Regulation of Insulin Gene Transcription by Multiple Histone Acetyltransferases

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Glucose-stimulated insulin gene transcription is mainly regulated by a 340-bp promoter region upstream of the transcription start site by beta-cell-enriched transcription factors Pdx-1, MafA, and NeuroD1. Previous studies have shown that histone H4 hyperacetylation is important for acute up-regulation of insulin gene transcription. Until now, only the histone acetyltransferase (HAT) protein p300 has been shown to be involved in this histone H4 acetylation event. In this report we investigated the role of the additional HAT proteins CREB binding protein (CBP), p300/CBP-associated factor (PCAF), and general control of amino-acid synthesis 5 (GCN5) in regulation of glucose-stimulated insulin gene transcription. Utilizing quantitative chromatin immunoprecipitation analysis, we demonstrate that glucose regulates the binding of p300, CBP, PCAF, and GCN5 to the proximal insulin promoter. siRNA-mediated knockdown of each of these HAT proteins revealed that depletion of p300 and CBP leads to a drastic decrease in histone H4 acetylation at the insulin promoter and in insulin gene expression, whereas knockdown of PCAF and GCN5 leads to a more moderate decrease in histone H4 acetylation and insulin gene expression. These data suggest that high glucose mediates the recruitment of p300, CBP, PCAF, and GCN5 to the insulin promoter and that all four HATs are important for insulin gene expression.

Introduction

GLUCOSE STIMULATES INSULIN gene transcription in pancreatic beta cells in a dose-dependent manner, raising preproinsulin mRNA transcript levels two- to three-fold above basal levels within 2 h of glucose treatment (Goodison *et al.*, 1992). Glucose-stimulated insulin gene transcription is regulated by a highly conserved 340 bp proximal promoter region that is upstream of the transcription start site (Walker *et al.*, 1983; German and Wang, 1994; German *et al.*, 1995; Melloul *et al.*, 2002; Andrali *et al.*, 2008). This promoter region contains A3/A4, C1, and E1/E2 enhancer elements, which are bound by the beta-cell-enriched transcription factors Pancreas and Duodenal Homeobox-1 (Pdx-1) (Melloul *et al.*, 1993; Petersen *et al.*, 1994), V-maf musculoaponeurotic fibrosarcoma oncogene homologue A (MafA) (Kataoka *et al.*, 2002; Olbrot *et al.*, 2002), and neurogenic differentiation 1 (NeuroD1) (Naya *et al.*, 1995, 1997), respectively, to synergistically activate transcription (Melloul and Cerasi, 1994; Sharma and Stein, 1994).

Histone modifications in the underlying chromatin of the insulin promoter are important for both epigenetic maintenance of euchromatin structure and for the rapid regulation of glucose-stimulated insulin gene transcription (Chakrabarti *et al.*, 2003; Mosley and Ozcan, 2003; Deering *et al.*, 2009). Stable epigenetic marks for the insulin promoter are histone H3-Lys⁴ methylation and H3 Lys^{9,14} acetylation, which are

enriched in the fully differentiated beta-cell (Chakrabarti *et al.*, 2003). The islet-enriched methyltransferase Set7/9 maintains beta-cell identity by maintaining euchromatin structure of beta-cell genes, including the insulin promoter and coding region, by promoting histone H3-Lys⁴ mono- and di-methylation (Francis *et al.*, 2005; Deering *et al.*, 2009). Similarly, histone H3 Lys^{9,14} acetylation of the promoter is also enriched in a glucose-independent manner, indicating that both histone H3 acetylation and methylation function as epigenetic marks of the active insulin gene in the beta-cell.

Importantly, glucose stimulation of insulin gene transcription correlates with hyperacetylation of histone H4-Lys^{5,8,12,16} at the insulin promoter. Under high glucose conditions histone H4 acetylation is catalyzed by the histone acetyltransferase (HAT) p300, which is recruited to the proximal insulin promoter through a direct protein-protein interaction with Pdx-1 (Peshavaria *et al.*, 2000; Mosley *et al.*, 2004; Mosley and Ozcan, 2004; Wang *et al.*, 2007). In the reciprocal regulation under low glucose conditions, a fraction of Pdx-1 remains bound to the insulin promoter and recruits the histone deacetylases HDAC-1 and HDAC-2, resulting in histone H4 hypoacetylation and downregulation of insulin gene transcription (Mosley and Ozcan, 2004). From these observations, Pdx-1 appears to act as a glucose-regulated molecular switch at the insulin promoter to regulate chromatin structure under appropriate glucose conditions.

Currently, only p300 has been implicated in regulation of histone acetylation at the insulin proximal promoter. However, several HAT proteins have been shown to interact with beta-cell-enriched transcription factors, including Pdx-1, MafA, and NeuroD1/Beta2. In addition to interacting with p300, Pdx-1 has also been shown to directly interact with the HAT CREB binding protein (CBP) through the N-terminal activation domain of Pdx-1 (Asahara *et al.*, 1999). Furthermore, the HAT p300/CBP-associated factor (PCAF) has also been shown to play a role in regulating the function of beta-cell-enriched transcription factors. The acetylation of NeuroD1/Beta2 by PCAF enhances its DNA-binding capacity (Qiu *et al.*, 2004). Furthermore, PCAF was shown in a recent study to enhance MafA protein stability through a direct protein-protein interaction (Rocques *et al.*, 2007). However, the exact role of these HAT proteins in insulin gene transcription and pancreatic beta-cell function remains to be investigated.

While p300, CBP, and PCAF have been implicated to directly or indirectly to be important for beta-cell gene transcription, there is no information on the function of general control of amino-acid synthesis 5 (GCN5) in beta cells. GCN5, a paralog of PCAF, has been shown to play a central role controlling glucose metabolism in liver (Liu and Montminy, 2006). GCN5 responds to the nutrient state of the liver cell and directly regulates the transcription of gluconeogenic genes by acetylating and inhibiting Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC) (Lerin *et al.*, 2006; Kelly *et al.*, 2009; Caton *et al.*, 2010; Dominy *et al.*, 2010).

In this study we demonstrate that glucose stimulates insulin gene transcription by inducing the recruitment of p300, CBP, PCAF, and GCN5 to the insulin promoter. Knockdown of these HATs using specific siRNAs causes a reduction in hyperacetylation of histone H4 at the insulin promoter leading to decreased insulin gene transcription. These data suggest that the recruitment of HATs and hyperacetylation of histone H4 at the insulin promoter are important events in initiation of insulin gene transcription.

Materials and Methods

Cell culture

MIN6 cell passages between 21 and 25 were cultured as previously described (Miyazaki *et al.*, 1990). Glucose regulation of HAT binding was studied by incubating MIN6 cells in 2.5 mM glucose overnight followed by stimulation with 2.5 mM or 25 mM glucose for 2 h before performing the described experiments.

Preparation of protein extracts and western blotting

Total lysate was extracted from MIN6 cells with standard lysis buffer (50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 0.1% NP-40, 5 mM ethylenediaminetetraacetic acid, and protease/phosphatase inhibitors). Western blot analysis of whole cell lysate was conducted as previously described (Vanderford *et al.*, 2007). Antibodies used are p300 (Santa Cruz, sc-584), CBP (Santa Cruz, sc-369), GCN5 (sc-6303), PCAF (Santa Cruz, sc-12124), and tetra-acetyl histone H4 (Upstate, 06-598). Densitometric analysis of western blots was conducted using Kodak 1D Image Analysis software. Relative quantification of band intensities was normalized to beta-actin

loading controls for each sample and calculated as fold difference relative to scrambled siRNA control, which was set to a value of 100%.

Chromatin immunoprecipitation

For each sample, $2-5 \times 10^6$ MIN6 cells were processed for chromatin immunoprecipitation (ChIP) analysis as previously described with modifications (Chakrabarti *et al.*, 2003; Mosley and Ozcan, 2003, 2004). In brief, cells were cross-linked in serum-free DMEM containing 1% formaldehyde for 10 min at room temperature and quenched with 10 mM glycine. Cell lysates were sonicated for 6 pulses (10 s each) using a Sonics Vibra-Cell Ultrasonic Processor at 50% amplitude setting. Lysate was pre-cleared with 30 μ L salmon-sperm blocked protein A or protein G sepharose beads for 1 h at 4°C with rotation. Lysate sample (5%) was set aside as total input. Samples were divided into two sample sets and used for immunoprecipitation with specific antibodies or with mouse, rabbit, or goat immunoglobulin G (IgG; 5 μ g) as a negative control for nonspecific binding for 8–12 h at 4°C with rotation. Thirty microliters of salmon sperm-blocked protein A or protein G beads was added to the diluted lysate and rotated at 4°C for 1 h. Immune complexes were collected by centrifugation and washed. Protein-DNA cross-links were reversed by incubating with 200 mM NaCl at 65°C overnight. Samples were treated with RNase (1 μ L of 10 mg/mL per sample), followed by Proteinase K (10 μ L of 10 mg/mL per sample). DNA was purified using the QIAquick PCR Purification Kit (Qiagen) according to manufacturer's protocols.

Real-time polymerase chain reaction quantitation of ChIP-purified promoter fragments

Quantitative analysis of ChIP-purified DNA was conducted using real-time polymerase chain reaction (PCR) on an Mx4000 instrument (Stratagene) (Christenson *et al.*, 2001; Chakrabarti *et al.*, 2002; Mosley and Ozcan, 2004b). Real-time PCR amplification was performed using SYBR Green QPCR Master Mix (Stratagene) according to manufacturer's protocols. PCR fragments were observed on a polyacrylamide gel electrophoresis to confirm the presence of a single PCR band migrating at the correct size.

Ct values for each sample were normalized to total input DNA and IgG controls using the formula: $2^{[(Ct \text{ IgG} - Ct \text{ Input}) - (Ct \text{ Ab} - Ct \text{ Input})]}$, where Ab = p300, CBP, PCAF, GCN5, or tetra-acetyl histone H4. This calculation determines the fold enrichment of DNA in experimental samples over their corresponding IgG control, which accounts for background/nonspecific signal. For siRNA experiments, scrambled siRNA control values were set to 100%, and the corresponding experimental samples were expressed as a percentage of scrambled. This allowed for comparison among sample sets while normalizing for variations of ChIP immunoprecipitation efficiency among independent experiments. The primers for the mouse *Ins2* promoter are 5' AGGGCCCCCTTGTTAAG ACTCTAA 3' and 5' ACTGGGTCCCCACTACCTTTAT 3'.

Real-time (RT) PCR analysis

Quantitative PCR (qPCR) reactions were conducted on the Mx4000 real-time PCR instrument (Stratagene) as previously published (Mosley and Ozcan, 2004; Finlin *et al.*, 2005;

Vanderford *et al.*, 2007). Cells were treated according to experimental conditions before harvesting of mRNA. RNA was isolated using the RNeasy Mini Kit (Qiagen) according to manufacturer's protocols. Isolated RNA was then treated with DNase I (Sigma) and used for cDNA synthesis with the Enhanced Avian HS RT-PCR Kit according to manufacturer's protocols. Insulin II pre-mRNA was amplified using SYBR Green qPCR master mix (Stratagene) with the following primers: 5' GGGGAGCGTGGCTTCTTCTA 3' and 5' GGGGACAGAAT TCAGTGGCA 3' as published previously (Evans-Molina *et al.*, 2007).

siRNA-mediated gene silencing

One microgram of each siRNA oligo for every 1×10^6 cells was electroporated into MIN6 cells using the Nucleofector Device (Lonza Corp., catalog #VCA-003) according to manufacturer's protocols. Cells were then incubated in DMEM containing 25 mM glucose and 15% fetal bovine serum (FBS). After 12 h incubation, cells were switched to DMEM containing 25 mM glucose, 15% FBS, and antibiotic/antimycotic solution for an additional 36–40 h. Cells were then processed for mRNA harvesting, western blot, or ChIP analysis. The predesigned siRNA oligos (Ambion) used are CBP (5' CCU AUCCGAGCAAACAUTT 3'), p300 (5' GCGUUGACUUC UCCAAACATT 3'), PCAF (5' GGAGUCCUGUAAAUGCA AUTT 3'), GCN5 (5' GGAUCCUGAUCAGCUAUAUTT 3'), and scramble control siRNA #1 (Ambion). All siRNA oligos were confirmed to be sequence specific for the intended target protein.

Statistical analysis

All quantifications represent the mean of at least three independent experiments $\pm$ standard deviation. Statistical significance was determined using a two-tailed Student's *t*-test. Sample sets with a *p*-value < 0.05 were deemed statistically significant.

Results

Glucose stimulates histone H4 acetylation at the insulin promoter

It has been previously shown that glucose regulates the initiation of insulin gene transcription. Consistent with this, we found that 1 h exposure of MIN6 cells to 25 mM glucose increased Insulin II pre-mRNA levels by more than 15-fold compared to 2.5 mM glucose incubated cells (Supplementary Fig. S1; Supplementary Data are available online at www.liebertonline.com/dna). In both, primary islets and MIN6 cells glucose stimulation of insulin gene expression correlated with hyperacetylation of histone H4-Lys^{5,8,12,16} (Chakrabarti *et al.*, 2003; Mosley and Ozcan, 2003, 2004). We found that exposure of MIN6 cells to 25 mM glucose leads to increased acetylation of histone H4 Lys^{5,8,12,16} compared to 2.5 mM glucose, where histone H4 acetylation was barely detectable (Supplementary Fig. S2A). As reported previously, histone H3-Lys^{9,14} acetylation and histone H3-Lys⁴ dimethylation were not altered by high glucose conditions (Supplementary Fig. S2B, C) (Mosley *et al.*, 2003; Francis *et al.*, 2005). These data indicate that histone H4 acetylation is increased in response to high glucose and correlates strongly with glucose-stimulated insulin gene transcription.

p300 and CBP are recruited to the insulin promoter and mediate histone H4 acetylation

Several published reports indicate that p300 is an essential co-regulator of insulin gene transcription and is recruited to the insulin gene promoter via the transcription factor Pdx-1 (Mosley *et al.*, 2004). To further investigate the specific role of p300 in regulation histone H4 acetylation and insulin gene transcription in MIN6 cells, ChIP analysis of p300 binding and histone H4 acetylation at the mouse proximal *Ins2* promoter was conducted using specific primers. ChIP analysis of MIN6 cells treated with 2.5 mM or 25 mM glucose showed about a twofold enrichment of p300 at the insulin promoter under high glucose conditions (Fig. 1A, Supplementary Fig. S3A). To assess the role of p300 in insulin gene transcription under 25 mM glucose conditions, p300 protein levels were depleted by 80% in MIN6 cells using a specific siRNA (Fig. 1B). siRNA-treated MIN6 cells were preincubated with 2.5 mM glucose overnight followed by 25 mM glucose treatment for 2 hours before ChIP analysis of histone H4-hyperacetylation. Ablation of p300 in MIN6 cells by RNAi resulted in an almost complete loss of detectable levels of histone H4 hyperacetylation at the proximal promoter region under high glucose conditions (Fig. 1C, Supplementary Fig. S4A). Furthermore, quantification of insulin II pre-mRNA transcript levels by quantitative real time (qRT)-PCR in MIN6 cells depleted for p300 indicated a 70% decrease in insulin gene transcription (Fig. 1D).

In addition to p300, CBP has also been previously reported to interact with the N-terminal activation domain of Pdx-1 (Asahara *et al.*, 1999). However, no prior information about the role of CBP in histone H4 acetylation at the insulin promoter is available. ChIP analysis of MIN6 cells treated with either 2.5 mM or 25 mM glucose indicated that CBP binds to the insulin promoter under both low and high glucose conditions. However, there was about a threefold enrichment of CBP at the insulin promoter under 25 mM glucose conditions, indicating that high glucose stimulates CBP binding (Fig. 2A; Supplementary Fig. S3B). siRNA-mediated ablation of CBP levels by 80% resulted in an 80% loss of glucose-stimulated histone H4 hyperacetylation of the proximal promoter region (Fig. 2B, C, Supplementary Fig. S4B). Furthermore, CBP depletion caused a $\sim 65\%$ reduction in insulin II pre-mRNA levels after stimulation with high glucose (Fig. 2D). These data, taken together, suggest that p300 and CBP both are required for histone H4 acetylation and insulin gene transcription initiation upon glucose stimulation.

PCAF associates with the insulin II promoter and mediates histone H4 acetylation

Previous data indicate that PCAF can enhance the DNA-binding capacity of BETA2/NeuroD1 and promote MafA stability by inhibiting the ubiquitination of MafA (Qiu *et al.*, 2004; Rocques *et al.*, 2007). However, it is unclear if PCAF is involved in insulin gene transcription. To investigate whether PCAF is recruited to the insulin promoter, we conducted ChIP analysis in MIN6 cells under 2.5 and 25 mM glucose conditions. As shown in Figure 3A, PCAF is recruited to the insulin promoter and there is an approximately sixfold enrichment of PCAF at the insulin promoter on 25 mM glucose compared to 2.5 mM glucose, where PCAF association was

FIG. 1. Glucose mediates p300 binding to the *Ins2* promoter to regulate histone H4 hyperacetylation. MIN6 cells were preincubated ~12 h with 2.5 mM glucose followed by 2-h stimulation with either 2.5 or 25 mM glucose. Cells were processed for ChIP analysis for p300 association with the *Ins2* proximal promoter (**A**). Data are represented as fold enrichment over background immunoglobulin. (**B**) p300 was knocked down in MIN6 cells and cells were cultured for ~48 h before western blotting with anti-p300 antibody (Upstate). p300 band densities were measured and normalized to beta-actin control. (**C**) siRNA-treated MIN6 cells were cultured for 48 h before 2-h stimulation with 25 mM glucose. ChIP analysis was conducted to measure histone H4 hyperacetylation (anti-Ac-H4, Upstate) at the proximal *Ins2* promoter. (**D**) siRNA-treated MIN6 cells were pretreated in a manner identical to panel C followed by measurement of insulin II mRNA (pre-mRNA) transcripts levels using qRT-PCR. All quantifications represent the mean of at least three independent experiments ($n=3$) $\pm$ SD. * $p<0.05$, ** $p<0.01$. ChIP, chromatin immunoprecipitation; quantitative real time (qRT)-PCR, qRT-polymerase chain reaction; SD, standard deviation.

barely detectable (Supplementary Fig. S3C). To specifically analyze the effects of PCAF on histone H4 hyperacetylation and insulin gene transcription, PCAF was ablated by about 70% in MIN6 cells via siRNA knockdown before ChIP analysis (Fig. 3B). Loss of PCAF protein resulted in a 60% reduction in histone H4 hyperacetylation. However, depletion of PCAF by siRNA led only to a 20% reduction in insulin II pre-mRNA levels (Fig. 3C, D, Supplementary Fig. S4C). These data suggest that although PCAF is associated with the insulin promoter at high glucose levels and contributes to some extent to histone H4 hyperacetylation, its effect on initiation of insulin gene transcription is minimal compared to p300 or CBP.

FIG. 2. CBP is associated with the *Ins2* promoter and regulates insulin gene transcription. (**A**) ChIP analysis of CBP association with the *Ins2* proximal promoter. (**B**) MIN6 cells were transfected with CBP siRNA and cultured for ~48 h before western blotting with anti-CBP antibody (Santa Cruz). CBP levels were normalized to beta-actin control. (**C**) siRNA-treated MIN6 cells were cultured for 48 h before 2-hour stimulation with 25 mM glucose and used for ChIP analysis of histone H4 acetylation levels at the *Ins2* promoter. (**D**) siRNA-treated MIN6 cells were incubated with 25 mM glucose for 2 h followed by measurement of insulin II pre-mRNA level. All quantifications represent the mean of at least three independent experiments ($n=3$) $\pm$ SD. ** $p<0.01$. CBP, CREB binding protein.

GCN5 is recruited to the insulin promoter and is required for glucose induction of insulin gene transcription

Several recent reports link GCN5 to regulation of glucose metabolism in liver by responding to nutrient conditions and regulating the transactivation potential of the transcription factor PGC1alpha at gluconeogenic gene targets (Lerin *et al.*, 2006; Kelly *et al.*, 2009; Caton *et al.*, 2010; Dominy *et al.*, 2010). Thus, we tested whether GCN5 regulates insulin gene transcription in response to glucose. As shown in Figure 4A, ChIP analysis with the GCN5 antibody indicates that GCN5 binds to the insulin promoter in a glucose-regulated manner. Binding of GCN5 to the insulin II promoter is about seven-fold increased on high versus low glucose conditions, where binding was almost undetectable (Fig. 4A, Supplementary Fig. S3D). siRNA-mediated silencing of GCN5 caused a 60% reduction in GCN5 protein levels (Fig. 4B). Surprisingly, however, loss of GCN5 did not cause a statistically

FIG. 3. Glucose causes the recruitment of PCAF to the *Ins2* promoter to regulate histone H4 hyperacetylation. (A) PCAF association with the *Ins2* promoter was determined in MIN6 cells incubated with 2.5 mM or 25 mM glucose for 2 h. (B) Depletion of PCAF after transfection of MIN6 cells with PCAF siRNA was determined using western blotting. Beta-actin was used as a loading control. (C) For ChIP analysis of histone H4 acetylation levels at the insulin II promoter, PCAF siRNA- or control siRNA-treated MIN6 cells were incubated with 25 mM glucose for 2 h. (D) Insulin II pre-mRNA levels were quantified in MIN6 cells treated with PCAF siRNA after incubation for 2 h in 25 mM glucose. The presented data represent the mean of at least three independent experiments ($n=3$) $\pm$ SD. * $p < 0.05$, ** $p < 0.01$. PCAF, p300/CBP-associated factor.

significant reduction of histone H4 hyperacetylation, but resulted in a 40% decrease in insulin II pre-mRNA levels under high glucose conditions (Fig. 4C, D, Supplementary Fig. S4D). These data indicate that GCN5 is to some extent important for insulin gene transcription, but this is independent of histone H4 hyperacetylation.

Discussion

There is accumulating evidence linking histone modification events to regulation of glucose homeostasis. However, the exact mechanisms by which chromatin modifications regulate gene expression to control glucose homeostasis are poorly understood. In the pancreatic beta-cell, reversible hyperacetylation of histone H4-Lys^{5,8,12,16} has been shown to contribute to acute glucose-stimulated insulin gene transcription (Mosley and Ozcan, 2003; Evans-Molina *et al.*, 2007). Previous data suggest that under high glucose con-

FIG. 4. GCN5 is recruited to the *Ins2* promoter in a glucose-dependent manner, but depletion of GCN5 has no effect on histone H4 acetylation. (A) GCN5 recruitment to *Ins2* promoter was quantified by ChIP in MIN6 cells incubated with 2.5 or 25 mM glucose for 2 h. (B) GCN5 protein levels were quantified and normalized to beta-actin after transfection of MIN6 cells with GCN5 siRNA. (C) Histone H4 acetylation levels in GCN5 siRNA-treated MIN6 cells were quantified by ChIP in MIN6 cells treated with GCN5 siRNA and incubated for 2 h with 25 mM glucose. (D) MIN6 cells treated with GCN5 siRNA were preincubated with 2.5 mM glucose and switched to 25 mM glucose for 2 h before quantification of Insulin II pre-mRNA levels by qRT-PCR. The data shown are representative of at least three independent experiments ($n=3$) $\pm$ SD. ** $p < 0.01$. GCN5, general control of amino-acid synthesis 5.

ditions, histone H4 hyperacetylation occurs in a process dependent on an interaction between p300 and the N-terminal activation domain of Pdx-1 (Mosley *et al.*, 2004a). Conversely, under low glucose conditions, it has been shown that Pdx-1 interacts with HDAC-1 and HDAC-2 to down-regulate insulin transcription by histone H4 deacetylation (Mosley and Ozcan, 2004b). A recent report demonstrates that the beta-cell-specific transcription factor insulin-associated antigen-1 (INSM1/A-1) is also able to mediate histone H4 acetylation at the insulin promoter and thereby promotes ductal cell transdifferentiation (Zhang *et al.*, 2010). Until now, only p300 has been implicated as a HAT protein required for glucose-regulated histone H4 hyperacetylation at the insulin promoter (Mosley *et al.*, 2004). This study provides new insight into the HATs that participate in this process. Using quantitative ChIP analysis, we found that all four HATs

(p300, CBP, PCAF, and GCN5) are recruited to the insulin promoter in a glucose-dependent manner in the MIN6 cell line. Depletion of all four HATs causes a reduction in insulin gene transcription to varying extent.

While depletion of p300, CBP, and PCAF leads to a significant reduction in histone H4 hyperacetylation, knockdown of PCAF reduced insulin gene transcription only by 20%. Thus, in this case there was no exact correlation between histone H4 acetylation and insulin II pre-mRNA levels. It is likely that a 40% reduction in histone H4 acetylation level as observed after depletion of PCAF is still sufficient to mediate the assembly of the transcription initiation complex at the insulin promoter. However, it is still surprising that the depletion of PCAF has only a minimal effect on insulin gene transcription given that PCAF has been shown to regulate the function of the insulin gene transcription factors NeuroD1/Beta2 and MafA. PCAF has been demonstrated to acetylate NeuroD1/Beta2 and to enhance its DNA-binding capacity (Qiu *et al.*, 2004). Furthermore, previous data indicate that MafA ubiquitination and degradation is prevented by its interaction with PCAF (Rocques *et al.*, 2007), though no data currently exist on MafA acetylation. The finding that depletion of PCAF only slightly affects insulin gene transcription may be explained by redundancy of HAT function. In the absence of PCAF, may be one or more of the remaining HATs replace PCAF function.

Another surprising result was that knockdown of GCN5 caused a 40% reduction in insulin gene expression without affecting histone H4 acetylation levels at the insulin promoter. This result indicates that GCN5 does not directly participate in acetylation of histone H4 and may regulate insulin gene transcription by acetylating of transcription factors that directly bind the proximal insulin promoter, such as Pdx-1, MafA, and/or NeuroD1/Beta2. Indeed, in liver GCN5 has been demonstrated to regulate gluconeogenic gene expression by acetylation of the transcriptional coactivator PGC1 alpha in response to the energy status of the cell (Lerin *et al.*, 2006; Liu and Montminy, 2006; Kelly *et al.*, 2009; Dominy *et al.*, 2010). Alternatively, histone H4 acetylation may only serve as a marker for recruitment of other transcriptional regulators to the insulin promoter and may not be directly involved in insulin gene transcription.

Our study suggests that glucose as a nutrient regulates the recruitment of HATs to the insulin gene promoter, thereby linking histone acetylation to nutrient status of the cell. A likely mechanism by which histone acetylation levels could be altered in dependence of the nutrient or energy state of the cell is via the intracellular pools of acetyl-CoA (Jeninga *et al.*, 2010). Interestingly, a recent report indicates that in mammalian cells, metabolism is directly linked to histone acetylation by the activity of a nuclear fraction of the enzyme ATP citrate lyase (ACL). This nuclear fraction of ACL uses glucose-derived citrate to produce a nuclear pool of acetyl-CoA, which then serves as a localized substrate for HAT activity (Wellen *et al.*, 2009). Based on this observation, it is likely that a similar mechanism is employed in beta cells to rapidly respond to glucose metabolism for the upregulation of histone H4 acetylation at the insulin gene promoter and other glucose-regulated targets. Additional studies are required to determine if nuclear ACL activity is required for glucose-stimulated histone H4 acetylation at the insulin promoter and for insulin gene transcription.

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Disclosure Statement

No competing financial interests exist.

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