Brief Communication

MOLECULAR METABOLISM

Transcriptional activation of the *Myc* gene by glucose in β-cells requires a ChREBP-dependent 3-D chromatin interaction between the *Myc* and *Pvt1* genes

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ABSTRACT

Objective: All forms of diabetes result from insufficient functional β-cell mass. Thus, achieving the therapeutic goal of expanding β-cell mass requires a better mechanistic understanding of how β-cells proliferate. Glucose is a natural β-cell mitogen that mediates its effects in part through the glucose-responsive transcription factor, carbohydrate response element binding protein (ChREBP) and the anabolic transcription factor, MYC. However, mechanistic details by which glucose activates *Myc* at the transcriptional level are poorly understood.
Methods: Here, siRNA was used to test the role of ChREBP in the glucose response of MYC, ChIP and ChIPseq to identify potential regulatory binding sites, chromatin conformation capture to identify DNA/DNA interactions, and an adenovirus was constructed to expresses x-dCas9 and an sgRNA that specifically disrupts the recruitment of ChREBP to a specific targeted ChoRE.
Results: We found that ChREBP is essential for glucose-mediated transcriptional induction of *Myc*, and for increases in *Myc* mRNA and protein abundance. Further, ChIPseq revealed that the carbohydrate response element (ChoRE) nearest to the *Myc* transcriptional start site (TSS) is immediately upstream of the gene encoding the lncRNA, *Pvt1*, 60,000 bp downstream of the *Myc* gene. Chromatin Conformation Capture (3C) confirmed a glucose-dependent interaction between these two sites. Transduction with an adenovirus expressing x-dCas9 and an sgRNA specifically targeting the highly conserved *Pvt1* ChoRE, attenuates ChREBP recruitment, decreases *Myc*-*Pvt1* DNA/DNA interaction, and decreases expression of the *Pvt1* and *Myc* genes in response to glucose. Importantly, isolated and dispersed rat islet cells transduced with the ChoRE-disrupting adenovirus also display specific decreases in ChREBP-dependent, glucose-mediated expression of *Pvt1* and *Myc*, as well as decreased glucose-stimulated β-cell proliferation.
Conclusions: The mitogenic glucose response of *Myc* is mediated *via* glucose-dependent recruitment of ChREBP to the promoter of the *Pvt1* gene and subsequent DNA looping with the *Myc* promoter.
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Keywords ChREBP; Myc; Pvt1; Beta cells; Glucose-mediated gene expression; Chromatin conformation capture; DNA looping; Glucose-stimulated beta cell proliferation

1. INTRODUCTION

There has been an enormous effort in the diabetes research community to better understand mechanisms that increase functional β-cell mass in the context of diabetes [1—7]. Investigated approaches include preservation of β-cell mass, neogenesis or trans-differentiation from other cell types, and promotion of β-cell replication. Glucose is a natural β-cell mitogen, and both MYC and carbohydrate response element binding protein (ChREBP) are essential for glucose-mediated β-cell proliferation and adaptive β-cell expansion after a high fat diet [8—13].

MYC is a master coordinator of β-cell proliferation and maturation [8,11,14]. Thus, mitogenic signals converge on *Myc*, following a common pathway for proliferation in insulinomas and in rodent models of β-cell proliferation [8]. In addition, MYC effects are classically dose-related in a bi-phasic manner. For example, levels typically rise with proliferative status in β-cells, as in the post-natal period when β-cells proliferate at their highest rate. In contrast, excessive MYC abundance correlates with decreased levels of β-cell maturity markers [11], and very high levels of MYC, as happens in hyperglycemia and diabetes, are associated with glucose toxicity, inhibition of insulin production, and β-cell death [15—18]. Thus, modest and transient increases in

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MYC are necessary for adaptive β-cell proliferation [12]. Increasing glucose metabolism in β-cells within physiological ranges results in increased expression of *Myc* mRNA and protein, in part by stabilizing the MYC protein and in part by increasing *Myc* transcription [12,15]. Thus, it is critically important to better understand the molecular mechanisms by which glucose increases the expression of the Myc gene in β-cells.

PVT1 is a lncRNA that resides approximately 60,000 bp downstream of the *MYC* gene. Its genomic location is syntenic between rodents and humans, and *PVT1* is often co-amplified with *MYC* in human cancers [19]. The upstream and downstream regulation of *PVT1* is poorly understood, but it has been described variably as a tumor promoter that supports MYC's expression, but also as a tumor suppressor, depending on cellular context [19—23].

Carbohydrate response element binding protein (ChREBP) is a glucose-responsive transcription factor that was originally described in liver as a driver of *de novo* lipogenesis [24—26]. ChREBP is expressed at levels comparable in liver and pancreatic β-cells and is essential for glucose-stimulated β-cell proliferation and for adaptive expansion of β-cells in response to a high fat diet [9,10,13]. There are two major isoforms of ChREBP, ChREBPα and ChREBPβ. ChREBPβ is induced *via* a feed-forward loop *via* a carbohydrate response element (ChoRE) that drives an alternative promoter upstream of the canonical full-length ChREBP gene. Alternative splicing results in a truncated form of ChREBP that lacks an inhibitory domain and nuclear export sequences, rendering ChREBPβ more transcriptionally active than its longer counterpart [27]. The feed-forward induction of ChREBP is necessary for a complete glucose response of ChREBP-dependent target genes and is necessary for adaptive expansion of β-cells in response to a high fat diet [9,13]. Notably, low, constant levels of Myc expression are required for ChREBP glucose-mediated DNA binding and glucose-mediated gene transcription in both hepatocytes and pancreatic β-cells [28—30], likely reflecting a requirement for enhanced metabolic flux and cellular energy [31].

In the present study, we explored the mechanisms by which glucose mediates the induction of the *Myc* gene. We found that ChREBP is necessary for a complete *Myc* response to glucose, and that the ChoRE nearest to *Myc* lies 60,000 bp downstream of the *Myc* gene, near the transcription start site of the *Pvt1* gene. Furthermore, using chromatin conformation capture (3C), we reveal that ChREBP mediates a glucose-dependent DNA/DNA interaction between *Myc* and *Pvt1*. Specific disruption of ChREBP recruitment to the *Pvt1* promoter using an adenovirally expressed, CRISPR-targeted sgRNA decreased glucose-mediated DNA looping between the *Pvt1* and *Myc* genes as well as the glucose-mediated expression of *Myc* and *Pvt1*. Importantly, targeting the *Pvt1* ChoRE with adenovirally expressed CRISPR-targeted sgRNAs prevented glucose-dependent *Myc* and *Pvt1* expression as well as glucose-mediated proliferation in primary β-cells isolated from rat islets.

2. RESULTS

Since glucose increases Myc expression in β-cells, and since both ChREBP and MYC are required for glucose-stimulated β-cell proliferation [9,12,30], we tested whether ChREBP is necessary for the glucose-mediated induction of the Myc gene. As shown in Figure 1A—B, the glucose-mediated induction of MYC protein was decreased by treatment with pooled siRNA directed against both isoforms of ChREBP

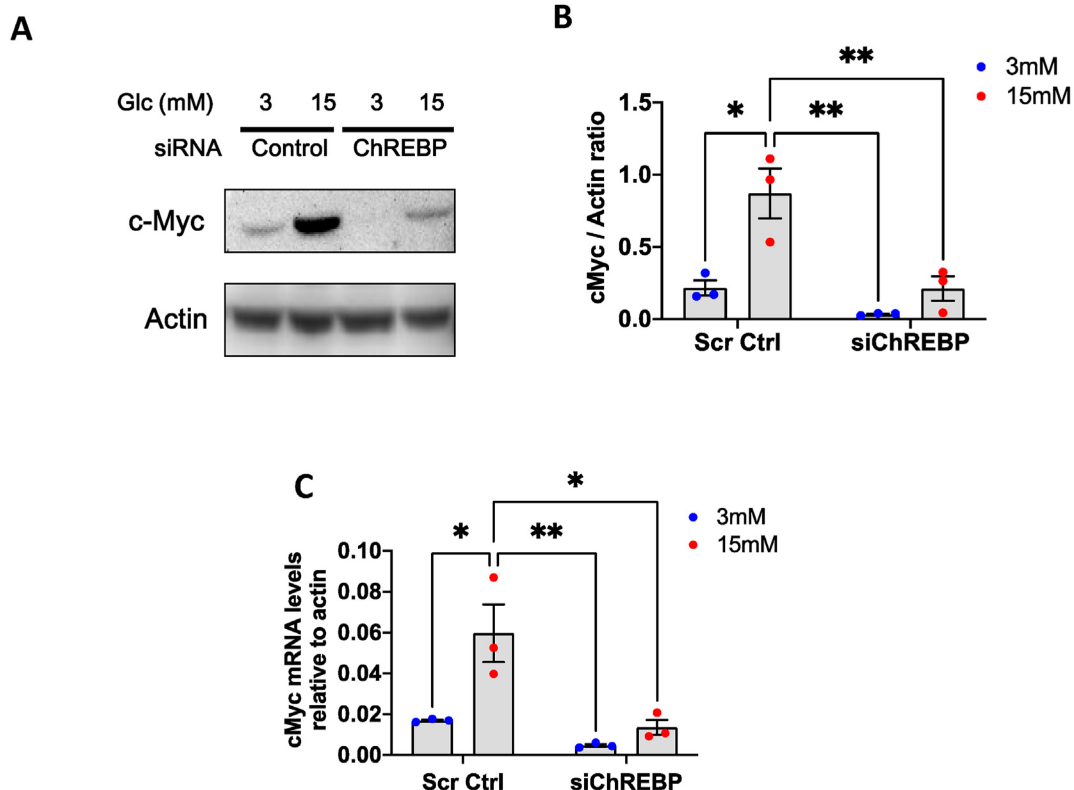

Figure 1: The glucose response of Myc requires ChREBP. INS-1 cells were cultured for 48 h in either low (2—5 mM glucose) or high (15—25 mM) glucose along with a Dharmacon SMARTpool siRNA directed against ChREBP or a scrambled SMARTpool control (Scr Ctrl). (A) Western blot of Myc and Actin. (B) Quantification of (A). (C) Relative mRNA levels of Myc normalized to actin using RT-PCR. Data shown are the means ± SEM, n = 3, *, **, $p < 0.05$ and $p < 0.01$, respectively.

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in 832/13 INS-1 rat β-cell-like insulinoma cells (henceforth INS-1 cells [32]). While the lack of ChREBP resulted in greatly attenuated MYC expression, there remained a small glucose-mediated induction of MYC, indicating a ChREBP-independent effect. In addition, the siRNA against ChREBP reduced the expression of *Myc* mRNA in response to glucose (Figure 1C). Thus, ChREBP is necessary for the glucose-mediated induction of the *Myc* gene.

The *Myc* promoter is extremely rich in transcription factor binding sites reflecting the fact that the *Myc* gene integrates signals from a plethora of environmental cues [33]. Therefore, using a promoter-scanning chromatin immunoprecipitation (ChIP) Rt-PCR approach, we searched for ChoRE sites in the *Myc* gene promoter region that might confer the ChREBP-dependent glucose response of the *Myc* gene (Figure 2A). INS-1 cells were cultured in either 2 or 20 mM glucose for 18 h and subjected to a ChIP assay using primers specific for the *Myc* promoter, and, as a positive control for the *Pklr* promoter [29]. No ChREBP recruitment was found 2000 bp upstream of the transcription start site. Interestingly, ChREBP-targeted ChIPseq studies from Schmidt et al. [34], wherein ChREBP recruitment was measured in INS-1 cells in either 2 or 25 mM glucose for 2 or 12 h, demonstrated a strong glucose-dependent ChREBP binding site approximately 60,000 bp downstream of the *Myc* gene that corresponded to the promoter region of the long non-coding RNA (lncRNA), *Pvt1* (Figure 2B). The binding of ChREBP is comparable to well-known glucose-responsive genes including *Pklr*, *Fasn*, and *Acaca* (Supplementary Figure 1). Since the *Myc*-*Pvt1* region is highly conserved and syntenic between rodents and primates [22], we examined the sequence under the 60,000 bp downstream ChREBP-targeted ChIPseq binding site. This revealed a potential ChoRE just upstream of the *Pvt1* transcription start site that is highly conserved among rodents and primates, and that was more GC-rich than other ChoRE motif analyses conducted in genome-wide ChIPseq studies [34—37], but still conformed to the basic pattern originally described by Towle and colleagues [38], consisting of two degenerate E-boxes separated by 5 bp (Figure 2C). The primate sequences better conform to consensus ChoREs if moved 2 bp to the "right" or 3′.

Since several studies have found long DNA loops interacting with, and regulating the expression of, the *Myc* gene [33,39,40], we hypothesized that ChREBP recruitment to the *Pvt1* promoter might create a DNA loop that interacts with a *Myc* regulatory region. Using a chromatin conformation capture (3C) assay, we found that increasing the culture medium glucose from 2 mM to 20 mM for 18 h significantly increased the interaction between the *Pvt1* and *Myc* promoters (Figure 2D).

Because we have found a close relationship between Myc and ChREBP in β-cells [28—30], and because they are both members of the extended network of MYC transcription factors [41], we tested if glucose alters the recruitment of MYC to either the *Myc* or the *Pvt1* loci by comparing it the ChREBP recruitment using a ChIP-PCR approach (Supplementary Figure 2). We found that MYC is recruited to the *Pvt1* gene locus over a broad region, ranging from the proximal ChoRE bound by ChREBP, well into the coding region of the gene. Scanning of the sequence revealed several E-boxes in the region. MYC is also increased over a broad region of the *Myc* gene, which does not contain perfect CACGTG E-boxes. Importantly, MYC interacts with several members of the transcriptional complex, including mediator, and so Myc may be interacting with the transcriptional machinery in a DNA-independent fashion, particularly since glucose stabilizes the MYC protein in β-cells [12]. We compared the ChIP-PCR data with ChIPseq and DNAse hypersensitivity data from Schmidt et al. ([34], [GEO GSE81628]) and found that glucose also increases the accessibility of the *Myc* locus. Together these results suggest that the binding of ChREBP (and Myc) on the *Pvt1* promoter initiates transcriptional activity on the *Myc* promoter.

We next devised an approach to test if recruitment of ChREBP to the *Pvt1* ChoRE was necessary for the glucose response of the *Myc* gene. Thus, we constructed an adenovirus expressing HA-tagged x-dCas9 and an sgRNA targeting the ChoRE in the *Pvt1* promoter [Figure 3A, and Methods]. The goal was to sterically hinder the recruitment of ChREBP to this specific ChoRE in a population of cells, taking advantage of the very high efficiency of adenoviruses transducing β-cells and the high affinity of sgRNAs to their target DNA sites [42,43]. Figure 3B illustrates the very high transduction efficiency of the ChoRE-disruptor adenovirus, with nearly every cell expressing the HA tag (the control adenovirus did not contain an HA tag). Using a chromatin immunoprecipitation assay, we found that INS-1 cells transduced with the ChoRE disruptor adenovirus significantly reduced glucose-induced recruitment of ChREBP to the *Pvt1* promoter (Figure 3C). Importantly, transduction with the adenovirus did not interfere with the recruitment of ChREBP to the LPK and ChREBPβ ChoREs, demonstrating its specificity (Figure 3D, E). Next, a 3C assay demonstrated that the disruption of ChREBP recruitment to the *Pvt1* promoter was associated with a profound decrease in the interaction frequency between regions near the *Pvt1* and *Myc* promoters (Figure 3G). Thus, the *Pvt1* ChoRE disruptor adenovirus specifically attenuates the recruitment of ChREBP to the *Pvt1* promoter ChoRE, and interferes with the formation of DNA loops between *Pvt1* and *Myc*.

Having established that the *Pvt1* ChoRE-disrupting adenovirus altered chromatin structure physically linking *Pvt1* and *Myc*, we asked how this affected glucose-mediated gene expression. As shown in Figure 4, blocking the interaction between *Pvt1* and *Myc* resulted in a decrease in glucose-mediated expression of *Pvt1* and *Myc*. This was a specific effect: the simultaneous glucose response in the same cells by ChREBPβ or *Txnip*, two other glucose-responsive genes was normal and unaffected. *Txnip* (thioredoxin interacting protein) is a ChREBP target gene whose product is implicated in exacerbating oxidative stress and glucose toxicity [44]. We also found that the Myc paralogs, MycN and MycL were not significantly affected by this treatment (Supplementary Figure 3). Furthermore, the same experiment was performed with isolated rat islet cells with the same result, with decreases in glucose-mediated *Pvt1* and *Myc* mRNA expression, but no changes in the glucose response of ChREBPβ or *Txnip* mRNA. Finally, as an additional readout of the functional consequences of interrupting *Pvt1* and *Myc* interactions, we tested if disrupting the recruitment of ChREBP to the *Pvt1* ChoRE is necessary for the glucose-responsive expression of *Myc* in primary rat islet cells. Rat islets were isolated, dispersed, and transduced with the ChoRE disruptor adenovirus and cultured in either 6 or 20 mM glucose for a total of 56 h. We found that adenoviral disruption of the *Pvt1* ChoRE resulted in attenuated glucose-stimulated β-cell proliferation (Figure 4I, J).

To explore if the ChREBP-*Pvt1*-*Myc* relationship is unique to β-cells or is a common feature of cancer we interrogated the Kaplan—Meier Plotter (https://kmplot.com/analysis/ [45]; Supplementary Figure 4). We found that high expression of all 3 genes corresponded significantly to poor outcomes in only head and neck squamous carcinoma, but not in 25 other human cancers. When comparing the genes one to one, *Myc* and *Pvt1* where often highly correlated, including in human insulinomas [46], but ChREBP and Myc or ChREBP and Pvt1 are not correlated in insulinomas and poorly correlated compared to Myc and Pvt1. Thus, the ChREBP-*Pvt1*-*Myc* relationship appears not to be common in human cancers.

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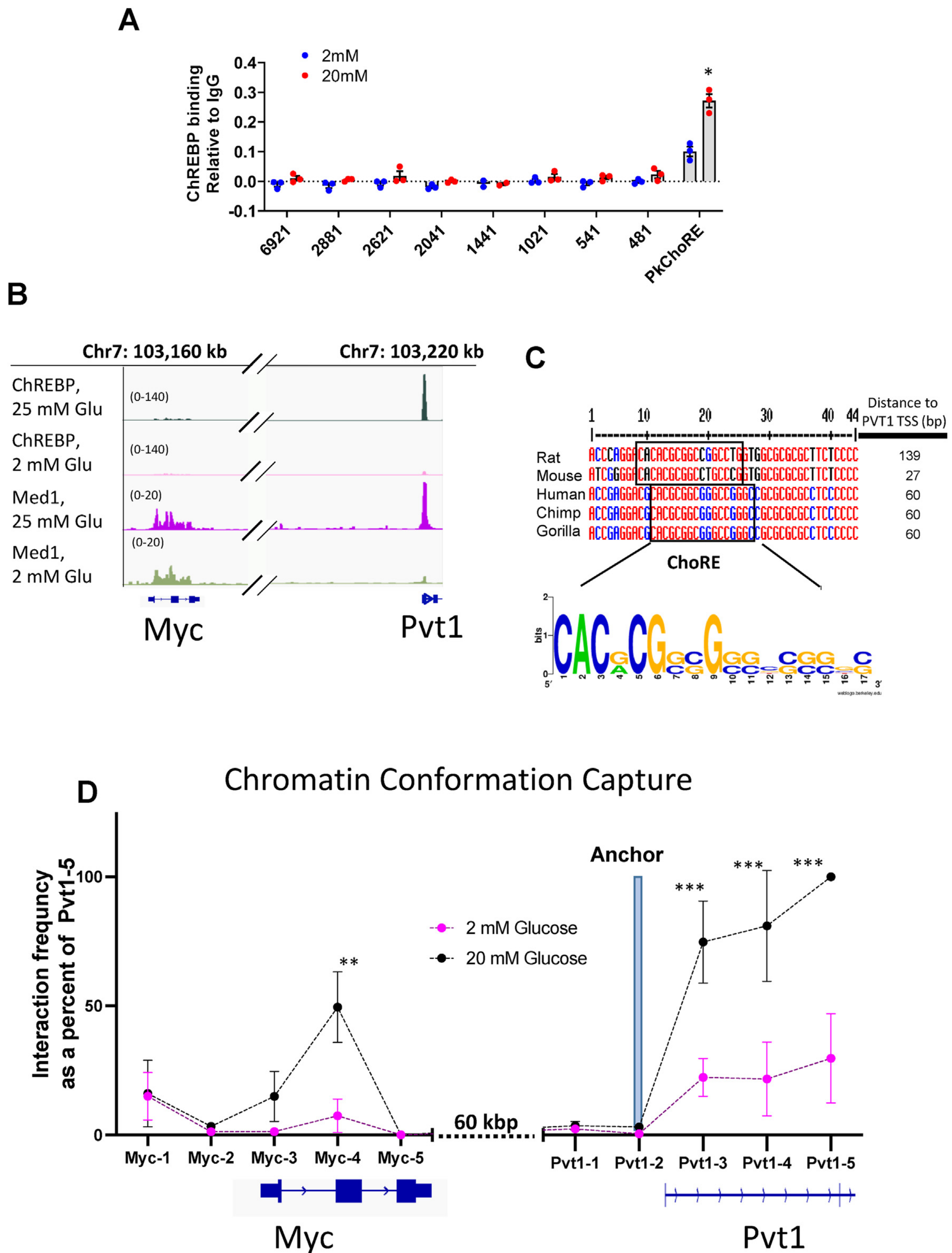

Figure 2: Glucose increases the interaction between the *Myc* and *Pvt1* promoters. (A) Using conventional ChIP-PCR, no ChoREs were found near the Myc transcription start site in INS-1 cells cultured in either 2 or 20 mM glucose. Numbers represent bp upstream of the Myc transcription start site. The *Pklr* ChoRE was used as a positive control (PKChoRE). (B) ChIPseq data from [34] (GEO GSE81628) demonstrating recruitment of ChREBP to the *Pvt1* promoter in INS-1 cells after 2 h of 25 mM glucose. (C) Alignment of rodent and primate sequences surrounding the *Pvt1* ChoRE with consensus ChoRE sequence. (D) Chromatin conformation capture from INS-1 cells treated with 2 or 20 mM glucose for 16 h. All data are means $\pm$ SEM. N = 3,4, *$p < 0.05$, **, $p < 0.01$, ***, $p < 0.005$.

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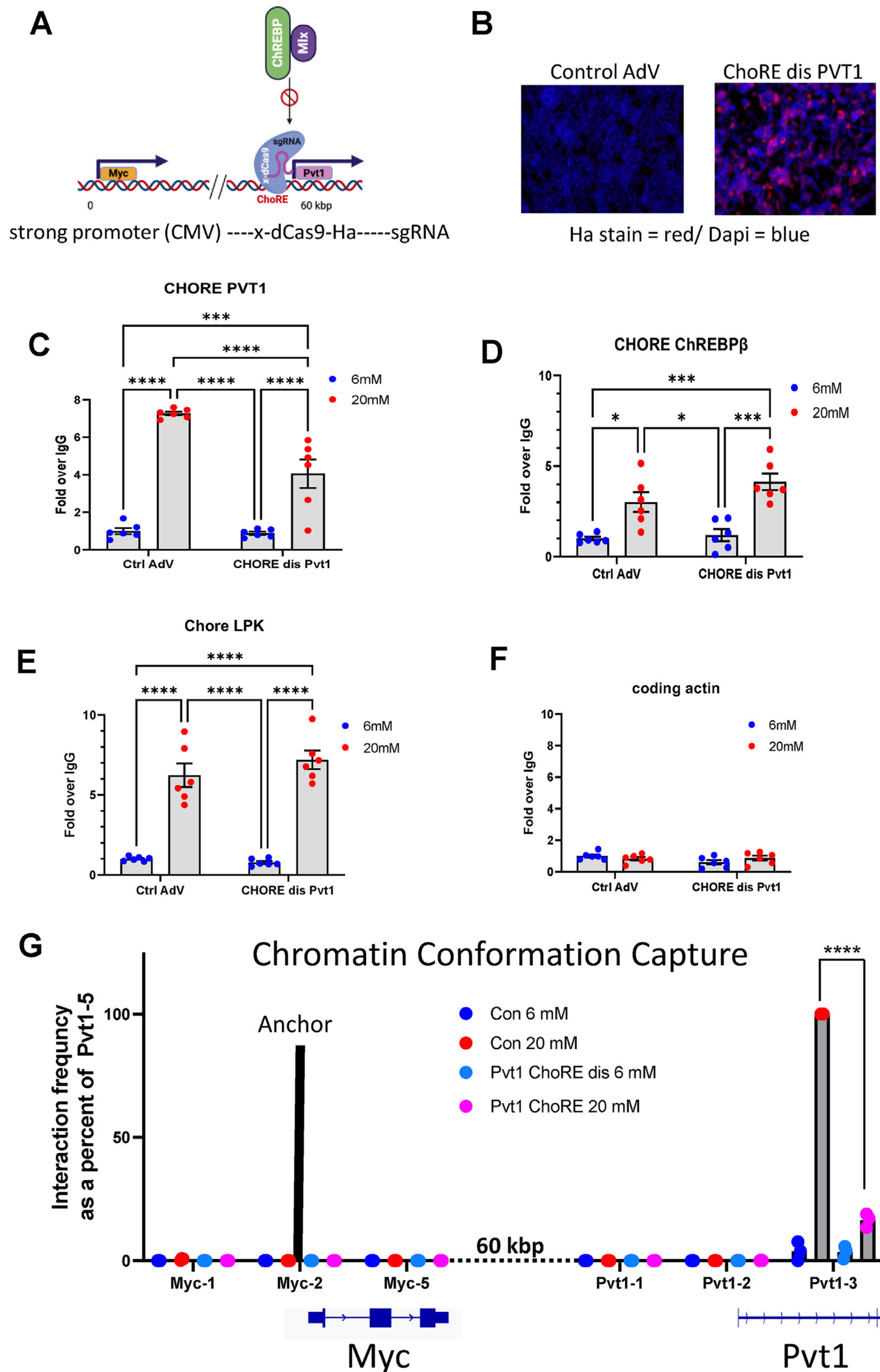

Figure 3: Specific disruption of the *Pvt1* ChoRE results in decreased interaction between the *Myc* and *Pvt1* promoters. (A) Schema showing construction of adenovirus expressing HA-tagged x-dCas9 and sgRNA and targeting the *Pvt1* ChoRE to block recruitment of ChREBP. Image created with BioRender.com (B) Indirect immunofluorescence using an antibody against the HA tag to determine transduction efficiency of the ChoRE disruptor adenovirus (ChoRE dis Pvt1) in INS-1 cells. The control adenovirus did not express HA tag. Transduction efficiency was near 100%. (C—F) INS-1 cells were transduced with a control adenovirus (Ctrl Adv) or the ChoRE disruptor adenovirus at an MOI of 150, cultured for 56 h, then cultured in either 6 or 20 mM glucose for an additional 16 h, and a ChIP assay was performed on ChoREs of the *Pvt1*, ChREBPβ, or *Pklr* (LPK) genes, or the coding region of α-Actin as a negative control. (G) Chromatin conformation capture assay with INS-1 cells treated with indicated adenovirus for 56 h, and then cultured in the indicated glucose concentrations for the final 16 h. All data are means ± SEM. N = 3—6, *p < 0.05, ***, p < 0.005, ****p < 0001.

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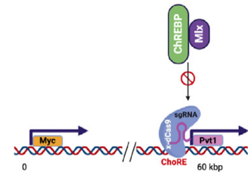

Figure 4: Specific disruption of the *Pvt1* ChoRE attenuates glucose-stimulated transcription and proliferation in β-cells. (A—D) INS-1 cells were transduced with a control adenovirus or the *Pvt1* ChoRE disruptor adenovirus at an MOI of 150 and cultured for 56 h and then treated with either 6 or 20 mM glucose for 16 h. RT-PCR was performed on isolated total RNA to determine relative mRNA abundance of the indicated glucose-responsive genes. (E—H) Isolated rat islets were dispersed, transduced with control or *Pvt1* ChoRE disruptor adenovirus, cultured for 56 h, and then treated with either 6 or 20 mM glucose, and mRNA abundance was determined as described above. (I—J) Isolated rat islets were dispersed and transduced with control or Pvt1 ChoRE disruptor adenovirus as described above, and then cultured in either 6 or 20 mM glucose for a total of 72 h, and were fixed and immunolabeled with antibodies against Ki67 and insulin. Insulin- and Ki67-positive cells were quantified in (J). Results shown are the means ± SEM. N = 4—6, *p < 0.05, **, p < 0.001, ***, p < 0.005, ****p < 0001.

Collectively, these findings support a novel mechanism for β-cell functional and mitogenic competence, in which glucose- and ChREBP-dependent DNA looping occurs *via* an interaction between a ChoRE in a distal downstream locus near the *Pvt1* gene, with the proximal *Myc* promoter. In keeping with current models of Myc function [47], glucose likely initiates a complex comprised of a phase-separated condensate containing mediator, Myc and ChREBP that interacts transiently with the Myc promoter in a "kiss and run" or "kiss and kick" manner [48,49] that ultimately provides the components and the energy to promote transcriptional cycling, resulting in increased Myc expression and glucose-stimulated β-cell proliferation (see Graphical abstract).

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3. DISCUSSION

MYC has been recognized as an important transcription factor in β-cell biology for over two decades. The upregulation of MYC in β-cells was first described as an important component of glucose toxicity [15,17,18,50]. We described MYC upregulation as essential for β-cell proliferation and adaptive expansion of β-cells in response to a high fat diet, but only if expressed at modest levels [8,12]. Thus, a 3- to 5-fold increase appears to be necessary for nearly all forms of increased β-cell proliferation. Indeed, harmine, the first-in-class drug that promotes human β-cell proliferation *in vitro* and *in vivo*, was originally identified in a *Myc* promoter screen [5]. Importantly, expression levels higher than the modest physiological induction leads to apoptosis, which likely explains the link between hyperglycemia, high MYC expression levels and glucose toxicity [8,18]. Since glucose is a natural and systemic β-cell mitogen, much effort has gone into describing mechanisms that drive the proliferative effect of glucose on β-cells [34,51–57]. We have reported previously that ChREBP and MYC are necessary both for glucose-mediated β-cell proliferation as well as for adaptive β-cell mass expansion in response to a high-fat diet [9,10,12,13]. Here, we describe the mechanistic relationship between ChREBP and MYC in the induction of the *Myc* gene by glucose. We found that: 1) the glucose response of the *Myc* gene requires ChREBP; 2) the nearest carbohydrate response element (ChoRE) is located in the promoter of the *Pvt1* gene, ∼60,000 bp downstream of the *Myc* gene, which is syntenic and highly conserved among rodents and primates; 3) expression of the *Pvt1* lncRNA is glucose-responsive; 4) glucose promotes increased interactions between the promoter regions of *Pvt1* and *Myc* that are dependent on the recruitment of ChREBP; and, 5) disruption of the *Pvt1*:*Myc* interaction attenuates glucose-stimulated β-cell proliferation in isolated rat islet cells.

The requirement for ChREBP in the glucose-mediated induction of the *Myc* gene has not been demonstrated previously. However, it is perhaps not surprising, considering that ChREBP, a transcription factor whose activity is activated by glucose, is necessary for glucose-stimulated β-cell proliferation as well as for adaptive expansion of β-cells in response to a high-fat diet [9,10,13,24]. Loss- and gain-of-function experiments suggest that ChREBP, originally cloned as a lipogenic regulator in liver, is necessary for the anabolic metabolism required for increasing the biomass of proliferating β-cells [9,58]. Furthermore, very low, constant levels of MYC activity are required for ChREBP activity in both liver and β-cells [28–30]. For example, genetic or pharmacological deletion or inhibition of MYC results in loss of the ability of ChREBP to be recruited to the ChoREs of glucose responsive genes including *Pklr*, *Prok2* and *Gpdh* [30]. In addition, conditional, β-cell-specific knockout of *Myc* prevents β-cell proliferation and β-cell mass expansion in response to a high-fat diet [12]. Thus, MYC and ChREBP are mutually dependent on each other's activities, and both are necessary for the adaptive and proliferative responses of β-cells to hypernutrition.

The relationship between MYC and ChREBP is not surprising considering the interrelated roles of the MYC and MLX network of transcription factors and their overlapping roles in anabolic cellular functions including metabolism, proliferation, and protein synthesis to name a few [41]. The MYC family of transcription factors, including MYC, MAX, MAD, MDI, MXD(1–4), form heterogeneous pairs of heterodimers with different transcriptional activities that regulate a large number of genes involved in the anabolic processes mentioned above [41,59]. The MLX family, which is closely related to the Myc family (indeed MLX stands for "MAX like"), including ChREBP, MondoA and their heterodimer pair, MLX, along with MXD(1–4) negative regulators that are shared with the MYC family, influence a much smaller number of genes that are broadly related to metabolism and more specifically related to *de novo* lipogenesis [24,41]. MYC family members bind to E-boxes (CACGTG) and MLX members bind to ChoREs, which are 2 degenerate E-boxes separated by 5 bp (roughly, CAYGNG[N5]CNCRTG) [36,38,41]. There is a large overlap in the genes that each family regulates, and indeed there is a non-random distribution of E-boxes and ChoREs across the genome such that many target genes contain both motifs [41,60]. MYC may bind to some ChoREs since MYC may bind to non-canonical E-boxes and some ChoREs have half sites that are perfect or near perfect E-boxes. Thus, some target genes are MYC-specific, some MLX-specific and some are regulated by both sets of transcription factors, and some elements may bind both MYC and MLX family members. In the present case, the *Myc* gene has no ChoREs, while the *Pvt1* promoter and gene body have numerous E-boxes and a single ChoRE in the proximal promoter. Note that we cannot exclude the possibility that MYC shares binding with the Pvt-1 ChoRE in response to glucose.

Pvt1 is a lncRNA most studied in the context of cancer biology [19,21–23], but is also implicated in end-stage diabetic renal disease [61,62]. *Pvt1* has up to 16 exons, several transcription start sites, dozens of alternatively spliced isoforms, and harbors a family of miRNAs and intragenic enhancers. It is often co-amplified with *Myc* in tumors [19,21–23]. Furthermore, Pvt1 lncRNA promotes the stability of Myc Protein as one of several post-transcriptional, Myc-promoting functions and so is considered an oncogene in some contexts [19]. By contrast, Chang and colleagues demonstrated a role for *Pvt1* as a tumor suppressor in the context of several human cancer cell lines [20]. In these cases, intragenic enhancers found in the very large (∼30,000 bp) coding region of the *Pvt1* gene body compete for interactions with either the *Myc* or the *Pvt1* promoter. Thus, in a setting where *Pvt1* acts as a tumor suppressor, silencing the *Pvt1* promoter increases the enhancer interaction with the *Myc* promoter, thereby increasing *Myc* transcription and downstream transformative properties.

In contrast to cancer biology, in the present case the interaction between *Pvt1* and *Myc* are in the context of a normal, physiological increase in *Myc* expression in response to glucose, that in turn results in modest expression of Myc that promotes a controlled, adaptive, proliferative response. Whereas *Myc* is an important oncogene, it is also an important anabolic transcription factor that plays crucial roles in normal physiology. Future studies should include extending the role of *Pvt1* in the glucose-response of human β-cells, and to determine if the expression of *Pvt1* lncRNA plays a role independent of *Myc* with respect to human β-cell biology. Overall, glucose-mediated induction of the Myc gene in β-cells is mediated by recruitment of ChREBP to the *Pvt1* promoter and requires a DNA looping between *Pvt1* and *Myc*.

4. MATERIALS AND METHODS

4.1. Cell culture and rat islet isolation

Isolated islets from three-month-old Sprague Dawley rats were obtained from the islet isolation core at Joslin. The islets were cultured as previously described [10]. INS-1–derived 832/13 rat insulinoma cells were maintained in RPMI 1640 medium with 10% FBS, 10 mM HEPES, 2 mM l-glutamine, 1 mM sodium pyruvate, and 50 mM β-mercaptoethanol, 100 U/mL penicillin, 100 mg/mL streptomycin and further supplemented with 11 mM glucose, at 37 °C in a 5% CO_2 incubator [29,32]. To study the glucose response, cells were incubated overnight

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in low glucose (5.5 mM), subsequent increases in glucose were added to achieve the indicated concentrations.

4.1.1. Quantitative reverse transcription PCR

Total RNA was extracted using the Qiagen RNeasy micro kit, reverse transcription was performed using the MMLV reverse transcriptase (Promega), following by real-time PCR with the SYBER-green reagent (BioRad) as previously described [28,29]. Quantitative PCR was performed on the QuantStudio5 using Syber-Green (BioRad) and analysis was performed using the $\Delta\Delta$Ct method. The primer sequences used are shown in Supplementary Table 1.

4.1.2. Western blots

INS-1 832/13 cell protein extracts were separated on SDS-PAGE and membranes incubated with cMyc antibody (LifeSpan Cat# LS-C49460) or β-actin (Sigma, Cat# A2066) at 1:500 and 1:1000 respectively, followed by peroxidase-conjugated secondary antibodies and chemiluminescence detection.

4.1.3. siRNA transfections

SMARTpool small interfering RNA (siRNA) duplexes (Dharmacon, Lafeyette, CO) were transfected into INS-1 cells for 48 h in the presence of Dharmafect Reagent 1 (Dharmacon). Duplexes were targeted to 19 to 21 bp regions of the rat ChREBP cDNA sequence (Cat. No. L-092970). A pool of duplexes with no known sequence homology or biological effect (siControl) was used as a control. Cells were cultured for 48 h in RPMI 1640 medium for INS-1 cells as described [10] with 5% FCS, followed by 6 h in low glucose (3 mmol/L). Glucose was then added to adjust the final glucose concentration to 15 mmol/L glucose for the high glucose groups, and the cells were cultured for an additional 18 h [10].

4.2. Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assays were performed as previously described in [63]. Briefly, 20 × 10́6 INS-1 cells were fixed and lysed to obtain chromatin using the Active Motif (Carlsbad, CA) chromatin shearing kit. Chromatin was precipitated by incubation with 3 μg of ChREBP antibody (Novus Biologicals), or c-Myc antibody (Santa Cruz Biotechnology), or rabbit IgG (Abcam) followed by separation with protein G magnetic beads (Active Motif). Binding was analyzed by real-time PCR. Primer sequences are shown in Supplementary Table 1.

4.3. Immunostaining

Cells were plated on glass bottom plated coated with Poly d-Lysine and Laminin. Isolated rat islet cells or INS-1 cells were transduced at a MOI of 150 of the adenoviruses indicated and were cultured as described above for 72 h. Cells were then rinsed with PBS and fixed in 4% paraformaldehyde, and β-cell proliferation was determined by immunolabeling for Ki67 (ThermoScientific, MA5-14520, 1:250), insulin (GeneTex, GTX27842, 1:1000), or HA (Cell Signaling, 2367S, 1:250). Confocal images were acquired using an Agilent BioTek Cytation 10 confocal imager.

4.4. Chromosome conformation capture (3C)

3C was performed as described in [64]. Briefly, 10́6 INS-1 cells were transduced with 150 MOI of the indicated adenovirus and cultured in the indicated concentrations of glucose. After 72 h, they were trypsinized and crosslinked in 2% formaldehyde. Chromatin was digested with Dpn II (NEB) and then ligated with the T4 ligase (Promega). DNA was then purified and processed for qPCR analysis with the primers in Supplementary Table 1.

4.5. Adenoviral-mediated ChoRE disruption

Pvt1 disruptor gRNA (Forward, CACCCAGGCCGGCCGCGTGTGTCCTGG; Reverse AAACCCAGGACACACGCGGCCGGCCTG) was cloned using Addgene kit# 1000000155 as described in [42]. Briefly, using the Bsa I (NEB) restriction enzyme and T4 ligase (Promega), plasmids KN701, HB401, KO401 and IQ013 were combined and a double stranded oligonucleotide using Gateway LR Clonase II Mix (Cat# 57028, Thermofisher) to generate the adenoviral disruptor adenovirus expressing x-dCas9 and an sgRNA targeting the *Pvt1* ChoRE. Adenovirus were transduced at an MOI of 150 with INS-1 and dispersed rat islets.

4.6. Statistics

All studies were performed with a minimum of three independent experiments. Data presented in this study are means ± standard error of the mean (SEM). Statistical analysis was performed using One- or Two-way ANOVA on GraphPad (Prism) V9.2.

AUTHOR CONTRIBUTIONS

Conceptualization, L.S.K. and D.K.S.; Methodology, L.S.K., G.B., P.W., J.M.H, C.B.N, D.K.S.; Investigation, L.S.K., G.B., P.W., P.Z., H.L., L.L.; Writing-Original Draft, L.S.K. and D.K.S.; Writing- Review and Editing, L.S.K., D.K.S., A.F.S., A.G-O; Supervision, C.B.N., A.F.S., A. G-O, D.K.S.; Funding Acquisition A.F.S., A.G-O., and D.K.S.

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DECLARATION OF COMPETING INTEREST

The Icahn School of Medicine at Mount Sinai has filed patents on related work on behalf of AFS, PW and AGO. The other authors declare that they have no conflicts of interest for this study.

DATA AVAILABILITY

Data will be made available on request.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molmet.2023.101848.

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