide an interesting model for examination of MAFA/MAFB interactions with coregulators, in order to determine whether coregulator interaction or recruitment is affected by this Cre-driven "maturation-like" process. Understanding the mechanisms of MafA transactivation could provide significant insight into β-cell maturation.

Concluding Remarks

This dissertation aimed to identify coregulators of the transcription factor MafA, and establish their role in β-cell maturation and function. MafA is critical for β-cell maturation, and its absence in mice leads to dysfunction in the islet.[56] Both human and mouse MAFA, as well as human MAFB, interact with the MLL3/4 complexes, histone 3 lysine 4 methyltransferases responsible for gene activation. In mice, the Mll3/4 complexes regulate a subset of MafA target genes, presumably through recruitment by MafA, and regulate H3K4me3 and levels of phosphorylated RNA Polymerase II at the TSS. However, *in vivo* deletion of *NCoA6* from β-cells in mice indicates that the Mll3/4 complexes are responsible for activation of a significant number of MafA target genes.

This is surprising, considering the large number of coregulators identified for MafA. However, when considered with data from cell line knockdowns, we propose that the Mll3/4 complexes regulate MafA/MafB target genes during development through interaction with both MafA and MafB (**Figure 18**). This leaves open the possibility that the Mll3/4 complexes "hand over" control of MafA target genes to other coregulators after β-cell maturation. Additionally, it is possible that the MLL3/4 complexes are dispensable due to a lack of a counter-regulatory repressive complex after β-cell maturation, and that under conditions such as chronic hyperglycemia, these complexes may be necessary to maintain MafA-mediated gene activation.

More broadly, these findings establish a method whereby coregulators of critical β-cell transcription factors can be identified and characterized. Recent work has shown that changes in histone methylation are an important part of either β-cell differentiation from embryonic stem cells (ESCs),[134] or β-cell transdifferentiation from α-cells.[163] However, while a plethora of islet-enriched transcription factors has been identified, the link between these factors and regulation of histone modifications remains undetermined. Additionally, our mouse models strongly indicate that not only are coregulatory interactions important, but the timing of these interactions may be critical in guiding their function. In order to fully understand β-cell development and how to produce mature β-cells, it will be critical to understand the transcriptional mechanism by which this development is controlled.

REFERENCES

1. Brissova M, Fowler MJ, Nicholson WE, et al. Assessment of human pancreatic islet architecture and composition by laser scanning confocal microscopy. *J Histochem Cytochem Off J Histochem Soc*. 2005;53(9):1087-1097. doi:10.1369/jhc.5C6684.2005.

2. Orci L, Stefan Y, Malaisse-Lagae F, Perrelet A. Instability of pancreatic endocrine cell populations throughout life. *Lancet*. 1979;1(8116):615-616.

3. Unger RH, Orci L. The role of glucagon in the endogenous hyperglycemia of diabetes mellitus. *Annu Rev Med*. 1977;28:119-130. doi:10.1146/annurev.me.28.020177.001003.

4. Unger RH, Orci L. Paracrinology of islets and the paracrinopathy of diabetes. *Proc Natl Acad Sci U S A*. 2010;107(37):16009-16012. doi:10.1073/pnas.1006639107.

5. Alberti K g. m. m., Zimmet P z. Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus. Provisional report of a WHO Consultation. *Diabet Med*. 1998;15(7):539-553. doi:10.1002/(SICI)1096-9136(199807)15:7<539::AID-DIA668>3.0.CO;2-S.

6. Wang M-Y, Yan H, Shi Z, et al. Glucagon receptor antibody completely suppresses type 1 diabetes phenotype without insulin by disrupting a novel diabetogenic pathway. *Proc Natl Acad Sci U S A*. 2015;112(8):2503-2508. doi:10.1073/pnas.1424934112.

7. Rhodes CJ. Type 2 diabetes-a matter of beta-cell life and death? *Science*. 2005;307(5708):380-384. doi:10.1126/science.1104345.

8. Butler AE, Janson J, Bonner-Weir S, Ritzel R, Rizza RA, Butler PC. Beta-cell deficit and increased beta-cell apoptosis in humans with type 2 diabetes. *Diabetes*. 2003;52(1):102-110.

9. Donath MY, Halban PA. Decreased beta-cell mass in diabetes: significance, mechanisms and therapeutic implications. *Diabetologia*. 2004;47(3):581-589. doi:10.1007/s00125-004-1336-4.

10. Guo S, Dai C, Guo M, et al. Inactivation of specific β cell transcription factors in type 2 diabetes. *J Clin Invest*. July 2013. doi:10.1172/JCI65390.

11. Harmon JS, Bogdani M, Parazzoli SD, et al. beta-Cell-specific overexpression of glutathione peroxidase preserves intranuclear MafA and reverses diabetes in db/db mice. *Endocrinology*. 2009;150(11):4855-4862. doi:10.1210/en.2009-0708.

12. Chua SC, Chung WK, Wu-Peng XS, et al. Phenotypes of mouse diabetes and rat fatty due to mutations in the OB (leptin) receptor. *Science*. 1996;271(5251):994-996.

13. Lin HV, Ren H, Samuel VT, et al. Diabetes in mice with selective impairment of insulin action in Glut4-expressing tissues. *Diabetes*. 2011;60(3):700-709. doi:10.2337/db10-1056.

14. Wang Z, York NW, Nichols CG, Remedi MS. Pancreatic β cell dedifferentiation in diabetes and redifferentiation following insulin therapy. *Cell Metab*. 2014;19(5):872-882. doi:10.1016/j.cmet.2014.03.010.

15. Talchai C, Xuan S, Lin HV, Sussel L, Accili D. Pancreatic β cell dedifferentiation as a mechanism of diabetic β cell failure. *Cell*. 2012;150(6):1223-1234. doi:10.1016/j.cell.2012.07.029.

16. Yoneda S, Uno S, Iwahashi H, et al. Predominance of β-cell neogenesis rather than replication in humans with an impaired glucose tolerance and newly diagnosed diabetes. *J Clin Endocrinol Metab*. 2013;98(5):2053-2061. doi:10.1210/jc.2012-3832.

17. American Diabetes Association. Standards of medical care in diabetes--2014. *Diabetes Care*. 2014;37 Suppl 1:S14-S80. doi:10.2337/dc14-S014.

18. Shapiro AM, Lakey JR, Ryan EA, et al. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. *N Engl J Med*. 2000;343(4):230-238. doi:10.1056/NEJM200007273430401.

19. Bellin MD, Barton FB, Heitman A, et al. Potent induction immunotherapy promotes long-term insulin independence after islet transplantation in type 1 diabetes. *Am J Transplant Off J Am Soc Transplant Am Soc Transpl Surg*. 2012;12(6):1576-1583. doi:10.1111/j.1600-6143.2011.03977.x.

20. Zwillich T. Diabetes research. Islet transplants not yet ready for prime time. *Science*. 2000;289(5479):531-533.

21. Pagliuca FW, Millman JR, Gürtler M, et al. Generation of Functional Human Pancreatic β Cells In Vitro. *Cell*. 2014;159(2):428-439. doi:10.1016/j.cell.2014.09.040.

22. D'Amour KA, Bang AG, Eliazer S, et al. Production of pancreatic hormone-expressing endocrine cells from human embryonic stem cells. *Nat Biotechnol*. 2006;24(11):1392-1401. doi:10.1038/nbt1259.

23. Baetge EE. Production of beta-cells from human embryonic stem cells. *Diabetes Obes Metab*. 2008;10 Suppl 4:186-194. doi:10.1111/j.1463-1326.2008.00956.x.

24. Bruin JE, Erener S, Vela J, et al. Characterization of polyhormonal insulin-producing cells derived in vitro from human embryonic stem cells. *Stem Cell Res*. 2014;12(1):194-208. doi:10.1016/j.scr.2013.10.003.

25. Pagliuca FW, Melton DA. How to make a functional β-cell. *Dev Camb Engl*. 2013;140(12):2472-2483. doi:10.1242/dev.093187.

26. Oliver-Krasinski JM, Stoffers DA. On the origin of the beta cell. *Genes Dev*. 2008;22(15):1998-2021. doi:10.1101/gad.1670808.

27. Nishizawa M, Kataoka K, Goto N, Fujiwara KT, Kawai S. v-maf, a viral oncogene that encodes a “leucine zipper” motif. *Proc Natl Acad Sci U S A*. 1989;86(20):7711-7715.

28. Swaroop A, Xu JZ, Pawar H, Jackson A, Skolnick C, Agarwal N. A conserved retina-specific gene encodes a basic motif/leucine zipper domain. *Proc Natl Acad Sci U S A*. 1992;89(1):266-270.

29. Cordes SP, Barsh GS. The mouse segmentation gene kr encodes a novel basic domain-leucine zipper transcription factor. *Cell*. 1994;79(6):1025-1034.

30. Benkhelifa S, Provot S, Lecoq O, Pouponnot C, Calothy G, Felder-Schmittbuhl MP. mafA, a novel member of the maf proto-oncogene family, displays developmental regulation and mitogenic capacity in avian neuroretina cells. *Oncogene*. 1998;17(2):247-254. doi:10.1038/sj.onc.1201898.

31. Ogino H, Yasuda K. Induction of lens differentiation by activation of a bZIP transcription factor, L-Maf. *Science*. 1998;280(5360):115-118.

32. Kannan MB, Solovieva V, Blank V. The small MAF transcription factors MAFF, MAFG and MAFK: current knowledge and perspectives. *Biochim Biophys Acta*. 2012;1823(10):1841-1846. doi:10.1016/j.bbamcr.2012.06.012.

33. Motohashi H, O’Connor T, Katsuoka F, Engel JD, Yamamoto M. Integration and diversity of the regulatory network composed of Maf and CNC families of transcription factors. *Gene*. 2002;294(1-2):1-12.

34. Nomoto H, Kondo T, Miyoshi H, et al. Inhibition of small Maf function in pancreatic beta cells improves glucose tolerance through the enhancement of insulin gene transcription and insulin secretion. *Endocrinology*. March 2015:en20141906. doi:10.1210/en.2014-1906.

35. Shimohata H, Yoh K, Morito N, Shimano H, Kudo T, Takahashi S. MafK overexpression in pancreatic beta-cells caused impairment of glucose-stimulated insulin secretion. *Biochem Biophys Res Commun*. 2006;346(3):671-680. doi:10.1016/j.bbrc.2006.05.184.

36. Matsushima-Hibiya Y, Nishi S, Sakai M. Rat maf-related factors: the specificities of DNA binding and heterodimer formation. *Biochem Biophys Res Commun*. 1998;245(2):412-418. doi:10.1006/bbrc.1998.8447.

37. Vinson C, Acharya A, Taparowsky EJ. Deciphering B-ZIP transcription factor interactions in vitro and in vivo. *Biochim Biophys Acta*. 2006;1759(1-2):4-12. doi:10.1016/j.bbaexp.2005.12.005.

38. Yang Y, Cvekl A. Large Maf Transcription Factors: Cousins of AP-1 Proteins and Important Regulators of Cellular Differentiation. *Einstein J Biol Med EJBM*. 2007;23(1):2-11.

39. Kataoka K, Noda M, Nishizawa M. Maf nuclear oncoprotein recognizes sequences related to an AP-1 site and forms heterodimers with both Fos and Jun. *Mol Cell Biol*. 1994;14(1):700-712.

40. Tennant BR, Robertson AG, Kramer M, et al. Identification and analysis of murine pancreatic islet enhancers. *Diabetologia*. 2013;56(3):542-552. doi:10.1007/s00125-012-2797-5.

41. Abate C, Luk D, Curran T. Transcriptional regulation by Fos and Jun in vitro: interaction among multiple activator and regulatory domains. *Mol Cell Biol*. 1991;11(7):3624-3632.

42. Rauscher FJ, Voulalas PJ, Franza BR, Curran T. Fos and Jun bind cooperatively to the AP-1 site: reconstitution in vitro. *Genes Dev*. 1988;2(12B):1687-1699.

43. Kerppola TK, Curran T. A conserved region adjacent to the basic domain is required for recognition of an extended DNA binding site by Maf/Nrl family proteins. *Oncogene*. 1994;9(11):3149-3158.

44. Kerppola TK, Curran T. Maf and Nrl can bind to AP-1 sites and form heterodimers with Fos and Jun. *Oncogene*. 1994;9(3):675-684.

45. Liu Q, Ji X, Breitman ML, Hitchcock PF, Swaroop A. Expression of the bZIP transcription factor gene Nrl in the developing nervous system. *Oncogene*. 1996;12(1):207-211.

46. Kataoka K, Shioda S, Ando K, Sakagami K, Handa H, Yasuda K. Differentially expressed Maf family transcription factors, c-Maf and MafA, activate glucagon and insulin gene expression in pancreatic islet alpha- and beta-cells. *J Mol Endocrinol*. 2004;32(1):9-20.

47. Lecoin L, Sii-Felice K, Pouponnot C, Eychène A, Felder-Schmittbuhl MP. Comparison of maf gene expression patterns during chick embryo development. *Gene Expr Patterns GEP*. 2004;4(1):35-46.

48. Kataoka K, Han S, Shioda S, Hirai M, Nishizawa M, Handa H. MafA is a glucose-regulated and pancreatic beta-cell-specific transcriptional activator for the insulin gene. *J Biol Chem*. 2002;277(51):49903-49910. doi:10.1074/jbc.M206796200.

49. Olbrot M, Rud J, Moss LG, Sharma A. Identification of beta-cell-specific insulin gene transcription factor RIPE3b1 as mammalian MafA. *Proc Natl Acad Sci U S A*. 2002;99(10):6737-6742. doi:10.1073/pnas.102168499.

50. Matsuoka T, Zhao L, Artner I, et al. Members of the large Maf transcription family regulate insulin gene transcription in islet beta cells. *Mol Cell Biol*. 2003;23(17):6049-6062.

51. Melloul D, Marshak S, Cerasi E. Regulation of insulin gene transcription. *Diabetologia*. 2002;45(3):309-326. doi:10.1007/s00125-001-0728-y.

52. Nishimura W, Kondo T, Salameh T, et al. A switch from MafB to MafA expression accompanies differentiation to pancreatic beta-cells. *Dev Biol*. 2006;293(2):526-539. doi:10.1016/j.ydbio.2006.02.028.

53. Slack JM. Developmental biology of the pancreas. *Dev Camb Engl*. 1995;121(6):1569-1580.

54. Pictet RL, Clark WR, Williams RH, Rutter WJ. An ultrastructural analysis of the developing embryonic pancreas. *Dev Biol*. 1972;29(4):436-467.

55. Zhang C, Moriguchi T, Kajihara M, et al. MafA is a key regulator of glucose-stimulated insulin secretion. *Mol Cell Biol*. 2005;25(12):4969-4976. doi:10.1128/MCB.25.12.4969-4976.2005.

56. Hang Y, Yamamoto T, Benninger RKP, et al. The MafA transcription factor becomes essential to islet β-cells soon after birth. *Diabetes*. 2014;63(6):1994-2005. doi:10.2337/db13-1001.

57. Jeon J, Correa-Medina M, Ricordi C, Edlund H, Diez JA. Endocrine cell clustering during human pancreas development. *J Histochem Cytochem Off J Histochem Soc*. 2009;57(9):811-824. doi:10.1369/jhc.2009.953307.

58. Dai C, Brissova M, Hang Y, et al. Islet-enriched gene expression and glucose-induced insulin secretion in human and mouse islets. *Diabetologia*. 2012;55(3):707-718. doi:10.1007/s00125-011-2369-0.

59. Artner I, Hang Y, Guo M, Gu G, Stein R. MafA is a dedicated activator of the insulin gene in vivo. *J Endocrinol*. 2008;198(2):271-279. doi:10.1677/JOE-08-0063.

60. Guo S, Vanderford NL, Stein R. Phosphorylation within the MafA N terminus regulates C-terminal dimerization and DNA binding. *J Biol Chem*. 2010;285(17):12655-12661. doi:10.1074/jbc.M110.105759.

61. Rocques N, Abou Zeid N, Sii-Felice K, et al. GSK-3-mediated phosphorylation enhances Maf-transforming activity. *Mol Cell*. 2007;28(4):584-597. doi:10.1016/j.molcel.2007.11.009.

62. Guo S, Burnette R, Zhao L, et al. The stability and transactivation potential of the mammalian MafA transcription factor are regulated by serine 65 phosphorylation. *J Biol Chem*. 2009;284(2):759-765. doi:10.1074/jbc.M806314200.

63. Scharfmann R, Pechberty S, Hazhouz Y, et al. Development of a conditionally immortalized human pancreatic β cell line. *J Clin Invest*. 2014;124(5):2087-2098. doi:10.1172/JCI72674.

64. Artner I, Hang Y, Mazur M, et al. MafA and MafB Regulate Genes Critical to {beta} Cells in a Unique Temporal Manner. *Diabetes*. 2010;59(10):2530-2539. doi:10.2337/db10-0190.

65. Sadl V, Jin F, Yu J, et al. The mouse Kreisler (Krml1/MafB) segmentation gene is required for differentiation of glomerular visceral epithelial cells. *Dev Biol*. 2002;249(1):16-29.

66. Blanchi B, Kelly LM, Viemari J-C, et al. MafB deficiency causes defective respiratory rhythmogenesis and fatal central apnea at birth. *Nat Neurosci*. 2003;6(10):1091-1100. doi:10.1038/nn1129.

67. Artner I, Blanchi B, Raum JC, et al. MafB is required for islet beta cell maturation. *Proc Natl Acad Sci U S A*. 2007;104(10):3853-3858. doi:10.1073/pnas.0700013104.

68. Jonsson J, Carlsson L, Edlund T, Edlund H. Insulin-promoter-factor 1 is required for pancreas development in mice. *Nature*. 1994;371(6498):606-609. doi:10.1038/371606a0.

69. Offield MF, Jetton TL, Labosky PA, et al. PDX-1 is required for pancreatic outgrowth and differentiation of the rostral duodenum. *Dev Camb Engl*. 1996;122(3):983-995.

70. Stoffers DA, Zinkin NT, Stanojevic V, Clarke WL, Habener JF. Pancreatic agenesis attributable to a single nucleotide deletion in the human IPF1 gene coding sequence. *Nat Genet*. 1997;15(1):106-110. doi:10.1038/ng0197-106.

71. Guz Y, Montminy MR, Stein R, et al. Expression of murine STF-1, a putative insulin gene transcription factor, in beta cells of pancreas, duodenal epithelium and pancreatic exocrine and endocrine progenitors during ontogeny. *Dev Camb Engl*. 1995;121(1):11-18.

72. Gu G, Dubauskaite J, Melton DA. Direct evidence for the pancreatic lineage: NGN3+ cells are islet progenitors and are distinct from duct progenitors. *Dev Camb Engl*. 2002;129(10):2447-2457.

73. Gao T, McKenna B, Li C, et al. Pdx1 maintains β cell identity and function by repressing an α cell program. *Cell Metab*. 2014;19(2):259-271. doi:10.1016/j.cmet.2013.12.002.

74. Gannon M, Ables ET, Crawford L, et al. pdx-1 function is specifically required in embryonic beta cells to generate appropriate numbers of endocrine cell types and maintain glucose homeostasis. *Dev Biol*. 2008;314(2):406-417. doi:10.1016/j.ydbio.2007.10.038.

75. Stoffers DA, Ferrer J, Clarke WL, Habener JF. Early-onset type-II diabetes mellitus (MODY4) linked to IPF1. *Nat Genet*. 1997;17(2):138-139. doi:10.1038/ng1097-138.

76. Yang Y-P, Thorel F, Boyer DF, Herrera PL, Wright CVE. Context-specific α- to-β-cell reprogramming by forced Pdx1 expression. *Genes Dev*. 2011;25(16):1680-1685. doi:10.1101/gad.16875711.

77. Zhou Q, Brown J, Kanarek A, Rajagopal J, Melton DA. In vivo reprogramming of adult pancreatic exocrine cells to beta-cells. *Nature*. 2008;455(7213):627-632. doi:10.1038/nature07314.

78. Mosley AL, Corbett JA, Ozcan S. Glucose regulation of insulin gene expression requires the recruitment of p300 by the beta-cell-specific transcription factor Pdx-1. *Mol Endocrinol Baltim Md*. 2004;18(9):2279-2290. doi:10.1210/me.2003-0463.

79. Mosley AL, Ozcan S. The pancreatic duodenal homeobox-1 protein (Pdx-1) interacts with histone deacetylases Hdac-1 and Hdac-2 on low levels of glucose. *J Biol Chem*. 2004;279(52):54241-54247. doi:10.1074/jbc.M410379200.

80. McKenna B, Guo M, Reynolds A, Hara M, Stein R. Dynamic recruitment of functionally distinct Swi/Snf chromatin remodeling complexes modulates Pdx1 activity in islet β cells. *Cell Rep*. 2015;10(12):2032-2042. doi:10.1016/j.celrep.2015.02.054.

81. Sommer L, Ma Q, Anderson DJ. neurogenins, a novel family of atonal-related bHLH transcription factors, are putative mammalian neuronal determination genes that reveal progenitor cell heterogeneity in the developing CNS and PNS. *Mol Cell Neurosci*. 1996;8(4):221-241. doi:10.1006/mcne.1996.0060.

82. Wang S, Jensen JN, Seymour PA, et al. Sustained Neurog3 expression in hormone-expressing islet cells is required for endocrine maturation and function. *Proc Natl Acad Sci U S A*. 2009;106(24):9715-9720. doi:10.1073/pnas.0904247106.

83. Akinci E, Banga A, Tungatt K, et al. Reprogramming of various cell types to a beta-like state by Pdx1, Ngn3 and MafA. *PloS One*. 2013;8(11):e82424. doi:10.1371/journal.pone.0082424.

84. Naya FJ, Huang HP, Qiu Y, et al. Diabetes, defective pancreatic morphogenesis, and abnormal enteroendocrine differentiation in BETA2/neuroD-deficient mice. *Genes Dev*. 1997;11(18):2323-2334.

85. Malecki MT, Jhala US, Antonellis A, et al. Mutations in NEUROD1 are associated with the development of type 2 diabetes mellitus. *Nat Genet*. 1999;23(3):323-328. doi:10.1038/15500.

86. Kornberg RD. Chromatin structure: a repeating unit of histones and DNA. *Science*. 1974;184(4139):868-871.

87. Levine M, Cattoglio C, Tjian R. Looping back to leap forward: transcription enters a new era. *Cell*. 2014;157(1):13-25. doi:10.1016/j.cell.2014.02.009.

88. Bernstein BE, Mikkelsen TS, Xie X, et al. A bivalent chromatin structure marks key developmental genes in embryonic stem cells. *Cell*. 2006;125(2):315-326. doi:10.1016/j.cell.2006.02.041.

89. Smith E, Shilatifard A. Enhancer biology and enhanceropathies. *Nat Struct Mol Biol*. 2014;21(3):210-219. doi:10.1038/nsmb.2784.

90. Wang Z, Schones DE, Zhao K. Characterization of human epigenomes. *Curr Opin Genet Dev*. 2009;19(2):127-134. doi:10.1016/j.gde.2009.02.001.

91. Bernstein BE, Meissner A, Lander ES. The mammalian epigenome. *Cell*. 2007;128(4):669-681. doi:10.1016/j.cell.2007.01.033.

92. Steward MM, Lee J-S, O'Donovan A, Wyatt M, Bernstein BE, Shilatifard A. Molecular regulation of H3K4 trimethylation by ASH2L, a shared subunit of MLL complexes. *Nat Struct Mol Biol*. 2006;13(9):852-854. doi:10.1038/nsmb1131.

93. Wysocka J, Swigut T, Xiao H, et al. A PHD finger of NURF couples histone H3 lysine 4 trimethylation with chromatin remodelling. *Nature*. 2006;442(7098):86-90. doi:10.1038/nature04815.

94. Kim D-H, Lee J, Lee B, Lee JW. ASCOM controls farnesoid X receptor transactivation through its associated histone H3 lysine 4 methyltransferase activity. *Mol Endocrinol Baltim Md*. 2009;23(10):1556-1562. doi:10.1210/me.2009-0099.

95. Yang Y, Chang BH-J, Samson SL, Li MV, Chan L. The Krüppel-like zinc finger protein Glis3 directly and indirectly activates insulin gene transcription. *Nucleic Acids Res*. 2009;37(8):2529-2538. doi:10.1093/nar/gkp122.

96. Aramata S, Han S, Yasuda K, Kataoka K. Synergistic activation of the insulin gene promoter by the beta-cell enriched transcription factors MafA, Beta2, and Pdx1. *Biochim Biophys Acta*. 2005;1730(1):41-46. doi:10.1016/j.bbaexp.2005.05.009.

97. Qiu Y, Guo M, Huang S, Stein R. Insulin gene transcription is mediated by interactions between the p300 coactivator and PDX-1, BETA2, and E47. *Mol Cell Biol*. 2002;22(2):412-420.

98. ZeRuth GT, Takeda Y, Jetten AM. The Krüppel-like protein Gli-similar 3 (Glis3) functions as a key regulator of insulin transcription. *Mol Endocrinol Baltim Md*. 2013;27(10):1692-1705. doi:10.1210/me.2013-1117.

99. Sampley ML, Ozcan S. Regulation of insulin gene transcription by multiple histone acetyltransferases. *DNA Cell Biol*. 2012;31(1):8-14. doi:10.1089/dna.2011.1336.

100. Ogihara T, Vanderford NL, Maier B, Stein RW, Mirmira RG. Expression and function of Set7/9 in pancreatic islets. *Islets*. 2009;1(3):269-272. doi:10.4161/isl.1.3.9779.

101. Liu A, Desai BM, Stoffers DA. Identification of PCIF1, a POZ domain protein that inhibits PDX-1 (MODY4) transcriptional activity. *Mol Cell Biol*. 2004;24(10):4372-4383.

102. Stanojevic V, Yao K-M, Thomas MK. The coactivator Bridge-1 increases transcriptional activation by pancreas duodenum homeobox-1 (PDX-1). *Mol Cell Endocrinol*. 2005;237(1-2):67-74. doi:10.1016/j.mce.2005.03.003.

103. Yamauchi T, Yamauchi J, Kuwata T, et al. Distinct but overlapping roles of histone acetylase PCAF and of the closely related PCAF-B/GCN5 in mouse embryogenesis. *Proc Natl Acad Sci U S A*. 2000;97(21):11303-11306. doi:10.1073/pnas.97.21.11303.

104. Falkenberg KJ, Johnstone RW. Histone deacetylases and their inhibitors in cancer, neurological diseases and immune disorders. *Nat Rev Drug Discov*. 2014;13(9):673-691. doi:10.1038/nrd4360.

105. Haumaitre C, Lenoir O, Scharfmann R. Histone deacetylase inhibitors modify pancreatic cell fate determination and amplify endocrine progenitors. *Mol Cell Biol*. 2008;28(20):6373-6383. doi:10.1128/MCB.00413-08.

106. Lenoir O, Flosseau K, Ma FX, et al. Specific control of pancreatic endocrine β- and δ-cell mass by class IIa histone deacetylases HDAC4, HDAC5, and HDAC9. *Diabetes*. 2011;60(11):2861-2871. doi:10.2337/db11-0440.

107. Turek-Plewa J, Jagodziński PP. The role of mammalian DNA methyltransferases in the regulation of gene expression. *Cell Mol Biol Lett*. 2005;10(4):631-647.

108. Dhawan S, Georgia S, Tschen S-I, Fan G, Bhushan A. Pancreatic β cell identity is maintained by DNA methylation-mediated repression of Arx. *Dev Cell*. 2011;20(4):419-429. doi:10.1016/j.devcel.2011.03.012.

109. Papizan JB, Singer RA, Tschen S-I, et al. Nkx2.2 repressor complex regulates islet β-cell specification and prevents β-to-α-cell reprogramming. *Genes Dev*. 2011;25(21):2291-2305. doi:10.1101/gad.173039.111.

110. Van Nuland R, Smits AH, Pallaki P, Jansen PWTC, Vermeulen M, Timmers HTM. Quantitative dissection and stoichiometry determination of the human SET1/MLL histone methyltransferase complexes. *Mol Cell Biol*. 2013;33(10):2067-2077. doi:10.1128/MCB.01742-12.

111. Glaser S, Schaft J, Lubitz S, et al. Multiple epigenetic maintenance factors implicated by the loss of Mll2 in mouse development. *Dev Camb Engl*. 2006;133(8):1423-1432. doi:10.1242/dev.02302.

112. Lee S, Lee J, Lee S-K, Lee JW. Activating signal cointegrator-2 is an essential adaptor to recruit histone H3 lysine 4 methyltransferases MLL3 and MLL4 to the liver X receptors. *Mol Endocrinol Baltim Md*. 2008;22(6):1312-1319. doi:10.1210/me.2008-0012.

113. Dou Y, Milne TA, Ruthenburg AJ, et al. Regulation of MLL1 H3K4 methyltransferase activity by its core components. *Nat Struct Mol Biol*. 2006;13(8):713-719. doi:10.1038/nsmb1128.

114. Hallson G, Hollebakken RE, Li T, et al. dSet1 is the main H3K4 di- and tri-methyltransferase throughout Drosophila development. *Genetics*. 2012;190(1):91-100. doi:10.1534/genetics.111.135863.

115. Ardehali MB, Mei A, Zobeck KL, Caron M, Lis JT, Kusch T. Drosophila Set1 is the major histone H3 lysine 4 trimethyltransferase with role in transcription. *EMBO J*. 2011;30(14):2817-2828. doi:10.1038/emboj.2011.194.

116. Wang P, Lin C, Smith ER, et al. Global analysis of H3K4 methylation defines MLL family member targets and points to a role for MLL1-mediated H3K4

methylation in the regulation of transcriptional initiation by RNA polymerase II. *Mol Cell Biol*. 2009;29(22):6074-6085. doi:10.1128/MCB.00924-09.

117. Herz H-M, Mohan M, Garruss AS, et al. Enhancer-associated H3K4 monomethylation by Trithorax-related, the Drosophila homolog of mammalian Mll3/Mll4. *Genes Dev*. 2012;26(23):2604-2620. doi:10.1101/gad.201327.112.

118. Hu D, Gao X, Morgan MA, Herz H-M, Smith ER, Shilatifard A. The MLL3/MLL4 branches of the COMPASS family function as major histone H3K4 monomethylases at enhancers. *Mol Cell Biol*. 2013;33(23):4745-4754. doi:10.1128/MCB.01181-13.

119. Lee J, Kim D-H, Lee S, et al. A tumor suppressive coactivator complex of p53 containing ASC-2 and histone H3-lysine-4 methyltransferase MLL3 or its paralogue MLL4. *Proc Natl Acad Sci U S A*. 2009;106(21):8513-8518. doi:10.1073/pnas.0902873106.

120. Lee S, Lee D-K, Dou Y, et al. Coactivator as a target gene specificity determinant for histone H3 lysine 4 methyltransferases. *Proc Natl Acad Sci U S A*. 2006;103(42):15392-15397. doi:10.1073/pnas.0607313103.

121. Lee SK, Anzick SL, Choi JE, et al. A nuclear factor, ASC-2, as a cancer-amplified transcriptional coactivator essential for ligand-dependent transactivation by nuclear receptors in vivo. *J Biol Chem*. 1999;274(48):34283-34293.

122. Li Q, Chu M-J, Xu J. Tissue- and nuclear receptor-specific function of the C-terminal LXXLL motif of coactivator NCoA6/AIB3 in mice. *Mol Cell Biol*. 2007;27(23):8073-8086. doi:10.1128/MCB.00451-07.

123. Surapureddi S, Rana R, Reddy JK, Goldstein JA. Nuclear receptor coactivator 6 mediates the synergistic activation of human cytochrome P-450 2C9 by the constitutive androstane receptor and hepatic nuclear factor-4alpha. *Mol Pharmacol*. 2008;74(3):913-923. doi:10.1124/mol.108.048983.

124. Wang H, Maechler P, Antinozzi PA, Hagenfeldt KA, Wollheim CB. Hepatocyte nuclear factor 4alpha regulates the expression of pancreatic beta -cell genes implicated in glucose metabolism and nutrient-induced insulin secretion. *J Biol Chem*. 2000;275(46):35953-35959. doi:10.1074/jbc.M006612200.

125. Gupta RK, Vatamaniuk MZ, Lee CS, et al. The MODY1 gene HNF-4alpha regulates selected genes involved in insulin secretion. *J Clin Invest*. 2005;115(4):1006-1015. doi:10.1172/JCI22365.

126. Miura A, Yamagata K, Kakei M, et al. Hepatocyte Nuclear Factor-4α Is Essential for Glucose-stimulated Insulin Secretion by Pancreatic β-Cells. *J Biol Chem*. 2006;281(8):5246-5257. doi:10.1074/jbc.M507496200.

127. Agger K, Cloos PAC, Christensen J, et al. UTX and JMJD3 are histone H3K27 demethylases involved in HOX gene regulation and development. *Nature*. 2007;449(7163):731-734. doi:10.1038/nature06145.

128. Zhou JX, Dhawan S, Fu H, et al. Combined modulation of polycomb and trithorax genes rejuvenates β cell replication. *J Clin Invest*. 2013;123(11):4849-4858. doi:10.1172/JCI69468.

129. Krishnamurthy J, Ramsey MR, Ligon KL, et al. p16INK4a induces an age-dependent decline in islet regenerative potential. *Nature*. 2006;443(7110):453-457. doi:10.1038/nature05092.

130. Thakker RV. Multiple endocrine neoplasia type 1 (MEN1) and type 4 (MEN4). *Mol Cell Endocrinol*. 2014;386(1-2):2-15. doi:10.1016/j.mce.2013.08.002.

131. Lemos MC, Thakker RV. Multiple endocrine neoplasia type 1 (MEN1): analysis of 1336 mutations reported in the first decade following identification of the gene. *Hum Mutat*. 2008;29(1):22-32. doi:10.1002/humu.20605.

132. Yeom S-Y, Kim GH, Kim CH, et al. Regulation of insulin secretion and beta-cell mass by activating signal cointegrator 2. *Mol Cell Biol*. 2006;26(12):4553-4563. doi:10.1128/MCB.01412-05.

133. Burghardt H, López-Bermejo A, Baumgartner B, et al. The nuclear receptor coactivator AIB3 is a modulator of HOMA beta-cell function in nondiabetic children. *Clin Endocrinol (Oxf)*. 2008;69(5):730-736. doi:10.1111/j.1365-2265.2008.03232.x.

134. Xie R, Everett LJ, Lim H-W, et al. Dynamic chromatin remodeling mediated by polycomb proteins orchestrates pancreatic differentiation of human embryonic stem cells. *Cell Stem Cell*. 2013;12(2):224-237. doi:10.1016/j.stem.2012.11.023.

135. Matsuoka T, Artner I, Henderson E, Means A, Sander M, Stein R. The MafA transcription factor appears to be responsible for tissue-specific expression of insulin. *Proc Natl Acad Sci U S A*. 2004;101(9):2930-2933. doi:10.1073/pnas.0306233101.

136. Ananthanarayanan M, Li Y, Surapureddi S, et al. Histone H3K4 trimethylation by MLL3 as part of ASCOM complex is critical for NR activation of bile acid transporter genes and is downregulated in cholestasis. *Am J Physiol Gastrointest Liver Physiol*. 2011;300(5):G771-G781. doi:10.1152/ajpgi.00499.2010.

137. Völkel P, Le Faou P, Angrand P-O. Interaction proteomics: characterization of protein complexes using tandem affinity purification-mass spectrometry. *Biochem Soc Trans*. 2010;38(4):883-887. doi:10.1042/BST0380883.

138. Smith AL, Friedman DB, Yu H, Carnahan RH, Reynolds AB. ReCLIP (reversible cross-link immuno-precipitation): an efficient method for interrogation of labile protein complexes. *PloS One*. 2011;6(1):e16206. doi:10.1371/journal.pone.0016206.

139. Efrat S, Linde S, Kofod H, et al. Beta-cell lines derived from transgenic mice expressing a hybrid insulin gene-oncogene. *Proc Natl Acad Sci U S A*. 1988;85(23):9037-9041.

140. Zhao L, Guo M, Matsuoka T-A, et al. The islet beta cell-enriched MafA activator is a key regulator of insulin gene transcription. *J Biol Chem*. 2005;280(12):11887-11894. doi:10.1074/jbc.M409475200.

141. Ravassard P, Hazhouz Y, Pechberty S, et al. A genetically engineered human pancreatic β cell line exhibiting glucose-inducible insulin secretion. *J Clin Invest*. 2011;121(9):3589-3597. doi:10.1172/JCI58447.

142. Matsuoka T, Kaneto H, Stein R, et al. MafA regulates expression of genes important to islet beta-cell function. *Mol Endocrinol Baltim Md*. 2007;21(11):2764-2774. doi:10.1210/me.2007-0028.

143. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods San Diego Calif*. 2001;25(4):402-408. doi:10.1006/meth.2001.1262.

144. Kawagoe J, Li Q, Mussi P, et al. Nuclear receptor coactivator-6 attenuates uterine estrogen sensitivity to permit embryo implantation. *Dev Cell*. 2012;23(4):858-865. doi:10.1016/j.devcel.2012.09.002.

145. Postic C, Shiota M, Niswender KD, et al. Dual roles for glucokinase in glucose homeostasis as determined by liver and pancreatic beta cell-specific gene knock-outs using Cre recombinase. *J Biol Chem*. 1999;274(1):305-315.

146. Stefan Y, Meda P, Neufeld M, Orci L. Stimulation of insulin secretion reveals heterogeneity of pancreatic B cells in vivo. *J Clin Invest*. 1987;80(1):175-183. doi:10.1172/JCI113045.

147. Eissenberg JC, Shilatifard A. Histone H3 lysine 4 (H3K4) methylation in development and differentiation. *Dev Biol*. 2010;339(2):240-249. doi:10.1016/j.ydbio.2009.08.017.

148. Lee J, Saha PK, Yang Q-H, et al. Targeted inactivation of MLL3 histone H3-Lys-4 methyltransferase activity in the mouse reveals vital roles for MLL3 in adipogenesis. *Proc Natl Acad Sci U S A*. 2008;105(49):19229-19234. doi:10.1073/pnas.0810100105.

149. Cal S, Peinado JR, Llamazares M, et al. Identification and characterization of human polyserase-3, a novel protein with tandem serine-protease domains in the same polypeptide chain. *BMC Biochem*. 2006;7:9. doi:10.1186/1471-2091-7-9.

150. Ansari KI, Hussain I, Kasiri S, Mandal SS. HOXC10 is overexpressed in breast cancer and transcriptionally regulated by estrogen via involvement of histone methylases MLL3 and MLL4. *J Mol Endocrinol*. 2012;48(1):61-75. doi:10.1530/JME-11-0078.

151. Kayton NS, Poffenberger G, Henske J, et al. Human islet preparations distributed for research exhibit a variety of insulin-secretory profiles. *Am J Physiol Endocrinol Metab*. 2015;308(7):E592-E602. doi:10.1152/ajpendo.00437.2014.

152. Sun G, Tarasov AI, McGinty J, et al. Ablation of AMP-activated protein kinase alpha1 and alpha2 from mouse pancreatic beta cells and RIP2.Cre neurons suppresses insulin release in vivo. *Diabetologia*. 2010;53(5):924-936. doi:10.1007/s00125-010-1692-1.

153. Zhang Z, Liew CW, Handy DE, et al. High glucose inhibits glucose-6-phosphate dehydrogenase, leading to increased oxidative stress and beta-cell apoptosis. *FASEB J Off Publ Fed Am Soc Exp Biol*. 2010;24(5):1497-1505. doi:10.1096/fj.09-136572.

154. Van der Zijl NJ, Moors CCM, Goossens GH, Blaak EE, Diamant M. Does interference with the renin-angiotensin system protect against diabetes? Evidence and mechanisms. *Diabetes Obes Metab*. 2012;14(7):586-595. doi:10.1111/j.1463-1326.2012.01559.x.

155. Wang W-L, Li Q, Xu J, Cvekl A. Lens fiber cell differentiation and denucleation are disrupted through expression of the N-terminal nuclear receptor box of NCOA6 and result in p53-dependent and p53-independent apoptosis. *Mol Biol Cell*. 2010;21(14):2453-2468. doi:10.1091/mbc.E09-12-1031.

156. Artner I, Le Lay J, Hang Y, et al. MafB: an activator of the glucagon gene expressed in developing islet alpha- and beta-cells. *Diabetes*. 2006;55(2):297-304.

157. Blum B, Hrvatin SSŠ, Schuetz C, Bonal C, Rezania A, Melton DA. Functional beta-cell maturation is marked by an increased glucose threshold and by expression of urocortin 3. *Nat Biotechnol*. 2012;30(3):261-264. doi:10.1038/nbt.2141.

158. Hussain MA, Habener JF. Glucagon gene transcription activation mediated by synergistic interactions of pax-6 and cdx-2 with the p300 co-activator. *J Biol Chem*. 1999;274(41):28950-28957.

159. Cole BK, Keller SR, Wu R, Carter JD, Nadler JL, Nunemaker CS. Valsartan protects pancreatic islets and adipose tissue from the inflammatory and metabolic consequences of a high-fat diet in mice. *Hypertension*. 2010;55(3):715-721. doi:10.1161/HYPERTENSIONAHA.109.148049.

160. Ashery-Padan R, Zhou X, Marquardt T, et al. Conditional inactivation of Pax6 in the pancreas causes early onset of diabetes. *Dev Biol*. 2004;269(2):479-488. doi:10.1016/j.ydbio.2004.01.040.

161. Du A, Hunter CS, Murray J, et al. Islet-1 is required for the maturation, proliferation, and survival of the endocrine pancreas. *Diabetes*. 2009;58(9):2059-2069. doi:10.2337/db08-0987.

162. Sander M, Sussel L, Conners J, et al. Homeobox gene Nkx6.1 lies downstream of Nkx2.2 in the major pathway of beta-cell formation in the pancreas. *Dev Camb Engl*. 2000;127(24):5533-5540.

163. Bramswig NC, Everett LJ, Schug J, et al. Epigenomic plasticity enables human pancreatic α to β cell reprogramming. *J Clin Invest*. 2013;123(3):1275-1284. doi:10.1172/JCI66514.

164. Schonhoff SE, Giel-Moloney M, Leiter AB. Neurogenin 3-expressing progenitor cells in the gastrointestinal tract differentiate into both endocrine and non-endocrine cell types. *Dev Biol*. 2004;270(2):443-454. doi:10.1016/j.ydbio.2004.03.013.

165. Söderberg O, Gullberg M, Jarvius M, et al. Direct observation of individual endogenous protein complexes in situ by proximity ligation. *Nat Methods*. 2006;3(12):995-1000. doi:10.1038/nmeth947.

166. Milewski WM, Temple KA, Wesselschmidt RL, Hara M. Generation of embryonic stem cells from mouse insulin I promoter-green fluorescent protein transgenic mice and characterization in a teratoma model. *In Vitro Cell Dev Biol Anim*. 2009;45(1-2):1-5. doi:10.1007/s11626-008-9142-9.

167. Wang C, Gong B, Bushel PR, et al. The concordance between RNA-seq and microarray data depends on chemical treatment and transcript abundance. *Nat Biotechnol*. 2014;32(9):926-932. doi:10.1038/nbt.3001.

168. Wicksteed B, Brissova M, Yan W, et al. Conditional gene targeting in mouse pancreatic ß-Cells: analysis of ectopic Cre transgene expression in the brain. *Diabetes*. 2010;59(12):3090-3098. doi:10.2337/db10-0624.

169. Surapureddi S, Rana R, Goldstein JA. NCOA6 differentially regulates the expression of the CYP2C9 and CYP3A4 genes. *Pharmacol Res Off J Ital Pharmacol Soc*. 2011;63(5):405-413. doi:10.1016/j.phrs.2011.01.013.

170. Kim GH, Park K, Yeom S-Y, et al. Characterization of ASC-2 as an antiatherogenic transcriptional coactivator of liver X receptors in macrophages. *Mol Endocrinol Baltim Md*. 2009;23(7):966-974. doi:10.1210/me.2008-0308.

171. Kelly RDW, Cowley SM. The physiological roles of histone deacetylase (HDAC) 1 and 2: complex co-stars with multiple leading parts. *Biochem Soc Trans*. 2013;41(3):741-749. doi:10.1042/BST20130010.

172. Cowley SM, Iritani BM, Mendrysa SM, et al. The mSin3A chromatin-modifying complex is essential for embryogenesis and T-cell development. *Mol Cell Biol*. 2005;25(16):6990-7004. doi:10.1128/MCB.25.16.6990-7004.2005.

173. Hendrich B, Guy J, Ramsahoye B, Wilson VA, Bird A. Closely related proteins MBD2 and MBD3 play distinctive but interacting roles in mouse development. *Genes Dev*. 2001;15(6):710-723. doi:10.1101/gad.194101.

174. Wang J, Scully K, Zhu X, et al. Opposing LSD1 complexes function in developmental gene activation and repression programmes. *Nature*. 2007;446(7138):882-887. doi:10.1038/nature05671.

175. Liu W-D, Wang H-W, Muguira M, Breslin MB, Lan MS. INSM1 functions as a transcriptional repressor of the neuroD/beta2 gene through the recruitment of cyclin D1 and histone deacetylases. *Biochem J*. 2006;397(1):169-177. doi:10.1042/BJ20051669.

176. Wang S, Hecksher-Sorensen J, Xu Y, et al. Myt1 and Ngn3 form a feed-forward expression loop to promote endocrine islet cell differentiation. *Dev Biol*. 2008;317(2):531-540. doi:10.1016/j.ydbio.2008.02.052.

177. Wang S, Zhang J, Zhao A, Hipkens S, Magnuson MA, Gu G. Loss of Myt1 function partially compromises endocrine islet cell differentiation and pancreatic physiological function in the mouse. *Mech Dev*. 2007;124(11-12):898-910. doi:10.1016/j.mod.2007.08.004.

178. Xu Y, Wang S, Zhang J, Zhao A, Stanger BZ, Gu G. The fringe molecules induce endocrine differentiation in embryonic endoderm by activating cMyt1/cMyt3. *Dev Biol*. 2006;297(2):340-349. doi:10.1016/j.ydbio.2006.04.456.

179. Tennant BR, Islam R, Kramer MM, et al. The transcription factor Myt3 acts as a pro-survival factor in β-cells. *PloS One*. 2012;7(12):e51501. doi:10.1371/journal.pone.0051501.

180. Xu C-R, Li L-C, Donahue G, et al. Dynamics of genomic H3K27me3 domains and role of EZH2 during pancreatic endocrine specification. *EMBO J*. August 2014. doi:10.15252/embj.201488671.

181. Sander JD, Joung JK. CRISPR-Cas systems for editing, regulating and targeting genomes. *Nat Biotechnol*. 2014;32(4):347-355. doi:10.1038/nbt.2842.

182. Pasquali L, Gaulton KJ, Rodríguez-Seguí SA, et al. Pancreatic islet enhancer clusters enriched in type 2 diabetes risk-associated variants. *Nat Genet*. 2014;46(2):136-143. doi:10.1038/ng.2870.