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Menin regulates pancreatic islet growth by promoting histone methylation and expression of genes encoding p27^{Kip1} and p18^{INK4c}

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Menin, the product of the *Men1* gene mutated in familial multiple endocrine neoplasia type 1 (MEN1), regulates transcription in differentiated cells. Menin associates with and modulates the histone methyltransferase activity of a nuclear protein complex to activate gene expression. However, menin-dependent histone methyltransferase activity in endocrine cells has not been demonstrated, and the mechanism of endocrine tumor suppression by menin remains unclear. Here, we show that menin-dependent histone methylation maintains the *in vivo* expression of cyclin-dependent kinase (CDK) inhibitors to prevent pancreatic islet tumors. *In vivo* expression of CDK inhibitors, including p27 and p18, and other cell cycle regulators is disrupted in mouse islet tumors lacking menin. Chromatin immunoprecipitation studies reveal that menin directly associates with regions of the *p27* and *p18* promoters and increases methylation of lysine 4 (Lys-4) in histone H3 associated with these promoters. Moreover, H3 Lys-4 methylation associated with *p27* and *p18* is reduced in islet tumors from *Men1* mutant mice. Thus, H3 Lys-4 methylation is a crucial function of menin in islet tumor suppression. These studies suggest an epigenetic mechanism of tumor suppression: by promoting histone modifications, menin maintains transcription at multiple loci encoding cell cycle regulators essential for endocrine growth control.

islet of Langerhans | Men1 | multiple endocrine neoplasia | tumor suppressor | diabetes mellitus

Mutation of the *Men1* tumor suppressor gene, which encodes the protein menin, promotes pathogenesis of type 1 multiple endocrine neoplasia (MEN1) syndrome and sporadic neuroendocrine tumors in humans (1, 2). The molecular basis for the endocrine tumor bias in MEN1 is unknown. Targeted heterozygous *Men1* inactivation in mice produces a spectrum of endocrine tumors similar to those observed in human patients with MEN1 syndrome (3–5). For example, *Men1*$^{+/-}$ mice develop pancreatic islet tumors, the most common enteroendocrine tumor in human MEN1 syndrome, as well as tumors in the anterior pituitary, adrenal cortex, and the stomach. In some cases, loss of chromosome 19, which harbors *Men1* in mice, was detected in islet tumors (5), consistent with loss of heterozygosity and the postulated tumor suppressor function of menin. Homozygous *Men1* loss in mice leads to embryonic lethality by midgestation [embryonic day (E) 11.5–13.5] and is found associated with a variety of developmental defects, including undergrowth and defective organogenesis of the neural tube, heart, and liver (6). Thus, menin may have general roles in cell growth and development.

Menin localizes to the nucleus and regulates gene transcription (7). Recent biochemical studies demonstrate that menin associates with a nuclear protein complex that includes the trithorax group (TrxG) members MLL, MLL2, and Ash2 (8–10). This complex promotes the methylation of histone H3 at lysine 4 (Lys-4), consistent with the hypothesized role of MLL as a histone methyltransferase (HMTase; ref. 11). Site-specific histone modification is a major epigenetic mechanism for maintaining stable gene transcription in terminally differentiated cells (12, 13). Thus, menin may function as a tumor suppressor by regulating histone methylation in promoters of specific target genes that govern neuroendocrine cell growth and differentiation. However, menin-dependent HMTase activity at endocrine cell promoters has not yet been demonstrated. Thus, it is unclear whether dysregulated gene expression promotes endocrine tumors, a defining phenotype of MEN1. Moreover, it has not yet been demonstrated that menin regulates histone H3 methylation *in vivo* to suppress endocrine tumor formation, for example, in mouse MEN1 models.

Clues to identifying relevant genes in endocrine cells that might be regulated by menin-dependent HMTase activity come from recent studies revealing the roles of cyclin-dependent kinases (CDKs) and their inhibitors in regulating endocrine cell growth. Mice homozygous for null alleles of *p18^{INK4C}* (which encodes a member of the Ink4 family of CDK inhibitors, hereafter called *p18*) or for *p27*^{Kip1} (which encodes a member of the Cip/Kip family of CDK inhibitors, hereafter called *p27*) had generalized organomegaly but did not develop spontaneous endocrine tumors until 10 months of age or later, when they developed pituitary adenomas (14–17). However, simultaneous loss of *p18* and *p27* was sufficient to produce a specific spectrum of endocrine tumors similar to that seen in human MEN1 and MEN2 syndromes, including tumors in the pituitary, parathyroid, thyroid, endocrine pancreas, stomach, and duodenum (18). p18 and p27 are known to inhibit functions of cell cycle regulators like CDK2 and CDK4 (19). Thus, these studies suggested that *Men1* inactivation might disrupt expression of *p18*, *p27*, and other established cell cycle regulators in endocrine cells. A recent study showed that menin can regulate *p18* and *p27* expression by fibroblasts *in vitro*, but menin-dependent changes in levels of histone methylation were not reported (10). Thus, the molecular basis for menin-dependent control of cell cycle regulators remained unclear. Here, we report that menin is required to regulate histone methylation in genes encoding specific cell cycle regulators, thereby maintaining the expression of these genes in endocrine cells, and that this property is the basis for endocrine tumor suppression by menin in a mouse model of MEN1.

Materials and Methods

Generation, Genotyping, and Physiologic Assessment of Men1$^{+/-}$ Mice. Generation, breeding, and genotyping of mice harboring a null allele of the *Men1* gene on the C57BL genetic background have

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Abbreviations: MEN, multiple endocrine neoplasia; TrxG, trithorax group; Lys-4, lysine 4; HMTase, histone methyltransferase; CDK, cyclin-dependent kinase; ChIP, chromatin immunoprecipitation; siRNA, small interfering RNA; PP, primer pair.

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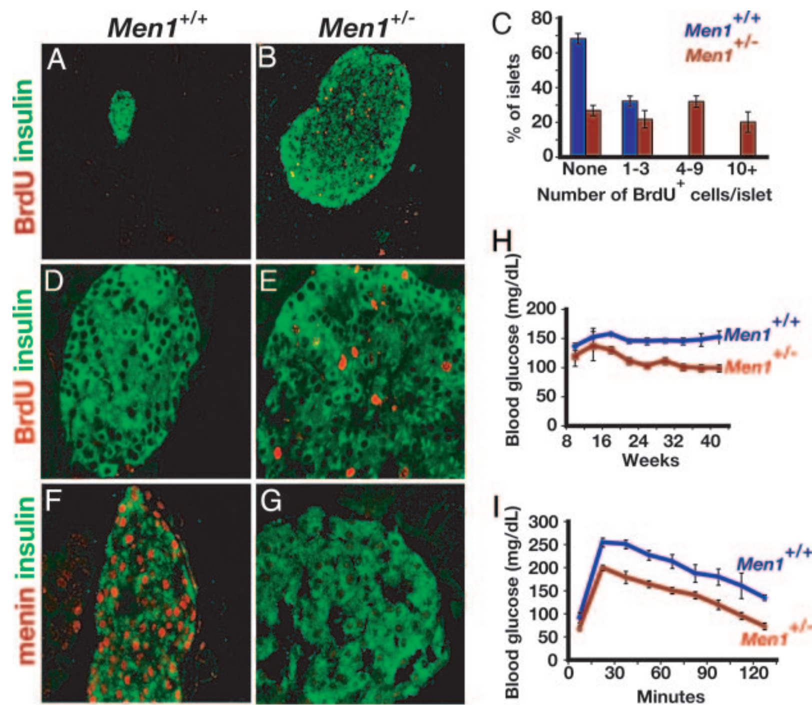

Fig. 1. Pancreatic β cell hyperplasia, increased BrdUrd incorporation, loss of menin expression, and hypoglycemia in *Men1*$^{+/-}$ mice. (*A*, *B*, *D*, and *E*) Immunostaining of BrdUrd (red) and insulin (green) in pancreatic tissue from 9-month-old *Men1*$^{+/+}$ (*A* and *D*) and *Men1*$^{+/-}$ mice (*B* and *E*). (*C*) Quantification of BrdUrd^{+} cells in islets (n = 100) from *Men1*$^{+/+}$ and *Men1*$^{+/-}$ mice. (*F* and *G*) Immunohistochemical detection of nuclear menin (red) and insulin (green) in pancreas from *Men1*$^{+/+}$ (*F*) and *Men1*$^{+/-}$ mice (*G*). (*H*) Blood glucose level during random feeding in mice with the indicated age and genotype (wild-type or *Men1*$^{+/-}$). n = 8 or more animals for each genotype. After 20 weeks of age, hypoglycemia in *Men1*$^{+/-}$ mice remained statistically significant at $P < 0.01$. (*I*) Glucose tolerance testing of 28-week-old wild-type (n = 11), and *Men1*$^{+/-}$ littermates (n = 10) after 14-h overnight fast. Data in *C*, *H*, and *I* are presented as average ± SEM. [Original magnification: ×16 (*A* and *B*) and ×63 (*D*–*G*).]

been described (8). Blood glucose levels during random feeding and glucose challenge were measured as described (20, 21). Serum glucose values are represented as the mean from at least eight animals per genotype per time point, with standard error of the mean indicated by a bar. Statistical significance was established here and elsewhere by using a two-tailed t test.

Histological Analysis. Immunohistochemical analyses were performed as described (20, 21). The antisera and detection methods used here are described in *Supporting Materials and Methods*, which is published as supporting information on the PNAS web site.

Isolation of Pancreatic Islet RNA, Protein, and RT-PCR Analysis. We isolated pancreatic islets by intraductal collagenase perfusion using standard methods (22). RNA was isolated from the purified islets and reverse-transcribed into cDNA by using a Retroscript Kit (Ambion, Austin, TX). RT-PCR conditions are described in *Supporting Materials and Methods*.

Chromatin Immunoprecipitation (ChIP) Assay. ChIP assays were performed on pancreatic islets and MIN6 cells by using a kit from Upstate Biotechnology (Lake Placid, NY). Briefly, DNA was crosslinked to protein with formaldehyde. Cellular lysates were obtained by scraping, followed by pulsed ultrasonication to shear cellular DNA. We performed overnight immunoprecipitations with the following antibodies: anti-menin (goat, 1:100 Santa Cruz Biotechnology), anti-dimethylated histone H3 Lys-4 (1:300 Upstate Biotechnology), or anti-trimethylated histone H3 Lys-4 (1:300, Upstate Biotechnology). On the next day, the crosslinks were reversed, and bound DNA was purified by phenol:chloroform extraction. We performed PCR using primers specific for *p18*, *p27*, *p15*, *p21*, and *cdk4* proximal promoter sequences. Primer sequences are available upon request.

Transfections and Western Blot Studies. MIN6 murine insulinoma cells were transfected with DNA encoding wild-type menin, with DNA encoding alleles of *Men1* that specify the menin missense forms A242V, L22R, and T344R (8), or with vector DNA lacking *Men1* sequences. In some experiments, these cells were simultaneously transfected with an small interfering RNA (siRNA) specific for menin or a scrambled siRNA control (23). After transfection, cells were grown for 48 h before the lysates were processed for Western blotting. Blots were developed as described (24). Probes for Western blotting are listed in *Supporting Materials and Methods*.

Luciferase Reporter Assays. Luciferase assays were performed according to the manufacturer's specifications (Promega). Additional details are in refs. 25 and 26 and in *Supporting Materials and Methods*.

Results

Menin Controls Pancreatic Islet Growth and Glucose Regulation. The strategy for generating mice harboring null alleles of *Men1* used in this study has been reported (8), but the endocrine cell phenotypes in adult mutants from these lines have not been described. Based on our ability to purify and assess fates of islet cells, we focused our studies on pancreatic islets. Mice heterozygous for the *Men1*-null allele (*Men1*$^{+/-}$) were indistinguishable from wild-type control littermates during the first several months of postnatal life. By 7 months of age, we observed several islet phenotypes in *Men1*$^{+/-}$ mice (Fig. 1). A majority of pancreatic islets appeared enlarged and hyperplastic in *Men1*$^{+/-}$ pancreata (Fig. 1 *A* and *B*) in all animals examined ($n > 20$). Immunostaining revealed that islet enlargement resulted from an increase in numbers of insulin^{+} β cells (Fig. 1 *A*, *B*, and *D*–*G*, and data not shown). Consistent with these results, we found increased *Men1*$^{+/-}$ pancreatic islet cell incorporation of bromodeoxyuridine (BrdUrd), a marker of DNA synthesis during the cell cycle S phase (Fig. 1 *A*–*E*). Islet and pancreatic architecture was not detectably altered in *Men1*$^{+/-}$ mice (data not shown). Immunohistochemistry revealed a significant reduction of menin protein in the β cells of these islets by 7 months (Fig. 1 *F* and *G*). Serial islet isolation and analyses of mRNA by RT-PCR demonstrated that levels of *Men1* mRNA from *Men1*$^{+/-}$ mice were reduced before the appearance of hyperplastic islets (Fig. 2*A*). Reduction of islet *Men1* mRNA likely reflected haploinsufficiency at earlier ages (6 weeks; Fig. 2*A*), but, by 18 weeks and later, we observed further reductions of islet *Men1* mRNA, coinciding with the loss of detectable nuclear menin in β cells. These islet pheno-

types mimic those reported in patients with MEN1 syndrome and in *Men1*$^{+/-}$ mice that developed insulin-producing tumors after loss of the other *Men1* allele (3, 5, 7, 27–29). Thus, this *in vivo* model provided an opportunity to elucidate the basis for islet tumor formation after loss of menin expression.

Men1$^{+/-}$ mice had mild but significant hypoglycemia during random feeding and after overnight fasting by 6–7 months (Fig. 1 *H* and *I*). During glucose challenge, *Men1*$^{+/-}$ mice had reduced blood glucose levels compared with matched wild-type littermate controls. Thus, heterozygosity for the *Men1*-null allele in our animal model resulted in a dominant transmission of phenotypes at high penetrance, including loss of detectable menin expression in islet β cells, islet hyperplasia, and systemic hypoglycemia.

Menin Is Required to Maintain Islet Expression of Multiple Genes Encoding CDK Inhibitors. Prior studies showed that menin is a transcriptional regulator (8–10, 30–34), but little is known about the molecular targets of menin activity in endocrine cells, and how these targets might mediate the tumor suppressor activity of menin. Recent reports showed that mutations disrupting genes encoding cell cycle regulators, including CDK4 and the CDK inhibitors p27 and p18, are sufficient to promote hyperplasia of pancreatic islets and other endocrine organs (17, 18, 35). Thus, gene loci encoding cell cycle regulators in endocrine cells are candidate targets of menin. Consistent with this possibility, p27, p18, and other members of the Cip/Kip and Ink4 family of CDK inhibitors are expressed in postnatal mouse islets (Fig. 2*A*).

To test the possibility that menin regulates islet expression of cell cycle regulators, we measured gene expression by RT-PCR and immunohistochemistry in the whole pancreas and in islets isolated from *Men1*$^{+/-}$ and control littermate mice at regular intervals for 1 year. By 18 weeks, islet expression of p18 and p27 was reduced in *Men1*$^{+/-}$ mice (Fig. 2*A*), a reduction that became more pronounced as these mice aged (Fig. 2 *A* and *B*). Consistent with these findings, Western blots revealed reduced levels of p27, p18, and menin protein in *Men1*$^{+/-}$ mouse islets at 40 weeks (Fig. 2*C*), and analysis of islets in sectioned pancreata revealed reduction of nuclear p27 in *Men1*$^{+/-}$ islets (Fig. 2 *D* and *E*). By 40 weeks, in *Men1*$^{+/-}$ mice, we also observed reduction of p15 and p21$^{Cip1/Waf1}$, two additional CDK inhibitors (Fig. 2*A*). Islet expression of the Cip/Kip member p57^{Kip2} was undetectable in these studies (data not shown). By contrast, expression of factors characteristic of differentiated β cells like insulin, Nkx6.1, Islet-1, Pdx1, and the Ink4 family member p19, was maintained in *Men1*$^{+/-}$ mice (Figs. 1 and 2 *A* and *F–I*, and data not shown). We also observed increased levels of CDK4 mRNA, starting at 18 weeks (Fig. 2 *A* and *B*), corroborated by Western blots showing increased CDK4 protein (Fig. 2*C*). Thus, reductions in islet menin expression preceded or accompanied the observed changes in islet p15, p18, p21, p27, and CDK4 expression (Fig. 2 and data not shown). Collectively, our data suggest that menin activity maintains the expression of specific genes encoding cell cycle regulators in adult pancreatic islet cells.

Menin Induces *p27* and *p18* Expression and Suppresses Growth of β Cells. To investigate the mechanisms underlying menin regulation of genes like *p27* and *p18* in differentiated endocrine cells like β cells, we tested whether menin was sufficient to induce expression of these putative target genes in β cells. MIN6 cells are a well established line derived from mouse pancreatic β cell tumors (36). Western blots revealed that MIN6 cells expressed nearly undetectable levels of menin (Fig. 4*A*, which is published as supporting information on the PNAS web site). Transient transfection of MIN6 cells with *Men1* increased levels of menin protein and mRNA (Fig. 4 *A* and *B*). Western blots also demonstrated that increased levels of p27 and p18 protein accumulated in MIN6 cells after *Men1* transfection (Fig. 4*A*). Consistent with these results, we observed increased expression of menin, p18, and p27 mRNA after MIN6 transfection with *Men1* (Fig. 4*B*). Cotransfection of a *Men1*-specific

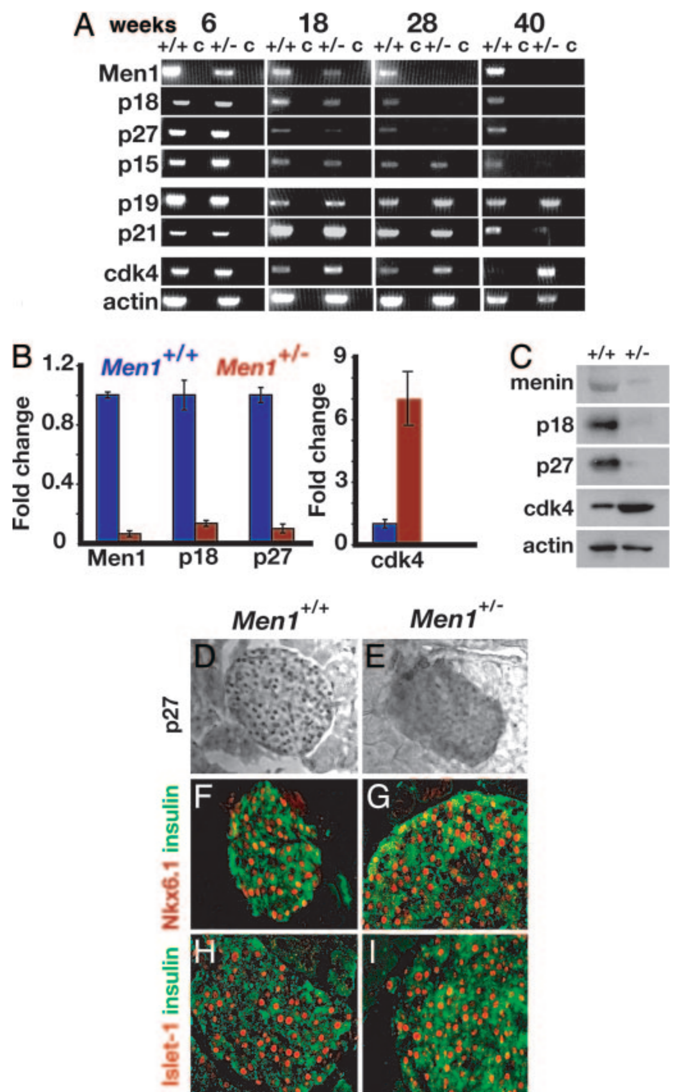

Fig. 2. Disrupted expression of genes encoding cell cycle regulators in *Men1*$^{+/-}$ pancreatic islets. (*A*) Conventional RT-PCR analysis of mRNA isolated from Ficoll-gradient-purified pancreatic islets for the indicated markers. Levels of mRNA specifying p19 and actin (a loading control) are not detectably altered in *Men1*$^{+/-}$ mice. (*B*) Quantitative RT-PCR on islets from 40-week-old *Men*$^{+/+}$ and *Men1*$^{+/-}$ mice for the indicated markers. (*C*) Western blot comparing expression of the indicated proteins in purified islets from 40-week-old *Men1*$^{+/+}$ and *Men1*$^{+/-}$ mice. Actin serves as a loading control. (*D* and *E*) Immunohistochemical detection of nuclear p27^{Kip1} (black dots) reveals reduced expression in pancreatic islets of *Men1*$^{+/-}$ mice. (*F* and *G*) Nuclear expression of the transcription factor Nkx6.1 (red) by insulin^{+} β cells (green) is maintained in *Men1*$^{+/-}$ mice. (*H* and *I*) Nuclear expression of the transcription factor Islet-1 (red) by insulin^{+} β cells (green) is maintained in *Men1*$^{+/-}$ mice. [Original magnification: ×40 (*D* and *E*) and ×63 (*F–I*).]

siRNA with *Men1*-encoding DNA strongly inhibited expression of menin, p27, and p18 protein (Fig. 4 *A* and *B*).

To determine whether menin regulates MIN6 cell growth, we monitored rates of cell accumulation. We transfected MIN6 cells with DNA encoding *IRES*:*GFP*, or *menin*:*IRES*:*GFP*. As an additional control, we cotransfected cells with *menin*:*IRES*:*GFP* and a *Men1*-specific siRNA. We then measured proliferation and BrdUrd incorporation on GFP^{+} cells purified by FACS (see *Materials and Methods*). We observed that *menin*:*IRES*:*GFP* transfection (compared with transfection with control DNA encoding *IRES*:*GFP*) impaired MIN6 cell accumulation (Fig. 4*C*). Cotransfection of MIN6 cells with *menin*:*IRES*:*GFP* and a *Men1*-specific siRNA

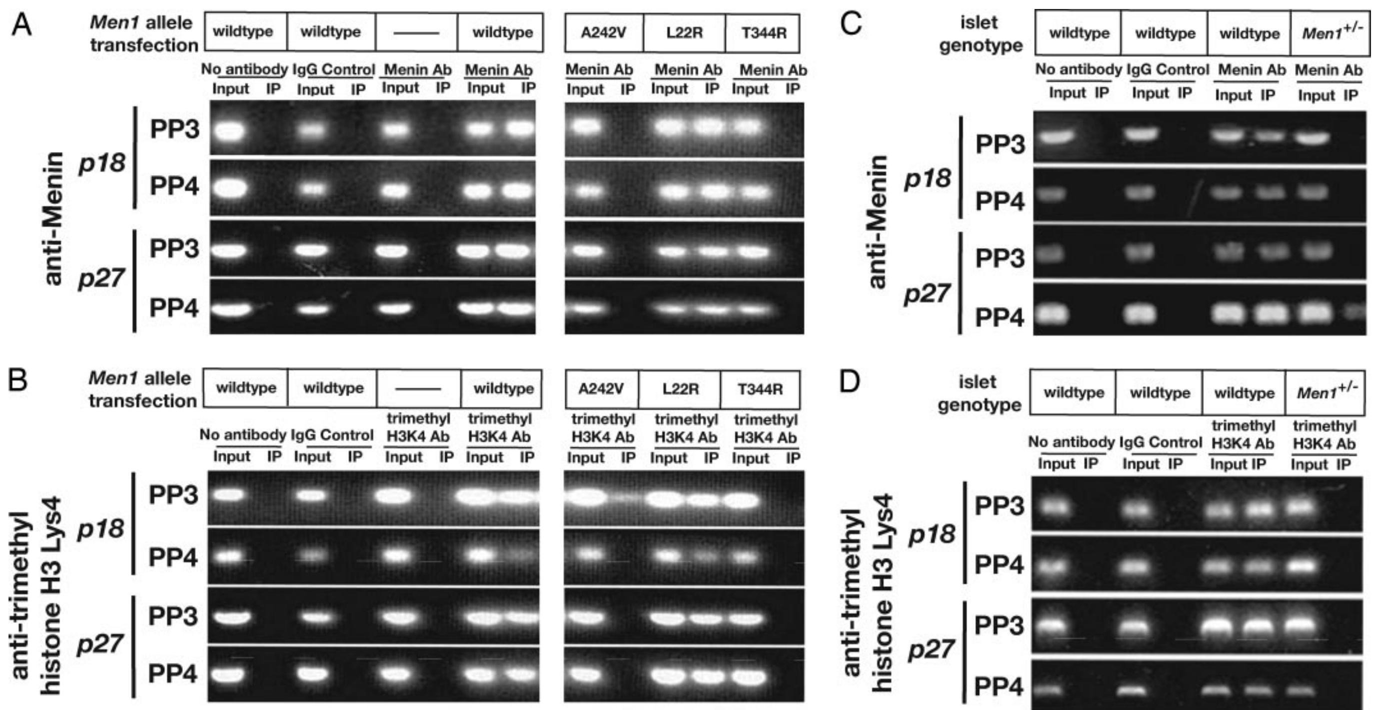

Fig. 3. Menin promotes methylation of histone H3 associated with chromatin at the *p18* and *p27* genes. MIN6 cells were transfected with DNA specifying wild-type menin (wild-type), or mutant missense alleles of *Men1* derived from human tumors that encode the amino acid substitutions A242V, L22R, or T344R. (*A*) Anti-menin ChIP was performed by using PP3 and PP4 specific for regions in the *p18* promoter, or PP3 and PP4 specific for regions in the *p27* promoter. Menin produced from alleles that retain histone methyltransferase activity (wild-type, L22R) associate with these promoters. (*B*) ChIP analysis using antibodies specific for trimethylated histone H3 Lys-4. (*C* and *D*) ChIP analysis using antibodies specific to menin and trimethylated histone H3 Lys-4 performed on isolated islets from $Men1^{+/+}$ and $Men1^{+/-}$ mice. (*E*) Comparison of trimethylated histone H3 Lys-4 levels in $Men1^{+/+}$ and $Men1^{+/-}$ islets by Western blotting. Actin protein served as a loading control.

restored MIN6 cell growth (Fig. 4*C*). Cotransfection with *menin*:*IRES*:*GFP* DNA and a control siRNA did not restore MIN6 cell growth (data not shown). To assess effects of *Men1* on proliferation further, we quantified BrdUrd incorporation in MIN6 cells. Nuclear labeling by BrdUrd after transfection with *menin*:*IRES*:*GFP* DNA was reduced compared with control MIN6 cells cotransfected with *menin*:*IRES*:*GFP* and a *Men1*-specific siRNA, or compared with MIN6 cells transfected with control DNA encoding *IRES*:*GFP* (Fig. 4*D*). These *in vitro* studies correlate well with our *in vivo* findings in $Men1^{+/-}$ islets and provide evidence that menin regulates the expression of p27 and p18, and growth of β cells.

If menin regulates growth by maintaining expression of cell cycle regulators like p18 and p27, we postulated that a subset of tumor-derived mutant menin proteins might not induce expression of these putative target loci in MIN6 cells. We analyzed levels of menin, p18, and p27 in MIN6 cells after transfection with wild-type *Men1*, or with patient-derived *Men1* missense mutant alleles that specify amino acid substitutions, designated A242V, and L22R. In prior studies (8), the A242V mutant form lacked histone methyltransferase-reconstituting activity, distinct from the L22R form, which retained this activity. MIN6 cells expressed equivalent levels of wild-type or mutant menin after transfection (Fig. 4*E*). However, the level of p18 and p27 protein induced by the A242V mutant form was reduced compared with those induced by the wild-type or L22R forms of menin (Fig. 4*E*). Compared with controls, growth of MIN6 cells transfected with the A242V allele was unperturbed, whereas cells transfected with the L22R allele grew more slowly (Fig. 4*F*). Consistent with this finding, we found that the percentage of BrdUrd incorporation was greater in MIN6 cells transfected with the A242V allele than in cells transfected with the L22R allele (48 ± 1 versus 32 ± 3; $n = 3$, $P < 0.01$). Collectively, these data support the conclusion that menin maintains expression of CDK inhibitors to suppress tumor formation in pancreatic islets.

Menin Associates with Regulatory Elements in the Mouse *p27* and *p18* Promoters. To determine whether menin directly regulates the expression of *p27* or *p18*, we performed ChIP assays with MIN6 cells, and with pancreatic islets isolated from $Men1^{+/-}$ and $Men1^{+/+}$ mice. Endogenous menin levels in MIN6 cells were nearly undetectable (Fig. 4). Thus, anti-menin ChIP studies in MIN6 cells required transfection of these cells with *Men1*-encoding DNA (Fig. 5 *B* and *D*, which is published as supporting information on the PNAS web site, compare + and − *Men1* transfection controls). This requirement for *Men1* transfection facilitated comparisons of wild-type and mutant menin activity in MIN6 cells.

Anti-menin ChIP revealed that menin associates with specific regions of the mouse *p18* and *p27* promoters (Fig. 5 *A*–*D*). For these studies, we used a set of primers directed to overlapping portions of the 1,000-bp immediately 5′ of the *p18* or *p27* transcription start sites (Fig. 5 *A* and *C*). Menin associates with sequences between −750 and −300 in the *p18* promoter (enriched by *p18*-specific primer pairs 3 and 4; Fig. 5*A*), and with sequences between −725 and −350 in the *p27* promoter (enriched by *p27*-specific primer pairs 3 and 4; Fig. 5*C*). Similar results were obtained from ChIP studies with pancreatic islets. Menin associated with specific regions proximal to the *p18* and *p27* promoters, and this association was lost in islets from $Men1^{+/-}$ mice (Fig. 3*C*). Thus, menin binds to specific promoter-proximal regions of the *p27* and *p18* loci, arguing that menin directly regulates the expression of *p27* and *p18*. Anti-menin antibodies did not coprecipitate sequences in *p18* or *p27* that

flanked these regions (Fig. 5 *B* and *D*) or sequences from *p15*, *p21*$^{Cip1/Waf1}$, and *CDK4* controls (Fig. 6, which is published as supporting information on the PNAS web site), suggesting that regulation of *p15*, *p21*$^{Cip1/Waf1}$, or *CDK4* expression by menin in islets (see Fig. 2*A*) may not be direct.

We performed transient transfection studies to confirm that the promoter-proximal elements in *p27* and *p18* that associate with menin in ChIP studies mediate menin-dependent transcription (Fig. 5 *E*–*H*). Compared with luciferase expression directed from a minimal promoter (pGL), which showed no detectable menin-dependence, expression directed from a synthetic transgene comprised of 1.5 kb of *p18* promoter-proximal DNA linked to luciferase or from a synthetic transgene comprised of 1.6 kb of *p27* promoter-proximal DNA linked to luciferase was clearly increased by menin (Fig. 5 *F* and *H*). Dissection of the *p18* regulatory region identified a minimal menin-responsive element in the region from −854 to −517 bp 5′ of an established *p18* transcriptional start site (p18 Luc20; Fig. 5 *E* and *F*). Dissection of the *p27* regulatory region using a similar strategy identified a menin-responsive element in the region from −936 to −502 bp 5′ of an established *p27* transcriptional start site (p27 Luc55; Fig. 5 *G* and *H*). Further parsing of this *p27* region into smaller elements led to reduced luciferase expression (Fig. 5*H*). Thus, defined promoter-proximal cis-regulatory elements immediately 5′ of known transcription start sites in *p27* and *p18* were sufficient for directing menin-dependent transcription. In some *in vitro* contexts, menin can bind directly to DNA (37). To determine whether menin might associate with a common specific sequence in *p27* and *p18*, we performed comparisons of DNA sequences in p27 Luc55 and p18 Luc20 (see *Supporting Materials and Methods*). This analysis (data not shown) did not reveal evidence for specific sequences of four or more consecutive bases common to the menin-responsive elements in *p27* and *p18*.

If association with target chromatin sequences is important for menin activity, we postulated that a subset of tumor-derived mutant menin protein would be unable to associate with cis-regulatory sequences in the *p27* and *p18* promoters. We performed anti-menin ChIP studies after transfection with patient-derived *Men1* missense mutant alleles that specify one of three amino acid substitutions: A242V, T344R, and L22R. Both A242V and T344R forms lack HMTase-reconstituting activity (8), and both failed to permit ChIP enrichment of specific sequences from the *p27* and *p18* promoters. By contrast, both L22R and wild-type menin, which retain HMTase-reconstituting activity, permitted ChIP enrichment of these sequences (Fig. 3*A*). These results suggest that the association of menin with *p27* and *p18* cis-regulatory chromatin is involved in the tumor suppressor function of menin.

Menin Promotes Methylation of Histone H3 Lys-4 Associated with the *p27* and *p18* Loci. Menin associates with a nuclear protein complex that includes the TrxG members MLL, MLL2, and Ash2 (8–10). Association of menin with this complex promotes the methylation of histone H3 at specific residues like Lys-4, consistent with the hypothesized role of MLL as a methyltransferase (11). Methylation of H3 Lys-4 has been correlated with activated expression of *Hox c8* (11) and other genes (13). Thus, we used ChIP analysis to test whether menin enhances methylation of H3 Lys-4 associated with the *p27* and *p18* promoter regions. ChIP assays using antibodies specific for tri-methyl H3 Lys-4 revealed a menin-dependent increase of H3 Lys-4 methylation in MIN6 cells associated with both the *p27* and *p18* promoters (Fig. 3*B*). Consistent with prior analyses demonstrating association of menin protein with these promoter sites, the PCR primers used in these trimethyl H3 Lys-4 ChIP studies spanned regions of the *p27* and *p18* promoters that associate with menin. Similar results were obtained in studies of pancreatic islets. H3 Lys-4 methylation associated with both the *p27* and *p18* promoters was readily detected in islets isolated from *Men1*$^{+/+}$ mice, but severely reduced or undetectable in *Men1*$^{+/-}$ islet tumors lacking menin (Fig. 3*D*). These results suggest that menin regulates H3 Lys-4 methylation at the *p27* and *p18* promoters. Consistent with these data, Western blotting analysis revealed a reduction of total trimethyl-histone H3 Lys-4 levels in these islet tumors (Fig. 3*D*).

If menin directs histone methyltransferase activity in cis-regulatory chromatin regions governing expression of genes like *p27* and *p18*, then we predicted that some tumor-derived mutant alleles of *Men1* would fail to enrich H3 Lys-4 methylation associated with these genes. As shown in Fig. 3*B*, MIN6 cells transfected with the wild-type or patient-derived L22R mutant form of menin had elevated levels of trimethyl H3 Lys-4 in association with *p27* and *p18* chromatin. In contrast, mutations in *Men1* producing the A242V and T344R forms completely failed to enrich trimethyl H3 Lys-4 in *p27* and in *p18* (Fig. 3*B*). These results are consistent with our ChIP studies demonstrating that L22R associates with the *p27* and *p18* promoters (Fig. 3*A*) and our analyses showing increased expression of p27 and p18 protein in MIN6 cells transfected with the L22R allele of *Men1* (Fig. 4*E*). Collectively, these data indicate that mutation of *Men1* disrupts menin HMTase complex activity at the *p27* and *p18* loci, impairing appropriate expression of these key endocrine cell cycle regulators.

Discussion

These studies reveal an epigenetic mechanism for tumor suppression by menin in pancreatic islets. One of our principal findings is that menin binds *in vivo* to specific regions of the *p27* and *p18* promoters in pancreatic endocrine cells. Prior studies (17, 18) have demonstrated that simultaneous loss of p27 and p18 function in mice leads to a spectrum of tumors similar to that seen in patients with type 1 MEN syndrome, suggesting that these cyclin-dependent kinase inhibitors serve as essential growth regulators of neuroendocrine tissues. Thus, our studies show that menin governs expression of at least two established regulators of endocrine cell proliferation. p18 and p27 are known to inhibit functions of CDK4 and other cyclin-dependent kinases (19). For pancreatic islets, this view is consistent with recent work showing that regulation of CDK4 is required for islet growth control (33, 35). Replacement of the endogenous *CDK4* gene with an Ink4-resistant activated *CDK4* allele resulted in mice with hyperplastic islets, comprised chiefly of insulin-producing β cells (35). Our data suggest that menin is required to control CDK4 expression in islets; however, results from ChIP studies suggest that menin effects on islet expression of CDK4 and of CDK inhibitors like p15 and p21 is indirect. Thus, further studies are required to reveal how menin controls expression of targets like p15, p21, and CDK4.

We found that menin associates with specific regions of the *p27* and *p18* proximal promoters. Prior studies showed that these regions contained or were adjacent to positive transcriptional regulatory elements (26, 27, 38). Here, we used luciferase-based assays to show that DNA encompassing these regions was sufficient for stimulating menin-dependent transcription. Based on data suggesting that menin forms a complex with MLL2 and regulates its histone methyltransferase activity (8, 9), we speculate that menin promotes histone methylation at 5′ promoter sites, and might function as a transcriptional coactivator at promoters near the transcriptional start site of specific targets like *p27* and *p18*. Consistent with this view are data from previous studies that demonstrate the direct association of menin with transcriptional activators and repressors (30–34). MLL and MLL2, TrxG members that associate with menin, have also been recently shown to bind promoter-proximal cis-regulatory elements to maintain expression of gene targets like *p27* and *p18* in terminally differentiated cells (10, 11, 39), including fibroblasts. Our analysis did not identify specific DNA sequence motifs common to regions in *p27* and *p18* that might mediate menin-dependent transcription, consistent with at least one prior study (37) that showed that menin can bind DNA in a sequence-independent manner. Thus, additional studies are

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needed to identify how menin, or other factors associated with menin, might localize to the *p27* and *p18* promoters.

The second principal finding in our study is that menin promotes methylation of histone H3 Lys-4 *in vivo* at *p27* and *p18*, genes crucial for islet growth control and tumor suppression. We showed that loss of menin expression by islet tumors in $Men1^{+/-}$ mice prevented Lys-4 methylation of histone H3 associated with *p27* and *p18*. Thus histone H3 Lys-4 methylation by menin is a possible mechanism for controlling expression of *p27* and *p18* by pancreatic endocrine cells *in vivo*. A subset of tumor-derived mutant forms of menin failed to associate with *p27* or *p18* cis-regulatory sequences or stimulate H3 Lys-4 methylation in cultured islet cell lines. These studies therefore provide evidence that some mutant forms of menin lack at least two activities (binding to specific promoters, and stimulating histone H3 Lys-4 methylation) that regulate tumorigenesis in islet cells. Both activities may be eliminated by mutations like A242V and T344R, but it remains unknown whether *Men1* mutations that affect promoter binding by menin invariably impair menin-dependent H3 methylation. Moreover, our work also reveals that some patient-derived mutant alleles of *Men1*, like L22R, specify forms of menin that retain both activities; thus, it is unclear whether dysregulated p27 or p18 expression serves as the basis for tumor pathogenesis in these cases.

Could menin regulate other epigenetic modifications that control neuroendocrine gene expression? Prior studies suggest that H3 Lys-4 methylation antagonizes CpG methylation (13), which is thought to be an important means for repressing promoter activity of *p27* and *p18* in some contexts (40). Histone H3 Lys-4 methylation also disrupts binding of the nucleosome remodeling and deactylase (NuRD) repressor complex to H3 tails (41, 42). Further studies may show whether menin-induced H3 Lys-4 methylation can alter promoter methylation or prevent histone deactylation at target loci like *p27* and *p18*. The association of menin with TrxG proteins like MLL (10) and the well established antagonism betweeen TrxG and polycomb group (PcG) proteins in maintenance of steady-state gene expression (39) raise the possibility that PcG proteins may regulate growth of islets and other neuroendocrine cells.

We propose that menin is required for specific methylation of histone H3 Lys-4 to ensure stable expression of target genes like *p27* and *p18* in endocrine cells. Thus, our data may also begin to explain the specific bias for endocrine tumor formation in MEN1, a hallmark feature of this familial tumor syndrome. Growth of mouse endocrine organs is sensitive to simultaneous loss of p27 and p18 activity (17, 18), suggesting that Ink4 family members like p18 and Cip/Kip members like p27 may have partially overlapping functions in maintaining growth control of diverse neuroendocrine cells. Thus, we speculate that the bias toward endocrine tumor pathogenesis in MEN1 results from a requirement for menin to maintain expression of CDK inhibitors in adult endocrine tissues. Understanding the molecular mechanisms underlying growth control by menin in endocrine cells may improve our ability to provide diagnoses, prognoses, and treatment for patients with MEN1 and diabetes.

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