MLL3 and MLL4 methyltransferases bind to the MAFA and MAFB transcription factors
to regulate islet β-cell function

David W. Scoville¹, Holly A. Cyphert², Lan Liao³, Jianming Xu³, Al Reynolds⁴, Shuangli Guo²,
and Roland Stein¹,²,⁵

¹Department of Cell and Developmental Biology, ²Department of Molecular Physiology and
Biophysics, and ⁴Department of Cancer Biology, Vanderbilt University School of Medicine,
Nashville, TN
³Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX
⁵Corresponding author: Roland Stein, Vanderbilt University Medical School, Department of
Molecular Physiology & Biophysics, 723 Light Hall, Nashville, TN 37232; Phone: 615-322-
7026, Facsimile: 615-322-7236.

Authors declare no financial conflict of interest.
Running Title: The MLL3/4 complexes interact with MAFA
Abstract

Insulin produced by islet β-cells plays a critical role in glucose homeostasis, with both type I and type II diabetes resulting from inactivation and/or loss of this cell population. Islet-enriched transcription factors regulate β-cell formation and function, yet little is known about the molecules recruited to mediate control. An unbiased in-cell biochemical and mass spectrometry strategy was utilized to isolate MafA transcription factor binding proteins. Among the many coregulators identified were all of the subunits of the Mixed-Lineage Leukemia 3 (Mll3) and 4 (Mll4) complexes, histone 3 lysine 4 (H3K4) methyltransferases strongly associated with gene activation. MafA was bound to the ~1.5 MDa Mll3 and Mll4 complexes in size fractionated β-cell extracts. Likewise, closely related human MAFB, which is important to β-cell formation and co-produced with MAFA in adult human islet β-cells, bound MLL3 and MLL4 complexes. Knockdown of NCOA6, a core subunit of these methyltransferases, reduced expression of a subset of MAFA and MAFB target genes in mouse and human β-cell lines. In contrast, a broader impact on MafA/MafB gene activation was observed in mice lacking NCoA6 in islet β-cells. We propose that MLL3 and MLL4 are broadly required for controlling MAFA and MAFB transactivation during development and postnatally.
Introduction

Diabetes mellitus is a disease that affects the body’s ability to maintain euglycemia, with type 1 (T1DM) characterized by a loss of insulin-producing islet β-cells and type 2 (T2DM) by peripheral insulin resistance and β-cell dysfunction. One proposed treatment for T1DM is to replace diseased β-cells with those generated from human Embryonic Stem Cells (hESCs) or induced Pluripotent Stem Cells (1). The principal limitation in producing functional β-cells has been directing the final postnatal maturation steps (2), which involves expression of proteins required for glucose sensitivity and insulin secretion (3).

Islet-enriched transcription factors are essential for embryonic formation and postnatal function of β-cells (4,5,6). For example, very early exocrine and endocrine pancreatic development is driven by Pdx-1 starting at embryonic day 8.5 (e8.5) in mice, with both mice and humans lacking a functional copy suffering from pancreatic agenesis (4,5). In contrast, Ngn3 expressed from e9.5 is only required in the formation of endocrine cell types (i.e. β, α (hormone glucagon producing), δ (somatostatin), ε (ghrelin), and PP (pancreatic polypeptide) (6). MafA is expressed even later during development and only in β-cells (i.e. e13.5), contributing in postnatal maturation steps (7). Interestingly, the induction of glucose-sensitive insulin secretion in vivo from transplanted hESC-derived endocrine progenitors correlates with MAFA expression (8). Moreover, the production of only Pdx-1, Ngn3, and MafA is sufficient to reprogram mouse exocrine, intestinal, and liver cells into insulin+ β-like cells in vivo (9,10,11). Although these examples clearly illustrate the fundamental importance of islet-enriched activators to β-cells, the transcriptional mechanisms involved are not well defined.
Transcription factors primarily regulate gene activation by recruitment of coregulators, which often influence expression by directly binding to the basal transcriptional machinery and/or through epigenetic remodeling of the chromatin structure. These coregulators can have positive (coactivator) and negative (corepressor) actions on target gene transcription (12), thus conferring a second level of specificity to the transcriptional response. 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 (13), Set7/9 (14), HDAC1/2 (15), PCIF1 (16), and Bridge-1 (17). In contrast, MafA has only been linked to p/CAF (18).

In this study, we used an ‘in cell’ Reversible Cross-link Immunoprecipitation (Re-CLIP) and mass spectrometry (MS) approach to isolate coregulators of MafA from mouse β-cells. Notably, 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 bind MAFB, a closely related transcription factor essential to mouse β-cell development and co-expressed with MAFA in adult human islets (19,20). Notably, mouse islet β-cell function is compromised in a heterozygous null mutant of NCoA6, a key subunit of Mll3/4 (21). Decreased NCOA6 levels was 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 \textit{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 25mM glucose, 10% fetal bovine serum, 100U/mL penicillin/streptomycin (22) while human EndoC-βH1 (23) cells were grown in DMEM containing 5.6mM glucose, 2% bovine serum albumin, 50µM 2-mercaptoethanol, 10mM nicotinamide, 5.5µg/mL transferrin, 6.7ng/mL selenite, 100U/mL penicillin, and 100U/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 (24). Nuclear extract was prepared 48 hours post-infection as described below. For immunoblotting (25), primary antibodies are listed in Supplementary Table 1. 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 (26). Briefly, ~10^9 βTC-3 cells were cross-linked using 0.1mM Dithiobis[succinimidyl propionate] (DSP) in PBS for 30 minutes at 37°C. Cells were then harvested, and nuclear extract prepared by high salt (400mM NaCl) extraction (25). Magnetic protein G beads (Life Technologies) were covalently cross-linked with MafA antibody (20µg), and either pre-incubated with a MafA blocking peptide (\textsuperscript{32}KKEPPEAERFC\textsuperscript{42}, 100-fold excess antibody) or with PBS alone. Extract and beads were incubated for 3 hours at 4°C, washed with RIPA buffer (10mM Tris pH=8.0, 140mM NaCl, 0.5% Triton X-100, 1% Sodium Deoxycholate, 0.1% SDS), and eluted in RIPA supplemented
with 200mM Dithiothreitol (DTT). The eluted proteins were visualized by PAGE/silver staining and protein identification determined by liquid chromatography/MS/MS (LC/MS/MS) or Multi-dimensional Protein Identification Technology (MudPIT) analysis in the Vanderbilt University Proteomics Core. Immunoprecipitation experiments were performed with the antibodies listed in Supplementary Table 1 at 10ug each as described above for Re-CLIP, omitting the DSP step and substituting PBS for RIPA.

**Sucrose gradient ultracentrifugation and Electrophoretic Mobility Shift Assays (EMSA)**

Sucrose gradients were performed as described earlier (25). Briefly, βTC-3 nuclear extract (600-1000 µg) was collected by high salt extraction (25) 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 (25), and immunoprecipitation analysis. HeLa cells were transfected with expression plasmids encoding human MAFA and/or MAFB (25). Antibodies used for gel shift are listed in Supplementary Table 1.

**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 RT² 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 Supplementary Table 2 on a LightCycler 480 II (Roche), and analyzed by the ∆∆CT method (27). Significance was calculated by comparing the ∆CT values in all but Figure 7 (A and B), wherein each mutant was normalized to a control littermate. The Qiagen RT² Profiler PCR array for human diabetes genes (Cat. No. 330231 PAHS-023ZA) was screened with 1 µg of cDNA from EndoC-βH1 cells following manufacture guidelines.

Chromatin Immunoprecipitation (ChIP)

Chromatin was prepared from βTC-3 cells and EndoC-βH1 cells by cross-linking for 10 minutes with 1% formaldehyde, followed by lysis in buffer containing 1% NP-40. The centrifuged pelleted nuclei were lysed in SDS buffer (50mM Tris, 10mM EDTA, 1% SDS), and chromatin sonicated using a Diaginode Bioruptor. Ten µg of DNA was incubated with 5 µg of antibody or species-matched IgG, and immunoprecipitation performed as described previously (28). Antibodies are listed in Supplementary Table 1, qPCR primers used for analysis are listed in Supplementary Table 2.
**Mouse lines, Intraperitoneal Glucose Tolerance Test (IPGTT), and Islet Isolation**

Floxed (fl) MafA (19) or NCoA6 (29) mice were crossed with rat insulin II promoter-driven Cre (RIP-Cre) (30) transgenic mice to delete MafA (termed MafA^{Δβ}) or NCoA6 (NCoA6^{Δβ}) specifically in β-cells. All the mice were maintained on a mixed background (C57BL/6J, 129, Balb/c, 129S6/SvEvTac, FVB/N), although MafA^{Δβ} mice were mostly C57BL/6J. MafA^{Δβ}, NCoA6^{Δβ}, MafA^{fl/fl} and/or NCoA6^{fl/fl} mice were fasted for 6 hours in the morning before the IPGTT. There was no change in total body weight in either MafA^{Δβ} or NCoA6^{Δβ} mice (Supplementary Figure 2A). 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 (31). 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 followed by paraffin embedding (32). The entire e15.5 pancreas was serial sectioned (6µm per section) and every 10th section analyzed by immunofluorescence. Three sections at least 120µm apart were examined at 8-weeks for islet architecture and for Slc30a8 and Slc2a2 expression. The primary antibody list is provided in Supplementary Table 1. 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

EndoC-βH1 cells (2x10^6) were placed in medium containing low glucose (1.1mM) for 12-14 hours. Low or high glucose (15.5mM) medium was then added for 1 hour. Medium and cell lysates (lysis buffer: 1M Tris, Triton x-100, glycerol, 5M NaCl, 0.2M EGTA, protease inhibitor tablet) were collected and analyzed for insulin content by the Vanderbilt Hormone Assay Core. Glucose stimulated insulin secretion 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 5.6mM or 16.7mM glucose for 1 hour. The insulin content in the medium and cell lysate was analyzed. 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 16.7mM glucose/5.6mM 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 proteins using Re-CLIP

MafA interacting proteins were isolated from mouse βTC-3 cells using a combined Re-CLIP/MS strategy (Figure 1A) (26). The Re-CLIP procedure was selected over other isolation methods (e.g. Tandem Affinity Purification (TAP) (33)), as the dimerization, DNA binding, and activation capacity of MafA is regulated by many phosphorylation events (25). 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 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 (22) and p/CAF (18), known MafA interacting proteins, were bound to MafA following Re-CLIP (Figure 1B). Moreover, many proteins were selectively bound to MafA in the immunoprecipitation conducted in the absence of blocking peptide (Figure 1C).

Numerous MafA bound proteins were detected upon MS analysis of the Re-CLIP products, with their activities consistent with a role in mediating MafA activity (Supplementary Table 3). It is notable that no other islet-enriched transcription factors or the p/CAF coactivator were identified by MS, presumably reflecting their relatively low abundance (e.g. Pdx-1, p/CAF) or inability to interact. This indicates that the results in Supplementary Table 3 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 (34). All nine proteins of the ~1.5 MDa Mll4 complex were found by MS (Figure 1D), and Mll3 was additionally detected via the more sensitive MudPIT analysis (Supplementary Table 3). 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, Set1B, Cfp1, Wdr82, Bod1, Bod1l) were detected. The ability of MafA to bind to Mll3/4 was also independently demonstrated in βTC-3 cells producing an adenovirus-driven Flag-tagged MafA (Supplementary Figure 1).

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 were surrounded by islet-enriched transcription factors of relatively low molecular weight (Figure 2A; 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 Rbp5 (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 2B). In addition, immunoprecipitation experiments conducted with antibodies to NCoA6 or MafA demonstrated that MafA interacted with several Mll3/4 components in
Fraction 14 (Figure 2C). These results strongly indicate that a significant fraction of MafA is associated with the Mll3/4 complex in β-cells.

A subset of MafA regulated genes are 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 (35,36,37,38). Furthermore, Mll3 and Mll4 have redundant roles in liver, and NCoA6 is required for activity of both methyltransferase complexes (36). Notably, mouse NCoA6+/2 mutants have impaired glucose clearance (21), while overexpression of NCoA6 in rat INS-1 β-cells increases cell function (39). In addition, polymorphisms in NCOA6 are associated with decreased human β-cell function (39). Our objective was to determine how closely linked Mll3/4 is to MafA-mediated transcriptional control.

Both MafA and NCoA6 protein levels in βTC-3 cells were effectively (>80%) and specifically depleted by siRNA treatment (Figure 3A). The effect of these conditions on expression of β-cell genes bound by MafA and down-regulated in pancreas-specific deletion MafAΔpanc mice was determined (40, 41, Supplementary Table 4). As expected, the expression of most of these genes was decreased upon depletion of MafA (Figure 3B; Slc2a2, Ins2, Lifr, Prss53, Atp2a2), while these conditions had no impact on NCoA6 levels. In contrast, only a small subset of these MafA-activated genes were affected in the NCoA6 knockdown (i.e. Slc2a2, Prss53). This outcome was considered likely as a number of other potential MafA coregulators were identified here and previously (e.g. p/CAF (18), Supplemental Table 1), 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 3C**). Moreover, H3K4 trimethylation and elongating, carboxy terminal tail phosphorylated RNA polymerase II levels 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 3D, E**).

**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. MafB is only produced in insulin+ cells during mouse embryogenesis, and plays an important role in β-cell formation (19). In contrast, human MAFB is expressed in developing and postnatal β-cells, being coproduced with MAFA in adult islet β-cells (20). These basic leucine-zipper proteins bind to DNA with identical specificity as homo- or hetero-dimers (32,42), as also observed in MAFA and MAFB transfected HeLa cell extracts (**Figure 4A**). Gel shift analysis indicates that both MAFA/MAFB and MAFB2 complexes are also formed in nuclear extracts prepared from the human EndoC-βH1 β-cell line (**Figure 4B**), 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 associations (**Figure 4A**: lane 3-5; **Figure 4B**: lane 3). Significantly, immunoprecipitation experiments demonstrated that both MAFA and MAFB were able to associate with multiple proteins of the MLL3/4 complex in EndoC-βH1 cells (**Figure 4C**; NCOA6, UTX, RBBP5), although whether this results from binding MAFB2 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 (Supplemental Table 1).

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

MAFA, MAFB, and NCOA6 were siRNA depleted from EndoC-βH1 cells to analyze their significance to human β-cell gene expression and function. Protein levels were reduced by ~50-70% under these conditions (Figure 5A). The impact on transcriptional control was determined on 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 5B). 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 several genes linked to T2DM and important to glucose stimulated insulin secretion were reduced upon NCOA6 depletion (Figure 5C), specifically AMP-kinase subunit *PRKAA1* (43), Glucose-6-Phosphate Dehydrogenase (44), and Angiotensin (*AGT*) (45). In addition, expression of each were 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 5D). Furthermore, glucose stimulated insulin secretion in EndoC-βH1 cells was compromised upon knockdown of MAFA, MAFB, or NCOA6 (Figure 5E). 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 phenotype of β-cell-specific loss of NCoA6 to that of MafA. Rat insulin II-driven Cre transgenic mice (30) were bred with MafA^{fl/fl} (19) and NCoA6^{fl/fl} (46) mice to remove these proteins specifically from insulin^+ cells in MafA^β and NCoA6^β mice during development (Figure 6A). Roughly 70% of mutant insulin^+ cells lacked the MafA or NCoA6 proteins by e15.5 (Figure 6B), a time at which MafB is still expressed in developing β-cells (47).

Like the pancreas-specific Pdx1-Cre removal of MafA (MafA^{Δpanc}) (41), MafA^{Δβ} mice were euglycemic and had impaired islet architecture, where glucagon^+ α-cells were no longer restricted to the outer mantle of the islet (Figure 6C). Adult NCoA6^{Δβ} mice were also euglycemic, but their islet architecture was unchanged. In addition, only MafA^{Δβ} mice displayed the impaired whole body glucose clearance observed in MafA^{Δpanc} mice (41, Supplementary Figure 2B-E).

Strikingly, most of the genes regulated by MafA in 8-week old MafA^{Δβ} mouse islets were now also significantly compromised in 8-week old NCoA6^{Δβ} islets (Figure 7A). Additionally, glucose stimulated insulin secretion was impaired in islets isolated from both MafA^{Δβ} and NCoA6^{Δβ} mice (Figure 7C), as found upon MAFA, MAFB or NCOA6 depletion in human EndoC-βH1 cells (Figure 5D). The basal insulin secretion level of NCoA6^{Δβ} islets was unusually high in relation to wild type controls and MafA^{Δβ} (Figure 7C), a sign of β-cell immaturity (48). The more penetrant Mll3/4 control properties observed in NCoA6^{Δβ} mice likely represent the combined actions of Mll3/4 on MafB activity during development and MafA postnatally.
Discussion

Gene induction involves the recruitment of coregulators by enhancer-bound transcription factors like MAFA and MAFB. These protein-bound effectors 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 are consistent with a model where MLL3/4 recruitment 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 2,4). Moreover, a significant fraction of MafA was bound to the ~1.5 MDa Mll3/4 complex by sucrose gradient centrifugation (Figure 2). 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 these islet-enriched complexes are 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 (49). Likewise, Pax6 co-migration with MafA:MLL3/4 could reflect binding to high molecular proteins like p300 (50). However, neither Nkx2.2, Nkx6.1, Pax6, nor any other islet-enriched transcription factors were detected by MafA antibody in Re-CLIP experiments (Supplemental Table 3), 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 4C). 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 5) and mouse (Figure 3) 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 co-regulated by MafA and Mll3/4 amongst a variety of tested MafA regulated genes in βTC-3 cells (Figure 3). 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 NCoA6Δβ islets (Figure 7A). Because of the early action of Cre-recombinase in developing MafA+ and MafB+ NCoA6Δβ β-cells, this more penetrant phenotype is likely due to the activity of Mll3/4 on MafB bound enhancers during development. Notably, this supposition is consistent with analysis of the
MafB<sup>−/−</sup> (19) and MafA<sup>Δpanc</sup> (41) mutants, which established that many genes first regulated by MafB developmentally were subsequently influenced by MafA after birth (41). In NCoA6<sup>Δβ</sup> mice, the lack of Mll3/4 activity on MafA and MafB bound enhancers during development may explain why glucose stimulated insulin secretion was differentially affected in NCoA6<sup>Δβ</sup> and MafA<sup>Δβ</sup> islets (Figure 7C). 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 glucose stimulated insulin secretion in both human and mouse β-cells, it did not have the same influential effect on islet cell architecture as MafA (Figure 7A).

Our understanding of the genes regulating glucose stimulated insulin secretion 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 (43), Glucose-6-Phosphatase Dehydrogenase (44), and Angiotensin (AGT) (45) expression may be important (Figure 5C). For example, Angiotensin appears to have a direct role in regulating β-cell activity (51). 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. This analysis should also be conducted with human islets, as it is possible the transformed and proliferative nature of the EndoC-βH1 cell line could influence how MAFA and MAFB regulate gene expression. It is also important to note how similar the phenotype of the MAFA, MAFB and NCOA6 regulatory response was in our β-cell line and mouse studies, and yet different from the other islet-enriched transcription factors present in high molecular weight complexes (Figure 2).

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 impact MAFA and MAFB control, like CoREST (LSD1, RCOR1, RCOR3, HDAC2) and histone deacetylase 6 (HDAC6), both of which are known to play a major regulatory role in other cell contexts (55,56). It will also be of interest to determine if coregulator recruitment is influenced by homodimeric (i.e. MAFA$_2$, MAFB$_2$) or heteromeric (MAFA/MAFB) transcription factor composition and the physiological or pathophysiological state of the β-cell.

Acknowledgements

This work was supported by grants from the National Institutes of Health (DK090570 to R.S.; F32 DK102283-01 and T32 DK007061 to H.A.C.; CA112403 and DK058242 to J.X.) and the Vanderbilt Diabetes Research and Training Center (DK20593). D.W.S. and H.A.C. researched data and wrote the manuscript. L.L. and J.X. provided animals. A.R. provided technical support. S.G. researched data. R.S. wrote the manuscript, and is the guarantor of this work. As such, he had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Adenoviral Flag-tagged MafA was kindly provided by Dr. Qiao Zhou, Harvard University. Imaging was performed with National Institutes of Health support from the Vanderbilt University Medical Center Cell Imaging Shared Resource (CA68485, DK20593, DK58404, HD15052, DK59637, EY08126) and Vanderbilt University Medical Center Islet Procurement and Analysis Core (DK20593).
References

1. Mayhew CN, Wells JM. Converting human pluripotent stem cells into beta-cells: recent advances and future challenges. Curr Opin Organ Transplant. 2010 Feb;15(1):54–60.

2. Pagliuca FW, Melton DA. How to make a functional β-cell. Dev Camb Engl. 2013 Jun;140(12):2472–83.

3. Aguayo-Mazzucato C, Koh A, El Khattabi I, Li W-C, Toschi E, Jermendy A, et al. Mafa expression enhances glucose-responsive insulin secretion in neonatal rat beta cells. Diabetologia. 2011 Mar;54(3):583–93.

4. Jonsson J, Carlsson L, Edlund T, Edlund H. Insulin-promoter-factor 1 is required for pancreas development in mice. Nature. 1994 Oct 13;371(6498):606–9.

5. 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 Jan;15(1):106–10.

6. Gradwohl G, Dierich A, LeMeur M, Guillemot F. neurogenin3 is required for the development of the four endocrine cell lineages of the pancreas. Proc Natl Acad Sci U S A. 2000 Feb 15;97(4):1607–11.

7. Nishimura W, Kondo T, Salameh T, El Khattabi I, Dodge R, Bonner-Weir S, et al. A switch from MafB to MafA expression accompanies differentiation to pancreatic beta-cells. Dev Biol. 2006 May 15;293(2):526–39.

8. D’Amour KA, Bang AG, Eliazer S, Kelly OG, Agulnick AD, Smart NG, et al. Production of pancreatic hormone-expressing endocrine cells from human embryonic stem cells. Nat Biotechnol. 2006 Nov;24(11):1392–401.

9. Zhou Q, Brown J, Kanarek A, Rajagopal J, Melton DA. In vivo reprogramming of adult pancreatic exocrine cells to beta-cells. Nature. 2008 Oct 2;455(7213):627–32.

10. Luo H, Chen R, Yang R, Liu Y, Chen Y, Shu Y, et al. Reprogramming of mice primary hepatocytes into insulin-producing cells by transfection with multicistronic vectors. J Diabetes Res. 2014;2014:716163.
11. Akinci E, Banga A, Tungatt K, Segal J, Eberhard D, Dutton JR, et al. Reprogramming of various cell types to a beta-like state by Pdx1, Ngn3 and MafA. PloS One. 2013;8(11):e82424.

12. Millard CJ, Watson PJ, Fairall L, Schwabe JWR. An evolving understanding of nuclear receptor coregulator proteins. J Mol Endocrinol. 2013 Dec;51(3):T23–36.

13. 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 Jan;22(2):412–20.

14. Ogihara T, Vanderford NL, Maier B, Stein RW, Mirmira RG. Expression and function of Set7/9 in pancreatic islets. Islets. 2009 Dec;1(3):269–72.

15. 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 Dec 24;279(52):54241–7.

16. Liu A, Desai BM, Stoffers DA. Identification of PCIF1, a POZ domain protein that inhibits PDX-1 (MODY4) transcriptional activity. Mol Cell Biol. 2004 May;24(10):4372–83.

17. 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 Jun 15;237(1-2):67–74.

18. Rocques N, Abou Zeid N, Sii-Felice K, Lecoin L, Felder-Schmittbuhl M-P, Eychène A, et al. GSK-3-mediated phosphorylation enhances Maf-transforming activity. Mol Cell. 2007 Nov 30;28(4):584–97.

19. Artner I, Hang Y, Mazur M, Yamamoto T, Guo M, Lindner J, et al. MafA and MafB Regulate Genes Critical to \{beta\} Cells in a Unique Temporal Manner. Diabetes [Internet]. 2010 Jul 13 [cited 2010 Jul 19]; Available from: http://www.ncbi.nlm.nih.gov/pubmed/20627934

20. Dai C, Brissova M, Hang Y, Thompson C, Poffenberger G, Shostak A, et al. Islet-enriched gene expression and glucose-induced insulin secretion in human and mouse islets. Diabetologia. 2012 Mar;55(3):707–18.

21. Yeom S-Y, Kim GH, Kim CH, Jung HD, Kim S-Y, Park J-Y, et al. Regulation of insulin secretion and beta-cell mass by activating signal cointegrator 2. Mol Cell Biol. 2006 Jun;26(12):4553–63.

22. Zhao L, Guo M, Matsuoka T-A, Hagman DK, Parazzoli SD, Poitout V, et al. The islet beta cell-enriched MafA activator is a key regulator of insulin gene transcription. J Biol Chem. 2005 Mar 25;280(12):11887–94.
23. Ravassard P, Hazhouz Y, Pechberty S, Bricout-Neveu E, Armanet M, Czernichow P, et al. A genetically engineered human pancreatic β cell line exhibiting glucose-inducible insulin secretion. J Clin Invest. 2011 Sep;121(9):3589–97.

24. Matsuoka T, Kaneto H, Stein R, Miyatsuka T, Kawamori D, Henderson E, et al. MafA regulates expression of genes important to islet beta-cell function. Mol Endocrinol Baltim Md. 2007 Nov;21(11):2764–74.

25. Guo S, Vanderford NL, Stein R. Phosphorylation within the MafA N terminus regulates C-terminal dimerization and DNA binding. J Biol Chem. 2010 Apr 23;285(17):12655–61.

26. 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.

27. 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 Dec;25(4):402–8.

28. Deramaudt TB, Sachdeva MM, Wescott MP, Chen Y, Stoffers DA, Rustgi AK. The PDX1 homeodomain transcription factor negatively regulates the pancreatic ductal cell-specific keratin 19 promoter. J Biol Chem. 2006 Dec 15;281(50):38385–95.

29. Kawagoe J, Li Q, Mussi P, Liao L, Lydon JP, DeMayo FJ, et al. Nuclear receptor coactivator-6 attenuates uterine estrogen sensitivity to permit embryo implantation. Dev Cell. 2012 Oct 16;23(4):858–65.

30. Postic C, Shiota M, Niswender KD, Jetton TL, Chen Y, Moates JM, 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 Jan 1;274(1):305–15.

31. Stefan Y, Meda P, Neufeld M, Orci L. Stimulation of insulin secretion reveals heterogeneity of pancreatic B cells in vivo. J Clin Invest. 1987 Jul;80(1):175–83.

32. Matsuoka T, Zhao L, Artner I, Jarrett HW, Friedman D, Means A, et al. Members of the large Maf transcription family regulate insulin gene transcription in islet beta cells. Mol Cell Biol. 2003 Sep;23(17):6049–62.

33. Völkel P, Le Faou P, Angrand P-O. Interaction proteomics: characterization of protein complexes using tandem affinity purification-mass spectrometry. Biochem Soc Trans. 2010 Aug;38(4):883–7.

34. Eissenberg JC, Shilatifard A. Histone H3 lysine 4 (H3K4) methylation in development and differentiation. Dev Biol. 2010 Mar 15;339(2):240–9.

35. 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 Oct;23(10):1556–62.
36. 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 Jun;22(6):1312–9.

37. Lee J, Saha PK, Yang Q-H, Lee S, Park JY, Suh Y, 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 Dec 9;105(49):19229–34.

38. Lee S, Lee D-K, Dou Y, Lee J, Lee B, Kwak E, et al. Coactivator as a target gene specificity determinant for histone H3 lysine 4 methyltransferases. Proc Natl Acad Sci U S A. 2006 Oct 17;103(42):15392–7.

39. Burghardt H, López-NBermejo A, Baumgartner B, Ibáñez L, Vendrell J, Ricart W, et al. The nuclear receptor coactivator AIB3 is a modulator of HOMA beta-cell function in nondiabetic children. Clin Endocrinol (Oxf). 2008 Nov;69(5):730–6.

40. Tennant BR, Robertson AG, Kramer M, Li L, Zhang X, Beach M, et al. Identification and analysis of murine pancreatic islet enhancers. Diabetologia. 2013 Mar;56(3):542–52.

41. Hang Y, Yamamoto T, Benninger RKP, Brissova M, Guo M, Bush W, et al. The MafA transcription factor becomes essential to islet β-cells soon after birth. Diabetes. 2014 Jun;63(6):1994–2005.

42. 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 Nov;9(11):3149–58.

43. Sun G, Tarasov AI, McGinty J, McDonald A, da Silva Xavier G, Gorman T, 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 May;53(5):924–36.

44. Zhang Z, Liew CW, Handy DE, Zhang Y, Leopold JA, Hu J, 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 May;24(5):1497–505.

45. 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 Jul;14(7):586–95.

46. 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 Jul 15;21(14):2453–68.

47. Artner I, Le Lay J, Hang Y, Elghazi L, Schisler JC, Henderson E, et al. MafB: an activator of the glucagon gene expressed in developing islet alpha- and beta-cells. Diabetes. 2006 Feb;55(2):297–304.
48. Blum B, Hrvatin SS, 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 Mar;30(3):261–4.

49. Papizan JB, Singer RA, Tschen S-I, Dhawan S, Friel JM, Hipkens SB, et al. Nkx2.2 repressor complex regulates islet β-cell specification and prevents β-to-α-cell reprogramming. Genes Dev. 2011 Nov 1;25(21):2291–305.

50. 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 Oct 8;274(41):28950–7.

51. 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 Mar;55(3):715–21.

52. Ashery-Padan R, Zhou X, Marquardt T, Herrera P, Toube L, Berry A, et al. Conditional inactivation of Pax6 in the pancreas causes early onset of diabetes. Dev Biol. 2004 May 15;269(2):479–88.

53. Du A, Hunter CS, Murray J, Noble D, Cai C-L, Evans SM, et al. Islet-1 is required for the maturation, proliferation, and survival of the endocrine pancreas. Diabetes. 2009 Sep;58(9):2059–69.

54. Sander M, Sussel L, Conners J, Scheel D, Kalamaras J, Dela Cruz F, 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 Dec;127(24):5533–40.

55. Qureshi IA, Gokhan S, Mehler MF. REST and CoREST are transcriptional and epigenetic regulators of seminal neural fate decisions. Cell Cycle Georget Tex. 2010 Nov 15;9(22):4477–86.

56. Dallavalle S, Pisano C, Zunino F. Development and therapeutic impact of HDAC6-selective inhibitors. Biochem Pharmacol. 2012 Sep 15;84(6):756–65.
Figure Legends

Figure 1. 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; treatment with (+) DTT breaks the MafA-protein disulfide cross-link and returns MafA to its normal molecular weight. 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) All of the proteins of the Mll4 complex were detected by Re-CLIP, a representative MS result is shown. Mll3 was also present in the MudPIT analysis (Supplementary Table 3).

Figure 2. 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. Rbp5, NCoA6, Wdr5, 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 Mll3/4 complexes. B) MafA2 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.

Figure 3. Knockdown of NCoA6 reduces MafA2 target gene expression in βTC-3 cells. Control siRNA (Controlsi) or siRNA specific to MafA (MafAsi) and 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. N=3. E) MafA and 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.

Figure 4. MAFA and MAFB associate with MLL3/4 in human EndoC-βH1 cells. A) MAFA2, MAFB2, and MAFA/B are produced in MAFA and MAFB transfected HeLa cells. Super-shift (S.S.) reveals three distinct complexes, with α-MAFA and α-MAFB altering homo- and
heterodimer formation. B) MAFB₂ and MAFA/B appear to be the predominant gel shift complexes formed in 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) Subunits of MLL3/4 were precipitated from EndoC-βH1 nuclear extracts with antibodies to MAFA and MAFB. Immunoblotting was performed for MLL3/4 subunits (NCOA6, RBBP5, UTX), islet-enriched transcription factors (PAX6, ISL1, HNF1α) and mouse MafA associated P/Caf (18). N=3, Representative blot shown.

**Figure 5. 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 RT² 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 6. Islet architecture is unchanged in NCoA6\textsuperscript{AB} mice. A) MafA\textsuperscript{AB} and NCoA6\textsuperscript{AB} mice were generated by breeding MafA\textsuperscript{fl/fl} and NCoA6\textsuperscript{fl/fl} mice with rat insulin II enhancer/promoter (RIP)-driven Cre transgenic mice. MafA protein coding sequence are only present within Exon 1 (19), while deletion of Exon 7 causes a frame-shift that prevents the production of NCoA6 (46).

B) Representative images illustrating the effectiveness of MafA and NCoA6 removal from insulin\textsuperscript{+} cells at e15.5; ~72% of insulin\textsuperscript{+} cells lack MafA or NCoA6. Grey arrows indicate insulin\textsuperscript{+} cells lacking NCoA6, Yellow arrow indicate cell still expressing NCoA6. N=3. C) Islet architecture is only distorted in MafA\textsuperscript{AB} mice at 8-weeks.

Figure 7. Glucose stimulated insulin secretion is impaired in MafA\textsuperscript{AB} and NCoA6\textsuperscript{AB} islets. A) Expression of all the direct targets of mouse MafA were decreased in 8-week old MafA\textsuperscript{AB} islets, but not B) those identified as human MAFA and/or MAFB regulated genes by Qiagen human diabetes RT\textsuperscript{2} profiler screening (see Figure 5C). Moreover, essentially all of these genes were also compromised in NCoA6\textsuperscript{AB} 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\textsuperscript{fl/fl}=3, MafA\textsuperscript{AB}=4, NCoA6\textsuperscript{fl/fl}=3, NCoA6\textsuperscript{AB}=3.

Supplementary Table 1. Antibodies.

Supplementary Table 2. Primers used in qPCR experiments.
**Supplementary Table 3. 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 bolded lettering depicts proteins in Mll3/4 complexes. All proteins listed had greater than two-fold peptide enrichment in the MafA Re-CLIP compared to control.

**Supplementary Table 4. Identification of direct targets of MafA.** This list was compiled upon comparing the MafA$^\Delta_{panc}$ microarray data (41) to mouse islet MafA ChIP-Seq results (40). Shown is the fold expression change seen in the original microarray data, as well as the nearest ChIP-Seq peaks for MafA.

**Supplementary Figure 1. Flag-tagged MafA is bound to 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.

**Supplementary Figure 2. NCoA6$^{\Delta\beta}$ mice do not develop glucose intolerance.** A) MafA$^{\Delta\beta}$ and NcoA6$^{\Delta\beta}$ body mass is not significantly different between 8-week old mutant and control mice. Intraperitoneal glucose tolerance tests were performed with 8-week old MafA$^{\Delta\beta}$, NCoA6$^{\Delta\beta}$, and control littermates. (B and C) Only MafA$^{\Delta\beta}$ mice display 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$^{\Delta\beta}$ (*p<0.05) and NCoA6$^{\Delta\beta}$ (p=0.84) mice.
Supplementary Figure 3. Slc2a2 and Slc30a8 protein levels are reduced in *MafA*Δβ and *NCoA6*Δβ mice. Immunofluorescence analysis of *MafA*Δβ, *NCoA6*Δβ, and control littermates at 8-weeks. All images are representative.
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; treatment with (+) DTT breaks the MafA-protein disulfide cross-link and returns MafA to its normal molecular weight. 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) All of the proteins of the Mll4 complex were detected by Re-CLIP, a representative MS result is shown. Mll3 was also present in the MudPIT analysis (Supplementary Table 3).
Sucrose gradient sedimentation reveals that MafA comigrates with the Mll3/4 complex. A) βTCP3 nuclear proteins were separated in a 5-35% sucrose gradient, and fractions were screened for MafA, Mll3/4 (i.e. Rbbp5, NCoA6, Wdr5, and Utx), p/CAF, PdxP1, Nkx6.1, Hnf1α, Nkx2.2, and Pax6 by immunoblotting. A significant portion of MafA was present in Fraction 14, which contains Mll3/4 complexes. B) MafA2 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.

234x314mm (300 x 300 DPI)
Figure 3
207x258mm (300 x 300 DPI)
MAFA and MAFB associate with MLL3/4 in human EndoC-βH1 cells. A) MAFA2, MAFB2, and MAFA/B are produced in MAFA and MAFB transfected HeLa cells. Super-shift (S.S.) reveals three distinct complexes, with α-MAFA and α-MAFB altering homo- and heterodimer formation. B) MAFB2 and MAFA/B appear to be the predominant gel shift complexes formed in 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) Subunits of MLL3/4 were precipitated from EndoC-βH1 nuclear extracts with antibodies to MAFA and MAFB. Immunoblotting was performed for MLL3/4 subunits (NCOA6, RBBP5, UTX), islet-enriched transcription factors (PAX6, ISL1, HNF1α) and mouse MafA associated P/CAF (18). N=3, Representative blot shown.
Figure 5
209x254mm (300 x 300 DPI)
Islet architecture is unchanged in NCoA6Δβ mice. A) MafAΔβ and NCoA6Δβ mice were generated by breeding MafAfl/fl and NCoA6fl/fl mice with rat insulin II enhancer/promoter (RIP)-driven Cre transgenic mice. MafA protein coding sequence are only present within Exon 1 (28), while deletion of Exon 7 causes a frame-shift that prevents the production of NCoA6 (45). 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. Grey arrows indicate insulin+ cells lacking NCoA6, Yellow arrow indicate cell still expressing NCoA6. N=3. C) Islet architecture is only distorted in MafAΔβ mice at 8-weeks.
Figure 7
206x247mm (300 x 300 DPI)
|           | Ad-GFP | Ad-Flag-MafA | + | + | + | + |
|-----------|--------|--------------|---|---|---|---|
| **βTC-3 Input** |        |              |   |   |   |   |
| **Eluate**    |        |              |   |   |   |   |
| Flag        | ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) | ![Image](image4.png) | ![Image](image5.png) | ![Image](image6.png) |
| NCoA6       | ![Image](image7.png) | ![Image](image8.png) | ![Image](image9.png) | ![Image](image10.png) | ![Image](image11.png) | ![Image](image12.png) |
| Rbbp5       | ![Image](image13.png) | ![Image](image14.png) | ![Image](image15.png) | ![Image](image16.png) | ![Image](image17.png) | ![Image](image18.png) |
| p/CAF       | ![Image](image19.png) | ![Image](image20.png) | ![Image](image21.png) | ![Image](image22.png) | ![Image](image23.png) | ![Image](image24.png) |
| Pdx-1       | ![Image](image25.png) | ![Image](image26.png) | ![Image](image27.png) | ![Image](image28.png) | ![Image](image29.png) | ![Image](image30.png) |
### Immunoprecipitation

| Target                  | Host        | 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        | Concentration | Company and Catalog # |
|-------------------------|-------------|---------------|-----------------------|
| MAFA                    | Rabbit      | 1:1000        | Bethyl #A300-BL1225   |
| p/CAF                   | 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        | Concentration | Company and Catalog # |
|-------------------------|-------------|---------------|-----------------------|
| Insulin                 | Guinea Pig  | 1:1000        | Dako #A0564           |
| MafA                    | Rabbit      | 1:500         | Novus #NBP1-00121     |
| NCoA6                   | Rabbit      | 1:1000        | Sigma #HPA00419B      |
| Glucagon                | Mouse       | 1:1000        | Sigma #G2654          |
| Slc2a2                  | Goat        | 1:1000        | Santa Cruz #7580      |
| Slc30a8                 | Rabbit      | 1:500         | Thermo #PA5-21010     |

### EMSA

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

### ChIP

| Target                  | Host        | Company and Catalog # |
|-------------------------|-------------|-----------------------|
| IgG                     | Rabbit      | Bethyl #P120-101      |
| MAFA (mouse and human)  | Rabbit      | Novus #NBP1-00121     |
| MAFB                    | Rabbit      | Bethyl A300-BL658     |
| MLL3 (mouse and human)  | Rabbit      | Santa Cruz #130173    |
| H3K3 trimethylation     | Rabbit      | Abcam #ab8580         |
| RNA Pol II (phosphorylated)  | Rabbit     | Abcam #ab5131         |
| Gene     | Species | Forward       | Reverse                  |
|----------|---------|---------------|--------------------------|
| MafA     | mouse   | CCTGTAGAGGAAGCCGAGGAA | CTCCTCCCAGTGCAGATAGC    |
| NCoA6    | mouse   | TTGCAGGAGGGCCCAAATACATG | TCCAGGTTTGGGTCTGTACATCAT |
| G6PC2    | mouse   | ACCTGGTCTTCTTGAGGTHTTT | TTCAAGGTCCTCCGATCGACAT    |
| preIns2  | mouse   | GGGGAGCGTGCTGTTTTCTCTT | GGGGACAGAATCTCAGTGCA    |
| Ins2     | mouse   | GCCCTGGCTAACATTCTAGCTA | CCAAGGTCTGAGCTCGACACT    |
| Slc2a2   | mouse   | GTTAATGGCCACCGCCAGAAACT | CATTTTCTGGTATGACATCGGT    |
| Slc30a8  | mouse   | AGCTCCGCTGTTTTCTGCTTAA | AATCATTCCCAGGGCTCCTCAT    |
| Lifr     | mouse   | GCTCGCAGGGTTAACAGTACAT | GAAAGGTATTGCCGATACAT    |
| Sytl4    | mouse   | TCAGTGGCTGAGTAGACTTC | CAGAGAATCTTCTGCAAGAAG    |
| Atp2a2   | mouse   | AGCCAGAGAGATGGCTCGTTAA | AGAGCGAGGAGCATTTGACACCA    |
| Prss53   | mouse   | ACACTGGTGCTTGGTGAATCAT | CCAAGAAGTCTGCGTGAAACAT    |
| Rab6     | mouse   | AGGCAAGTGGTCAATTGGAGGGG | TTCTCTGCTTTCTGCTGTGCT    |
| CyclinD2 | mouse   | GAGTGGAGAAGGTAGGGCTTTT | CACACAGGAGCATTTGCAAG    |
| Prkaa1   | mouse   | CATGGTGCTGGTGTTGCAAAG | CAGAGGAGGAGTTAAAGAGAAG    |
| G6pdx    | mouse   | CCACCTGCCACCCACAGTAAA | GAAAGATGGTGCGTCAATAGGG    |
| Agt      | mouse   | GGCCTGAGGATACACAGAGA | CCAGGTCAAGATGCAAGAGATG    |
| MAF     | Human   | TGACGCAGAAGCCGAGTATTTTCAAAGG | GGAACGCGAGAACCACTTCACCT |
| MAFB     | Human   | ACCTGGTCAAGGCGAGATAG | CTTCCAGCTGGAGGAGTTACTC    |
| NCOA6    | Human   | TCCTCTGGTGCCTCATATAC | GCTGGTTCATTGTGCTTCT    |
| G6PC2    | Human   | GCAAGGGCTTATGGGGCTATT | AGGTCAATTCCCACAGGTCAG    |
| INS      | Human   | AGAAGGGCCCTGCAAGCTCTTGATG | AGGTGTTGGTTCACAGGCT    |
| ROBO2    | Human   | AAGCAACATGTCTTCTCCACATT | TGGGATGCAGTCTGGTACACT |
| SLC2A1   | Human   | GGAACAGCTGCAAAGGTTAAG | AGGAAGTGGTGAGTTAAAT |
| SLC2A2   | Human   | CTAGGGCCAGCTGCTGGGAAATAA | CTAAGGTCAGCTGCTGCAATT |
| PRKAA1   | Human   | CAGGTCATAGTCATGACCTTAAGTGA | TCAATTACGCCCTCCATTCAGT |
| G6PD     | Human   | GTAGGGCAAGCTCCTCTGCTTAAA | TGGGCTGTTGGCGAGTTAAA |
| AGT      | Human   | AAAGGCCAGCAGCAGATAACAA | CAGCAGACTTACAGGCAAGGAAA    |
| ChIP Ins2| mouse   | CAGGGGAAATGGTCGCGGGA | GCTGGGCTTTATAGTCTCAGAGC    |
| ChIP Slc2a2| mouse | TATTAGCCCTTTTCCATGCCTGCTT | ATCAACATGAGACCAGTCACCT |
| ChIP Prss53| mouse | GGCCTGCTGACTCTGAAATGAGCCTCACA | TGGGCTCATCTGTGACTGGCTA |
| ChIP Prss53 TSS | mouse | CTAGCAGCTCCCTGTAAGTGC | GACAAATGGCTCGTTGACTTACCT |
| ChIP Albumin | mouse | CCCTCCCTCATACATACCTTTC | CATGGAAGCATGCGACATTTTA    |
| ChIP AGT | Human   | GTCCAGGAGGACTTGGTCTAG | CTGCGAATGGGTTAGTGGA    |
| ChIP GAPDH | Human | Cell signaling #4471 |  |
| Transcription factors | Co-regulators | Other proteins of various functions |
|-----------------------|---------------|-------------------------------------|
| Cux1                  | Ash2l         | Otud4                               |
| Zbtb20                | Mll4          | MCM7                                |
| Trps1                 | Mll3          | HDAC6                               |
| Zfhx3                 | Utx           | Wiz                                 |
| Dach2                 | Rbbp5         | Uty                                 |
| Dach1                 | NCoA6         | Chd4                                |
| Myt1                  | Ptip          | Rbbp7                               |
| Myt3                  | Fmr1          | Cdyl                                |
| Myt1l                 | Ehmt2         | Rcor3                               |
| Hmbox1                | Gm10093       | Rcor1                               |
| znf516                | Ehmt1         | LSD1                                |
| Zfp644                | Pa1           | Smarca4                             |
| Trp53                 | Wdr5          | Smarce1                             |
| Zfhx2                 | Ogt           | Smarca5                             |
| Srfbp1                | Chd7          | Smarcc2                             |
| Nfic                  | Sin3a         | Taf5l                               |
| Baz2b                 | Hcfc1         | Ctbp1                               |
| GATAD2A               | Trim28        | Arid1a                              |
| Cdc5l                 | Smarcb1       |                                     |
| Mox2                  |                |                                     |
| Dido1                 |                |                                     |
| Ubn1                  |                |                                     |
| HIRA                  |                |                                     |
| Tjp2                  |                |                                     |
| Tjp1                  |                |                                     |
| COE2                  |                |                                     |
| Gtf2i                 |                |                                     |
| Cnot1                 |                |                                     |
| Trim56                |                |                                     |

Diabetes
| Name     | Gene Name  | Fold Change | ChIP-seq Peak Height | Peak Distance 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                  |
| CyclinD2 | NM_009829  | -2          | 7                    | -68388                 |