Dynamic Epigenetic Regulation by Menin During Pancreatic Islet Tumor Formation

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Abstract

The tumor suppressor gene MEN1 is frequently mutated in sporadic pancreatic neuroendocrine tumors (PanNET) and is responsible for the familial multiple endocrine neoplasia type 1 (MEN-1) cancer syndrome. Menin, the protein product of MEN1, associates with the histone methyltransferases (HMT) MLL1 (KMT2A) and MLL4 (KMT2B) to form menin–HMT complexes in both human and mouse model systems. To elucidate the role of methylation of histone H3 at lysine 4 (H3K4) mediated by menin–HMT complexes during PanNET formation, genome-wide histone H3 lysine 4 trimethylation (H3K4me3) signals were mapped in pancreatic islets using unbiased chromatin immunoprecipitation coupled with next-generation sequencing (ChIP-seq). Integrative analysis of gene expression profiles and histone H3K4me3 levels identified a number of transcripts and target genes dependent on menin. In the absence of Men1, histone H3K27me3 levels are enriched, with a concomitant decrease in H3K4me3 within the promoters of these target genes. In particular, expression of the insulin-like growth factor 2 mRNA binding protein 2 (IGF2BP2) gene is subject to dynamic epigenetic regulation by Men1-dependent histone modification in a time-dependent manner. Decreased expression of IGF2BP2 in Men1-deficient hyperplastic pancreatic islets is partially reversed by ablation of RBP2 (KDM5A), a histone H3K4-specific demethylase of the jumonji, AT-rich interactive domain 1 (JARID1) family. Taken together, these data demonstrate that loss of Men1 in pancreatic islet cells alters the epigenetic landscape of its target genes.

Implications: Epigenetic profiling and gene expression analysis in Men1-deficient pancreatic islet cells reveals vital insight into the molecular events that occur during the progression of pancreatic islet tumorigenesis. Mol Cancer Res; 13(4); 689–98. ©2014 AACR.

Introduction

Multiple endocrine neoplasia type 1 (MEN-1) is an autosomal-dominant syndrome, characterized by multiple tumors in endocrine tissues such as the pituitary gland, parathyroid gland, and pancreatic islets (1). Linkage studies and positional cloning identified the causative gene, MEN1, for this disorder. Over 1,300 mutations, typically truncating, have been identified in MEN1 (2, 3). The importance of MEN1 inactivation in tumorigenesis is highlighted by the frequency of MEN1 mutations in sporadic endocrine tumors—44% in pancreatic neuroendocrine tumors and 35% in parathyroid adenomas (4, 5). Heterozygous and conditional Men1 knockout mice develop tumors in multiple neuroendocrine tissues, recapitulating the spectrum of tumors in MEN-1 syndrome, with Men1 conditional knockout animals demonstrating a shorter latency (6–9). Although Men1 mutations are primarily associated with neuroendocrine cancers, several lines of evidence demonstrate that Men1 can also be dysregulated in non-neuroendocrine tumors such as lung cancer, melanoma, and liver cancer (10–12).

Several studies have implicated that menin, the protein product of MEN1, is involved in transcriptional regulation, cell-cycle control, protein degradation, and genome instability through interaction with a number of transcription factors such as JunD, NF-kB, and members of the Smad family (3, 13–15). In addition, we and others have shown that menin is physically associated with Trihomolog-like complexes containing the histone methyltransferases MLL1 (KMT2A) and MLL4 (KMT2B, previously MLL2), to promote trimethylation of histone H3 at lysine 4 (H3K4me3; refs. 16, 17). Surprisingly, menin also binds to the MLL fusion protein in leukemia cells to upregulate HoxA9 gene expression, thus promoting oncogenic activity in MLL-associated leukemogenesis (18). Genome-wide analysis by chromatin immunoprecipitation coupled with DNA microarray analysis (ChIP-chip; ref. 19) has revealed that menin colocalizes with MLL at gene promoters in various cell types, suggesting that menin regulates transcription in cooperation with MLL in multiple tissues (20).

Currently, there are no targeted therapies directed toward patients harboring pancreatic neuroendocrine cancer tumors with MEN1 mutations. Thus, there is a critical need to deepen our understanding of the biology of these cancers to develop more effective therapeutic approaches. Given the inactivation of menin...
in multiple endocrine cancers and the reversibility of histone H3K4me3, we were interested in the role of enzymes that potentially antagonize the histone methylation activity of menin. Rbp2 (Kdm5a, Jarid1a), initially identified as Retinoblastoma-binding protein 2, is a member of the Jumonji (JMJ) domain-containing family of histone demethylases, with roles in chromatin modification and transcriptional regulation (21). Loss of Rbp2 recruitment to the CDKN1B gene is highly correlated with increased histone H3K4me3 levels and elevated gene expression (21–23).

We have previously demonstrated in murine models that inactivation of the histone demethylase Rbp2 significantly inhibits tumor growth in Men1-deficient mice (24). We also demonstrated that alterations in gene expression patterns upon Men1 loss in pancreatic islets are partially reversed by Rbp2 loss in these cells (24). Collectively, these observations support the notion that (i) histone methylation plays a key role in Men1 deletion-mediated tumorigenesis in neuroendocrine cells, and (ii) the demethylase enzyme activity of Rbp2 antagonizes the histone methyltransferase activity associated with menin at gene loci such as CDKN1B. Although loss-of-function of menin is known to play an important role in tumor initiation and progression in endocrine tissues (7), there is limited information on the mechanisms linking menin–HMT complexes to neuroendocrine-specific hyperplasia and tumorigenesis.

Men1-deficient mice can take up to a year to accumulate the numerous genetic and epigenetic alterations that result in tumor formation, a slow process during which pancreatic islet cells transform from normal to a hyperplastic and finally a malignant state (7). Thus, this period represents a window of opportunity to investigate early events leading to tumorigenesis and to address the role of menin–HMT complexes in modulating cell proliferation and behavior at this precancerous stage.

To investigate tumor formation mediated by alterations in H3K4me3 levels and to identify gene targets of menin–HMT complexes, we conducted epigenetic profiling of Men1-deficient pancreatic islets in 2-month-old Men1 conditional knockout mice and control wild-type littermates. Using ChiP techniques coupled with next-generation sequencing (ChiP-seq), we found that Men1 loss lowered H3K4me3 levels at select target gene promoters, resulting in downregulation of gene expression. In addition, loss of H3K4me3 correlated with increased H3K27me3 levels, consistent with the known association of H3K27me3 with gene repression (25).

Our study is the first to identify gene targets of menin–HMT complexes in mouse pancreatic islets in vivo along with the time course of the epigenetic changes accompanying mouse pancreatic neuroendocrine tumor formation.

Materials and Methods

Mouse experiments

Creation and genotyping of RIP-Cre mice, Men1 KO mice, and Men1/Rbp2 KO mice has been described previously (24). Mice were maintained on a mixed 129s6, FVB/N, and C57BL/6 background. All procedures were carried out in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the Dana-Farber Cancer Institute (Boston, MA).

Isolation of mouse pancreatic islets

Pancreatic islets were isolated as previously described (24).

Histologic and immunohistochemical analysis for pancreatic tissues

Pancreata were collected from mice at indicated time points and fixed in 4% paraformaldehyde for 2 hours followed by dehydration and paraffin embedding. Histopathologic analysis was carried out on 5 μm sections stained with hematoxylin and eosin. Islet morphology and tumors were examined in at least three cut sections for each pancreas after staining with hematoxylin and eosin. Appropriate positive and negative controls were run on matched sections for all applied antibodies. Immunohistochemical staining was performed on serial sections using antibodies against H3K4me3 (Active Motif, catalog no. 36159, 1:500), H3K27me3 (Cell Signaling Technology, catalog no. 9733, 1:100) and IgG2b (Abcam, Ab124930, 1:1,000). Sections were counterstained in Meyer hematoxylin, mounted, and photographed using an Olympus microscope.

ChiP-seq

For each ChiP experiment, islets from at least 4 adult mice were purified by collagenase digestion and gradient centrifugation, with subsequent hand picking. ChiP was performed as described (26) using 4 μg of anti-H3K4me3 (Active Motif, catalog no. 36159) or anti-H3K27me3 antibodies (Cell Signaling Technology, catalog no. 9733). Five to 50 ng of DNA was used for library construction. DNA was prepared for sequencing by Illumina cluster generation using a SPIRI-work system with 100–300 bp size selection followed by enrichment with barcoded PCR primers for multiplexing. Sequencing was performed on a HiSeq2000 machine for 40 nucleotides from a single end, at the MIT BioMicro Center. Barcode-separated FASTQ files were generated from QSEQ files.

ChiP-Seq data analysis

Forty nucleotides of sequenced reads were aligned to the mouse reference genome (mm9 assembly), using Bowtie aligner (27). Only those reads/tags that mapped to unique genomic locations with at most two mismatches were retained for further analysis. Histone mark peaks were detected using MACS (version 1.4.2) as previously described (28), with a P value cutoff of 10−6 and with default values for other parameters. Quantitative changes in H3K4me3 upon Men1 deletion was assessed using MAnorm algorithm (29) with a P value cutoff of 10−3 and >1 log2 fold change. H3K4me3 peaks that significantly decreased in Men1-deficient compared with RIP-Cre control islets were assigned to the most adjacent gene within 30 kb; these genes were assessed for overlap with genes downregulated in Men1-deficient mice. H3K4me3 ChiP signals were plotted around transcription start sites (TSS) of both upregulated and downregulated genes in Men1-deficient islets. H3K27me3 ChiP signals were plotted around the peaks of either unchanged or decreased levels of H3K4me3 in Men1-deficient compared with RIP-Cre control islets.

Data availability

All ChiP-Seq data generated in this study have been deposited in the NCBI GEO repository (accession number GSE63020).

RNA isolation and qRT-PCR

Total RNA was isolated using the RNeasy Kit (Qiagen) from 100 to 300 mouse pancreatic islets purified from 2 mice with different genotypes. RNA quality was assessed on the Agilent Bioanalyzer.
Epigenetic Regulation by the Men1 Tumor Suppressor Gene

Results

Inactivation of Men1 does not alter global H3K4me3 levels in pancreatic beta cells

To address whether Men1 loss causes global changes in histone H3K4 methylation, we assessed H3K4me3 levels in Men1-deficient mouse pancreatic islets and control RIP-Cre islets by immunohistochemistry (IHC). We detected no significant change in the overall levels of H3K4me3 in Men1-deficient islets compared with wild-type islets (Fig. 1A). Our finding is consistent with earlier studies demonstrating that in contrast to Set1a and Set1b, the major H3K4 trimethylases in mammalian cells (30), MLL1 and MLL4, the HMTs known to associate with menin, are responsible for H3K4 trimethylation of only a subset of loci (31). Our observations are also in line with studies showing that MLL1 loss decreased H3K4me3 levels in less than 5% of genes in mouse embryonic fibroblasts (MEFs) and that MLL4 knockdown had no impact on overall H3K4me3 levels in mouse embryonic stem cells (31, 32). We previously reported that inactivation of Rbp2 could partially rescue the tumor phenotype in Men1-deficient mice (24). Immunohistochemistry revealed no appreciable change in overall H3K4me3 levels in Men1/Rbp2 double knockout islets compared with wild-type or Men1-deficient islets (Fig. 1A, right panel), suggesting that menin-HMT complexes potentially regulate histone modifications for only a subset of genes in mouse pancreatic islets.

Menin-dependent H3K4me3 is altered during early stages of pancreatic neuroendocrine tumor formation

To investigate locus-specific H3K4 trimethylation potentially regulated by menin-HMTs in pancreatic islets, we performed ChIP-seq to identify genome-wide H3K4me3 occupancy in islets from 2-month-old RIP-Cre (control) mice or Men1-deficient mice. We identified >22,000 H3K4me3 peaks, using the MACS algorithm (28). As expected, H3K4me3 marks were most frequently observed at proximal promoter regions, near TSSs; ref. 16; Supplementary Tables S1 and S2). Comparable profiles were observed in two independently purified batches of islets from RIP-Cre mice (data not shown). We next compared H3K4me3 signals in Men1-deficient pancreatic and control islets. Consistent with our predictions from the IHC results described above, we observed differential H3K4me3 signals only in a subset of regions (Fig. 1B). Among the total 1565 differential peaks identified in control cells (P < 10^-5, fold change >2), only 815 peaks (~3.5% of total H3K4me3 peaks) showed a decrease in H3K4me3 signals in Men1-deficient islets (Fig. 1B, and Supplementary Table S3), representing potential menin-HMT targets.

Integrative analysis of H3K4 trimethylation and gene expression identifies menin-HMT gene targets

To determine whether the regulation of differentially expressed genes in Men1-deficient islets is dependent on menin-mediated H3K4me3, we integrated gene expression data (24) with

For RT-PCR, DNase I (Qiagen)-treated RNA samples were reverse transcribed using oligo-dT and SuperScript III (Invitrogen), with first strand cDNA used for PCR using SYBR Green PCR Mix (Qiagen) in an Applied Biosystems 7300 Real Time-PCR system. First strand cDNA was used for PCR using SYBR Green PCR Mix (Qiagen) in an Applied Biosystems 7300 Real Time-PCR system. Standard ChIP with H3K4me3 and H3K27me3 antibodies was performed on mouse pancreatic islets in duplicate. PCR primer pairs were designed to amplify 150- to 200-bp fragments from select genomic regions. Primer sequences are listed in Supplementary Table S5.

Figure 1.
H3K4me3 profiles in mouse pancreatic islets. A, total H3K4me3 levels were evaluated in pancreatic islet cells by IHC. Islets were purified from 2-month-old mice in which Men1 was deleted using an islet-specific Cre driver, either alone (Men1f/f; RIP-Cre) or in combination with Rbp2 (Men1f/f; Rbp2f/f; RIP-Cre). Islets from mice bearing the Cre driver alone (RIP-Cre) were used as controls. B, volcano plot illustrating differential changes in H3K4me3 levels in Men1-deficient islets versus wild-type controls (left, green dots represent decreases in the Men1 KO while red dots represent increases). Heatmap of those loci from the volcano plot showing significant alterations of H3K4me3 levels in Men1-null islets compared with wild-type islets (right).
H3K4me3 profile data. Genes whose expression was downregulated at least two-fold in Men1-deficient islets showed a significant reduction in H3K4me3 levels (Fig. 2A). In contrast, H3K4me3 levels were unchanged at genes upregulated at least two-fold upon menin loss (Fig. 2B). Notably the H3K4me3 signal in downregulated loci was also generally lower than for genes showing increased expression upon Men1 loss (compare profiles of Fig. 2A and B). These data suggest a direct role for menin-dependent H3K4me3 in activating gene expression from its target promoters in mouse pancreatic islets. The role of H3K4me3 in menin-independent repression of gene expression however remains to be elucidated.

To identify genes that were downregulated and associated with reduced H3K4me3 levels in Men1-deficient islets we intersected these two datasets, revealing that 50 out of 365 genes showing reduced gene expression (14% overlap rate vs 1.7% expected random overlap rate; \( P < 0.001 \)) also exhibited lower H3K4me3 (Fig. 2C). Many of these targets are linked to type 2 diabetes and beta cell functions (also see Supplementary Table S4), with \( \text{Igf2bp2} \) (insulin-like growth factor 2 mRNA binding protein 2) emerging as one of the most significant genes in this category. In contrast, of the 1227 genes that showed increased expression upon Men1 loss, only 40 (3.1% overlap rate vs 5.7% expected random overlap rate; not enriched) showed a corresponding increase in H3K4me3 (Supplementary Fig. S1). These data indicate that gene repression mediated by menin under wild-type conditions likely occurs by mechanisms other than via reduction of H3K4 tri-methylation.

\( \text{Men1-loss does not change histone modifications at known menin gene targets in mouse pancreatic islets, at age of 2 months} \)

A number of genes including \( \text{Cdkn1b, Cdkn2c, Mnx1, Hoxa9, and Hoxc6} \), have been previously shown to be regulated by menin or menin-HMT complexes [16, 18, 20, 33, 34]. We, however, did not observe a significant difference in enrichment of H3K4me3 or reduction of H3K27me3 at the \( \text{Cdkn1b, Cdkn2c, and Mnx1} \) promoters in Men1-deficient islets (Supplementary Fig. S2), despite \( \text{Cdkn1b and Cdkn2c} \) being highly expressed in mouse pancreatic islets (WL, unpublished observations). These findings, although consistent with our prior observations (24) where we reported no significant difference in expression of these genes upon Men1 loss, are at odds with other studies using mouse embryonic fibroblasts (MEF) and pancreatic islets.
that have demonstrated the role of trimethylation of H3K4 in transcriptional activation of Cdkn1b and Cdkn2c (34). It is possible that expression of Cdkn1b and Cdkn2c does not require menin–HMT complexes at this stage. Menin is also known to regulate the majority of Hox genes in MEFs (31). Hox genes are typically bivalently modified in embryonic stem cells (32); however, we did not observe strong H3K4me3 signals at these loci in pancreatic islets (Supplementary Fig. S3) although we detected strong H3K27me3 association with four Hox gene clusters (Supplementary Fig. S3). As H3K27me3 is associated with repression of gene expression, our results are consistent with microarray data showing that most Hox genes were silenced or expressed at very low levels (data not shown). Thus, at the time point being evaluated, menin-driven gene expression appears to occur via both H3K4me3-dependent and -independent mechanisms.

Loci with increased H3K27me3 signals in Men1-deficient islets are associated with decreased H3K4me3

Trimethylation of histone H3K4 is mediated by trithorax group proteins including MLL1 and MLL4 and can antagonize H3K27 trimethylation mediated by polycomb group complexes such as PRC2 (35, 36). To investigate this potential inverse correlation, we evaluated H3K27me3 levels by IHC in control, Men1 single knockout and Men1/Rbp2 double knockout islets. As observed for H3K4me3, we did not detect significant differences in overall expression of H3K27me3 (Fig. 3A).

We next asked whether loss of H3K4me3 in genes regulated by menin–HMT complexes altered H3K27me3 levels. We compared H3K27me3 signals on H3K4me3-marked regions classified as unchanged or decreased by Men1 loss (Figs. 1B, gray and green points and 3B). We detected enrichment of H3K27me3 at promoters that showed decreased levels of H3K4me3 in D. 

**Figure 3.** Increased H3K27me3 levels are observed in regions with decreased H3K4me3. A, H3K27me3 levels were evaluated in pancreatic islet cells by immunohistochemical analysis. Islets were purified from mice in which Men1 was deleted using an islet-specific Cre driver either alone (Men1f/f, RIP-Cre) or in combination with Rbp2 (Men1f/f, Rbp2f/f, RIP-Cre). Islets from mice bearing the Cre driver alone (RIP-Cre) served as controls. B, regions of H3K4me3 occupancy were classified according to changes in baseline levels occurring upon Men1 loss. C and D, H3K27me3 levels were assessed within a 20 kb window spanning the center of the H3K4me3 signal in loci exhibiting decreased H3K4 trimethylation (red trace) or no change (purple) in RIP-Cre (C) or Men1-depleted (D) islet cells. A significant enrichment in H3K27me3 signal was observed in genes associated with decreased H3K4me3 level in Men1 KO cells (red peaks in area defined by green dotted lines in D), but not in wild-type cells (C).
Men1-deficient islets, but not at promoters with unaltered H3K4me3 occupancy (compare red tracings in Fig. 3C and D). In total, 37 of 50 genes with both decreased expression and decreased H3K4me3 in Men1-deficient islets showed enhanced H3K27me3 (Supplementary Fig. S4).

Dynamic time-dependent changes in H3K4me3 and H3K27me3 at the Igf2bp2 promoter

Next, we sought to establish how H3K4me3 and H3K27me3 signals change over time during pancreatic islet tumorigenesis. We focused on the Igf2bp2 locus as Igf2bp2 was one of the two most downregulated genes in Men1-deficient islets compared with RIP-Cre islets based on analysis of microarray data, and because the Igf2bp2 promoter showed the most dramatic change in H3K4me3 signal, of the 50 genes identified in our integrative analysis. We isolated pancreatic islets from control and Men1-deficient mice at 2, 6, and 12 months of age and tumors from Men1-deficient animals, with a barely detectable Peak 2 in tumors (Fig. 4A).

In contrast, H3K27me3 occupancy at the Igf2bp2 promoter exhibited an inverse trend over time. We detected no appreciable H3K27me3 signal in RIP-Cre islets from 2-month-old mice, whereas Men1-deficient islets showed enhanced H3K27me3, in regions distinct from H3K4me3-occupied areas. We observed a slight increase in H3K27me3 signals in the RIP-Cre islets and strong H3K27me3 signals in the Men1-deficient islets from 6-month-old mice, including an overlap with H3K4me3 Peak 2. This trend continued at 12 months, where we observed further enhancement of the H3K27me3 signal in control islets and tumors from Men1-deficient animals, with a robust H3K27me3 signal in 12-month-old mice, spreading over the Igf2bp2 promoter, upstream regulatory and coding regions (Fig. 4B). Many other genes also show a similar inverse relationship between H3K4me3 and H3K27me3 signals that was consistent during the progression of pancreatic islet tumorigenesis; examples include Gata6 and Oxtr (Supplementary Fig. S5).
Igf2bp2 expression is epigenetically regulated during pancreatic islet tumor formation

Analysis of our previously published microarray data demonstrated that Igf2bp2 expression in islets from wild-type, Men1−/−, and Men1−/−; Rbp2−/− mice as determined by microarray analysis, and subsequent validation by qPCR (B). C, ChIP-qPCR showing H3K4me3 levels at the Igf2bp2 locus in control, Men1−/− or Men1−/−;Rbp2−/− islet cells. Data are the mean ± SEM of three independent experiments. **, P < 0.01. D, detection of Igf2bp2 by IHC in wild-type, Men1−/− and Men1−/−; Rbp2−/− pancreatic islets in 2-month-old mice.

Collectively, these data reveal that Igf2bp2 is epigenetically regulated by menin–HMT complexes in mouse pancreatic islets and that epigenetic changes occurring as a consequence of Men1 loss are partially restored by ablation of the Rbp2 histone demethylase.

Discussion

Menin–HMT targets associated with tumor progression

Few genes to date have been established as targets of menin; these include Hoxc6, Hoxa8, Hoxa9, Cdh23c, Cdh11b, and Mnx1 (16, 20, 33, 34). Menin is known to associate with a number of histone methyltransferases including MLL1 and MLL4 (16, 17). Here, we have investigated menin-induced epigenetic modifications during pancreatic neuroendocrine tumorigenesis. We sought to identify direct targets of menin–HMT complexes in mouse pancreatic islets via integrative analysis of ChIP-seq and gene expression data and have identified a number of genes regulated by menin-mediated H3K4 trimethylation in pancreatic
islets. A similar approach has been employed by others (37), using mouse embryonic stem cells and mouse pancreatic islet-like endocrine cells (PILEC) as models to study menin-mediated H3K4me3 during ES cell differentiation. Consistent with our observations, the authors did not observe a correlation between downregulation of gene expression and decreased H3K4me3 in ES cells upon Men1 loss. However, upon differentiation of ES cells into PILEC, decreased H3K4me3 levels were associated with downregulated genes, indicating a role for menin–HMT complexes during ES cell differentiation and lineage specification upon Men1 loss (37).

In contrast to previous studies in different experimental systems (33, 34), we did not find trimethylation of H3K4 at the 
\textit{Cdh12c} and 
\textit{Cdh11b} genes to be dependent on menin–HMTs. Thus, regulation of Cdk inhibitor genes at this stage may be driven by other mechanisms or may be independent of menin. Menin has also been reported to play a critical role during embryogenesis and MLL1-mediated leukemogenesis via regulation of 
\textit{Hox} gene expression (31).

Although several previous studies have focused on chromatin and gene expression targets of menin (20, 37), we have identified different targets in our study. Previous studies utilized different experimental systems, either nonhyperplastic islets (20) as opposed to the hyperplastic islets in our study, or Men1–deficient mESCs and mouse pancreatic islet-like endocrine cells (PILEC; 37). Furthermore, in studies of mouse embryos, we previously identified 
\textit{HoxC6} and 
\textit{HoxC8} as menin targets (16), but did not find these genes as targets in the current study, suggesting the possibility that 
\textit{Hox} genes might be the targets of menin during embryonic stages rather than in adult stages. Indeed, in adult mouse pancreatic islets, we did not observe significant expression of 
\textit{Hox} genes or significant trimethylation of H3K4me3 at 
\textit{Hox} gene promoters but did observe high levels of H3K27 methylation at 
\textit{Hox} gene promoters (Supplementary Fig. S3). Thus, we believe that the differences between the current and previous studies are likely to represent the effect of Men1 under different biologic circumstances, although we cannot exclude the possibility that some of these differences represent the effects of variation in experimental conditions. Further independent studies in consistent cell types and tissues will be required to clarify this issue in the long term.

\textbf{Anticorrelation between H3K4me3 and H3K27me3 signals}

We observed that H3K27me3 signals were enriched in regions showing decreased H3K4me3. This may arise from either a mixed population of individual cells with each feature or represent truly bivalent domains within a single cell. In the former case, this likely reflects an indirect effect of Men1 loss as menin has not been known to associate with any histone K27 demethylase. However, as decreased gene expression is accompanied by decreased H3K4me3 at menin target gene promoters (Fig. 2A), it is conceivable that these epigenetic marks may coexist within a given cell.

Genes regulating cell fate decisions during embryonic development are often characterized by dual H3K4me3 and H3K27me3 marks (38), a bivalent mark indicating a ‘poised’ state which can either be activated or repressed during lineage specification. The MLL4 methyltransferase that interacts with menin (16, 17) has been reported to regulate bivalent promoters in mouse embryonic stem cells (32). During pancreatic islet cell differentiation some bivalent marks may keep genes silent and poised, capable of switching to either an activated or repressed state in response to the appropriate signal, genes associated with H3K4me3 alone, however, are inactivated by additional trimethylation of H3K27 (ref. 38; Fig. 6). To our knowledge, this is the first study providing evidence that a significant number of menin-dependent mouse genes are subject to bivalent histone modification and regulation under physiologic conditions.
**Igf2bp2 functions during cell differentiation and tumorigenesis**

We have identified Igf2bp2 as a menin gene target that shows an increase in H3K4me3 levels and reduction in H3K27me3, upon Men1 loss. We also observe that this inverse correlation is enhanced with increasing age and is most dramatic in Men1-deficient tumors. We found that Igf2bp2 is the first gene that shows an inverse correlation between H3K4me3 and H3K27me3 levels in neuroendocrine tumor formation in vivo and thus may represent a new class of menin-regulated genes associated with this pattern of dual histone modifications. Accordingly, Igf2bp2 expression can be adjusted not only by a decrease in H3K4me3 but also by enhancement of H3K27me3. Igf2bp2 is a developmental gene highly expressed during embryogenesis, and gradually silenced in the adult (39, 40). It is the major Igf2 binding protein family member expressed in adult pancreatic islets (WL, unpublished observations). Although the precise function of Igf2bp2 is unclear, it has been reported that Igf2bp2 interferes with Igf2 translation by associating with the 5’ end of the Igf2 transcript during embryonic development (39). It is unknown whether Igf2bp2 also functions as an inhibitor of Igf2 or other genes in the adult. Thus, misregulation of Igf2bp2, as observed for other developmental genes, might play a role in tumorigenesis.

**Dynamic epigenetic regulation of Igf2bp2 by Men1 and Rbp2 under physiologic conditions**

Interestingly, menin has previously been shown to bind the promoter of the Igf2bp2 (insulin-like growth factor binding protein 2) and to repress Igf2bp2 expression in MEFs (41, 42), in addition to the effect on Igf2bp2 that we report here. We do not see significant changes for either H3K4me3 or H3K27me3 levels at the Igf2bp2 locus in pancreatic islets harvested from mice at age of 2 months, suggesting that the mechanism for Igf2bp2 expression by menin is different from that for Igf2bp2 expression. In contrast, Igf2bp2 expression is dynamically regulated by epigenetic changes driven by menin–HMT complexes, and also modulated by the Rbp2 histone demethylase. We observed that Igf2bp2 expression is decreased during the hyperplasia stage in Men1-deficient pancreatic islets and is accompanied by changes in H3K4 and H3K27 histone methylation at the Igf2bp2 promoter. These effects are partially reversed by deletion of the Rbp2 histone demethylase, implying that inactivation of Rbp2 counteracts epigenetic changes induced by menin–HMT complexes. One explanation for this phenomenon may be that Rbp2, in association with the PRC2 complex (43), binds the Igf2bp2 promoter to reduce its expression; loss of Rbp2 may relieve this repression, resulting in gene activation.

In conclusion, we have identified several genes, notably Igf2bp2, as being regulated by menin-mediated H3K4me3 and observe epigenetic changes in these targets over time, strongly suggesting a role for these genes in pancreatic islet tumorigenesis induced upon ablation of Men1.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

Concept and design: W. Lin, H. Watanabe, M. Meyerson

Development of methodology: W. Lin, H. Watanabe, N. Kaplan

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): W. Lin, H. Watanabe, J.M. Francis, N. Kaplan, A. Agoston

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): W. Lin, H. Watanabe, J.M. Francis, N. Kaplan, C.S. Pedamallu, A.J. Bass

Writing, review, and/or revision of the manuscript: W. Lin, H. Watanabe, N. Kaplan, C.S. Pedamallu, A. Ramachandran, M. Meyerson

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): N. Kaplan

Study supervision: M. Meyerson

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