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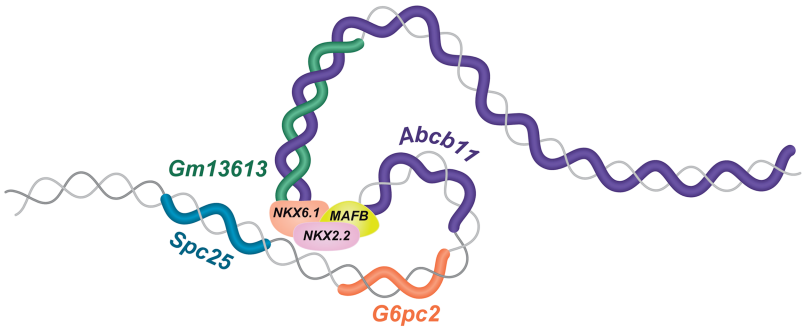

An Enhancer Within *Abcb11* Regulates *G6pc2* in C57BL/6 Mouse Pancreatic Islets

Mark P. Keller,[1] Emily M. Hawes,[2] Kathryn L. Schueler,[1] Donnie S. Stapleton,[1] Kelly A. Mitok,[1] Shane P. Simonett,[1] James K. Oeser,[2] Leesa L. Sampson,[3] Alan D. Attie,[1,4,5] Mark A. Magnuson,[2,3] and Richard M. O'Brien[2]

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***G6PC2* is predominantly expressed in pancreatic islet β-cells where it encodes a glucose-6-phosphatase catalytic subunit that modulates the sensitivity of insulin secretion to glucose by opposing the action of glucokinase, thereby regulating fasting blood glucose (FBG). Prior studies have shown that the *G6pc2* promoter alone is unable to confer sustained islet-specific gene expression in mice, suggesting the existence of distal enhancers that regulate *G6pc2* expression. Using information from both mice and humans and knowledge that single nucleotide polymorphisms (SNPs) both within and near *G6PC2* are associated with variations in FBG in humans, we identified several putative enhancers 3′ of *G6pc2*. One region, herein referred to as enhancer I, resides in the 25th intron of *Abcb11* and binds multiple islet-enriched transcription factors. CRISPR-mediated deletion of enhancer I in C57BL/6 mice had selective effects on the expression of genes near the *G6pc2* locus. In isolated islets, *G6pc2* and *Spc25* expression were reduced ∼50%, and *Gm13613* expression was abolished, whereas *Cers6* and *nostrin* expression were unaffected. This partial reduction in *G6pc2* expression enhanced islet insulin secretion at basal glucose concentrations but did not affect FBG or glucose tolerance in vivo, consistent with the absence of a phenotype in *G6pc2* heterozygous C57BL/6 mice.**

ARTICLE HIGHLIGHTS

- Genome-wide association study data suggest that single nucleotide polymorphisms (SNPs) in distal *G6PC2* enhancers may contribute to the association signal between *G6PC2* and fasting blood glucose (FBG); thus, the aim of this study was to identify these distal enhancers.
- We identified a strong distal enhancer in the neighboring *ABCB11* gene, showed that its deletion reduces *G6pc2* expression in mice, but found that a SNP within this particular enhancer does not contribute to the association signal between *G6PC2* and FBG.
- Several other distal enhancers were identified in addition to the enhancer in the neighboring *ABCB11* gene.
- SNPs within these other enhancers likely contribute to the association signal between *G6PC2* and FBG.

G6PC2 catalyzes the hydrolysis of glucose-6-phosphate to glucose and inorganic phosphate within the lumen of the endoplasmic reticulum (1). *G6PC2* is mainly expressed in pancreatic islet β-cells (2) where it has been shown to oppose glucokinase, the islet glucose sensor, by creating a futile substrate cycle (3). Deletion of *G6pc2* shifts the dose-response curve for glucose-stimulated insulin secretion (GSIS) to the left, resulting in decreased fasting blood glucose (FBG) in *G6pc2* knockout (KO) mice (4–6), an observation that is consistent with genome-wide association studies (GWAS) in humans that have identified strong association signals in and near *G6PC2* that are linked with variations in FBG (7,8).

Prior studies, which sought to identify the regulatory elements controlling *G6pc2* expression, identified multiple

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islet-enriched transcription factors that bind near the proximal *G6pc2* promoter and drive expression in β-cell lines (9–11). While the proximal ∼300-base-pair (bp) *G6pc2* promoter region is sufficient to drive islet-specific expression of a transgene in newborn mice (12), expression was not sustained in adult animals. Similarly, while fusion gene analyses have identified several weak enhancers 5′ of the *G6pc2* transcription start site (13), an ∼15-kbp transgene containing those enhancers, even though it exhibits strong islet-specific expression in newborn mice, was unable to sustain that expression in adult animals (13). These data led us to consider that one or more distal enhancers may also contribute to *G6pc2* expression in adult mouse islets.

In this study, we describe the identification and analysis of several putative enhancers 3′ of the *G6PC2* gene. Through a combination of fusion gene studies in a β-cell line and clustered regularly interspaced short palindromic repeats (CRISPR) deletion in C57BL/6 mice, we determine that one of these regions, designated enhancer I, which is located within intron 25 of the neighboring gene *Abcb11*, functions as a distal enhancer for *G6pc2*.

RESEARCH DESIGN AND METHODS

CRISPR Mutagenesis and Mouse Husbandry

All animal experimentation was approved by the Vanderbilt University or University of Wisconsin animal care and use committees. The *G6pc2* enhancer I KO allele Rr327em1Mgn (MGI:7407248), was produced by CRISPR gene editing as described in the Supplementary Material. Mice were fed a regular rodent chow diet (Purina 5008) or a Western-style diet (WD) high in fat and sucrose (TD.08811; Evigo Teklad) from weaning to ∼18 weeks of age when they were sacrificed for islet isolation. Food and water were provided ad libitum for all mice.

In Vivo Physiological Measurements and Procedures on Isolated Pancreatic Islets

Oral glucose tolerance tests, pancreatic islet isolation, ex vivo insulin secretion measurements, RNA sequencing, and assay for transposase-accessible chromatin sequencing (ATAC-seq) were all performed as previously described (14–16).

Fusion Gene Analyses

The design of fusion genes containing putative *G6PC2* enhancer sequences is described in the Supplementary Material. βTC-3 cells were cultured and transfected using lipofectamine (Promega) as previously described (17).

Analysis of Islet Gene Expression

cDNA generation using islet RNA and quantitation of gene expression using PCR were performed as previously described (16,18). PCR primer sequences are provided in Supplementary Material.

Statistical Analysis

Fusion gene and gene expression data were analyzed using a two-sample Student *t* test, assuming equal variance. Islet insulin secretion and insulin content measurements for wild-type (WT) mice and mice that were homozygous null for the *G6pc2* enhancer I deletion ($G6pc2^{\Delta EnhI/\Delta EnhI}$); body weight, glucose, and insulin measures at multiple ages; and oral glucose tolerance test curves were analyzed using a repeated-measures two-way ANOVA with the Geisser-Greenhouse correction and Šidák multiple comparisons test, with individual variances computed for each comparison within sex. Areas under the curve were analyzed using unpaired *t* tests with Welch correction.

Data and Resource Availability

All data generated or analyzed during this study are included in the published article and its online supplementary files.

RESULTS

Multiple Putative Enhancers Are Located 3′ of the *G6PC2* Gene

A regional association plot using human GWAS data for FBG identifies several SNPs strongly associated with FBG at the *G6PC2/ABCB11* gene locus (Fig. 1*A*). By making symbol sizes proportional to the $-\log_{10} P$ value, these data emphasize those SNPs with the strongest association and demonstrate that the linkage broadly spans the *G6PC2/ABCB11* locus.

The ATAC-seq assay has previously identified regions of open chromatin at the *G6PC2/ABCB11* gene locus in human islets (19) (Fig. 1*B*) and the equivalent *G6pc2/Abcb11* locus in islets from eight genetically distinct mouse strains (16) (Fig. 1*C*). Based on sequence conservation between species, earlier studies had identified four putative enhancers 5′ and within the mouse *G6pc2* gene, designated enhancers A–D, as well as two putative enhancers 3′ of the last *G6PC2* exon, designated enhancers E and F (13) (Fig. 1*C*). ATAC-seq analyses demonstrated that the chromatin in the vicinity of enhancers E and F was open in human (Fig. 1*B*) and mouse islets (Fig. 1*C*). In human islets, ATAC-seq identified an additional open region 3′ of enhancer F, designated enhancer G (Fig. 1*B*). In addition, in both human (Fig. 1*B*) and mouse (Fig. 1*C*) islets, ATAC-seq identified two open chromatin regions in the 25th intron of the *ABCB11* gene, which we designated as putative enhancers H and I. The ATAC peak at enhancer I showed remarkable differences in amplitude among the eight mouse strains, suggesting that genetic differences among the mice result in differential chromatin accessibility. Taken together, the landscape of open chromatin at the *G6PC2/ABCB11* locus is highly conserved between mouse and human.

To compare the relative ability of putative human *G6PC2* enhancers E–I to promote gene transcription, these regions were isolated and ligated 5′ of a *G6PC2*-luciferase fusion

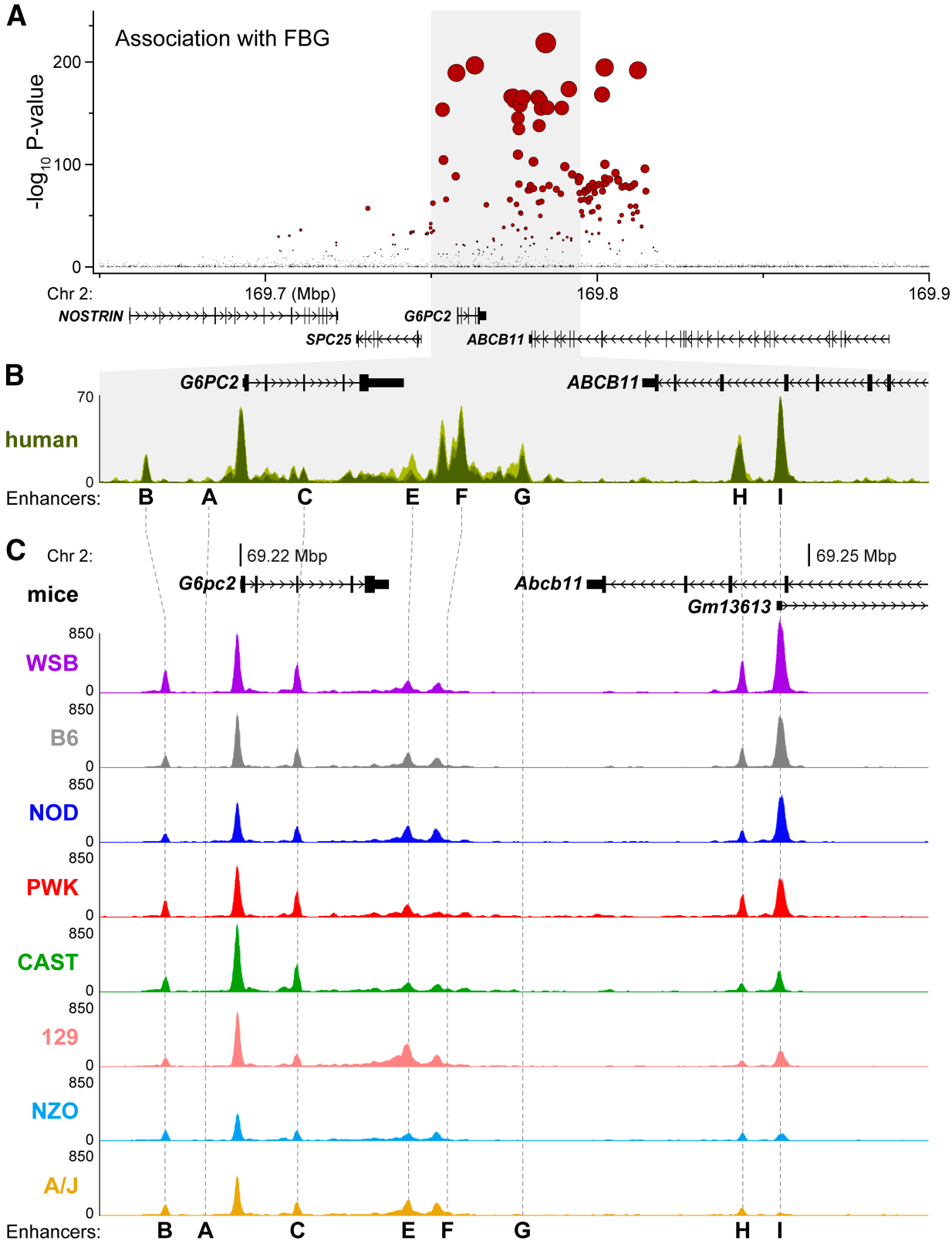

Figure 1—ATAC-seq reveals the existence of multiple putative enhancers 3′ of the *G6PC2* gene in mouse and human islets. *A*: Regional association plot from human GWAS for FBG at the *G6PC2/ABCB11* gene locus from the Type 2 Diabetes Knowledge Portal (https://t2d.hugeamp.org/). Symbol size is proportional to the $-\log_{10} P$ value, emphasizing SNPs with the strongest association. *B* and *C*: ATAC-seq was used to identify regions of open chromatin at the *G6PC2/ABCB11* gene locus in islets from human donors (19) or eight genetically distinct mouse strains. Putative *G6PC2* enhancers are denoted by the letters A, B, C, E, F, G, H, and I (*B*). Regions that are syntenic to these

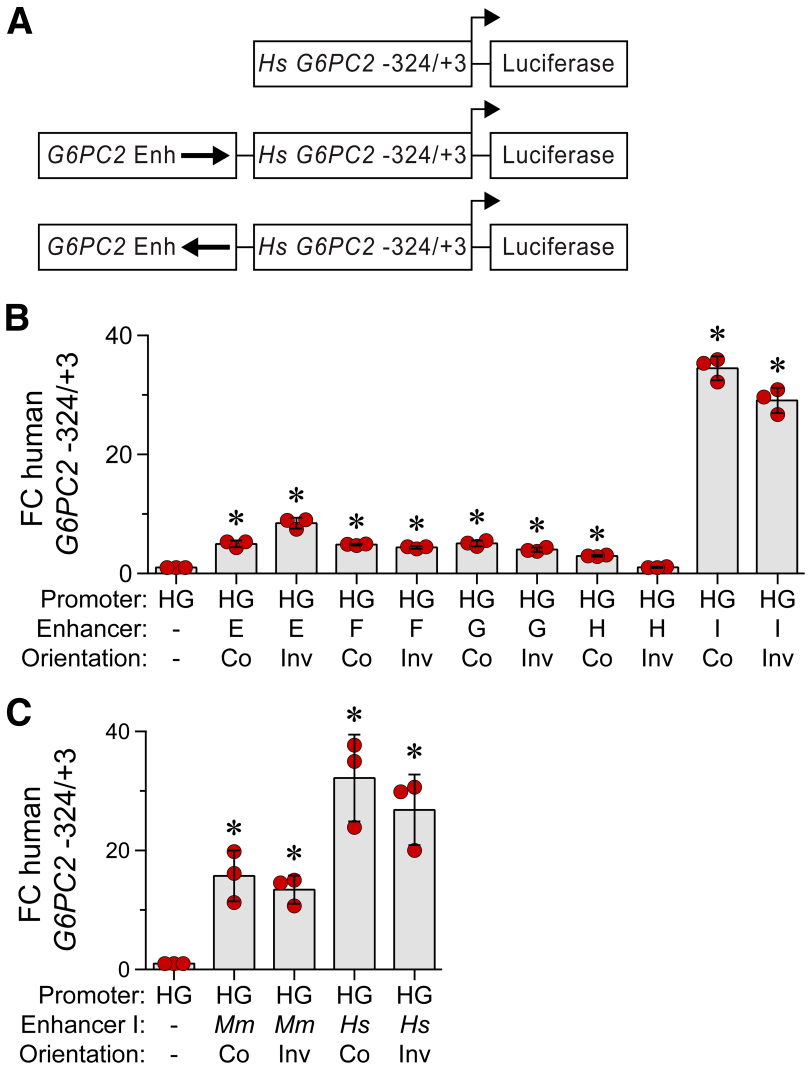

Figure 2—Multiple putative enhancers are located 3′ of the *G6PC2* gene. *A*: βTC-3 cells were transiently cotransfected, as described in the *Research Design and Methods*, with an expression vector encoding Renilla luciferase (0.5 μg) and the indicated firefly luciferase fusion genes (2 μg) in which putative human and mouse enhancer sequences were ligated to the proximal human *G6PC2* promoter in the correct (Co) or inverted (Inv) orientation relative to their orientation in human chromosome 2. Following transfection, cells were incubated for 18–20 h in serum-containing medium. The cells were then harvested, and luciferase activity was assayed as described in the *Research Design and Methods*. *B* and *C*: Results are presented as the ratio of firefly:Renilla luciferase activity relative to the vector control, are expressed as fold change (FC), and represent the mean ± SEM of three experiments. *B* and *C*: Significant differences were found between the transcriptional activity of the proximal human *G6PC2* promoter and fusion genes containing putative enhancer sequences. *$P < 0.05$ by Student *t* test. *Hs*, *Homo sapiens*; Enh, enhancer; HG, *H. sapiens G6PC2*; *Mm*, *Mus musculus*.

gene containing the proximal −324 to +3 promoter sequence (Fig. 2*A*). Luciferase expression directed by these fusion genes was then analyzed by transient transfection of islet-derived βTC-3 cells. All five regions enhanced reporter gene expression beyond that observed using only the −324/+3 *G6PC2* promoter, with enhancer I demonstrating the strongest effect (∼30-fold increase) (Fig. 2*B*). The effects were independent of orientation consistent with the classical definition of an enhancer (20,21) (Fig. 2*B*). Sequence analyses revealed only ∼65% conservation of enhancer I in mice (Supplementary Fig. 1). Nonetheless, the mouse enhancer I sequence also exhibited strong transcriptional activity when ligated to the proximal human *G6PC2* promoter (Fig. 2*C*).

G6PC2 Enhancer I Binds Multiple Islet-Enriched Transcription Factors

Human islet chromatin immunoprecipitation sequencing data demonstrate that multiple islet-enriched transcription

enhancer regions in mouse are shown by vertical dotted lines (*C*), illustrating that ATAC peaks at enhancer loci are conserved in human and mouse islets. Putative *G6pc2* enhancer D, which is located 5′ of enhancer B and not shown in panels *B* and *C*, is conserved between mice and rats but not humans. Chr, chromosome.

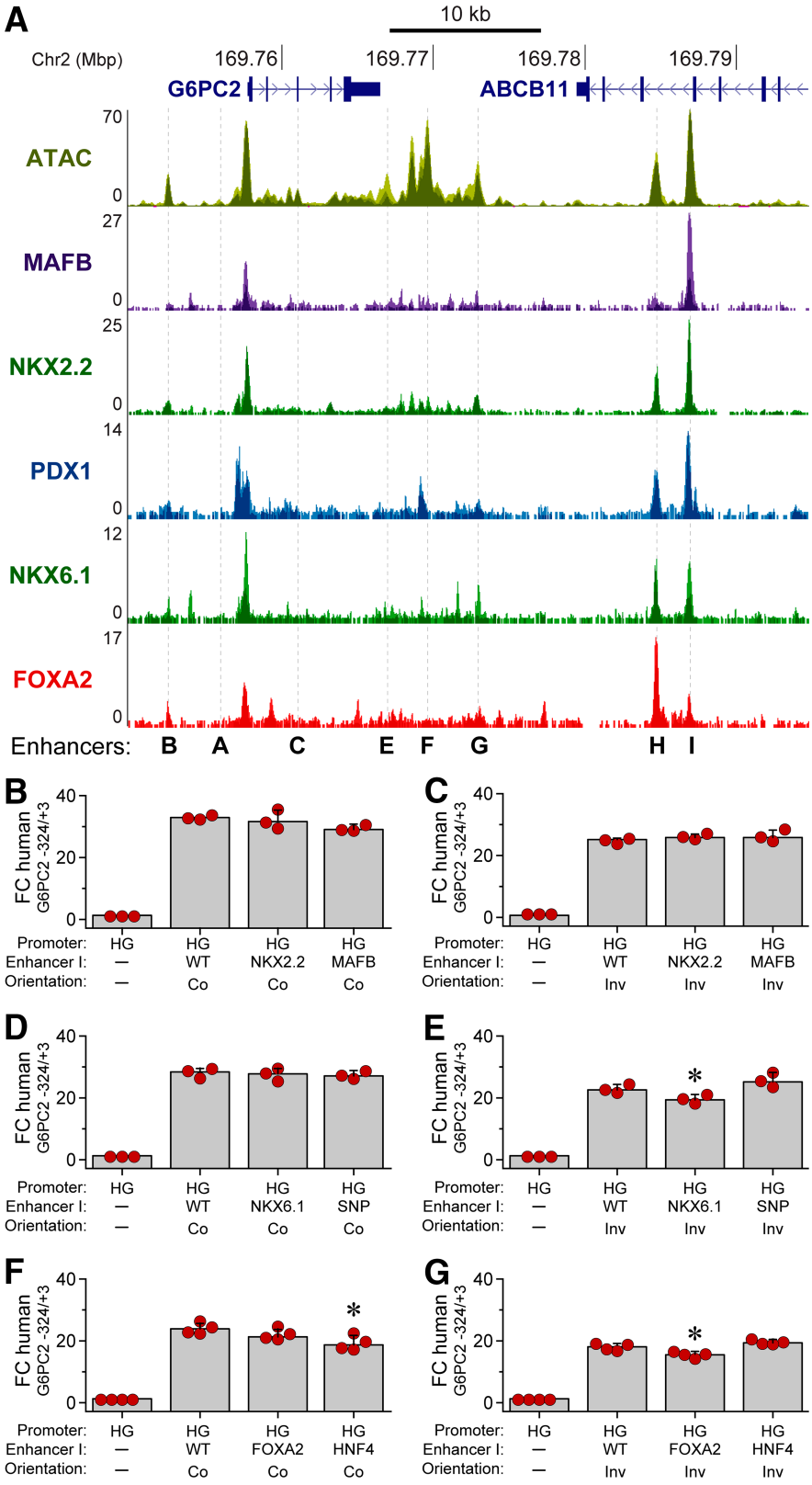

Figure 3—*G6PC2* enhancer I binds multiple islet-enriched transcription factors. *A*: The top trace represents the results of the ATAC-seq that was used to identify regions of open chromatin at the *G6PC2/ABCB11* gene locus in islets from human donors (19). The lower traces show the results of chromatin immunoprecipitation sequencing for the indicated key transcription factors in islets from human donors (22). The data show that enhancer I is a strong binding site for MAFB, NXK2.2, NKX6.1, PDX1, and FOXA2. Enhancer H is also strongly bound by all these factors, with the exception of MAFB. All these transcription factors bind to the proximal promoter of *G6PC2*. *B–G*: βTC-3

factors bind enhancer I (22) (Fig. 3*A*). Sequence analyses revealed that enhancer I contains perfect consensus binding sites for MAFB, NKX2.2, NKX6.1, FOXA2, and HNF-4 (Supplementary Fig. 1). In the context of the proximal *G6PC2* promoter, mutation of the MAFB and NKX2.2 binding sites did not impair the transcriptional activity of enhancer I (Fig. 3*B* and *C*), while mutation of the NKX6.1, FOXA2, and HNF-4 binding sites only mildly affected enhancer activity and only in one orientation (Fig. 3*D*–*G*). These results strikingly contrast with *G6pc2* promoter mutations, where the loss of individual transcription factor binding sites markedly impairs promoter activity (1).

The rs56100844 SNP Does Not Affect Enhancer I Activity

Using Type 2 Diabetes Knowledge Portal (https://t2d.hugeamp.org/) data, a single SNP, rs56100844, was identified in enhancer I that is strongly associated with variations in random glucose ($P < 2.37E-52$), HbA_{1c} ($P < 6.99E-39$), FBG adjusted for BMI ($P < 2.07E-41$), and FBG alone ($P < 2.15E-6$). However, rs56100844 did not affect the activity of enhancer I when assessed in the context of the proximal *G6PC2* promoter (Fig. 3*D* and *E*).

Deletion of *G6pc2* Enhancer I Impairs Endogenous *G6pc2* Gene Expression

Since these data indicated that enhancer I was a strong candidate for a 3′ *G6pc2* enhancer, we performed CRISPR mutagenesis in C57BL/6J mouse embryos to delete a 653-bp region containing enhancer I (Supplementary Fig. 2*A*–*C*). *G6pc2*$^{\Delta EnhI/\Delta EnhI}$ mice were viable, and the allele passed in the expected Mendelian ratios.

The effect of deleting enhancer I on endogenous *G6pc2* gene expression was assessed using real-time PCR in islets isolated from *G6pc2*$^{\Delta EnhI/\Delta EnhI}$ mice after 18 weeks on a WD. We also quantitated the expression of *Cers6*, *nostrin*, *Spc25*, *Abcb11*, and *Gm13613*, a noncoding RNA, to determine whether enhancer I regulates the expression of other genes in the vicinity of the *G6pc2* locus (Supplementary Fig. 2*D*). Deletion of enhancer I reduced both *G6pc2* and *Spc25* expression without affecting *Cers6* or *nostrin* expression (Fig. 4*A*). The expression of *Gm13613* was abolished (Fig. 4*A*), suggesting that enhancer I also contains the *Gm13613* promoter (Supplementary Fig. 2*D*). Islet expression of *Abcb11* was undetected by quantitative PCR (data not shown). Similar trends were observed in islets isolated from chow-fed mice, although the decrease in *G6pc2* expression was not statistically significant (Supplementary Fig. 3). Hepatic *Abcb11* expression was unchanged in *G6pc2*$^{\Delta EnhI/\Delta EnhI}$ mice (Supplementary Fig. 4), suggesting that this enhancer regulates islet rather than hepatic gene expression.

Deletion of *G6pc2* Enhancer I Enhances GSIS at Basal Glucose

The shift to the left in the dose-response curve for GSIS that occurs following *G6pc2* deletion results not only in decreased FBG in *G6pc2* KO mice (4–6) but also in enhanced GSIS in *G6pc2* KO islets at basal, but not high, glucose levels (5). While total islet insulin levels were unchanged in both male and female *G6pc2*$^{\Delta EnhI/\Delta EnhI}$ mouse islets (Fig. 4*B*), islets from WD-fed male *G6pc2*$^{\Delta EnhI/\Delta EnhI}$ mice showed enhanced insulin secretion evoked by basal (3.3 mmol/L) glucose levels relative to islets from WT mice (Fig. 4*C*). Insulin secretion in response to higher glucose concentrations (8.3 or 16.7 mmol/L) or other conditions (amino acids, glucagon-like peptide 1, and fatty acid) was not different between male WT and *G6pc2*$^{\Delta EnhI/\Delta EnhI}$ mice (Fig. 4*C*). Female *G6pc2*$^{\Delta EnhI/\Delta EnhI}$ mice did not show enhanced secretion in response to submaximal glucose (Fig. 4*C*). Similar trends were observed in islets isolated from chow-fed mice, although the increase in GSIS at submaximal glucose was not statistically significant (Supplementary Fig. 5).

Deletion of *G6pc2* Enhancer I Does Not Markedly Affect Glucose Homeostasis In Vivo

To examine the effect of enhancer I deletion in vivo, multiple parameters were measured in WD-fed mice. FBG trended lower in male WT versus *G6pc2*$^{\Delta EnhI/\Delta EnhI}$ mice, but overall, deletion of enhancer I did not markedly affect glucose homeostasis (Supplementary Fig. 6). These results were expected because heterozygous deletion of *G6pc2* in C57BL/6J mice, which presumably reduces *G6pc2* expression to a similar level as enhancer I deletion, is insufficient to influence in vivo physiological parameters (5). Similar results were also observed in chow-fed mice (Supplementary Fig. 7).

DISCUSSION

The experiments described here demonstrate that several distal enhancers exist 3′ of the *G6pc2* gene and that one specific region, enhancer I, which lies nearly 30 kbp downstream of the start site for *G6pc2*, contributes substantially to the expression of *G6pc2*, both in reporter gene assays and in mice. The absence of this enhancer in previous transgenic mouse experiments may explain why the

cells were transiently cotransfected, as described in the *Research Design and Methods*, with an expression vector encoding Renilla luciferase (0.5 μg) and the indicated firefly luciferase fusion genes (2 μg) in which WT or human *G6PC2* enhancer I sequences containing mutations in the indicated binding sites or the alternate allele of rs56100844 were ligated to the proximal *G6PC2* promoter in the correct (Co) or inverted (Inv) orientation relative to their orientation in human chromosome (Chr) 2. Following transfection, cells were incubated for 18–20 h in serum-containing medium. The cells were then harvested, and luciferase activity was assayed as described in the *Research Design and Methods*. Results are presented as the ratio of firefly:Renilla luciferase activity relative to the vector control, are expressed as fold change (FC), and represent the mean ± SEM of three to four experiments. Significant differences were found between the transcriptional activity of the *G6PC2* promoter ligated to the WT enhancer I sequence and fusion genes containing mutated enhancer I sequences. *$P < 0.05$ by Student *t* test. HG, *Homo sapiens G6PC2*.

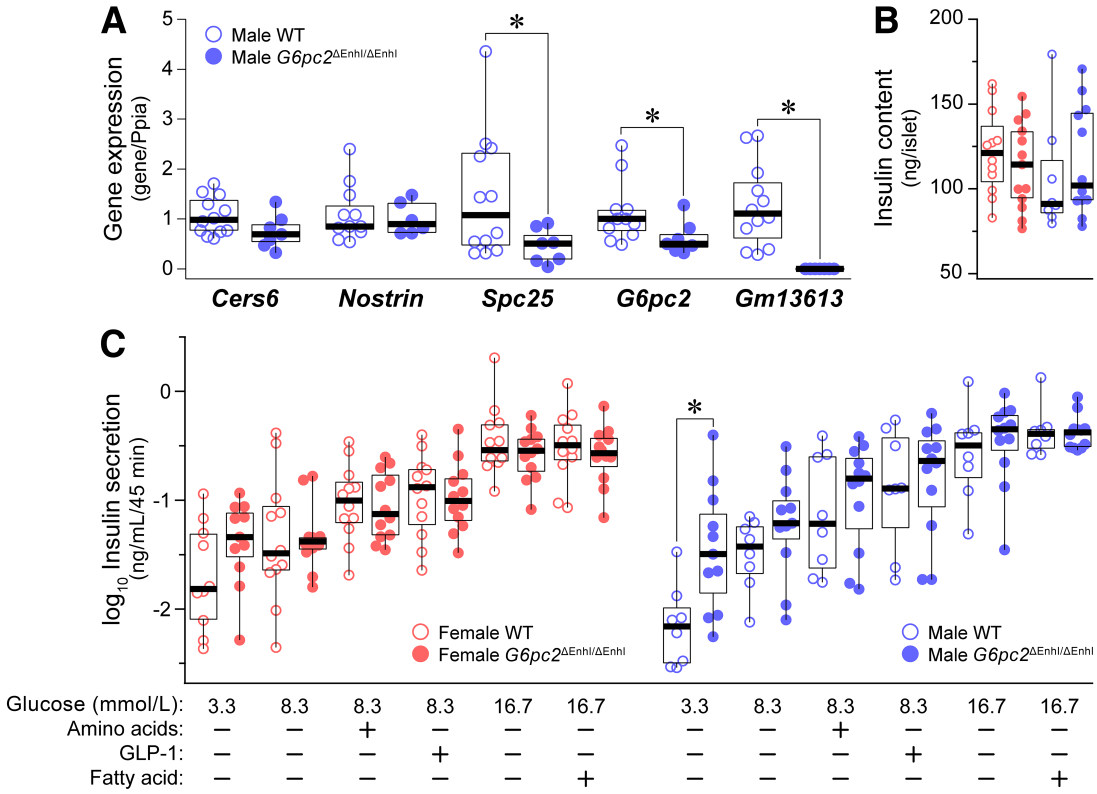

Figure 4—Deletion of *G6pc2* enhancer I impairs endogenous *G6pc2* gene expression and enhances GSIS at basal glucose in islets isolated from WD-fed male mice. *A*: Comparison of *Cers6*, *nostrin*, *Spc25*, *G6pc2, Abcb11*, and *Gm13613* gene expression in islets isolated from 18-week-old WD-fed WT and male *G6pc2*$^{\Delta Enhl/\Delta Enhl}$ mice. Gene expression was quantitated relative to *Ppia* (cyclophilin A) expression, and data are mean ± SEM, with the genotype of animals indicated. *$P < 0.05$ between WT and *G6pc2*$^{\Delta Enhl/\Delta Enhl}$ mice (Student *t* test). *B*: Insulin content in islets used for insulin secretion studies. *C*: Insulin secretion evoked by varying glucose concentrations, amino acids (0.5 mmol/L leucine, 1.25 mmol/L alanine, 2 mmol/L glutamine), the incretin hormone glucagon-like peptide 1 (GLP-1) (100 nmol/L), or a fatty acid (0.5 mmol/L palmitate conjugated to 0.67% BSA) in cultured islets from female and male WT and *G6pc2*$^{\Delta Enhl/\Delta Enhl}$ mice. A significant increase in insulin secretion was observed for islets collected from male *G6pc2*$^{\Delta Enhl/\Delta Enhl}$ mice at 3.3 mmol/L glucose. *$P < 0.05$.

transgenes studied did not maintain expression in adult islets (12,13).

The existence of enhancers 3′ of the *G6PC2* gene was strongly suggested by genetic studies indicating that additional causative SNPs may exist 3′ of the *G6PC2* gene that are not in high linkage disequilibrium with the known causative SNPs (https://t2d.hugeamp.org/) (Fig. 1*A*). Our data suggest that the rs56100844 SNP in enhancer I is not a causative SNP and, instead, that its association with FBG may arise because it is in linkage disequilibrium (R^2 = 0.49) with rs2232323, a nonsynonymous SNP that markedly affects G6PC2 protein stability (23). However, we recognize that a major limitation of our plasmid-based fusion gene analyses in insulinoma cells is that they do not reflect the complexity of enhancer architecture in vivo (24). Therefore, this conclusion will require analyzing the impact of this SNP on *G6PC2* expression in the context of the endogenous locus. While *G6PC2* enhancers E, F, and G were less active than enhancer I in our transcriptional assays (Fig. 2), a key future direction will be investigating whether common SNPs in these enhancers affect enhancer activity and therefore contribute to the association between *G6PC2* and variations in FBG.

While deletion of *G6pc2* enhancer I affects *G6pc2*, *Spc25*, and *Gm13613* expression (Fig. 4), multiple studies have shown that *G6PC2* is the effector gene at this locus that regulates FBG in humans (23,25). However, future studies will be required to determine whether enhancer I regulates *G6pc2* expression directly, the simplest possibility, or whether disruption of enhancer I results in altered *G6pc2* gene expression as a result of indirect effects of altered *Spc25* and *Gm13613* expression.

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Author Contributions. M.P.K., A.D.A., M.A.M., and R.M.O. jointly conceived the study, and each contributed to writing the manuscript. E.M.H. and J.K.O. performed the gene expression and fusion gene analyses and participated in manuscript writing. K.L.S. and K.A.M. performed the in vivo physiological measurements on the mice. D.S.S. and S.P.S. performed ex vivo insulin secretion studies on isolated islets. L.L.S. designed the *G6pc2* enhancer I deletion mouse CRISPR strategy, characterized the deletion, and participated in manuscript writing. M.P.K., A.D.A., M.A.M., and R.M.O. are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of data and the accuracy of the data analysis.

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