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Accessibility
Kmt2a cooperates with menin to suppress tumorigenesis in mouse pancreatic islets

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ABSTRACT
The reported incidence of pancreatic neuroendocrine tumors (PanNETs) has increased, due in large part to improvements in detection and awareness. However, therapeutic options are limited and a critical need exists for understanding more thorough characterization of the molecular pathology underlying this disease. The Men1 knockout mouse recapitulates the early stage of human PanNET development and can serve as a foundation for the development of advanced mouse models that are necessary for preclinical testing. Menin, the product of the MEN1 gene, has been shown to physically interact with the KMT2A and KMT2B histone methyltransferases. Both the KMT2A and MEN1 genes are located on chromosome 11q, which frequently undergoes loss of heterozygosity (LOH) in PanNETs. We report herein that inactivation of Kmt2a in Men1-deficient mice accelerated pancreatic islet tumorigeneration and shortened the average lifespan. Increases in cell proliferation were observed in mouse pancreatic islet tumors upon inactivation of both Kmt2a and Men1. The Kmt2a/Men1 double knockout mouse model can be used as a mouse model to study advanced PanNETs.

Introduction
Pancreatic neuroendocrine tumors (PanNETs) arise from the endocrine cells of the pancreas. The World Health Organization (WHO) guidelines grade these tumors as well-differentiated neuroendocrine tumors, well-differentiated neuroendocrine carcinomas and poorly differentiated neuroendocrine carcinomas.1 Compared to the more common form of pancreatic cancer, adenocarcinoma, which arises in exocrine cells, PanNETs comprise only 2% to 5% of new pancreatic neoplasms according to current diagnostic procedures.1,2 However, recent epidemiological studies also support that the overall incidence of PanNETs has increased at a statistically significant rate due to improvements in radiological imaging and clinician awareness.3,4 In addition, the prevalence of PanNETs observed in autopsy studies has been shown to be as high as 10%, suggesting that there may be a great number of undiagnosed, nonfunctional PanNETs in the general population.

The current approach for the treatment of PanNETs is surgical resection and management of hormone hyperscertainment if feasible. PanNETs tend to grow at a slower rate and with an overall better prognosis than exocrine tumors.1 The 5-year survival of PanNETs can be as high as 55% when the tumors are detected before metastasis and are amenable to surgical resection. Once the tumor becomes metastatic, the median overall survival rate is approximately 2 years.5 The molecular mechanisms underlying this malignancy are poorly understood, and the 5-year survival rate has not significantly improved over the past several decades. The FDA recently approved 2 targeted therapies, sunitinib malate and everolimus, both of which have only shown a modest benefit to patients with PanNETs.6,7 These shortcomings highlight the need for a better understanding of the molecular pathology of this disease and the urgency of appropriate pre-clinical models to validate potential targets to this type of cancer.

A number of signaling pathways have been shown to be involved in the tumorigenesis of pancreatic neuroendocrine cells such as PI3K signaling, mTOR pathway components and cell cycle regulators. MEN1 is one of the most commonly mutated genes in PanNETs, with an up to 36% mutation frequency in sporadic localized neuroendocrine tumors and an up to 56% in metastatic neuroendocrine tumors.8 Recent exon sequencing identified mutations of ATRX/DAXX both of which are involved in chromatin regulation, providing additional insights into the pathogenesis of PanNETs.8

The Men1 knockout (KO) mouse mimics the phenotypes observed in patients with PanNETs. Studies on the Men1 KO mouse show that pancreatic neuroendocrine tumors progress though 4 different stages: normal, hyperplasia, atypia and adenoma.9 In the Men1 conditional KO mouse model, menin is lost in pancreatic islets during embryogenesis, and hyperplastic islets can be observed as early as...
2 months. However, frank tumors do not develop until 8 to 10 months of age. In addition, even though hyperplasia is observed in most, if not all of the pancreatic islets, only a small percent of hyperplastic islets ultimately develop tumors. The long tumor latency and sporadic tumor formations in the Men1 KO mouse model indicate that deletion of Men1 is sufficient to induce hyperplasia, but that additional somatic events may be required for further tumor progression. LOH analyses also support this notion. Loss of heterozygosity (LOH) analysis on tumors from MEN-1 patients as well as sporadic tumors found that up to 68% of PanNETs exhibit losses of large parts of chromosome 11q.10

Kmt2a (MLL) encodes a histone H3 lysine 4 specific methyltransferase. It is ubiquitously expressed and plays important roles in many mouse tissues and at different tumor stages.11-13 Rearrangements of the human KMT2A gene by chromosomal translocation are associated with a variety of acute myeloid and lymphoid leukemias.14 However, the functions of KMT2A in solid tumors have not been well characterized. Interestingly, KMT2A is physically associated with menin in conjunction with a number of other proteins to form a COMPASS-like complex that promotes histone H3 methylation.15 The KMT2A gene is located on chromosome segment 11q23 which also frequently undergoes LOH in PanNETs. Based on these observations, we hypothesized that Kmt2a might be involved in tumor suppression in pancreatic islet tumors. In this study, we aim to define the role of the TrxG protein Kmt2a in pancreatic islet tumorigenesis using genetically modified mouse models.

Results

Pancreatic islet specific loss of Kmt2a leads to hyperplasia

To address whether Kmt2a is required for islet development and homeostasis of adult islets, we crossed the Kmt2a<sup>fl/fl</sup> mouse with a RIP-Cre transgenic mouse to generate Kmt2a<sup>fl/fl</sup>;RIP-Cre mice. Immunohistochemical (IHC) analysis for Kmt2a was performed on 2-month old mice to assess Kmt2a protein level changes in mouse pancreatic islets. The results were consistent with Kmt2a<sup>fl/fl</sup>;RIP-Cre pancreatic islets upon introduction of RIP-Cre (Fig. 1B), suggesting that RIP-Cre could efficiently delete the Kmt2a allele in pancreatic islets. Islet-specific Kmt2a knockout mice were viable and did not show a reduced lifespan compared to control mice (data not shown). The pancreata from Kmt2a<sup>fl/fl</sup>;RIP-Cre mice and control mice were collected at 2 months, 6 months, and 10 months and

Figure 1. Kmt2a inactivation leads to mild islet hyperplasia in mouse pancreatic islets. (A) Kmt2a expression was evaluated over time in mouse pancreatic islet cells by RT-PCR. Men1 expression served as the control. (B) Detection of Kmt2a by immunohistochemistry in RIP-Cre, Kmt2a<sup>fl/fl</sup>;RIP-Cre, Men1<sup>fl/fl</sup>;RIP-Cre and Kmt2a<sup>fl/fl</sup>;Men1<sup>fl/fl</sup>;RIP-Cre pancreatic islets in 2-month old mice. (C) Representative H&E staining of pancreata from mice with the indicated genotypes. (Scale bar: 100μm) (D) Average pancreatic islet size. Islet size was estimated by measuring the area of an islet using Image J. The 10 largest islets were measured in each mouse, and the average islet size was generated from 80 islets for each genotype.
were examined for islet morphology by histological analysis. At the 2-month stage and 6-month stage, the morphology and size of islets from Kmt2a<sup>f/f</sup>;RIP-Cre mice were almost indistinguishable from those in control mice, indicating that Kmt2a is not required for pancreatic islet development. However, at 10-months the islets in Kmt2a<sup>f/f</sup>;RIP-Cre mice were significantly larger than those in control mice, suggesting that Kmt2a inactivation causes a hyperplastic phenotype (Figs. 1C and 1D). Further analysis of Kmt2a<sup>f/f</sup>;RIP-Cre mice at 12 months and 16 months confirmed the presence of hyperplasia, but no frank tumors were detected in Kmt2a<sup>f/f</sup>;RIP-Cre mice (data not shown). This study indicates that ablation of Kmt2a can cause modest hyperplasia in mouse pancreatic islets even though Kmt2a loss does not lead to pancreatic islet tumors on its own.

**Ablation of Kmt2a in the Men1-deficient mouse leads to reduced survival through hyperinsulinemia and hypoglycemia**

To address whether Kmt2a inactivation has biological consequences in mice with a Men1 null background, we generated Kmt2a<sup>f/f</sup>;Men1<sup>f/f</sup>;RIP-Cre mice. Kmt2a IHC on islets from Kmt2a<sup>f/f</sup>;Men1<sup>f/f</sup>;RIP-Cre mice showed that the Kmt2a signal is almost completely lost (Fig. 1B). Over time, the median survival time for Men1<sup>f/f</sup>;RIP-Cre mice (N = 25) was 313 days, while the median survival time for Kmt2a<sup>f/f</sup>;Men1<sup>f/f</sup>;RIP-Cre mice (N = 16) was markedly shorter at 258 days, indicating that Kmt2a loss shortens the life span of Men1-deficient mice (Fig. 2A). Similarly, within the same littersmates the median survival time for Kmt2a<sup>f/f</sup>;Men1<sup>f/f</sup>;RIP-Cre mice (N = 10) was 274 d compared to 334 d for Men1<sup>f/f</sup>;RIP-Cre mice (N = 12) when both Kmt2a<sup>f/f</sup>;Men1<sup>f/f</sup>;RIP-Cre mice and Men1<sup>f/f</sup>;RIP-Cre mice were generated from the same littersmates (Fig. S1).

The plasma insulin levels were measured at 2 months, 4 months, 6 months, 8 months and 10 months to assess hypoglycemia within the cohort. In Men1<sup>f/f</sup>;RIP-Cre mice the plasma insulin levels began to gradually increase at ~4 months of age and became significantly elevated by 6 months, whereas the plasma insulin levels in Kmt2a<sup>f/f</sup>;Men1<sup>f/f</sup>;RIP-Cre mice began to rise at 4 months and then quickly surpassed Men1<sup>f/f</sup>;RIP-Cre mouse insulin levels (Fig. 2B). These results suggest that the combined loss of Kmt2a and Men1 leads to an earlier onset of tumor formation as evidenced by hyperinsulinemia.

**Ablation of Kmt2a promotes tumorigenesis in Men1-deficient pancreatic islets**

We harvested mouse pancreata from Men1<sup>f/f</sup>;RIP-Cre and from Kmt2a<sup>f/f</sup>;Men1<sup>f/f</sup>;RIP-Cre mice and conducted histological analysis to examine pancreatic islet size. We found that the islet cell morphology and islet size in Kmt2a<sup>f/f</sup>;Men1<sup>f/f</sup>;RIP-Cre mice were indistinguishable from that in Men1<sup>f/f</sup>;RIP-Cre mice at age of 2 months and 4 months (Fig. 3A). However, by 6 months of age, the islets in Kmt2a<sup>f/f</sup>;Men1<sup>f/f</sup>;RIP-Cre mice were larger than those in Men1<sup>f/f</sup>;RIP-Cre mice. When the pancreas was harvested from both strains at the 10-month stage, the islet tumor size and the islet cell morphology in Kmt2a<sup>f/f</sup>;Men1<sup>f/f</sup>;RIP-Cre mice were consistently different from that in the Men1<sup>f/f</sup>;RIP-Cre mice (Fig. 3A). The average islet tumor size was 2 times larger in Kmt2a<sup>f/f</sup>;Men1<sup>f/f</sup>;RIP-Cre mice than in Men1<sup>f/f</sup>;RIP-Cre mice (Fig. 3B). Additionally, the size of both the nucleus and cytoplasm were larger in the Kmt2a<sup>f/f</sup>;Men1<sup>f/f</sup>;RIP-Cre mice than that in Men1<sup>f/f</sup>;RIP-Cre mice (Fig. 3A). We also observed that there were a greater number of tumors detected in the Kmt2a<sup>f/f</sup>;Men1<sup>f/f</sup>;RIP-Cre mice than that in Men1<sup>f/f</sup>;RIP-Cre mice. When we defined abnormal islets as being larger than 0.5 mm with showing strong vascularization, we found 5 tumors in Kmt2a<sup>f/f</sup>;Men1<sup>f/f</sup>;RIP-Cre mice on average compared to only 1.3 tumors on average in Men1<sup>f/f</sup>;RIP-Cre mice (Fig. 3C). In general, the tumor phenotypes in Kmt2a<sup>f/f</sup>;Men1<sup>f/f</sup>;RIP-Cre mice were more advanced than the tumors in Men1<sup>f/f</sup>; RIP-Cre mice (Table S1). Thus, inactivation of Kmt2a in pancreatic islets promotes tumor progression rather than facilitating tumor initiation. We also observed that there were more blood vessels in tumors from the Kmt2a<sup>f/f</sup>;Men1<sup>f/f</sup>;RIP-Cre mice compared to Men1<sup>f/f</sup>;RIP-Cre mice (Figs. S2A and S2B).

**Kmt2a loss results in more cell proliferation in hyperplastic islets and tumors in Kmt2a<sup>f/f</sup>; Men1<sup>f/f</sup>;RIP-Cre mice**

We next utilized immunohistochemical analysis to investigate whether the larger hyperplastic islets and increased number of tumors observed in Kmt2a<sup>f/f</sup>;Men1<sup>f/f</sup>;RIP-Cre mice were a consequence of increased cell proliferation or reduced apoptosis. We found that there were elevated numbers of mitotic cells in

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**Figure 2.** Loss of Kmt2a shortens life span in Men1-deficient mice. (A) Kaplan-Meier survival curves comparing Men1<sup>f/f</sup>;RIP-Cre mice (n = 24) with Kmt2a<sup>f/f</sup>;Men1<sup>f/f</sup>;RIP-Cre mice (n = 16; P < 0.0001). (B) Circulating insulin levels in mice with the indicated genotypes (paired sample t test: p = 0.03 at 6 months).
Kmt2a;Men1;RIP-Cre mice compared to Men1;RIP-Cre mice (Figs. 4A and 4B). A PanNET is considered to be a malignant tumor if there are more than 20 mitotic cells in 10 high power fields. There were approximately 20 more mitotic cells in one Kmt2a;Men1;RIP-Cre tumor than that in a comparable Men1;RIP-Cre tumor (Fig. 4C), indicating that tumors caused by Men1 and Kmt2a loss are more proliferative than those caused by loss of Men1 alone.

Next we performed IHC staining for the mitotic marker H3S10P in islets harvested from RIP-Cre, Men1;RIP-Cre and Kmt2a;Men1;RIP-Cre mice. As expected, the Men1;RIP-Cre islets showed more H3S10P positive cells than RIP-Cre control islets (Fig. 5A). However, in Kmt2a;Men1;RIP-Cre islets, there were many more H3S10P positive cells when compared with Men1;RIP-Cre islets, thus demonstrating increased mitosis (Fig. 5A). Quantification of H3S10P positive cells showed that there were 8 times more mitotic cells in Kmt2a;Men1;RIP-Cre islets than that in Men1;RIP-Cre islets (Fig. 5C). Additionally, H3S10P staining in 10-month islets/tumors consistently showed more H3S10P positive cells in Kmt2a;Men1;RIP-Cre tumors compared to that in Men1;RIP-Cre tumors (Figs. S3A and S3C).

TUNEL assays performed on islets/tumors from RIP-Cre, Men1;RIP-Cre and Kmt2a;Men1;RIP-Cre mice revealed that there were similar numbers of apoptotic cells in all 3 of the tested trains (Figs. 5B and 5D). Cleaved Caspase-3 (CC3) IHC staining was also performed in these islets; similar numbers of CC3 positive cells were observed in the islets (Figs. S3B and S3D). Statistical analyses did not show a significant difference between islets. These studies indicate that the more highly mitotic tumor phenotypes observed in Kmt2a;Men1;RIP-Cre mice were more likely due to increased cell proliferation rather than decreased apoptosis.

Discussion

Kmt2a function during islet development and islet function in the adult stage

Kmt2a functions are not only critical for embryonic development, but are also essential for the maintenance of homeostasis in particular lineage/tissues such as haematopoietic stem cells as well as during neurogenesis in mouse postnatal brain. Kmt2a is also highly expressed in mouse pancreatic islets. Our study reveals that Kmt2a is dispensable for islet development as there is no detectable difference in islet numbers in Kmt2a-deficient mice compared with RIP-Cre control mice. However, Kmt2a is required for adult islet homeostasis as the loss of Kmt2a was found to lead to advanced hyperplasia in adult mice. Our
investigation indicates that Kmt2a is not a neuroendocrine-specific tumor suppressor as no tumors were observed in Kmt2a-deficient mice. Consistent with this observation, we did not observe any survival disadvantage upon Kmt2a loss in islets. Possible explanations include: Kmt2a function might indeed be dispensable for islet cell differentiation and/or Kmt2a loss might be compensated by Kmt2a family members such as Kmt2b, Kmt2c, or Kmt2d.

Figure 4. Kmt2a loss leads to a robust increase in mitotic cells. Mitotic cells were observed from Kmt2a<sup>f/f</sup>;Men1<sup>f/f</sup>;RIP-Cre but not in Men1<sup>f/f</sup>;RIP-Cre islets in (A) (lower magnification, Scale bar: 100 μm) and (B) (higher magnification, Scale bar: 100 μm). Arrow indicates cells undergoing mitosis. (C): Quantification of mitotic cells from hyperplastic islets/tumors harvested from mice with the indicated genotypes at an age of 10 months.
Kmt2a cooperates with MEN1 to prevent tumor formation

*Men1*-deficient islets quickly become hyperplastic at 2 to 4 months of age. However, it takes 6 to 8 months for the hyperplastic islet to develop detectable tumors. Thus, other genetic or epigenetic alterations must likely occur to facilitate tumor progression. During human MEN-1 syndrome, *MEN1* is mutated at the germline level and the second allele is frequently lost through LOH, which often involves a broad region of 11q. As *KMT2A* is located in 11q23 within 10 Mb of the *MEN1* locus, *KMT2A* is frequently lost in tumors from patients with MEN-1 syndrome. Although *Kmt2a* loss in mice by itself does not lead to tumor formation, *Kmt2a* inactivation accelerates islet tumor progression in the *Men1*-deficient mouse. This study indicates that *Men1* is a driver and initiator of islet tumors from patients with MEN-1 syndrome. Although *Kmt2a* loss in mice by itself does not lead to tumor formation, *Kmt2a* inactivation accelerates islet tumor progression in the *Men1*-deficient mouse. This study indicates that *Men1* is a driver and initiator of islet tumors whereas *Kmt2a* loss can facilitate tumor progression. *Kmt2a* might be involved in islet tumorigenesis in both a menin-dependent and a menin-independent manner. As a menin-associated protein, *Kmt2a* might partially compensate for menin dysfunction and maintain key downstream targets. Whereas when *Kmt2a* is inactivated, menin might counteract the effect caused by *Kmt2a* loss. On the other hand, although menin is a component of the COMPASS-like complex that promotes histone H3 methylation under certain circumstance, *Kmt2a* might be critical for certain important genes in pancreatic islets which are independent of menin. Our preliminary ChIP-seq and RNA-Seq data (unpublished) show that the expression of several islet-specific transcription factors is positively regulated by *Kmt2a* enzymatic activity, but not menin activity. Further experiments should be conducted to address these remaining questions.

Advanced mouse models for molecular pathology studies and preclinical studies

Currently two targeted therapies for pancreatic neuroendocrine tumors have been approved by the FDA, indicating that targeted therapies can be developed for the treatment of pancreatic neuroendocrine cancer. However, current targeted therapies only prolong progression-free survival for approximately 6 months, providing only a modest benefit to patients with this type of cancer. Additionally the response rates are very low when patients with advanced PanNETs were treated with the tyrosine kinase inhibitor sunitinib and the mTOR inhibitor everolimus. Targeted therapeutic approaches which combine the effect of both mTOR inhibition and angiogenesis inhibition have shown substantial anti-cancer activity, and new potential approaches have been proposed. In principle, better mouse models will help to develop improved targeted therapies. The *Kmt2a*;*Men1*;RIP-Cre mouse has more advanced tumor phenotypes than other models, so this mouse model may be more suitable as a preclinical mouse model for testing new targeted therapies for pancreatic neuroendocrine...
tumors. In addition, menin is physically associated with Kmt2a, which is critical in leukemia mediated by Kmt2a fusion proteins. Several inhibitors that specifically disrupted the menin-Kmt2a interaction have been developed and pharmacologic inhibition has shown a very promising response. Disruption of Kmt2a does not lead to phenotypes in early stage of islets whereas Men1 deletion causes a hyperplastic phenotype, suggesting that menin has Kmt2a-independent roles in the maintenance of homeostasis in mouse pancreatic islets, which is consistent with a recent study in the haematopoietic system. This observation may alleviate some concern regarding the potential strong side effect on pancreatic function when applying these MI-2 and related inhibitors in patients with Kmt2a-associated leukemia.

**Materials and methods**

**Generation of mouse strains**

The creation and genotyping of RIP-Cre mice, Men1f/f; RIP-Cre mice has been described previously. Kmt2afl mice were crossed with RIP-Cre mice to obtain Kmt2afl; RIP-Cre mice. Kmt2afl; RIP-Cre mice were crossed with Men1fl; RIP-Cre mice to obtain Kmt2afl; Men1fl; RIP-Cre, Kmt2afl; RIP-Cre mice were then crossed with Kmt2afl; Men1fl mice to obtain Kmt2afl; Men1fl; RIP-Cre mice. Mice were maintained on a mixed 129S6, FVB/N, and C57BL/6 background. All mice were maintained in the research animal facility of the Dana-Farber Cancer Institute and Hefei Institutes of Physical Science Laboratory Animal Center, Chinese Academy of Sciences. All procedures were performed 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 both the Dana-Farber Cancer Institute and Hefei Institutes of Physical Science, Chinese Academy of Sciences.

**Isolation of mouse pancreatic islets**

Pancreatic islets were isolated as previously described.

**Circulating insulin measurement**

Blood was collected via the submandibular vein method after the mice were fasted for 16h. Plasma was prepared by spinning a tube of fresh blood containing an anticoagulant. The circulating insulin level was measured using an ultra sensitive Mouse ELISA kit (Crystal Chemical, Downers Grove, IL, USA) according to the manufacturer’s instructions.

**Histological and immunohistochemical analysis of 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. Histopathological analysis was carried out on 5-micrometer sections stained with hematoxylin and eosin. Islet morphology and tumors were examined in at least 3 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 Kmt2a (GeneTax, GTX17959, 1:400), Anti-Histone H3(Phospho S10) (Abcam, Ab14955, 1:180), and CC3 (Thermo, PA1-24473, 1:100). Sections were counterstained in Meyer’s hematoxylin, mounted and photographed using an Olympus microscope.

**RNA isolation and RT-qPCR**

Total RNA was extracted using the RNaseq kit (Qiagen) from 100 to 300 mouse pancreatic islets purified from 2 mice with different genotypes according to the manufacturer’s instructions. For RT-qPCR, DNase I (Qiagen)-treated RNA samples were reverse transcribed using oligo-dT and SuperScript III (Invitrogen), with first strand cDNA for PCR created using SYBR green PCR mix (Qiagen, Valencia, California, United States). PCR primer pairs were designed to amplify 150- to 200-bp fragments from select genomic regions. Quantification of mRNA expression in each sample was performed by normalizing values to the expression values for Gapdh. Primer sequences for Kmt2a are 5'-CATTCCCGAAATGGAGCGAG-3' (forward) and 5'-TAGAGGAGGTGCTCATGT GT-3' (reverse); primer sequences for Men1 are 5'-GATGGACATCTCTGAGACCCA-3' (forward) and 5'-CCAGTCCCTCTCGACTCCA-3' (reverse); primer sequences for Gapdh are 5'-TCAATGAAGGGGTCTTGAT-3' (forward) and 5'-CGTCCCAGTACAAAATGGT-3' (reverse).

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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