aspects of root development. In the future, it will be interesting to compare the spatial and temporal transcriptional complexity that underlies organ development in other multicellular organisms.

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Menin Controls Growth of Pancreatic β-Cells in Pregnant Mice and Promotes Gestational Diabetes Mellitus

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During pregnancy, maternal pancreatic islets grow to match dynamic physiological demands, but the mechanisms regulating adaptive islet growth in this setting are poorly understood. Here we show that menin, a protein previously characterized as an endocrine tumor suppressor and transcriptional regulator, controls islet growth in pregnant mice. Pregnancy stimulated proliferation of maternal pancreatic islet β-cells that was accompanied by reduced islet levels of menin and its targets. Transgenic expression of menin in maternal β-cells prevented islet expansion and led to hyperglycemia and impaired glucose tolerance, hallmark features of gestational diabetes. Prolactin, a hormonal regulator of pregnancy, repressed islet menin levels and stimulated β-cell proliferation. These results expand our understanding of mechanisms underlying diabetes pathogenesis and reveal potential targets for therapy in diabetes.

Maternal pancreatic islet expansion in rodents and humans (*1–3*) suggests that adaptive islet cell growth is a mechanism for ensuring metabolic balance in pregnancy, a physiological state marked by increased insulin demand. Descriptive studies with rats (*2*, *3*) support the hypothesis that proliferation of insulin-secreting islet β-cells is the principal mechanism of β-cell expansion in pregnancy, but the molecular basis of facultative maternal β-cell proliferation is unknown. Moreover, it is unclear if impaired maternal β-cell proliferation leads to reduced insulin levels and gestational diabetes (*4*).

To investigate the mechanisms controlling maternal islet expansion, we examined β-cell mass in pregnant C57Bl6 mice. We found that maternal β-cell mass increased by twofold (fig. S1A), accommodating increases in maternal body mass (fig. S1B). After parturition, maternal β-cell mass and body mass returned to prepartum levels (fig. S1, A and B). To assess maternal islet cell proliferation, we performed labeling studies with bromodeoxyuridine (BrdU). β-cell proliferation increased in pregnant mice until 15 days postcoitum (dpc) and then declined to prepartum levels (Fig. 1, A to C). Thus, maternal islet β-cell expansion and mass are dynamic in mice.

Hyperplasia of the maternal pituitary (*5*) and islets in pregnancy is reminiscent of endocrine proliferation in multiple endocrine neoplasia type 1 (MEN1), a human cancer syndrome characterized by synchronous tumors of the pituitary, endocrine pancreas, and parathyroid. Most MEN1 cases result from mutation of *Men1*, whose protein product is menin (*6*, *7*). In mice and humans, mutation and pathological *Men1* loss promote neuroendocrine tumors, including islet β-cell tumors (*7*, *8*). Thus, we postulated that physiological changes in *Men1* expression might regulate facultative maternal β-cell growth in pregnancy. Immunohistology, Western blotting, and real-time reverse transcription polymerase chain reaction (RT-PCR) studies of maternal islets isolated during gestation revealed that islet levels of *Men1* mRNA and menin decreased in pregnancy (Fig. 1, D to G), then increased to prepartum levels by 1 week after birth of the pups (Fig. 1, F and G). By contrast, we did not detect changes in maternal islet levels of mRNA encoding mixed lineage leukemia-1 (MLL1), a protein that associates with menin (Fig. 1F). Thus, attenuated *Men1* expression corresponded with increased β-cell proliferation in maternal islets.

Menin functions in a histone methyltransferase protein complex containing MLL (*9*, *10*). This complex promotes trimethylation of histone H3 on lysine 4 (H3K4), an epigenetic mark associated with transcriptionally active chromatin. Menin-dependent histone methylation maintains expression of $p27^{Kip1}$ and $p18^{INK4C}$ (hereafter, *p27* and *p18*), which encode cyclin-dependent kinase (CDK) inhibitors that prevent islet proliferation (*11–14*). Consistent with our previous findings in islet tumors (*11*), reduced islet menin levels in pregnancy after 8 dpc were accompanied by reduced *p27* and *p18* mRNA and protein levels (Fig. 1G and fig. S1C) and, as revealed by chromatin immunoprecipitation (ChIP), by decreased levels of menin and trimethyl H3K4 associated with *p27* and *p18* (Fig. 1H). Thus, attenuated menin levels and function reduced pancreatic islet *p27* and *p18* expression in pregnant mice.

To determine whether adaptive maternal β-cell proliferation might require reduced *Men1* expression, we generated mice that permitted conditional *Men1* expression in β-cells. Transgenic mice producing hemagglutinin-tagged menin under control of the tetracycline response element (TRE-*Men1*) (*15*) were generated and mated with mice expressing the reverse tetracycline trans-activator (rtTA) in β-cells directed by the rat insulin promoter (RIP) (*16*). In bi-transgenic RIP-*rtTA*, TRE-*Men1* mice (abbreviated βMen1), administration of doxycycline (Dox) allows rtTA binding to the TRE element and stimulates β-cell expression of *Men1* mRNA and menin protein (fig. S2, A and B). Exposure of RIP-*rtTA* or TRE-*Men1* single transgenic mice to Dox did not induce changes in menin levels (fig. S2, A

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and B). In islets isolated from male or virgin female βMen1 mice continuously exposed to Dox after 10 weeks of age, we detected increased levels of *Men1*, *p27*, and *p18* mRNA. Thus, conditional induction of menin in β-cells stimulated expression of known menin-target genes. However, TRE-*Men1* expression in male or virgin female βMen1 mice did not alter serum insulin or glucose control (fig. S2, C to E), indicating that TRE-*Men1* expression alone did not disrupt β-cell function.

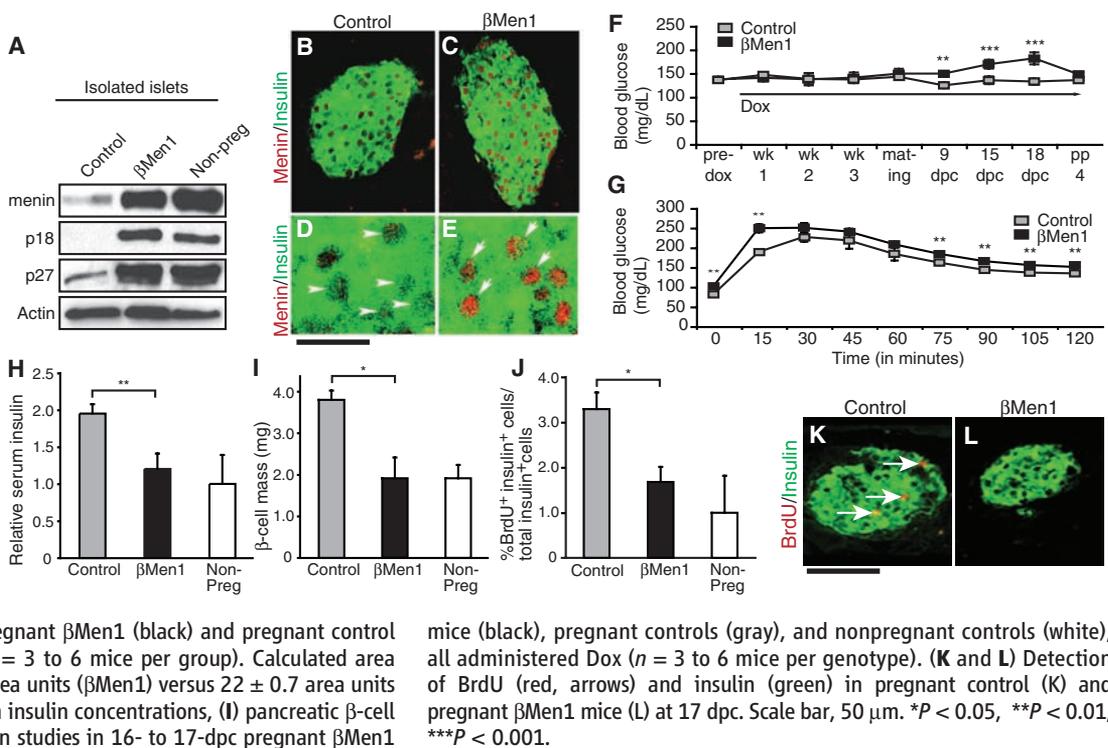To assess the consequences of menin misexpression during pregnancy, we continuously exposed 10-week-old βMen1 females to Dox for 8 weeks, a period encompassing mating, gestation, and delivery. Western blot and RT-PCR analysis revealed that *Men1* mRNA and menin levels were increased in islets from Dox-exposed pregnant βMen1 females compared to controls, and immunohistology confirmed that menin was increased in islet β-cells from βMen1 mothers (Fig. 2, A to E, and fig. S2F). *p27* and *p18* mRNA and protein in βMen1 islets also increased to levels indistinguishable from those in prepartum islets (Fig. 2A and fig. S2F). By contrast, mRNAs encoding *Insulin1* (*Ins1*), *Insulin2* (*Ins2*), *Glut2*, and *Pdx1*, factors that govern β-cell function, were unchanged in

βMen1 islets (fig. S2G). We measured glucose regulation, islet gene expression, and β-cell growth in female βMen1 mice starting at 10 weeks of age. Unlike age-matched pregnant controls or virgin βMen1 females exposed to Dox, pregnant βMen1 females exposed to Dox and fed ad libitum developed moderate hyperglycemia by 9 dpc, which worsened until delivery (Fig. 2F and fig. S3, A and B). Compared to controls, mean glucose levels in fasted βMen1 mothers on Dox were also significantly higher (Fig. 2G and fig. S3C). Intraperitoneal glucose challenge similarly revealed impaired glucose tolerance in pregnant βMen1 females on Dox

Fig. 1. Dynamic regulation of menin and its target genes during facultative islet growth in pregnant mice. (**A**) Quantification of BrdU incorporation by maternal islet β-cells (*n* = 3 to 6 mice sampled per time point). (**B** and **C**) Detection of insulin (green) and BrdU (red) in islets from nonpregnant (B) and 17-dpc pregnant mice (C). (**D** and **E**) Detection of insulin (green) and menin (red) in islets from nonpregnant (D) and 17-dpc pregnant female mice (E). Scale bar, 50 μm. (**F** and **G**) Real-time PCR analysis of *Men1* and *Mll* (F) and Western blot analysis of the indicated proteins (G) from isolated maternal islets. (**H**) ChIP studies of menin and trimethylated H3K4 associated with *p18* and *p27* from nonpregnant, pregnant, and postpartum maternal islets (*n* = 3 to 6 mice). Data here and in Figs. 2 to 4 are presented as the means ± SD. *$P < 0.05$, **$P < 0.01$. Ab, antibody; IgG, immunoglobulin G; Menin, anti-menin Ab; Methyl, anti-H3K4 Ab; In, input DNA; IP, immunoprecipitate.
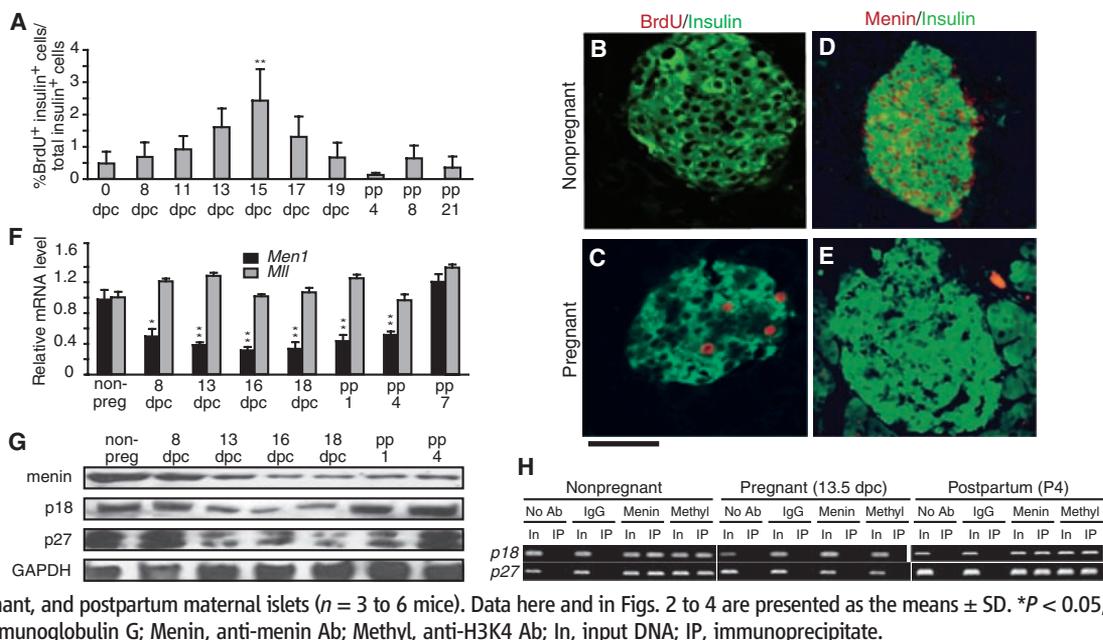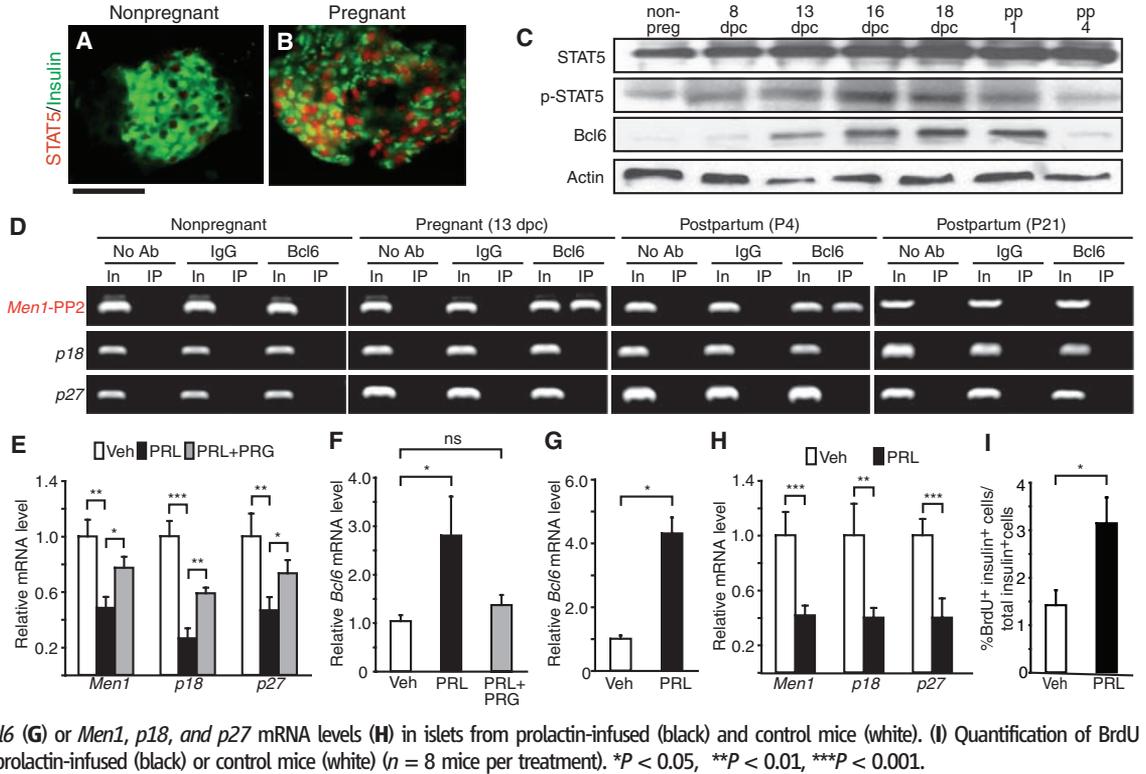

Fig. 2. Phenotypes from misexpression of menin in islets of pregnant mice. (**A**) Western blot analysis of the indicated proteins in islets from pregnant control and pregnant βMen1 mice, both at 17 dpc, and from nonpregnant control mice. (**B** to **E**) Immunohistologic detection of nuclear menin [red, arrowheads in (D) and arrows in (E)] in islet β-cells (green) from pregnant control (B and D) and pregnant βMen1 (C and E) mice, both at 17 dpc. Scale bar, 50 μm (B and C) and 20 μm (D and E). (**F**) Blood glucose levels in βMen1 mice (black) and controls (gray) fed ad libitum and exposed to Dox before, during, and after pregnancy (*n* = 10 to 15 mice per group). (**G**) Intraperitoneal glucose tolerance tests of Dox-exposed pregnant βMen1 (black) and pregnant control mice (gray), both at 16 dpc (*n* = 3 to 6 mice per group). Calculated area under the curve was 25 ± 0.4 area units (βMen1) versus 22 ± 0.7 area units (controls), *P* < 0.005. (**H**) Serum insulin concentrations, (**I**) pancreatic β-cell mass, and (**J**) BrdU incorporation studies in 16- to 17-dpc pregnant βMen1 mice (black), pregnant controls (gray), and nonpregnant controls (white), all administered Dox (*n* = 3 to 6 mice per genotype). (**K** and **L**) Detection of BrdU (red, arrows) and insulin (green) in pregnant control (K) and pregnant βMen1 mice (L) at 17 dpc. Scale bar, 50 μm. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Fig. 3. Stat5 and Bcl6 regulation of menin in maternal islets. (**A** and **B**) Detection of Insulin (green) and Stat5 (red) in islets from nonpregnant (A) and pregnant (B) mice. Scale bar, 50 μm. (**C**) Western blot detection of Stat5, phospho-Stat5, and Bcl6 in extracts of maternal islets. (**D**) ChIP analysis of Bcl6 association with the *Men1*, *p18*, and *p27* loci in islets isolated from nonpregnant, pregnant, postpartum day 4, and postpartum day 21 mice. Primer pair 2 (PP2) for *Men1* has been described previously (*7*). (**E** and **F**) Real-time RT-PCR analysis of *Men1*, *p18*, and *p27* (E) or *Bcl6* mRNA levels (F) in islets exposed to prolactin (black), vehicle (white), or to both prolactin and progesterone (gray) (*n* = 3 independent experiments). Real-time RT-PCR analysis of *Bcl6* (**G**) or *Men1*, *p18, and p27* mRNA levels (**H**) in islets from prolactin-infused (black) and control mice (white). (**I**) Quantification of BrdU incorporation by islet β-cells in prolactin-infused (black) or control mice (white) (*n* = 8 mice per treatment). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

(Fig. 2G). Consistent with these findings, serum insulin levels were reduced in pregnant βMen1 mice (Fig. 2H). Other phenotypes, including body mass, litter size, and average birth weight of pups, were indistinguishable for βMen1 mothers and controls (fig. S4, A to C). In women with gestational diabetes, serum glucose concentration often returns to normal after delivery. Likewise in βMen1 mothers on Dox, blood glucose levels decreased after birth of the pups and were indistinguishable from those of control mothers (Fig. 2F). Thus, βMen1 mice recapitulated features of human gestational diabetes.

To investigate whether impaired insulin production or secretion might underlie hypoinsulinemia in βMen1 mice, we measured insulin content and secretion in isolated islets. Insulin content per islet cell was similar in pregnant βMen1 and control mice (fig. S4D). Likewise, insulin secretion by βMen1 islets after stimulation with glucose or arginine (fig. S4, E and F) was indistinguishable from that of controls. To determine if gestational hypoinsulinemia and hyperglycemia in βMen1 mice reflected impaired β-cell expansion, we assessed the pancreata of pregnant βMen1 and control mice. In Dox-treated pregnant βMen1 mice at the end of gestation, β-cell mass was significantly reduced compared to pregnant controls and appeared indistinguishable from β-cell mass in prepartum βMen1 mice (Fig. 2I). In pregnant βMen1 mice, BrdU studies revealed reduced β-cell proliferation at 17 dpc (Fig. 2, J to L). Thus, gestational reduction of menin, p27, and p18 levels was prevented in βMen1 islets, leading to impaired β-cell expansion. Moreover, we did not detect differences in islet cell apoptosis in βMen1 and control littermate mice (fig. S3G), suggesting that reduced β-cell mass in βMen1 mice did not
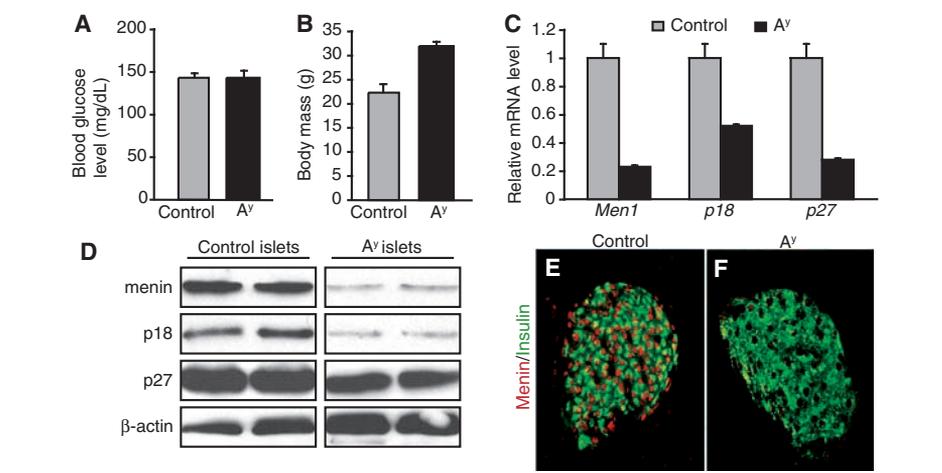
Fig. 4. Dynamic regulation of menin and its target genes during facultative islet growth in obese *A^y* mice. (**A**) Blood glucose concentration and (**B**) body mass in 12-week-old control and *A^y* littermate mice fed ad libitum. (**C**) Real-time RT-PCR analysis of *Men1*, *p18*, and *p27* mRNA levels in islets from control or *A^y* littermate mice. (**D**) Western blot analysis of the indicated proteins in islets from control and *A^y* littermate mice. (**E** and **F**) Detection of insulin (green) and menin (red) in islet β-cells from control (E) and *A^y* littermate (F) mice. Scale bar, 50 μm.

result from cell death. Collectively, these findings suggest that attenuation of maternal islet menin levels permits adaptive β-cell expansion in pregnancy.

Prolactin and placental lactogens are hormonal regulators of pregnancy that stimulate β-cell proliferation in rodent and human islets (*17–19*), but the molecular basis for their mitogenic effect is unknown. We investigated if prolactin signaling regulates *Men1* in β-cells. Lactogenic hormones stimulate phosphorylation and nuclear accumulation of signal transducer and activator of transcription 5 (STAT5), which induces expression of targets like *Bcl6* (*20*). In islets from pregnant mice, nuclear

STAT5 accumulated in β-cells (Fig. 3, A and B), and levels of phospho-STAT5 and STAT5 occupancy at consensus STAT5 binding sequences (*21*) in *Bcl6* increased (Fig. 3C and fig. S5A). *Bcl6* mRNA and protein content increased (Fig. 3C and fig. S5B), and ChIP revealed direct association of Bcl6, a transcriptional repressor (*21*, *22*), with *Men1* during pregnancy (Fig. 3D). Misexpression of Bcl6 in MIN6 cells, a murine insulinoma–derived β-cell line responsive to normal growth cues (*11*, *23*), was sufficient to reduce expression of endogenous *Men1*, *p18*, and *p27* mRNA (fig. S5C). Moreover, *Bcl6* expression reduced transcription of *Men1*-

luciferase reporters harboring consensus Bcl6 binding sequences (fig. S5, D and E). Thus, Bcl6 directly associated with and repressed *Men1* transcription in β-cells. Previous studies have shown that steroids like progesterone and dexamethasone can inhibit the mitogenic effects of prolactin on β-cells (*24*), but the underlying mechanism is unknown. Simultaneous exposure of isolated mouse islets to prolactin and progesterone attenuated changes in *Men1*, *p18*, *p27*, and *Bcl6* expression provoked by prolactin alone (Fig. 3, E and F). Thus, multiple hormonal inputs likely regulate β-cell *Men1* expression. Bcl6-dependent changes in *Men1*, *p18*, and *p27* expression provoked by prolactin in MIN6 cells or in cultured human islets (figs. S6 to S8) corroborated these findings and showed that *Men1* regulation by lactogen signaling is evolutionarily conserved.

To test if lactogen signaling was sufficient to reduce *Men1* expression and increase β-cell proliferation in vivo, we transplanted mice with osmotic micropumps to deliver prolactin for 6 days (*25*). Compared with islets from vehicle-infused controls, islets from prolactin-infused mice had a fourfold increase in *Bcl6* expression, a 50% reduction of *Men1*, *p18*, and *p27* mRNA, and a 2.5-fold increase of BrdU incorporation by β-cells (Fig. 3, G to I). Thus, short-term prolactin infusion was sufficient to reduce *Men1* expression in vivo and to stimulate proliferation of adult islet β-cells. Additional studies are needed to determine if lactogenic hormone regulation of *Men1* governs other features that affect β-cell expansion, such as β-cell size and survival (*26*).

To determine if menin might regulate adaptive β-cell expansion in obesity, another common physiological state that stimulates adaptive islet expansion, we measured islet menin levels in A^y mice, a well-characterized model of hyperphagic obesity [reviewed in (*27*)]. At 3 months, when A^y mice are obese but normoglycemic (Fig. 4, A and B), A^y islet levels of *Men1* mRNA, menin, and *p27* and *p18* mRNA and protein were reduced compared to islets from wild-type controls (Fig. 4, C to E). These results suggest that in obesity, menin attenuation regulates adaptive β-cell proliferation.

Studies of endocrine neoplasias in MEN1 syndrome and other cancers have defined menin roles solely in the context of tumor pathogenesis. Our work expands this view, showing that menin functions as a physiological regulator of adaptive β-cell expansion in pregnancy and possibly other common states linked to type 2 diabetes, such as obesity. We speculate that menin may integrate β-cell growth signals in physiological islet expansion, controlling dynamic histone modifications that govern β-cell fate and proliferation. Menin-independent control of maternal β-cell expansion is not excluded by our study, and investigating the role of other islet tumor suppressors, like von Hippel–Lindau protein (*28*), in physiological β-cell expansion might be fruitful. Our finding that *Men1* expression is regulated by prolactin and progesterone raises the possibility that defects in signaling pathways regulated by lactogenic or steroid hormones might underlie specific forms of type 2 diabetes, including gestational diabetes, and endocrine neoplasias linked to

Men1 inactivation, including carcinoid and insulinoma (*7*). Our work also suggests that manipulation of regulators, cofactors, and targets of menin might be a therapeutic strategy for expanding functional pancreatic islets in diabetes.

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Ordered Phosphorylation Governs Oscillation of a Three-Protein Circadian Clock

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The simple circadian oscillator found in cyanobacteria can be reconstituted in vitro using three proteins—KaiA, KaiB, and KaiC. The total phosphorylation level of KaiC oscillates with a circadian period, but the mechanism underlying its sustained oscillation remains unclear. We have shown that four forms of KaiC differing in their phosphorylation state appear in an ordered pattern arising from the intrinsic autokinase and autophosphatase rates of KaiC and their modulation by KaiA. Kinetic and biochemical data indicate that one of these phosphoforms inhibits the activity of KaiA through interaction with KaiB, providing the crucial feedback that sustains oscillation. A mathematical model constrained by experimental data quantitatively reproduces the circadian period and the distinctive dynamics of the four phosphoforms.

Circadian clocks coordinate metabolism and behavior with diurnal cycles in the environment (*1*). These clocks traditionally have been understood as transcriptional feedback oscillators in which clock genes repress their own synthesis, creating negative feedback that drives oscillation (*1*). However, pioneering work by Kondo and colleagues has shown that the circadian clock of the cyanobacterium *Synechococcus elongatus* requires neither transcription nor translation (*2*), and circadian oscillations can be reconstituted in vitro using only three proteins: KaiA, KaiB, and KaiC (*3*).

KaiC is a hexameric enzyme (*4*) that can autophosphorylate (*5*) and autodephosphorylate (*6*) at both serine 431 (S431) and threonine 432 (T432) (*7*, *8*). The dimeric KaiA (*9*, *10*) enhances the autophosphorylation of KaiC (*11*), whereas KaiB antagonizes the activity of KaiA (*11–13*). In the absence of KaiA, KaiC fully dephospho-

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Science

Menin Controls Growth of Pancreatic ß-Cells in Pregnant Mice and Promotes Gestational Diabetes Mellitus

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