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The Nucleosome Binding Protein HMGN3 Modulates the Transcription Profile of Pancreatic β Cells and Affects Insulin Secretion▽

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Improper glucose-stimulated insulin secretion from pancreatic β cells is a major factor in the onset of type 2 diabetes. We now report that HMGN3, a nuclear protein that binds to nucleosomes and affects chromatin function, is highly expressed in β cells and that in mice, loss of HMGN3 impairs glucose-stimulated insulin secretion and leads to a diabetic phenotype. In pancreatic β cells, loss of HMGN3 affects the transcription of several genes involved in glucose-stimulated insulin secretion, including that of the *Glut2* glucose transporter. Chromatin immunoprecipitation reveals that HMGN3 and the transcription factor PDX1 mutually reinforce their specific binding to the chromatin in the promoter of the *Glut2* gene, thereby regulating GLUT2 protein levels in pancreatic islets and in β cells. Our results identify a new regulator of glucose homeostasis and demonstrate a link between the activity of a nucleosome binding structural protein and the regulation of insulin secretion.

Proper secretion of insulin from the β cells located in pancreatic islets is a key element in maintaining glucose homeostasis and preventing the onset of diabetes mellitus, a severe and prevalent metabolic disorder (17, 28, 29). Despite intensive studies, the full spectrum of genetic factors leading to diabetes is still not fully understood (43). β cells secrete insulin in response to elevated levels of glucose which can occur after eating, a response named glucose-stimulated insulin secretion (GSIS). The entry of glucose into β cells is mediated by type 2 glucose transporters (GLUT2) (15, 23). The internalized glucose is phosphorylated by glucokinase, leading to an increase in ATP levels, closure of the K^+ channels, plasma membrane depolarization, and activation of voltage-gated Ca^{2+} channels, thereby triggering exocytosis of insulin-storing granules from these cells (28, 32).

Inadequate progression of the GSIS pathway, due to impaired signaling or improper protein functionality, may lead to diabetes mellitus (12, 16, 22, 41, 42). Accordingly, transcription factors such as HNF1α, HNF4α, PDX1, FoxA2, and NeuroD, which regulate the expression of the protein components involved in maintaining specific β-cell functions, have been shown to play an important role in insulin secretion and in the pathogenesis of diabetes (9). The transcription network of β cells functions in the context of chromatin and is affected by changes in chromatin structure (11); therefore, nucleosome binding proteins that affect the structure and activity of the chromatin fiber may also play a role in β-cell function and insulin secretion. We now report that the nucleosomal binding protein HMGN3 is highly expressed in pancreatic islets and that loss of this protein impairs insulin secretion, thereby leading to a diabetic phenotype.

HMGN (high-mobility group N) proteins are a family of structural proteins that bind specifically to the 147-bp nucleosome core particle, the building block of the chromatin fiber, without any obvious specificity for the underlying DNA sequence (6). The binding of these proteins to nucleosomes induces structural changes in chromatin, alters the levels of posttranslational modifications in the tail of the nucleosomal histones, and affects transcription, replication, and DNA repair of chromatin templates (6, 36). In living cells, the interaction of HMGN with chromatin is highly dynamic: the proteins move continuously among nucleosomes, and their residence time at any specific site is short. HMGN proteins compete with other architectural proteins for chromatin binding sites and therefore can be viewed as functioning within a dynamic network of proteins that continuously move among nucleosomes, constantly reconfiguring their chromatin binding sites (8). The three members of this family, HMGN1, HMGN2, and HMGN3, have a highly conserved domain structure and very similar biochemical properties (6); however, their expression levels vary among tissues (26, 40).

Here we demonstrate that HMGN3 is highly expressed in the insulin-producing cells of the pancreatic islets in both mice and humans. To test whether the protein affects the function of pancreatic β cells, we generated *Hmgn3*$^{-/-}$ mice. These mice are viable and appear normal; however, compared to their *Hmgn3*$^{+/+}$ littermates, their blood glucose levels are elevated, their serum insulin levels are reduced, and their glucose tolerance is impaired. To test the molecular mechanisms leading to this diabetic phenotype, we analyzed the role of HMGN3 in the function of MIN6 cells, a mouse insulinoma cell line that has been extensively used for functional studies of pancreatic β cells (27). We found that HMGN3 modulates the expression of

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TABLE 1. Primers used in this study

Application and primer	Location with respect to TSS[a] (bp)	Sequence		PCR product size (bp)
		Forward	Reverse	
ChIP				
Glut2-P18	−8855 to −8656	GTTCGCCTCAGAAGTTGGAG	AAGCCCAGATGGTTTCACC	200
Glut2-P17	−8233 to −8035	TGCTGTTGTACTCAGGCAATG	CAGGAAGGATGCTGTCTCTAGG	199
Glut2-P16	−7902 to −7753	GCTTTGGACTTCCCTCTGTTAAG	GTCCCTCAGTGTCTTCCATGTAG	150
Glut2-P15	−7458 to −7309	TCAGTGGGAGGTGGATAACTG	CCCATTGATGCCAGATAAGG	150
Glut2-P14	−6931 to −6784	TCTGGATCTTCGCTATCAGACA	AAGGCTCAGTGACTTTGGAATC	148
Glut2-P13	−6469 to −6353	GAGCCGATCCTGCAATAAAG	CAGCTTCAGGTCTCCGTAATC	117
Glut2-P12	−5700 to −5607	ACCACACCCAGCTTTGAGTT	TGGGCCCACTGTGTTGTAT	94
Glut2-P11	−5323 to −5174	TAAGAGCCAGATGGGATGGA	CACAACCAGGCTATTAGTGCAA	150
Glut2-P10	−4704 to −4555	AGGCTGCTATCTACCTATCCACTC	ACATCTTCCCTCATTCCTCTCTC	150
Glut2-P9	−4227 to −4078	AGCGAGATGGGACTGAACA	TAAGATACCACCTCCCACAGG	150
Glut2-P8	−3885 to −3736	GTGGAGAAGAGTGTGTCTGATTC	ACTTAAGTGTATCCCGGACCA	150
Glut2-P7	−3158 to −3009	AGTACTCTGCTGCCTCTTTGGT	CAGGGTTCAGCCATCTACCT	150
Glut2-P6	−2712 to −2564	GAACACAGCAGGGTTCTCTGA	GGAAGCGCCTGAAACTAAATG	149
Glut2-P5	−2388 to −2241	ATGGATCATGAGCAGAGTTGG	AGAGGAGCTGCGAGTTTCTG	148
Glut2-P4	−1918 to −1769	CTGATCGGCACAAGTGTGTT	CCGCTTTGGTATTAGGCTTTC	150
Glut2-P3	−1200 to −1051	TGGCCTTCACACAGAGGAGTA	GAAGGTCGGAAGGAAACTGG	150
Glut2-P2	−984 to −835	ACTGTGTGGATGGCAGTGAC	TGGCTTTCCAGTGAATTCTG	150
Glut2-P1	−218 to −70	AAATGGGTCTGTCTCTGGTTTG	GGAGCAAAGAGAGGTAGTTCTGAC	149
Glut2-P0	+27 to +176	TCCACACACACACAATGTCAG	AAGCAAAGAGGTAGCCTGGAC	150
Glut2-G1	+696 to +845	GGAGCAAAGTGAAGGCTGTG	TACACCATTGCCGTATGCAG	150
Glut2-G2	+1255 to +1402	GCAGGGAGAACTGTAATGATGA	CTGTTAAGCACCGTCCCTTC	148
Glut2-G3	+1571 to +1718	GGTGGTGCTCAGGAGAGTTAGA	TGCAAGGGAACTTAAGCCATAG	148
Glut2-G4	+2012 to +2156	ATGGTTGTTGATAAGCCCAGGT	AGCAGGTGGCAGGAGGATTA	145
Glut2-G5	+2800 to +2949	GTCTGTGTGTGGTATGTGCAGT	AGTGAGAACTGACTCCTGGAAG	150
Glucagon promoter	−125 to −3	GAGTGGGCGAGTGAAATCAT	CCAGGGTGCTGTGCTTTTATA	122
Insulin 2 promoter	−160 to +8	GACCTAGCACCAGGGAAGTG	TTAGGGCTGGTGGTTACTGG	168
Q-RT-PCR of mRNA				
HMGN3		GGAAATTTCCATCATCCTCAAGTC ACGC	CTCAGTAAAAACACCACAGTCTGC ACAG	
Insulin total		GTGGAACAACTGGAGCTGGGAG	CTTAGTTGCAGTAGTTCTCCAGCTG	
Insulin 1		CCAGCTATAATCAGAGACCA	CCAGGTGGGGACCACAAAGA	
Insulin 2		GGCTTCTTCTACACACCCAT	CCAAGGTCTGAAGGTCACCT	
Glucagon		ATCTTGCCACCAGGGACTT	GTTGGGTTACACAATGCTAGAGG	
PDX-1		CAGCTCCCTTTCCCGTGGAC	CAACATCACTGCCAGCTCCA	
NeuroD		GCCTTTACCATGCACTACCC	GTGAAACTGACGTGCCTCTAATC	
MafA		CATCCGACTGAAACAGAAGC	CCGCCAACTTCTCGTATTT	
FoxA2		CTACGCCAACATGAACTCGAT	AGAGGTCCATGATCCACTGATAG	
Pax4		AGAGAAGCTGAAATGGGAAGC	AGGTGTCACTGGAACATCTGC	
Pax6		ACACCTGTCTCCTCCTTCACAT	TCATAACTCCGCCCATTCA	
HNF1α		TTGGCCATGGACACCTATAAC	GCCGCAGACACTGTGACTAA	
Insulin receptor		AGGCTAACCTTGGCCTCATT	GATGGTGAGGTTGTGTTTGCT	
Glut2		GCCTGTGTATGCAACCATTG	TGGCAGTCATGCTCACGTA	
Glucose kinase		GGACAAGCATCAGATGAAACAC	CCACCACATCCATCTCAAAGT	
Sur1		ATTGCCTCAGGACAAGCAAC	GCTTATTGAAGGGACTCTCGAA	

[a] TSS, transcription start site.

several genes involved in insulin secretion, including that for the glucose transporter GLUT2. In both MIN6 cells and *Hmgn3*$^{-/-}$ mice, loss of HMGN3 protein reduces the levels of GLUT2. HMGN3, but not the HMGN1 or HMGN2 variant, binds specifically to chromatin in the promoter region of the *Glut2* gene and enhances its expression by facilitating the recruitment of PDX1 and additional transcription factors to the *Glut2* promoter. Chromatin immunoprecipitation (ChIP) experiments reveal that HMGN3 enhances the acetylation of H3 at the *Glut2* promoter and suggest that HMGN3 and PDX1 mutually reinforce their interaction with the chromatin at the *Glut2* promoter but not throughout the chromatin or in the nucleoplasm. Given that the human and mouse HMGN3 amino acid sequences are identical and that this protein is also highly expressed in the β cells of humans, our findings may have relevance to the understanding of the molecular mechanisms regulating the transcriptional network of human pancreatic β cells. Our studies provide insight into the mechanism of action of HMGN proteins and identify a new factor which, either by itself or in conjunction with additional genetic defects, may play a role in the pathogenesis of type 2 diabetes.

MATERIALS AND METHODS

Materials. The following antibodies were used: anti-PDX1 (Upstate and Chemicon), anti-HNF1α (Santa Cruz), anti-HNF4α (Santa Cruz), anti-FoxA2 (Santa Cruz), anti-Glut2 (Millipore), anti-actin (Sigma), anti-rabbit insulin (Santa Cruz), anti-mouse insulin (Zymed), and Alexa Fluor 488 or Alexa Fluor 568 goat anti-rabbit immunoglobulin G (IgG; Molecular Probes). Affinity-puri-

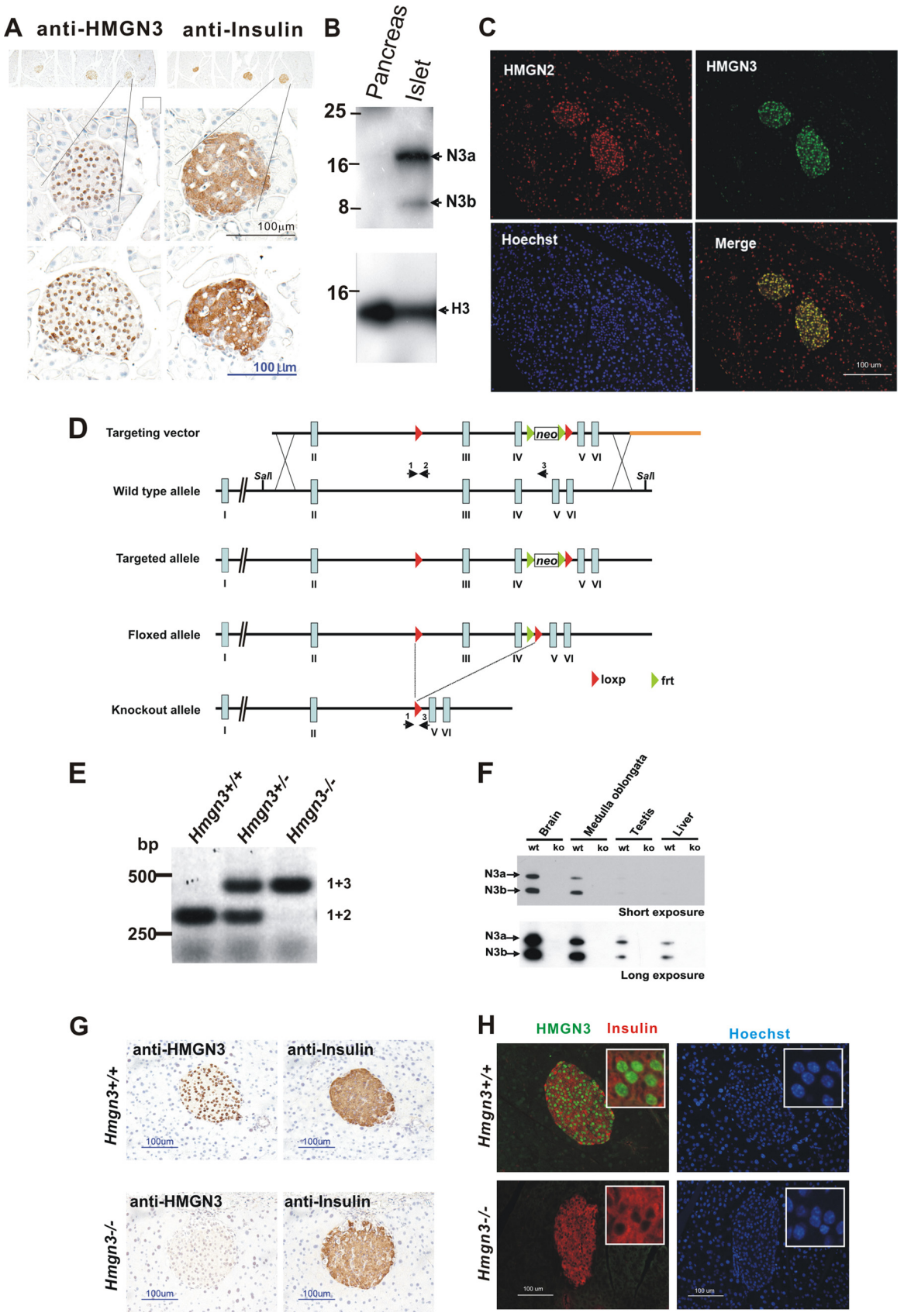


FIG. 1. Elevated levels of HMGN3 in pancreatic β cells. (A) Immunostaining of HMGN3 or insulin in the pancreases of wild-type mice. The upper panel shows a low magnification of a region in the pancreas containing three distinct islets that stain with both antibody to HMGN3 (left)

fied antibodies to HMGN variants and to histone H3 elicited in either rabbits or goats were prepared as described previously (7). The antibody to HMGN3 was previously characterized (40). Prepared solutions of insulin (10 mg/ml) and glucose (45%) were purchased from Sigma.

Generation of *Hmgn3* knockout mice. The targeting vector to conditionally remove the gene for HMGN3 was constructed by a recombinogenic cloning strategy (25) with a murine bacterial artificial chromosome clone, RP24-388H18. The vector was constructed to remove exons III and IV, which code for the nucleosomal binding domain of HMGN3 (Fig. 1D). A 21-kb fragment containing the gene for HMGN3 (except exon I) was retrieved from the bacterial artificial chromosome clone and inserted into the targeting vector PL253 by recombination in bacterial strain DY380. The *neo* gene with the phosphoglycerate kinase 1 promoter (pGKneo) was employed as a positive selectable marker, and the pGK-thymidine kinase cassette was used as a negative selectable marker (34). The *loxP*/*Frt*-flanked positive selectable marker and the *loxP* site for conditional deletion of the gene for HMGN3 were inserted as described in Fig. 1. Electroporation and selection were performed by using the v6.4 embryonic stem (ES) cell line as described elsewhere (34). DNAs derived by G418/1,2′-deoxy-2′-fluoro-1-β-D-arabinofuranosly-5-iodo-uracil-resistant ES cell clones were screened by diagnostic BclI or NheI restriction enzyme digestion by using the 5′ and 3′ probes external to the targeting vector sequence. Two independent ES cell clones targeted to the gene for HMGN3 and injected into C57BL/6 blastocysts generated chimeras that transmitted the mutated allele to the progeny (5). The neomycin resistance (*neo*) cassette was removed by crossing with FLP mice, and the genomic fragment containing exons III and IV of the *Hmgn3* gene was removed by crossing with EIIA-Cre mice. The mice containing the targeted allele were backcrossed into the C57BL/6 background for at least five generations. Mice were bred in a specific-pathogen-free facility with food and water ad libitum.

Immunostaining of pancreatic section. The immunofluorescence assay was performed as previously described (13). The primary antibodies used were rabbit anti-HMGN3 (prepared in our laboratory), rabbit anti-insulin (0.4 μg/ml; Santa Cruz Biotechnology), and mouse anti-insulin (0.2 μg/ml; Zymed) antibodies. Alexa Fluor 488 or Alexa Fluor 568 goat anti-rabbit IgG (Molecular Probe) secondary antibodies were used at 8 μg/ml.

Glucose and insulin measurements. Blood glucose levels were measured with an Ascensia CONTOUR blood glucose meter (Bayer). Blood was collected from the tail vein. Serum insulin levels were measured with a rat/mouse insulin enzyme-linked immunosorbent assay kit (Millipore).

Glucose and insulin tolerance tests. Mice were fasted for 16 h before injection. Glucose (2 g/kg) or insulin (0.75 U/kg) was injected intraperitoneally, and glucose levels were measured at 0, 15, 30, 60, 120, and 180 min for the glucose tolerance test and at 0, 15, 30, 45, and 60 min for the insulin tolerance test.

Cell culture, small interfering RNA (siRNA)-mediated knockdown of HMGN proteins, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. MIN6 cells (obtained from A. L. Notkins, National Institute of Dental and Craniofacial Research, NIH) were maintained in Dulbecco modified Eagle medium (DMEM) containing 15% fetal bovine serum and 25 mM HEPES. ON-target plus SMART pool siRNAs for HMGN1, HMGN2, and HMGN3 and control siRNA were purchased from Dharmacon. An additional set of siRNAs for HMGN3 was purchased from Qiagen. The siRNA (final concentration, 20 nM) was transfected into MIN6 cells, cultured in DMEM containing 15% (vol/vol) fetal bovine serum, by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Four days posttransfection, the medium was changed and then the cells were retransfected and cultured for an additional 4 days. Efficiency of HMGN knockdown was monitored by Western blotting (protein level) and quantitative real-time PCR (Q-RT-PCR; RNA level). Cell viability was monitored with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay kit (Roche) according to the manufacturer's instructions.

Insulin secretion from MIN6 cells. MIN6 cells transfected with siRNA were cultured in DMEM containing 15% fetal bovine serum and 2 mM glucose overnight. Cells were washed twice with phosphate-buffered saline and cultured in KRBH (25 mM HEPES [pH 7.4], 125 mM NaCl, 1.3 mM $CaCl_2$, 5 mM $NaHCO_3$, 5.9 mM KCl, 1.2 mM $MgCl_2$, 0.1% [wt/vol] bovine serum albumin) containing 2 mM glucose for 2 h. After 2 h, the cells were washed twice with phosphate-buffered saline and induced to secrete insulin by exposure to various concentrations of either glucose or arginine in KRBH. After 10 min, supernatants were collected. Insulin concentrations were measured by enzyme-linked immunosorbent assay (Millipore).

Q-RT-PCR analysis. Total RNA from MIN6 cells or isolated islets was purified with an RNeasy mini plus or an RNeasy micro kit (Qiagen), respectively. cDNA was prepared from purified RNA with an iScript cDNA synthesis kit (Bio-Rad). Q-RT-PCR analysis was performed with the ABI PRISM 7900 system and Power SYBR green PCR master mix (ABI) according to the manufacturer's recommendations. The sequences of the primers used are shown in Table 1.

Western blot analyses. The polyclonal antibody to mouse HMGN3 was made in goats (Rockland) and affinity purified on immobilized HMGN3. Cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membrane (Millipore), and then stained with goat anti-HMGN3 antibody. Bound antibody was detected with horseradish peroxidase-conjugated anti-goat IgG antibody (Pierce) and ECL plus reagent (GE Healthcare).

ChIP assay and coimmunoprecipitation. Cells were fixed with 0.6% formaldehyde for 10 min, sonicated to produced ~500-bp DNA fragments, and then subjected to ChIP assay. ChIP assays were performed with a ChIP IT express kit (Active Motif) according to the manufacturer's instructions. ChIP experiments were performed with affinity-purified anti-HMGN3 and anti-PDX1 antibodies (Upstate). Immunoprecipitated DNA was analyzed, and the relative enrichment value was calculated as described previously (24). For coimmunoprecipitation, 0.35 M NaCl extracts from either MIN6 cells or MIN6 cells that were cotransfected with plasmids expressing HMGN3 and PDX1 were prepared. The extracts were dialyzed to 0.15 M NaCl-20 mM Tris (pH 7.5)-0.2% NP-40-1 mM EDTA and treated with antibodies to either HMGN3 or PDX1.

RESULTS

Specific enrichment of HMGN3 in pancreatic islets. The expression of HMGN3 is tissue specific and differs from that of the closely related but more abundant and ubiquitous HMGN1 and HMGN2 variants (40). In the pancreas, HMGN3 is specifically enriched in the islets (Fig. 1A) while HMGN2 (Fig. 1C) and HMGN1 (data not shown) are more broadly expressed throughout the entire pancreas, including the islet cells. In the islets, antibodies to HMGN3 prominently stain the

and antibody to insulin (right). Magnified images of the stained islets are shown below. (B) Western blot analysis of HMGN3 in extracts prepared from whole pancreas tissue or from isolated islets. N3a and N3b refer to HMGN3a and HMGN3b. Histone H3 served as a loading control. The values on the left are molecular sizes in kilodaltons. (C) Double immunostaining with anti-HMGN2 and anti-HMGN3 antibodies reveals that both proteins are expressed in the islets but only HMGN2 is detected in the exocrine cells. (D) Strategy for generating *Hmgn3* knockout mice. Black arrowheads with numbers show the positions of the primers used for genotyping of mice. A first *loxP* site was placed upstream of exon III of the *Hmgn3* gene. The *neo* cassette flanked by both *loxP* and *Frt* sites was placed downstream of exon IV. Following removal of the *neo* cassette together with exons III and IV of *Hmgn3*, breeding of $Hmgn3^{+/-}$ mice gave rise to homozygous mutants lacking the nucleosomal binding domain of the protein. (E) Genotyping of mice. The primers used to amplify the bands are shown on the right. (F) Global loss of HMGN3 protein in $Hmgn3^{-/-}$ mice, demonstrated by Western analyses. Short exposure demonstrates loss of HMGN3 in tissues that express HMGN3 highly, such as the brain and the medulla oblongata, while longer exposures demonstrate loss of HMGN3 proteins from the testis and liver, where there are low levels of this protein. wt, wild type; ko, knockout. (G) Immunostaining of the pancreas reveals the absence of HMGN3 protein from the islets of $Hmgn3^{-/-}$ mice. (H) Confocal immunofluorescence reveals the presence of HMGN3 in the nuclei of cells containing insulin in $Hmgn3^{+/+}$ mice but not in $Hmgn3^{-/-}$ mice. Red demonstrates immunolocalization of insulin in the cytoplasm, and green visualizes the localization of HMGN3 in the nucleus. The nuclei are visualized by Hoechst staining. The magnified insert demonstrates loss of HMGN3 in the nuclei of the insulin-positive β cells of $Hmgn3^{-/-}$ mice.

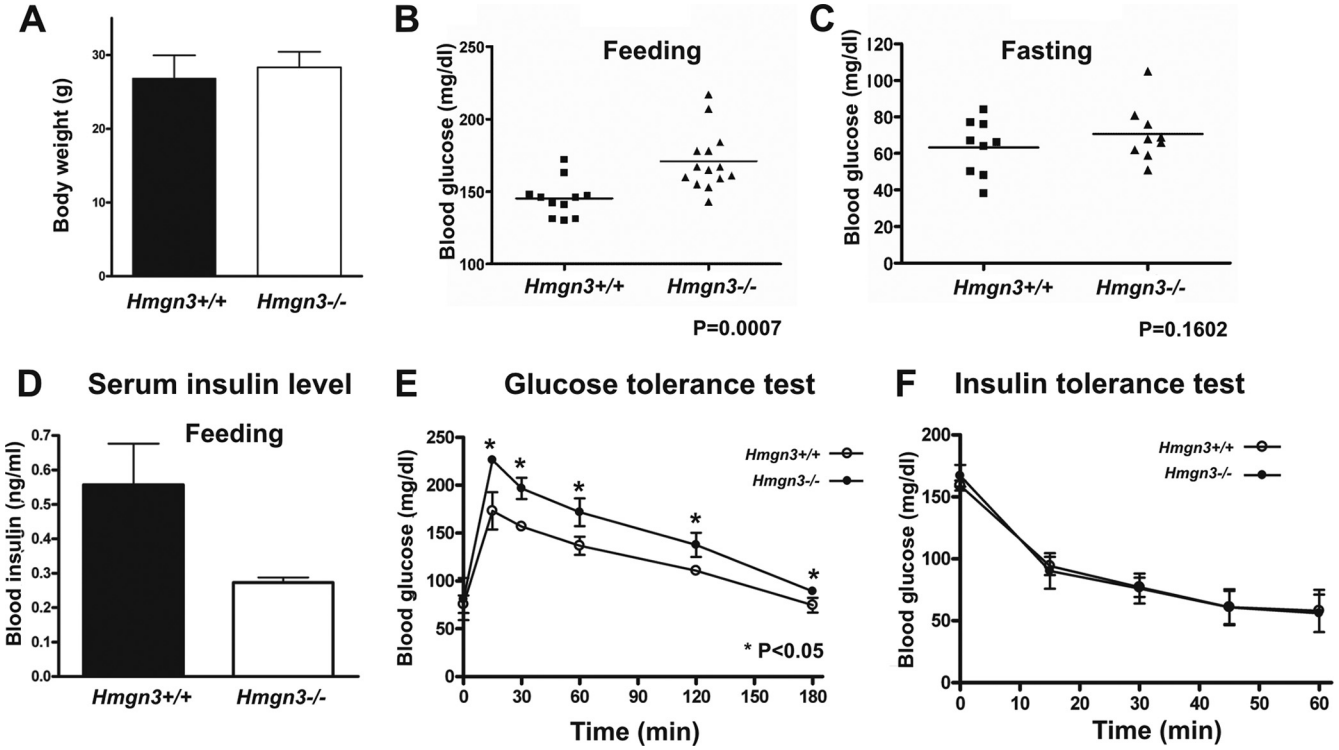


FIG. 2. Reduced insulin secretion and impaired glucose tolerance in *Hmgn3*$^{-/-}$ mice. (A) Body weights of 3-month-old male mice. (B) Increased blood glucose levels in feeding mice. (C) Glucose levels in fasting mice. (D) Decreased serum insulin levels in feeding mice. (E) Intraperitoneal glucose tolerance test. Glucose (2 g/kg body weight) was injected intraperitoneally into mice fasted for 16 to 18 h, and blood glucose levels were measured at 0, 15, 30, 60, 120, and 180 min after injection. (F) Insulin tolerance test. Insulin (0.75 U/kg body weight) was injected into mice fasted for 6 h, and blood glucose levels were measured at 0, 15, 30, 45, and 60 min after injection. All comparisons were done with *Hmgn3*$^{+/+}$ and *Hmgn3*$^{-/-}$ littermate mice derived from several pairs of *Hmgn3*$^{+/-}$ breeders.

nuclei of cells expressing insulin, an indication that β cells are highly enriched in HMGN3 protein (Fig. 1A). Western blot analyses verified that the expression of HMGN3 in mouse islets is significantly higher than that in the surrounding exocrine cells (Fig. 1B), and double-immunofluorescence analysis reveals that while HMGN3 is highly expressed in the islets, the HMGN2 variant is expressed both in the islets and in the exocrine pancreatic cells (Fig. 1C). High HMGN3 expression in β cells of pancreatic islets is not unique to mice, since the expression of HMGN3 is also highly elevated in the β cells of the human pancreas (data not shown). The high level of HMGN3 in the insulin-producing cells of the pancreatic islets raises the possibility that this nucleosomal binding protein affects β-cell function.

Impaired glucose tolerance and low serum insulin level in *Hmgn3*$^{-/-}$ mice. To test whether the HMGN3 protein could affect β-cell function, we generated *Hmgn3*$^{-/-}$ mice (Fig. 1D to H). To minimize genomic alterations, we excised from the *Hmgn3* gene only the region that codes for the nucleosomal binding domain of HMGN3, which is highly conserved among all HMGN variants (35). Genomic analysis with appropriate primers verified complete loss of exons III and IV of *Hmgn3* (Fig. 1E), and Western blot assays of extracts from several tissues obtained from *Hmgn3*$^{+/+}$ and *Hmgn3*$^{-/-}$ mice revealed complete loss of the HMGN3 protein in the knockout mice (Fig. 1F). Immunostaining of pancreatic sections verified the absence of HMGN3 from the pancreatic islets and from the nuclei of insulin-producing cells (Fig. 1G and H). The *Hmgn3*$^{-/-}$ mice are viable and fertile.

A comparison of male *Hmgn3*$^{-/-}$ and *Hmgn3*$^{+/+}$ mice, obtained by crossing *Hmgn3*$^{+/-}$ mice, did not reveal significant differences in the body weights of the mice (Fig. 2A). However, the glucose levels of *Hmgn3*$^{-/-}$ mice were 12% higher during feeding, but not at fasting, than those of their *Hmgn3*$^{+/+}$ littermates (Fig. 2B and C), suggesting possible effects of HMGN3 on glucose metabolism. The insulin levels in the serum of fasting mice were not significantly affected by HMGN3 (data not shown), while the insulin levels in the serum of feeding *Hmgn3*$^{-/-}$ mice were approximately 50% of those of their *Hmgn3*$^{+/+}$ littermates (Fig. 2D), providing a possible explanation for the elevated glucose levels in their blood. Further analyses revealed that glucose tolerance (Fig. 2E), but not insulin sensitivity (Fig. 2F), was impaired in the *Hmgn3*$^{-/-}$ mice. Taken together, these findings suggest that the altered glucose homeostasis in feeding *Hmgn3*$^{-/-}$ mice could be due to the effects of HMGN3 on β-cell function, perhaps due to faulty insulin secretion in response to increased levels of blood glucose during feeding.

HMGN3 regulates GSIS in pancreatic MIN6 cells. To examine the molecular mechanism underlying the phenotype of *Hmgn3*$^{-/-}$ mice, we analyzed the function of HMGN3 in mouse insulinoma MIN6 cells, which have been extensively used to study the properties of pancreatic β cells, including their pathway of insulin synthesis and secretion (27). Like

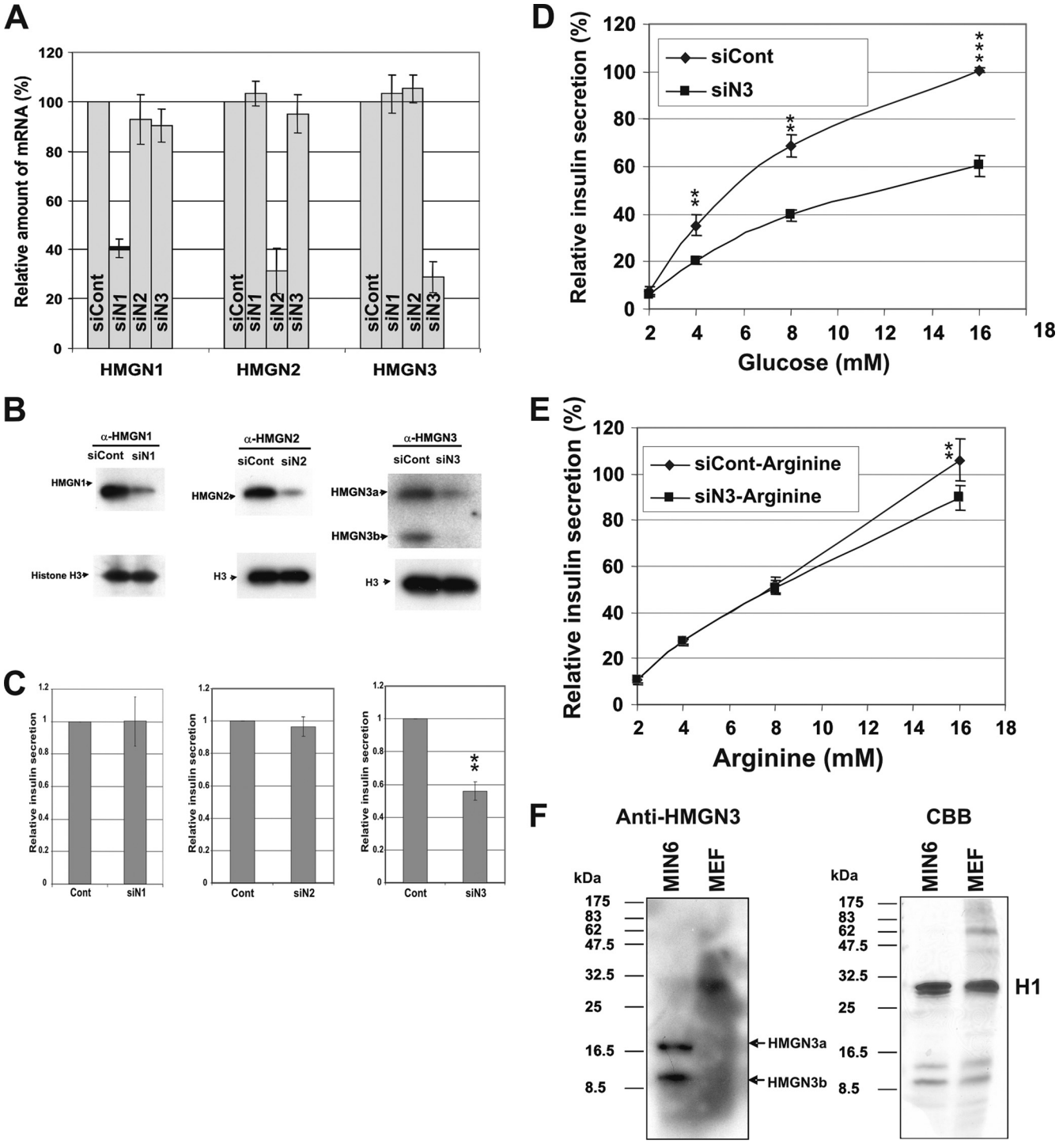
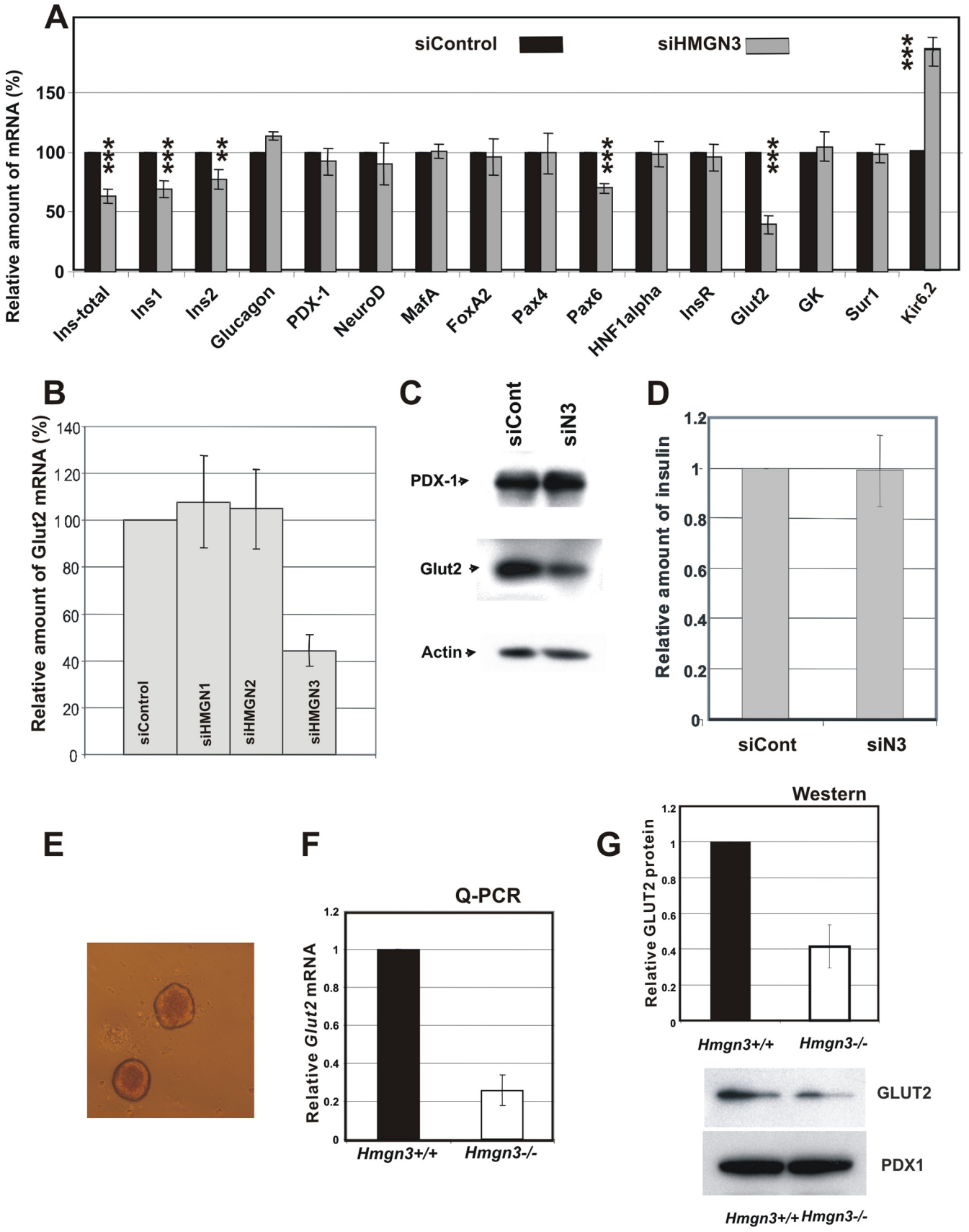


FIG. 3. HMGN3 enhances the rate of GSIS. (A) Specific siRNA-mediated downregulation of *Hmgn* variant transcripts in MIN6 cells. Each type of siRNA downregulated only the corresponding transcript. siCont, control siRNA. (B) Efficient downregulation of HMGN variant protein levels by siRNA treatments. Shown are Western blot analyses that indicate that the protein levels of the HMGN variants are reduced by >80%. A Western blot analysis with histone H3 served as a loading control. (C) Downregulation of HMGN3, but not of HMGN1 or HMGN2, impairs GSIS. Cells were induced to secrete insulin by exposure to 15 mM glucose (see Materials and Methods) Cont, control. (D, E) Reduced levels of HMGN3 decrease GSIS from MIN6 cells more significantly than arginine-stimulated insulin secretion. (F) High HMGN3 expression in MIN6 cells. Western blot assays of 5% perchloric acid extracts from MIN6 or mouse embryonic fibroblasts (MEF). CBB, Coomassie blue staining indicating equal loading. The prominent band is histone H1. Statistical analyses were done by the Student *t* test. **, $P < 0.01$; ***, $P < 0.001$.


A
siControl
siHMGN3
Relative amount of mRNA (%)
150
100
50
0
Ins-total
Ins1
Ins2
Glucagon
PDX-1
NeuroD
MafA
FoxA2
Pax4
Pax6
HNF1alpha
InsR
Glut2
GK
Sur1
Kir6.2
B
Relative amount of Glut2 mRNA (%)
140
120
100
80
60
40
20
0
siControl
siHMGN1
siHMGN2
siHMGN3
C
siCont
siN3
PDX-1
Glut2
Actin
D
Relative amount of insulin
1.2
1
0.8
0.6
0.4
0.2
0
siCont
siN3
E
F
Q-PCR
Relative Glut2 mRNA
1.2
1
0.8
0.6
0.4
0.2
0
Hmgn3+/+
Hmgn3-/-
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Western
Relative GLUT2 protein
1.2
1
0.8
0.6
0.4
0.2
0
Hmgn3+/+
Hmgn3-/-
GLUT2
PDX1
Hmgn3+/+ Hmgn3-/-

pancreatic β cells, MIN6 cells also had a high content of HMGN3 and the amount of HMGN3 in these cells is significantly higher than that of somatic cells such as mouse embryonic fibroblasts (Fig. 3F). Treatment of these cells with siRNA targeted to *Hmgn3* (siN3) specifically downregulated *Hmgn3* transcripts (Fig. 3A) and reduced the levels of HMGN3a protein and its splice variant HMGN3b by >80% (Fig. 3A and B) without affecting cell viability (not shown). As a control, cells were treated with siRNAs targeted to *Hmgn1* (siN1) or *Hmgn2* (siN2). These siRNAs were specific for their target genes and had no effect on *Hmgn3* transcript levels (Fig. 3A).

To test whether HMGN3 affects β-cell function, we exposed MIN6 cells to either glucose or arginine; two compounds known to stimulate insulin secretion by different pathways (28). In both pathways, insulin release from β cells is associated with membrane depolarization; however, only the glucose-stimulated pathway involves facilitated import of glucose into the cells (33). We found that siN3-mediated depletion of HMGN3 decreased GSIS from MIN6 cells significantly more than arginine-stimulated insulin secretion (Fig. 3D and E). Reduction in GSIS was also observed with another siN3 sequence (data not shown), an indication that the effects are HMGN3 specific and not due to off-target action of the siRNAs. Furthermore, control experiments with siRNAs to the *Hmgn1* and *Hmgn2* variants, which efficiently reduced the levels of the corresponding proteins (Fig. 3A and B), also did not affect glucose-mediated insulin secretion (Fig. 3C). We therefore conclude that HMGN3 specifically enhances GSIS from β cells.

HMGN3 enhances *Glut2* expression. HMGN variants are nucleosome binding proteins known to affect the structure and function of chromatin, including transcription. Hence, reduction in HMGN3 protein may alter the levels of the various components involved in the GSIS pathway. Q-RT-PCR revealed that, indeed, reduction in the levels of HMGN3 protein led to a decrease in the transcript levels of *insulin*, *Pax6*, and *Glut2* (glucose transporter 2 gene) and to an increase in the levels of *Kir6.2*, all of which are known to be involved in GSIS (Fig. 4A). The changes in the transcript levels of several components of the GSIS pathway suggest that the diabetic phenotype of the *Hmgn3*$^{-/-}$ mutant mice and the impaired insulin secretion from MIN6 cells result from HMGN3-mediated effects on the transcription of specific genes.

The initial phase of GSIS involves the export of insulin-storing granules and is dependent on the amount of stored insulin in the granules rather than on the steady-state levels of insulin transcription (31). We found that the total insulin content of MIN6 cells treated with siN3 was indistinguishable from that of cells treated with control siRNAs (Fig. 4D). Thus, the decreased level of insulin secretion from the glucose-stimulated MIN6 cells depleted of HMGN3 is not majorly due to the decreased levels of insulin mRNA. Likewise, since the transcription factor Pax6 is not directly involved in the early phases of GSIS, it is unlikely that the HMGN3-mediated decrease in *Pax6* transcripts is a major factor in the observed effects on insulin secretion. Kir6.2, a component of the ATP-sensitive potassium channel, plays a role in several physiological processes, and genetic mutations in this protein affect insulin secretion (19). Our analysis of arginine-stimulated insulin secretion in MIN6 cells suggests that, by themselves, the changes in Kir6.2 levels do not have major effects on insulin secretion. GLUT2 plays an important metabolic function; however, its role in GSIS is not fully understood (23, 30). Given the metabolic significance of this gene and our observation that downregulation of HMGN3 affected GSIS more profoundly than arginine-stimulated insulin secretion (Fig. 3D and E), we focused on the role of HMGN3 in regulating *Glut2* expression.

Western blot analyses of MIN6 cell extracts verified that the decrease in *Glut2* transcripts resulted in a significant reduction in GLUT2 protein levels (Fig. 4C). Furthermore, the level of *Glut2* mRNA was not affected by the siRNA-mediated downregulation of either *Hmgn1* or *Hmgn2* (Fig. 4B). This finding supports our observation that siRNA-mediated knockdown of HMGN1 or HMGN2 has no effect on insulin secretion (Fig. 3C). Taken together, these results support a specific role for the HMGN3 variant in GSIS and in β-cell function.

Decreased *Glut2* expression in *Hmgn3*$^{-/-}$ pancreatic islets. Since our analysis of MIN6 cells indicates that loss of HMGN3 downregulates GLUT2, we tested whether loss of HMGN3 also affects *Glut2* expression in mice. To this end, we determined the levels of *Glut2* mRNA and GLUT2 protein in pancreatic islets (Fig. 4E) isolated from *Hmgn3*$^{-/-}$ and *Hmgn3*$^{+/+}$ littermates. Q-RT-PCR (Fig. 4F) and Western blot analyses (Fig. 4G) revealed that loss of HMGN3 leads to an 80% reduction in the amount of *Glut2* transcripts and a 50% reduction in the amount of GLUT2 protein. Taken together, our results indicate that HMGN3 enhances GLUT2 expression both in pancreatic islets and in MIN6 cells. We therefore investigated the mechanism whereby HMGN3 affects *Glut2* expression.

HMGN3 enhances the binding of transcription factors to the *Glut2* promoter. To gain insights into the mechanism whereby HMGN3 affects *Glut2* transcription, we first tested whether HMGN3 interacts with the chromatin in the regulatory region of the gene. ChIP analyses of an 11-kb region (from −9 kb to +2.5 kb relative to start of transcription) with antibodies to HMGN3 revealed a two- to threefold enrichment of

FIG. 4. Altered levels of GSIS-related transcripts in siN3-treated MIN6 cells. (A) Q-RT-PCR analysis of mRNA levels of genes coding for components of the GSIS pathway in HMGN3 knockdown or control cells. Statistical analysis was done with the Student *t* test. **, $P < 0.01$; ***, $P < 0.001$. (B) Knockdown of HMGN3, but not that of the HMGN1 or HMGN2 variant, downregulates *Glut2* expression. (C) Western analyses demonstrate reduced GLUT2, but not PDX1, protein in HMGN3 knockdown cells. Actin served as a loading control. siCont, control siRNA. (D) Knockdown of HMGN3 does not affect the amount of insulin stored in MIN6 cells. (E to G) Reduced *Glut2* expression in pancreatic islets of *Hmgn3*$^{-/-}$ mice. (E) Photomicrograph of isolated islets. (F) Q-RT-PCR (Q-PCR) analysis of *Glut2* mRNA in islets. (G) Decreased GLUT2 protein levels in pancreatic islets of *Hmgn3*$^{-/-}$ mice. The bar graph represents data obtained from islets of four different *Hmgn3*$^{-/-}$ and *Hmgn3*$^{+/+}$ mice that were separately prepared and separately analyzed. Protein extracts were analyzed by Western blot assays for GLUT2 protein and PDX1, which served as a loading control to verify that equal amounts of islet extracts were applied to the gels. A representative Western blot assay is shown under the bar graph.

HMGN3 over a 3-kb region 5′ to the start of transcription (Fig. 5A). Consistent with the finding that only the HMGN3 variant affects *Glut2* transcription and insulin secretion, this region is not enriched in either HMGN1 or HMGN2. Taken together, the findings indicate that HMGN3 enhances *Glut2* transcription by binding to the chromatin regions containing regulatory sequences for this gene. Since HMGN proteins are known to affect chromatin structure, we first tested whether depletion of HMGN3 alters the chromatin structure of the *Glut2* promoter. Detailed analysis of the kinetics of DNase I digestion of this region did not reveal any major HMGN3-dependent effects on the chromatin structure of the *Glut2* chromatin (Fig. 5B to D).

Although HMGN3 did not affect the chromatin structure of the *Glut2* promoter, it had major effects on the binding of several transcription factors known to affect *Glut2* transcription, such as HNF1α, FoxA2, HNF4α, and PDX1 (2, 10, 38, 39), to the promoter region of this gene (Fig. 6A). Depending on the transcription factor tested, siRNA-mediated downregulation of HMGN3, which did not affect the transcript levels of these factors (Fig. 4A), decreased the relative amounts of the factors bound to the *Glut2* promoter by three- to eightfold. These data indicate that HMGN3 facilitates the recruitment of transcription factors to the *Glut2* promoter and is the first example of an HMGN variant facilitating the binding of specific transcription factors to a specific chromatin region. PDX1, the factor most prominently affected by the siRNA treatment, plays a major role in the maintenance of adult β cells, where it regulates the transcription of several genes, including *Glut2* (9). We therefore focused on the possible mechanism whereby HMGN3 enhances the binding of PDX1 to the *Glut2* promoter.

HMGN3 and PDX1 colocalize to *Glut2* promoter chromatin. To explore the mechanism by which HMGN3 promotes the interaction of PDX1 with *Glut2* chromatin, we first tested whether the binding of HMGN3 precedes that of PDX1. If this were the case, downregulation of HMGN3 would affect the chromatin binding of PDX1 (Fig. 6A) but the downregulation of PDX1 would not affect the interaction of HMGN3 with the *Glut2* promoter. Surprisingly, we found that the efficient siRNA-mediated downregulation of PDX1 (Fig. 6C) downregulated the binding of HMGN3 to the promoter region but did not alter the background signals at an upstream region (region P17) of *Glut2* (Fig. 6B). Thus, PDX1 and HMGN3 mutually reinforce each other's interactions with the *Glut2* promoter.

A possible explanation for the mutual effects of PDX1 and HMGN3 is that these two proteins preassemble into a regulatory macromolecular complex prior to their binding to chromatin. We found that anti-HMGN3 antibody efficiently precipitated HMGN3 from a nuclear extract of MIN6 cells (Fig. 6D, lower panel); however, the precipitate did not contain PDX1 (Fig. 6D, upper panel). Likewise, anti-PDX1 antibody efficiently immunoprecipitated PDX1, but these precipitates did not contain HMGN3 (Fig. 6D, right panels). Thus, the two proteins do not assemble into a macromolecular complex in the nucleoplasm. We next tested whether the two proteins interact globally throughout the entire chromatin. ChIP analyses indicate that antibodies to both HMGN3 and PDX1 efficiently immunoprecipitate chromatin containing the respective proteins (Fig. 6E); however, the chromatin enriched for HMGN3 is not enriched for PDX1 (Fig. 6E, upper left panel) and the chromatin enriched in PDX1 does not contain HMGN3 (Fig. 6E, lower right panel). Thus, HMGN3 and PDX1 do not interact with each other in nuclear extracts and globally do not share a chromatin binding site.

In contrast, sequential ChIP analysis revealed that chromatin that was first precipitated with anti-HMGN3 antibody and then reprecipitated with anti-PDX1 antibody was significantly enriched in DNA of the *Glut2* proximal promoter (Fig. 6F, left). Likewise, the *Glut2* proximal promoter DNA was enriched in chromatin that was sequentially immunoprecipitated with anti-PDX1 and anti-HMGN3 antibodies (Fig. 6F, right). Thus, the two proteins colocalized specifically and mutually reinforce their binding to the chromatin in the promoter region of *Glut2*. Although this interaction does not lead to detectable changes in the chromatin structure, as assessed by DNase I digestion (Fig. 5), ChIP analysis indicates that the presence of HMGN3 increases the overall acetylation levels of histone H3 in the promoter region of the gene (Fig. 6G). HMGN proteins were shown to modulate histone modifications in a variant-specific fashion (36); the exact modification(s) affected by HMGN3 remains to be determined.

DISCUSSION

The regulation of glucose and insulin levels in the blood involves the coordinated action of several organs and numerous regulatory factors (9, 17, 28). Our studies indicate that the chromatin binding protein HMGN3 impairs glucose homeostasis by affecting GSIS from pancreatic β cells, thereby raising the possibility that misexpression of, or specific mutations in, HMGN3 may be a contributing factor in diabetes.

Several types of experiments link the expression of HMGN3 to GSIS. First, the protein is highly expressed in pancreatic islets, where it localizes to the nuclei of insulin-producing β cells. The expression pattern of HMGN3 is clearly different from that of the major members of the HMGN protein family HMGN1 and HMGN2, which are highly expressed both in the islets and in the exocrine cells of the pancreas (Fig. 1C) and ubiquitously detected in most tissues (6). Furthermore, while the expression of HMGN1 and HMGN2 is generally downregulated during differentiation (4, 13, 18), our preliminary analyses indicate that HMGN3 protein is first expressed in a few pancreatic cells only after embryonic day 14.5 and the levels of the protein increase during differentiation (data not shown). HMGN3 is highly expressed in the terminally differentiated β cells of both mice and humans. The relative abundance of HMGN3 in adult β cells suggests that the protein plays a role in the specialized function of these cells.

Second, loss of HMGN3 increases the glucose levels and impairs the glucose tolerance of feeding, but not fasting, mice. Nutritional signals are known to affect the levels of insulin in the blood, a key regulator of glucose homeostasis (28). The decreased insulin levels in the serum of feeding *Hmgn3*$^{-/-}$ mice provide a direct link between HMGN3 expression and glucose homeostasis. Third, loss of HMGN3, but not loss of either HMGN1 or HMGN2, impairs GSIS. Each of the specific siRNAs siN1, siN2, and siN3 efficiently downregulated the expression of its target protein, but only siN3 treatments reduced the GSIS from MIN6 cells (Fig. 3). The levels of

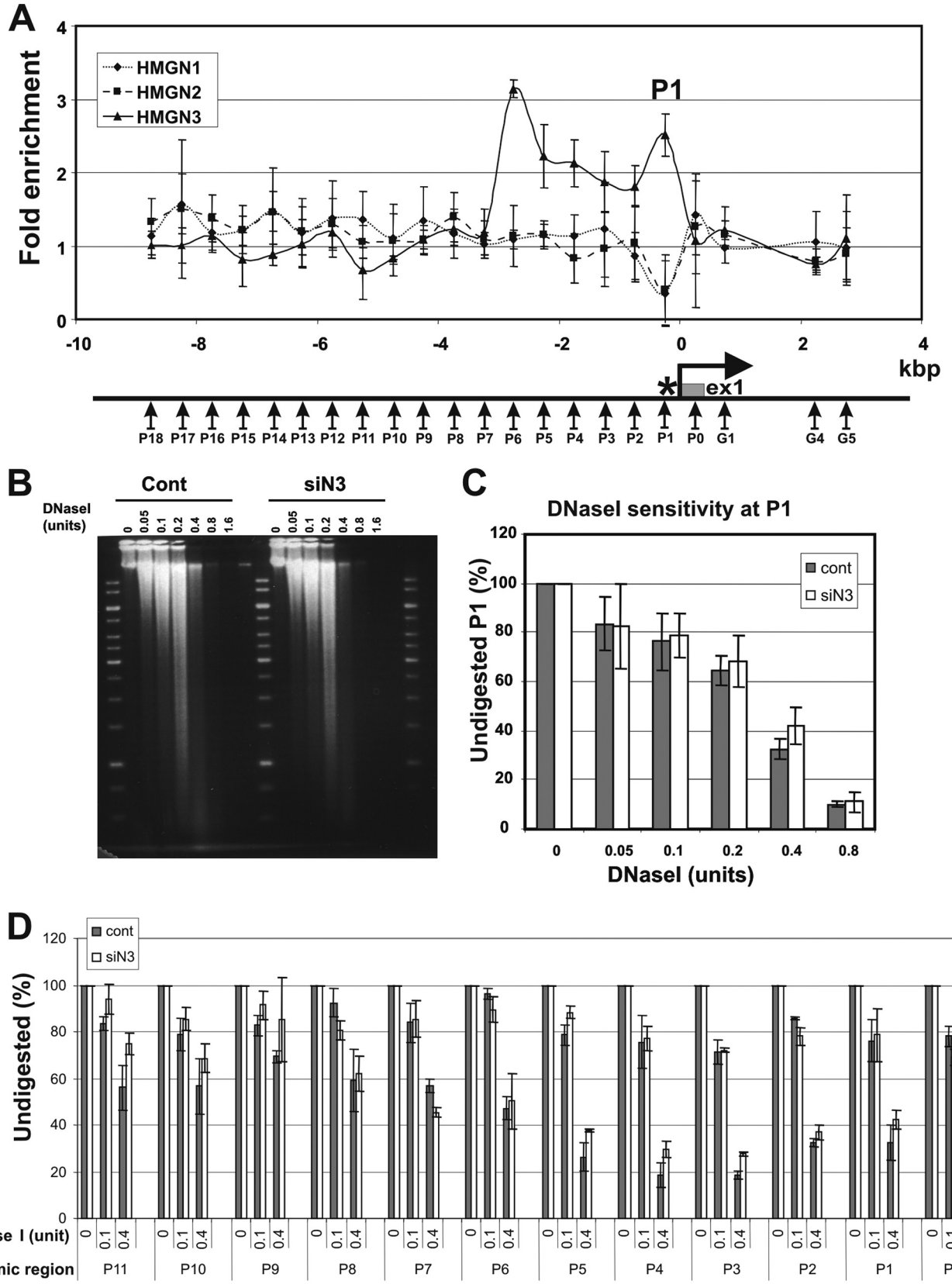

FIG. 5. HMGN3 is enriched in *Glut2* promoter chromatin. (A) ChIP assays demonstrating that HMGN3, but not the HMGN1 or HMGN2 variant, is enriched in the *Glut2* promoter. P_0 to P_{18} and G_1 to G_5 denote the regions amplified in the ChIP assay (see Table 1). The P1 fragment (marked by the asterisk) contains PDX1 binding sites. ex1, exon I. (B) DNase I chromatin sensitivity assays of nuclei isolated from control and HMGN3 knockdown MIN6 cells. Shown are ethidium bromide-stained gels of the digested DNAs. Cont, control. (C) Plot demonstrating that downregulation of HMGN3 levels does not affect the chromatin structure of the P1 region in the *Glut2* promoter. cont, control. (D) Downregulation of HMGN3 levels does not affect the chromatin in the 5′ region of the *Glut2* gene.

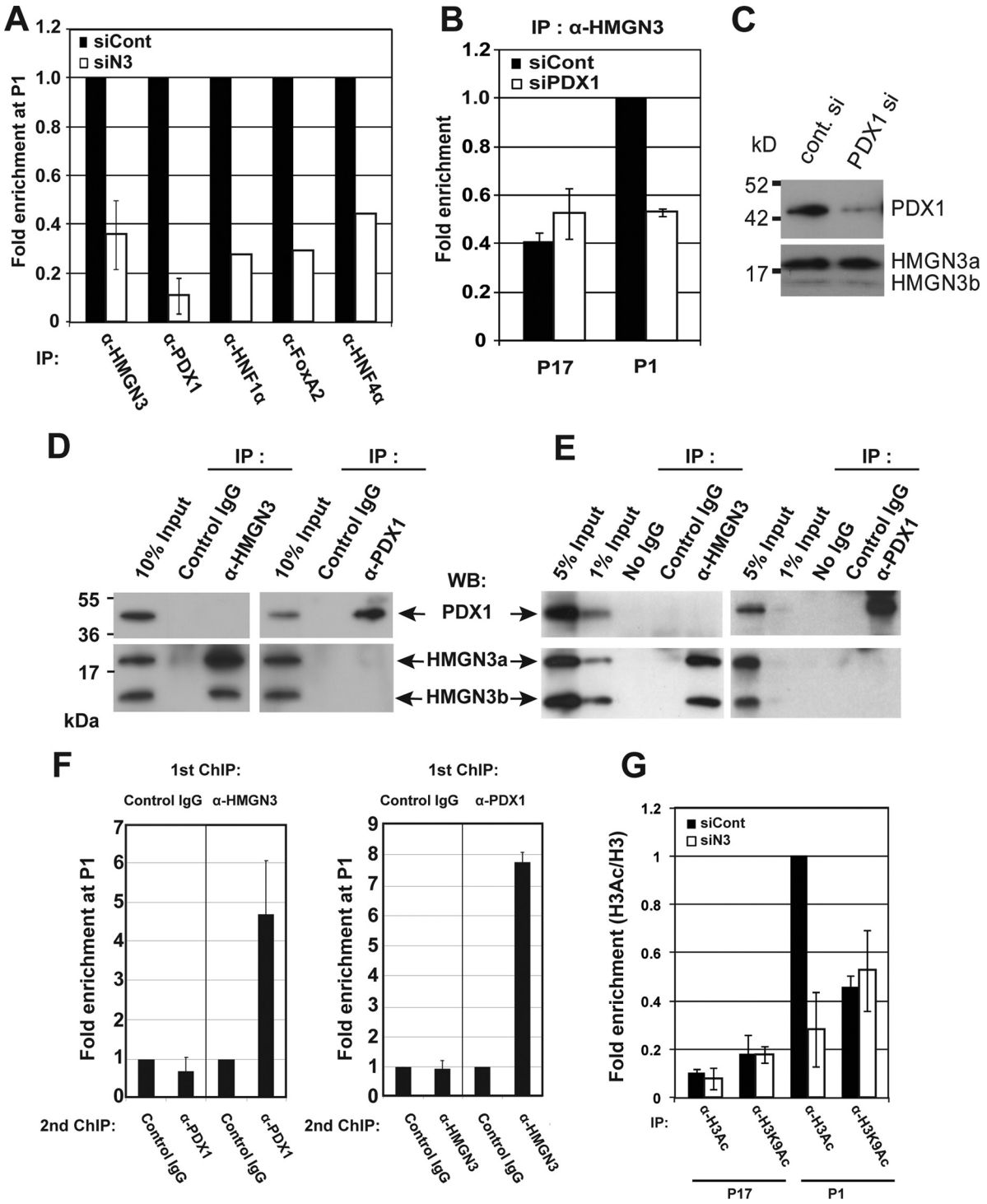

FIG. 6. HMGN3 and PDX1 colocalize on the *Glut2* proximal promoter. (A) Downregulation of HMGN3 reduces the binding of transcription factors to the *Glut2* promoter. siCont, control siRNA; IP, antibody used for immunoprecipitation. (B) Downregulation of PDX1 reduces the binding of HMGN3 to the Glut2 promoter. (C) Efficient downregulation of PDX1 expression by siRNA treatment. Shown are Western blot assays of extracts prepared from MIN6 cells treated with either control siRNA (cont. si) or PDX1-specific siRNA. A Western blot assay with HMGN3 served as a loading control. (D) HMGN3 and PDX1 do not form a complex in the nucleoplasm. Shown are Western blot assays of immunoprecipitates of nuclear extracts treated with antibodies to either HMGN3 or PDX1. WB, antibody used for Western blot assays. (E) HMGN3 and PDX1 do not colocalize on chromatin globally. Shown are Western blot assays of ChIP experiments with either antibodies to HMGN3 or antibodies to PDX1. (F) HMGN3 and PDX1 colocalize in the promoter region of *Glut2* chromatin. Left panel: first ChIP, anti-HMGN3 antibody; second ChIP, anti-PDX1 antibody. Right panel: first ChIP, anti-PDX1 antibody; second ChIP, anti-HMGN3 antibody. Note the lack of enrichment when control IgG was used. The enrichment of the immunoprecipitated DNAs with the P1 region of *Glut2* was analyzed by Q-RT-PCR. (G) HMGN3 enhances the global acetylation of histone H3 in the promoter but not in the upstream region of the *Glut2* gene. The locations of the P1 and P17 regions are shown in Fig. 5A.

HMGN3 expression in β cells and in MIN6 cells are high relative to those in other mouse tissues, but they are comparable to those of HMGN1 or HMGN2 (Fig. 1C and 3B). Yet, in spite of similar protein levels, only HMGN3 affects insulin secretion, suggesting a specific role for HMGN3 in β-cell function. Fourth, while loss of HMGN3 impairs GSIS, it does not impair arginine-stimulated insulin secretion. Arginine stimulates insulin secretion by affecting membrane polarization directly, independently of glucose sensing and import into β cells (28). Taken together, the findings indicate that HMGN3 plays a role in glucose secretion both in mice and in isolated β cells.

GLUT2 is a membrane protein that facilitates the entry of glucose into various mammalian cells, including insulin-secreting pancreatic β cells (15, 23, 30, 38). Although loss of GLUT2 has severe metabolic consequences and affect insulin secretion, several studies, including analysis of *Glut2*$^{-/-}$ mice, suggest that partial downregulation of GLUT2 levels in β cells does not lead to a strong diabetic phenotype and does not significantly impact insulin secretion from isolated cells (15, 16, 21, 37). Thus, by itself, the partial downregulation of GLUT2 levels in β cells does not fully explain the diabetic phenotype of *Hmgn3*$^{-/-}$ mice. HMGN3 is expressed in all of the various pancreatic islet cell types, including glucagon-secreting alpha cells (data not shown), as well as in several other tissues, including the brain (20, 40), all of which impact insulin levels and glucose homeostasis. Furthermore, we also observed that the siRNA-mediated downregulation of HMGN3 in MIN6 cells alters the expression of several genes involved in GSIS (Fig. 4A), suggesting that loss of HMGN3 may have a widespread effect on the transcription profile of β cells. While the transcription level of a single gene may not be severely affected, the cumulative changes in the expression levels of several genes impair the normal function of β cells and impact the GSIS pathway. Recent studies on the role of the histone methyltransferase Set7/9 in β-cell function demonstrate that changes in histone modification and chromatin structure alter the expression of several genes and lead to impaired GSIS (11). Members of the HMGN protein family have been shown to affect the structure of chromatin and the modification levels of histone tails. Thus, HMGN3 may affect the expression levels of β-cell genes by altering their chromatin structure and affecting the recruitment of transcription factors to specific sites.

Our ChIP analyses (Fig. 5A) indicate that HMGN3 enhances *Glut2* transcription directly by binding to the chromatin in the promoter region of this gene. Significantly, only HMGN3, and not the closely related proteins HMGN1 and HMGN2, is enriched in the *Glut2* promoter, a finding consistent with the observation that these two HMGN variants do not affect *Glut2* transcription (Fig. 4B) or GSIS (Fig. 3C). Interestingly, HMGN3 does not have significant effects on the kinetics of DNase I digestion of *Glut2* (Fig. 5B to D), an indication that the HMGN3-mediated transcriptional enhancement of this gene does not involves major changes in chromatin structure. On the other hand, HMGN3 alters the levels of histone modification in this gene (Fig. 6G). Previous studies demonstrated that HMGN1 and HMGN2 affect the levels of several histone modifications in a variant-specific manner (36). Given the large number of histone modifications, it remains to be seen which specific modification is affected by HMGN3.

We found that loss of HMGN3 significantly decreased the binding of several transcription factors, including PDX1, to *Glut2* chromatin (Fig. 6A), thereby providing a mechanism whereby HMGN3 regulates *Glut2* transcription. PDX1 is a key transcription factor involved in the development and maintenance of mature β cells, where it affects the transcription of key genes, including *Glut2* (9). In considering the mechanism whereby HMGN3 promotes the binding of PDX1 to *Glut2*, we found that these two proteins specifically associate on the chromatin of the *Glut2* promoter, as indicated by our reciprocal sequential precipitation experiments (Fig. 6F). Furthermore, we found that while HMGN3 affects the binding of PDX1, PDX1 in turn affects the binding of HMGN3 to the same region (Fig. 6B). Our studies clearly demonstrate that the two proteins do not form a complex in the nucleoplasm and do not bind to the same binding sites throughout the entire chromatin (Fig. 6D and E). It follows that the two proteins mutually strengthen their interaction with a specific chromatin locus. This mode of binding is reminiscent of that of the glucocorticoid receptor (GR) and the chromatin binding protein HMGB1, which have been shown to mutually stabilize each other's chromatin interactions (1).

Stochastic assembly of transcription factors at their target sites has been described in several systems (14). For HMGB1 and GR, Agresti et al. argue that in living cells the stochastic assembly of rapidly moving chromatin binding protein at specific binding sites mutually reinforces their chromatin interactions (1). It is well documented that, just like GR and HMGB1, HMGN and various transcription factors move rapidly through the nucleus, continuously sampling nucleosomes for optimal binding sites (8). We therefore propose that the stochastic assembly of HMGN3 with PDX1 and perhaps other transcription factors at the *Glut2* promoter reinforces their chromatin interaction, thereby enhancing *Glut2* transcription.

Together with recent studies on the effect of HMGN1 on estrogen-mediated transcriptional activation (44) and with previous analyses of the role of HMGN in gene expression, the emerging picture suggests that HMGN proteins modulate the interaction of specific transcription factors at distinct genes in the context of chromatin (3, 18, 24).

In summary, we identify HMGN3 as a chromatin binding protein that modulates the interaction of a transcription factor with chromatin and in pancreatic β cells affects the expression of several genes, including *Glut2*. The protein is present in all of the cell types of pancreatic islets and in several tissues known to be involved in regulating glucose homeostasis. In mice, loss of HMGN3 leads to an impaired GSIS response and to changes in glucose and insulin levels. The human and mouse HMGN3 amino acid sequences are very similar, and human β cells express significant levels of HMGN3. Our findings raise the possibility that impaired function of HMGN3 is a contributing factor in the etiology of diabetes.

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