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## Epigenetic regulation in the tumorigenesis of MEN1-associated endocrine cell types

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### Abstract

Epigenetic regulation is emerging as a key feature in the molecular characteristics of various human diseases. Epigenetic aberrations can occur from mutations in genes associated with epigenetic regulation, improper deposition, removal or reading of histone modifications, DNA methylation/demethylation, and impaired non-coding RNA interactions in chromatin. Menin, the protein product of the gene causative for the multiple endocrine neoplasia type 1 (MEN1) syndrome, interacts with chromatin-associated protein complexes and also regulates some non-coding RNAs, thus participating in epigenetic control mechanisms. Germline inactivating mutations in the *MEN1* gene that encodes menin predispose patients to develop endocrine tumors of the parathyroids, anterior pituitary and the duodenopancreatic neuroendocrine tissues. Therefore, functional loss of menin in the various MEN1-associated endocrine cell types can result in epigenetic changes that promote tumorigenesis. Because epigenetic changes are reversible, they can be targeted to develop therapeutics for restoring the tumor epigenome to the normal state. Irrespective of whether epigenetic alterations are the cause or consequence of the tumorigenesis process, targeting the endocrine tumor-associated epigenome offers opportunities for exploring therapeutic options. This review presents epigenetic control mechanisms relevant to the interactions and targets of menin, and the contribution of epigenetics in the tumorigenesis of endocrine cell types from menin loss.

### Keywords

Neuroendocrine; Pancreas; Parathyroid; Pituitary

### Introduction

Epigenetic changes observed in various human diseases have been shown to play important roles as disease drivers, biomarkers and therapeutic targets. Epigenetic changes can occur due to mutations in factors that control epigenetic modifications or in response to metabolic or environmental signals, that lead to improper deposition, removal or reading of histone

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modifications, DNA methylation/demethylation, and impaired non-coding RNA interactions in chromatin (Allis et al. 2015). The evidence for the role of epigenetic regulation in tumor etiology is highlighted by the discovery of mutations in genes encoding factors that participate in epigenetic regulatory mechanisms in various cancers (Koschmann et al. 2017). Menin, the protein encoded by the gene responsible for causing the multiple endocrine neoplasia type 1 (MEN1) syndrome participates in epigenetic regulation (Shilatifard 2012, van Nuland et al. 2013). Patients with the MEN1 syndrome carry germline heterozygous inactivating mutations in the *MEN1* gene located at chromosome 11q13, that encodes menin, which predisposes to the development of tumors in multiple endocrine organs (Chandrasekharappa et al. 1997, Lemmens et al. 1997). The main MEN1-associated endocrine cell types that form tumors are located in the parathyroids, anterior pituitary and the duodenopancreatic neuroendocrine tissues (Thakker et al. 2012). Other MEN1-associated endocrine tumors include foregut carcinoids that arise from neuroendocrine cells of the lung, thymus or gastrointestinal (GI) tract. The tumors are classified as functioning when they hypersecrete the hormones characteristic of the endocrine cell types affected, or non-functioning when they do not hypersecrete any hormones. Parathyroid tumors are functioning causing primary hyperparathyroidism due to excessive parathyroid hormone (PTH) secretion. Pituitary and duodenopancreatic neuroendocrine tumors in MEN1 patients are observed to be either functioning (prolactinoma, 20%; somatotropinoma, 10%; gastrinoma, 40%; insulinoma, 10%), or non-functioning (pituitary, 5%; PNETs, 20–55%) (Marini et al. 2017, Schussheim et al. 2001, Thakker et al. 2012). In the context of these endocrine tumors, *MEN1* acts as a classic tumor suppressor gene because human tumors show loss of heterozygosity (LOH) at 11q13 resulting in biallelic loss/inactivation of *MEN1*. (Chandrasekharappa et al. 1997, Lemmens et al. 1997). Similarly, in genetically engineered mouse models, germline targeted deletion of both copies of the *Men1* gene causes embryonic lethality, while germline targeted deletion of one copy of the *Men1* gene generates live mice that later in life develop endocrine tumors similar to those seen in the human MEN1 syndrome with LOH at the *Men1* locus (Agarwal 2014, Agarwal 2017b).

Interestingly, *MEN1* mutations are also detected in 30–40% of sporadic tumors of MEN1-associated endocrine cell types - parathyroid adenomas, gastrinomas, pancreatic neuroendocrine tumors (PNETs), and lung neuroendocrine tumors (Cromer et al. 2012, Debelenko et al. 1997, Jiao et al. 2011, Newey et al. 2012, Scarpa et al. 2017, Simbolo et al. 2017, Zhuang et al. 1997). Among sporadic PNETs, *MEN1* mutations are detected in the non-functioning PNETs, but rarely in the functioning PNETs (insulinomas) (Cao et al. 2013, Chou et al. 2016, Jiao et al. 2011, Lichtenauer et al. 2015, Scarpa et al. 2017, Wang et al. 2017). Also, *MEN1* mutations are rarely detected in sporadic pituitary tumors (Bi et al. 2017, Marques and Korbonits 2017, Newey et al. 2013, Poncin et al. 1999). Because menin participates in epigenetic regulation, functional loss of menin in the various MEN1-associated endocrine cell types would lead to epigenetic changes that could potentially promote tumorigenesis. Furthermore, evidence from genome and exome sequencing efforts of sporadic non-functioning and functioning PNETs, parathyroid adenoma, and lung neuroendocrine tumors have shown additional mutations in other genes associated with epigenetic control mechanisms that predict epigenetic changes to promote tumorigenesis in

these endocrine cell types (Chou et al. 2016, Cromer et al. 2012, Jiao et al. 2011, Romano et al. 2017, Scarpa et al. 2017, Simbolo et al. 2017, Wang et al. 2017).

Menin is a 67 kDa ubiquitously expressed predominantly nuclear protein, with no intrinsic enzymatic activity. As stated above, menin is a tumor suppressor in the context of MEN1-associated endocrine cell types; however, menin has been shown to act as a pro-oncogenic co-factor in mixed lineage leukemia (Muntean and Hess 2012, Yokoyama et al. 2005). The crystal structure of menin shows a deep pocket that can act as a binding site for interacting proteins (Protein Data Bank No. 3RE2 and 3U84) (Huang et al. 2012, Murai et al. 2011). Evidence from protein-protein interaction studies have shown that menin is involved in epigenetic regulation and gene transcription through interaction with proteins in chromatin-associated protein complexes and transcription factors, affecting the expression of target genes such as genes that control cell proliferation (Karnik et al. 2005, Wu and Hua 2011). Interacting partners of menin also include proteins that participate in cell adhesion, cell division, cell motility, cell signaling, cytoskeletal structure, DNA repair in response to DNA damage, and studies conducted in *Drosophila melanogaster* have shown a role of menin in the maintenance of genomic stability (Agarwal 2017a, Busygina et al. 2004, Feng et al. 2017a, Henty et al. 2009).

Investigations to elucidate the biological function of menin through protein-protein interaction studies have significantly advanced our knowledge about the potential roles of menin in various cellular pathways, but they have been mainly conducted using cell lines (HEK293, HeLa, HepG2, K562, and Mouse Embryo Fibroblasts (MEFs)) that are unrelated to MEN1-associated endocrine cell types. Follow-up functional characterization of some interactions of menin have been performed in endocrine/endocrine-related cell lines ( $\beta$ lox5, BON1, INS1, MIN6, NIH-H720, NIH-727, and QGP1) albeit not in endocrine cell lines with *MEN1* mutations or that lack menin because no such cell lines are currently available. The focus of this review will be on studies conducted using endocrine/endocrine-related cell lines, or menin-null endocrine tumors from mouse models. This review excludes the discussion of menin's interaction with transcription factors because they were recently well-covered elsewhere (Agarwal 2017a, Dreijerink et al. 2017, Feng et al. 2017a). In this review, we present a survey of the published literature to describe epigenetic control mechanisms that are relevant to the interactions and targets of menin, the contribution of epigenetics in the tumorigenesis of endocrine cell types from menin loss, and experimental therapeutic strategies to potentially correct the tumor-specific epigenetic changes.

## Epigenetic Mechanisms

Eukaryotic DNA with core histone proteins (H2A, H2B, H3, and H4) is packaged into nucleosomes to form chromatin fibers (Olins and Olins 2003). This packaging regulates access to DNA for various biological processes such as DNA replication, DNA repair and transcription into RNA. Modifications to chromatin are essential to permit or restrict access, for example, to the transcriptional machinery including transcription factors for gene expression (Flavahan et al. 2017). Chromatin modifiers and remodelers constitute 'epigenetic factors' that function in multi-protein complexes that may also include non-coding RNAs, to modify the DNA or histones contained in nucleosomes (Allis and Jenuwein

2016). The multi-protein complexes contain enzymes and proteins referred to as the 'writers', 'readers' and 'erasers' of chromatin modifications (Allis and Jenuwein 2016). Modifications to DNA include methylation, hydroxymethylation and further oxidation. Histones can undergo a variety of post-translational modifications (PTMs) such as methylation, acetylation, phosphorylation and ubiquitination to generate an active, repressive or poised state to control gene expression; and the array of PTMs that govern a specific chromatin state is referred to as the 'histone code' (Huang et al. 2014). Also, chromatin interactions can occur over short and long distances thereby generating conditions that enhance gene transcription or insulate the regions of gene transcription (Rowley and Corces 2016). 'Epigenetic changes' through alterations in DNA and histone modifications, chromatin remodeling, and alterations in non-coding RNA interactions, create a positive or negative environment affecting the outcome of gene transcription and chromatin structure (Allis et al. 2015). The 'epigenetic marks' on DNA and histones that are generated as a result of the enzymatic activity of various chromatin modifying enzymes can survive successive cell divisions, and they are reversible, which presents an opportunity for correction to the normal state, for example when such changes promote disease processes (Zoghbi and Beaudet 2016). Technological advances in chromatin immunoprecipitation and next-generation sequencing have allowed comprehensive characterization of genome-wide epigenetic marks to reveal the status of the epigenome in normal and pathological conditions that can help to identify epigenomic changes that are disease drivers, or to serve as biomarkers and therapeutic targets. Below we describe the various epigenetic mechanisms and chromatin modifications that are relevant to MEN1-associated endocrine cell types (Figure 1).

## Histone methylation and demethylation

Methylation of histones at lysine or arginine residues in the chain of amino acids that protrude from the nucleosome, referred to as the 'histone tail', is achieved by histone methyltransferases (KMTs) that exist in multi-subunit protein complexes (Cheng 2014). KMTs methylate histone H3 on lysine residues at positions 4, 9, 27, 36, and 79 in the mono-, di-, or tri-methylated form depending on whether one, two, or three methyl groups are added, respectively (Chi et al. 2010). In general, the histone H3 lysine-4 trimethyl mark (H3K4me3) specifies a state of transcriptional activation, that is located at the transcriptional start site (TSS) of actively transcribed genes. On the other hand, H3K9me3 and H3K27me3 are associated with transcriptional silencing. When both H3K4me3 and H3K27me3 occur together, the gene is said to be bivalently poised - a state maintained, for example, by embryonic stem cells during lineage differentiation and development, to enable rapid switching between active and repressive gene transcription states (Voigt et al. 2013). Eraser KDMs such as lysine specific demethylase 1 (LSD1/KDM1A), lysine specific demethylase 2 (LSD2/KDM1B), and Jumonji AT-rich interacting domain 1A (JARID1A/KDM5A/RBP2), remove mono- and di-methylation of H3K4 and H3K9 (Cheng 2014). Depending on the specific amino acid residue methylated or demethylated, and the level of methylation, the process of histone methylation and demethylation generates active or repressive chromatin states.

### H3K4me3 and H3K27me3

There are at least six different protein complexes that contain KMTs for catalyzing the gene activating histone modification H3K4me3 (Dreijerink et al. 2017). Menin has been shown to interact with KMTs, MLL1 (KMT2A) and MLL2 (KMT2B), in two of those multi-subunit protein complexes, and studies using chromatin immunoprecipitation have shown that menin is present at the TSS with H3K4me3 (Hughes et al. 2004, Scacheri et al. 2006, Yokoyama et al. 2004). Several lines of evidence have established the importance of this interaction of menin involving H3K4me3 and the target genes affected by using MEN1-associated endocrine cell types, mainly pancreatic islets.

Mice with targeted deletion of *Men1* in the germline (*Men1<sup>+/-</sup>*) or specifically in the pancreatic islet beta-cells (*Men1<sup>f/f</sup>;RIP-Cre*) develop insulin-secreting pancreatic islet tumors (insulinomas) (Agarwal 2014, Agarwal 2017b). Interestingly, both *KMT2A* and *MEN1* co-localize at human chromosome 11q and mouse chromosome 19. LOH at this locus in *MEN1* mutation-positive human PNETs and mouse PNETs (insulinomas) in *Men1<sup>+/-</sup>* mice contributes to the tumorigenesis process. Targeted conditional deletion of both *Kmt2a* and *Men1* in mice (*Kmt2a<sup>f/f</sup>;Men1<sup>f/f</sup>;RIP-Cre*) led to the acceleration of pancreatic islet tumor formation, and also shortened the average life span of these mice (Lin et al. 2016). This study showed that MLL1 can cooperate with menin to prevent tumor formation. In another study, targeted conditional deletion in mice of *Men1* and *Rbp2* which encodes a H3K4me3 demethylase (*Rbp2<sup>f/f</sup>;Men1<sup>f/f</sup>;RIP-Cre*) decreased the rate of tumor formation and prolonged survival (Lin et al. 2011). These data showed that the histone demethylase activity of RBP2 is linked to islet tumorigenesis downstream of menin loss.

To investigate the contribution of menin-MLL1 mediated epigenetic regulation in tumor formation, genome-wide distribution of the gene activation mark H3K4me3 and its counterpart recessive mark H3K27me3 was examined in menin-deficient pancreatic islets of 2-month-old *Men1<sup>f/f</sup>;RIP-Cre* and control wild-type mice (Lin et al. 2015). This study found that within the same set of genes, loss of H3K4me3 correlated with increased H3K27me3 in menin-deficient islets, and the expression of such genes was downregulated. The most significantly downregulated gene in menin-deficient islets was *Igfbp2*, that also displayed loss of H3K4me3 together with gain of H3K27me3. Interestingly, the decline in *Igfbp2* expression in menin-deficient islets could be reversed with simultaneous deletion of *Rbp2* (Lin et al. 2015). This observation further confirms that the histone demethylase activity of RBP2 is linked to islet tumorigenesis downstream of menin loss.

Genome-wide occupancy patterns of menin, MLL1 and another component of the MLL-protein-complex (RBBP5) in human pancreatic islet cells, combined with gene expression analysis in menin-null islets from *Men1<sup>f/f</sup>;RIP-Cre* mice showed upregulation of the pancreatic islet  $\beta$ -cell differentiation factor HLXB9 (Scacheri et al. 2006). Candidate gene analysis using menin-null islets from *Men1<sup>f/f</sup>;RIP-Cre* mice showed downregulation of cell cycle inhibitory genes in menin-null islet tumors compared to normal islets, mainly the cyclin-dependent kinase inhibitor (CDKI) genes *CDKN2C/p18* and *CDKN1B/p27*, with concomitant loss of H3K4me3 in their promoter regions (Karnik et al. 2005). In another study, a long non-coding RNA, MEG3 was identified as an epigenetic target of menin (Agarwal and Jothi 2012). This study used genome-wide assessment of H3K4me3 and gene

expression from wildtype mouse embryonic stem cells (mESCs) and menin-null mESCs. Loss of H3K4me3 was observed in menin-null mESCs at the *Meg3* gene, together with downregulation of the *Meg3* transcript. Further studies showed decreased MEG3 levels in mouse and human islet tumors (Modali et al. 2015). Also, loss of MEG3 expression was reported in sporadic non-functioning pituitary tumors (Zhao et al. 2005). When mESCs were in vitro differentiated into pancreatic-islet like endocrine cells (PILECs), reduced H3K4me3 and reduced gene expression was observed of the *Hox* genes in PILECs derived from menin-null mESCs (Agarwal and Jothi 2012). *HOX* genes have been identified as the direct target of the MLL1-complex in many different cell types, particularly in hematopoietic cells where the menin-MLL association partner as an oncogenic complex in MLL-fusion leukemia (Muntean and Hess 2012). It is important to note that the PILECs were heterogeneous in terms of the cell population as assessed by the expression of pancreatic marker genes, and not entirely consisted of pancreatic endocrine cells (Agarwal and Jothi 2012). Whether *HOX* genes play a role in MEN1-associated endocrine tumorigenesis remains to be determined.

### H3K9me3

Another histone lysine modification regulated by menin is the repressive H3K9me3, through interaction with a lysine methyltransferase, suppressor of variegation 3–9 homolog 1 (SUV39H1) (Yang et al. 2013). In INS-1 rat insulinoma cells (Merglen et al. 2004), enhanced H3K9me3 was detected at the promoter region of the membrane metallo-proteinase (*Mme*) gene with combined presence of menin, SUV39H1 and the chromatin remodeling protein DAXX (Feng et al. 2017b). The *DAXX* gene is frequently mutated in sporadic non-functioning PNETs (Chou et al. 2016, Jiao et al. 2011, Scarpa et al. 2017). Therefore, this study helps to integrate the potential involvement of multiple epigenetic regulators to prevent tumorigenesis.

### H4R3me2s

PRMTs methylate arginine residues in histone H3 at positions 2, 8, 17, and 26; and histones H4 and H2A at positions 3 and 11, respectively (Yang and Bedford 2013). PRMTs are classified into two families. The type I family comprises the members 1, 2, 3, 4, 6 and 8, which catalyze mono-methylation and asymmetric dimethylation of arginines (Yang and Bedford 2013). The type II family consisting of PRMTs 5, 7, and 9 mediate monomethylation and symmetric dimethylation of arginines. In mammalian cells, PRMT5 is the predominant type II arginine methyltransferase which acts as a transcriptional repressor specifying H3R2me2 and H4R3me2 (Stopa et al. 2015). Menin has been shown to interact with PRMT5, coinciding with a repressive chromatin mark, symmetric dimethylation of H4R3 (H4R3me2s), at the promoter of the growth arrest specific 1 (*Gas1*) gene (Gurung et al. 2013). GAS1 has been shown to direct the binding of the sonic hedgehog (SHH) ligand to its receptor patched 1 (PTCH1) to activate the pro-proliferative and oncogenic Hedgehog signaling pathway (Martinelli and Fan 2007). Thus, menin loss would coincide with increased GAS1 protein and oncogenic Hedgehog signaling. Further characterization of menin/PRMT5 showed that pharmacologic inhibition of Hedgehog signaling with the drug GDC-0449 reduced cell proliferation in insulinoma tumors of *Men1<sup>fl/fl</sup>*;RIP-Cre mice (Gurung et al. 2013). These data show that inhibition of Hedgehog signaling in PNETs could

target the downstream effects from loss of menin/PRMT5 interaction, revealing a potential target for treating MEN1 tumors.

## Histone acetylation and deacetylation

Histone acetyl transferases (HATs) catalyze acetylation at lysine residues in histone tails. There are five well characterized HAT families that can acetylate specific lysine residues of mammalian histones - p300/CBP, Gcn5-related N-acetyltransferase (GNAT) (GCN5/PCAF), MYST (for 'MOZ, Ybf2/Sas3, Sas2 and Tip60) related HATs, the general transcription factor HATs which include the TFIID subunit TAF250, and nuclear receptor coactivators (SRCs) (Marmorstein and Zhou 2014). The acetylation marks on histones are biologically read by the bromodomain (BRD) contained in the bromodomain and extraterminal (BET) proteins comprising members BRD2, BRD3, BRD4, and BRDT. Histone acetylation generally favors active transcription that is reversed by the activity of histone deacetylases (HDACs) (Marmorstein and Zhou 2014). There are 18 HDACs that belong to four different subtypes classified on sequence similarities, shared catalytic mechanisms, and active sites (Seto and Yoshida 2014). Small molecule inhibitors (CPI-203, JQ1 and PFI-1) of bromodomain interactions with acetylated histone tails have been developed as potential therapeutics (Perez-Salvia and Esteller 2017). JQ1 binds to the acetyl-lysine recognition pocket of bromodomains 1 and 2 on BRD4 which displaces BRD4 from chromatin, thus preventing the 'reading' of the acetyl mark and blocking active transcription. CPI-203 is a primary amide analog of JQ1, and PFI-1 inhibits BRD2 and BRD4 (Perez-Salvia and Esteller 2017).

There is no direct evidence in MEN1-associated endocrine cells for the interaction of menin in protein complexes that are involved in specific histone acetylation or deacetylation. However, there is indirect evidence for the involvement of histone acetylation/deacetylation in endocrine cells that comes from the demonstration of growth inhibitory effects of HDAC inhibitors (HDACi) (trichostatin-A (TSA), thailandepsin-A (TDP-A), sodium butyrate (NaB), valproic acid (VPA), and MS-275), and BET protein bromodomain inhibitors (BETi) (CPI201, PFI-1 and JQ1) in neuroendocrine tumor cell lines BON1, NCI-H720, NCI-H727, and QGP1 (Adler et al. 2009, Arvidsson et al. 2016, Baradari et al. 2006, Lines et al. 2017, Wong et al. 2014). BON1 is a human cell line derived from a peripancreatic lymph node metastasis of a pancreatic neuroendocrine carcinoid tumor (Townsend et al. 1993). NCI-H720 and NCI-H727 are derived from human atypical and typical bronchial carcinoid tumors, respectively (ATCC). QGP-1 is a carcinoembryonic antigen-producing cell line from a human pancreatic islet carcinoma (Kaku et al. 1980). Anti-tumor activity of the HDACi VPA and BETi CPI203 was also shown in a BON1 xenograft model (Greenblatt et al. 2007, Wong et al. 2014). Also, in vivo anti-tumor activity of JQ1 has been demonstrated in primary tumors (insulinomas) of the *Men1<sup>fl/f</sup>;RIP-Cre* mouse model (Lines et al. 2017). These studies highlight the potential of targeting the epigenetic histone acetyl marks in treating MEN1-associated neuroendocrine tumors.

## Chromatin remodeling

Histones and nucleosomes are subjected to remodeling by protein-complexes that contain ATP-dependent chromatin remodeling activity. Also, eukaryotic cells express histone variants, in addition to the “canonical” core histones, providing another layer of epigenetic regulation. Chromatin remodeling activity facilitates transcriptional initiation and elongation for the passage of RNA polymerase through nucleosomal arrays (Clapier and Cairns 2009). This is achieved by displacing histone dimers H2A/H2B or H3/H4 leading to the substitution of the core histones in the dimers with histone variants such as histone H2A.Z or H3.3 (Clapier and Cairns 2009, Szenker et al. 2011). In addition to controlling gene transcription, histone variants and their PTMs also contribute to nucleosome and chromatin structure and function (Szenker et al. 2011).

The evidence for the involvement of histone remodeling in MEN1-associated endocrine cells comes from sporadic non-functioning PNETs that show somatic mutations in death-domain-associated protein (*DAXX*) and its interacting partner, alpha thalassemia/mental retardation syndrome X-linked (*ATRX*) (Chou et al. 2016, Jiao et al. 2011, Scarpa et al. 2017). *ATRX* with its intrinsic ATPase activity, in collaboration with its partner *DAXX*, a highly specific histone chaperone, deposit the histone variant H3.3 in chromatin at telomeric and pericentromeric regions (Amorim et al. 2016). Telomeres protect chromosome ends to prevent the loss of DNA, and in neoplastic cells the maintenance of telomeres is required for immortality. Lengthening of telomeres in tumor cells can occur from excessive production of the telomerase enzyme or through alternative lengthening of telomeres (ALT), a process similar to homologous recombination (Amorim et al. 2016). *ATRX/DAXX* mutations, or loss of their expression in sporadic PNETs correlate with increased telomere length that can potentially support tumor progression (Kim et al. 2017, Marinoni et al. 2017, Scarpa et al. 2017, Singhi et al. 2017, VandenBussche et al. 2017). Some level of ALT was also detected in *MEN1* mutation-positive sporadic PNETs (Scarpa et al. 2017). Whether ALT plays a role in the endocrine tumors of patients with the MEN1 syndrome remains to be determined.

## DNA methylation and demethylation

DNA methylation is an epigenetic modification that occurs predominantly at CpG sites where a cytosine nucleotide is followed by a guanine nucleotide in which the cytosine is methylated to 5-methylcytosine (5-mC) (Li and Zhang 2014). Methylation at CpG islands (GC-rich DNA regions), is often located in promoter regions of genes. Aberrant hypo- or hyper- methylation of these promoter region CpG islands can result in gene overexpression or gene silencing, respectively. A family of DNA methyl transferases (DNMTs), designated DNMT1, DNMT3A, and DNMT3B mediate CpG methylation. While DNMT3A and DNMT3B establish de novo DNA methylation, DNMT1 serves to propagate and stably maintain the DNA methylation mark (Li and Zhang 2014). DNA methylation can be reversed by the ten-eleven translocation (TET) family of methylcytosine hydroxylases that convert 5mC to 5-hydroxymethylcytosine (5-hmC) (Li and Zhang 2014). Also, drugs such as 5-azacitidine (5AC) and 5-aza-2'-deoxycytidine (DAC) can block DNA methylation by directly inhibiting DNMTs (Sato et al. 2017).



The histone modification H3K4me3 has been shown to protect CpG islands from DNA methylation resulting in an antagonism between H3K4me3 and DNA methylation to regulate gene transcription (Cedar and Bergman 2009). Also, DNA methylation in transcription factor recognition sequences can alter transcription factor binding (Barlow and Bartolomei 2014). A study conducted in MIN6 mouse insulinomas cells (Miyazaki et al. 1990) showed that overexpression of menin increased the expression of the lncRNA Meg3 (Modali et al. 2015). This effect coincided with H3K4me3 at the Meg3 promoter, and hypomethylation of the central CpG of a cAMP response element (CRE) that binds the transcription factor CREB; the DNA hypomethylation prevented CREB binding (Modali et al. 2015, Zhao et al. 2006). Whether similar instances of reciprocal H3K4me3 and DNA methylation together with altered transcription factor binding are present in MEN1-associated endocrine cells remains to be determined.

Several studies have shown indirect evidence for the participation of DNA methylation in MEN1-associated endocrine cell types. DNA hypermethylation has been shown to occur in a subset of inherited MEN1 tumors, sporadic PNETs, sporadic pituitary tumors or sporadic parathyroid tumors at the promoter region of different genes (*APC*, *CTNNB1*, *CDKN2A/p16*, *CDKN2B/p15*, *HIC1*, *MEG3*, *MLH1*, *RASSF1A*, and *RIZ1*), with some also correlating with respective reduction of gene expression (Carling et al. 2003, Juhlin et al. 2010, Lindberg et al. 2008, Modali et al. 2015, Svedlund et al. 2012, Zhao et al. 2005). A genome-wide analysis of quantitative DNA methylation in parathyroid tissue samples showed global DNA hypermethylation in menin-null parathyroid tumors from MEN1 patients, and increased transcript levels of DNMT1, predicting increased DNMT1 activity in menin-null parathyroid cells accounting for the hypermethylated DNA (Yuan et al. 2016). Another study showed altered DNA methylation profiles in normal and pathologic parathyroid tissue with gene expression changes, and restored expression of hypermethylated genes upon treatment of primary cell cultures of parathyroid tumors with the DNA methylation inhibitor DAC (Starker et al. 2011). A few studies in endocrine/endocrine-related cell lines (BON1, H727, MIN6) have reported growth inhibition upon treatment with DNA methylation inhibitors 5AC or DAC (Alexander et al. 2010, Modali et al. 2015). These studies suggest DNA hypermethylation as an oncogenic event in MEN1-associated endocrine cell types, and therefore the potential benefit of DNA methylation inhibitors in treating PNETs, pituitary tumors and parathyroid tumors.

## Non-coding RNA interactions

Non-coding RNAs (ncRNAs), as the name indicates, lack open reading frames (ORFs) and do not encode proteins. Short ncRNAs such as microRNA (miRNA) are processed from longer primary transcripts into small single-stranded molecules (20–24 nucleotides). They perform RNAi-mediated (RNA interference-mediated) gene silencing by complementary base-pair interactions targeting DNA in chromatin for RNA-induced transcriptional silencing (RITS), or messenger RNAs (mRNAs) post-transcriptionally for degradation, or by blocking translation (Cech and Steitz 2014). Long non-coding RNAs (lncRNAs) are transcripts of 200 bp or more in length that can interact with DNA and proteins whereby they also participate in epigenetic regulation (Kopp and Mendell 2018). Some lncRNAs have been shown to reside in chromatin-modifying and chromatin-remodeling protein complexes

that silence gene expression. The eukaryotic ncRNAs play a dual role in epigenetic regulation - some miRNAs can silence genes that encode epigenetic factors such as DNMTs, and some lncRNAs can directly participate in chromatin modifying epigenetic control mechanisms such as in the chromatin inhibitory complex polycomb group (PcG) repressive complex 2 (PRC2) (Chen et al. 2015, Fabbri et al. 2007, Kaneko et al. 2014, Mondal et al. 2015).

In MEN1-associated endocrine cells (MIN6 and  $\beta$ lox5 cells), microRNA-24 (miR24) has been shown to target menin, and increased miR24 level was also shown to correlate with decreased menin level in the islets of *Men1<sup>f/f</sup>*;RIP-Cre mice and human parathyroid tumors (Grolmusz et al. 2017, Luzi et al. 2017, Luzi et al. 2012, Vijayaraghavan et al. 2014). This silencing of menin by a microRNA predicts downstream effects on chromatin modifications because menin acts as an epigenetic co-factor in chromatin modifying protein complexes. Low levels of the lncRNA MEG3 have been observed in mouse and human MEN1-associated PNETs, human sporadic PNETs, and in human sporadic pituitary tumors (Modali et al. 2015, Zhao et al. 2005). In MIN6 mouse insulinoma cells, Meg3 was demonstrated to co-immunoprecipitate with Ezh2 which is part of PRC2 for H3K27me3 establishment (Iyer et al. 2017). How these ncRNA interactions affect endocrine tumor epigenetics should be further investigated.

## Targeting the pathologic epigenome of MEN1-associated endocrine cell types

Epigenome profiling for global epigenetic changes, such as in DNA methylation or histone modifications between normal and pathological samples, has demonstrated the presence of epigenetic changes in tumors and other disease states that could be exploited as targets for therapy (Jones et al. 2016). Epigenetic therapies that are under investigation to revert specific disease epigenomes to normal are designed to block the enzymatic activity of epigenetic regulators, disrupt interactions in chromatin modifying protein-complexes, or target specific epigenetic factors for degradation (Ahuja et al. 2016). However, designing of such therapeutics has to overcome several issues and challenges such as cell-specific targeting due to heterogeneity of the affected tissues, side effects, drug resistance, and achieving constant, consistent and long-lasting inhibition of the target (Dawson 2017). Potential therapies in the form of various small molecule chemical inhibitors that target epigenetic enzymes (e.g., DNMTi, HDACi, KTMi, KDMi, and BETi) have been developed. DNA hypomethylating agents (decitabine and azacitidine) that target DNMT1 and DNMT3A, and a few HDACi (panobinostat, vorinostat, and romidepsin) were approved by the US Food and Drug Administration (FDA) for the treatment of specific hematologic malignancies (Ahuja et al. 2016).

As discussed above, various HDACi, BETi and DNMTi have been shown to inhibit the growth of endocrine/endocrine-related cells lines, and intraperitoneal injection of the BETi JQ1 has been shown to reduce cell proliferation and increase apoptosis in insulinomas of the *Men1<sup>f/f</sup>*;RIP-Cre mouse model (Adler et al. 2009, Alexander et al. 2010, Arvidsson et al. 2016, Baradari et al. 2006, Lines et al. 2017, Modali et al. 2015, Wong et al. 2014). Another

study showed that lack of a histone demethylase Rbp2 in the *Men1<sup>fl/fl</sup>;RIP-Cre* mouse background could decrease insulinoma development (Lin et al. 2011). Rbp2 loss could potentially restore H3K4me3 that occurred in the insulinoma cells due to menin-deficiency. Furthermore, overexpression of RBP2 was detected in human PNETs or their metastases, implicating a target for histone demethylase inhibitor therapy (Maggi et al. 2016). These studies suggest the potential of epigenetic drug therapy of tumors arising in MEN1-associated endocrine cell types irrespective of whether the epigenetic status of the tumor cells is a direct consequence of menin loss or a consequence of the tumorigenesis process.

## Perspectives

In this review, we aimed to survey the literature for evidence of epigenetic regulation in “MEN1-associated endocrine cell types” which mainly included information from the parathyroids, pituitary, pancreatic islets, and lung neuroendocrine cells with the intention of highlighting the direct effects of menin as an epigenetic co-factor or functionally related epigenetic effects. Whole genome/exome sequencing efforts have identified mutations in the *MEN1* gene and/or genes associated with epigenetic control mechanisms in sporadic endocrine tumors - non-functioning and functioning PNETs, parathyroid adenoma, and lung neuroendocrine tumors. Future studies directed towards identifying the downstream effects from these mutations will help to understand the contribution of epigenetic changes in the pathogenesis of the specific endocrine tumors. Work conducted in cell lines derived from pancreatic or lung neuroendocrine tumors or their metastasis, and in vivo work in insulinomas of *Men1<sup>fl/fl</sup>;RIP-Cre* mice have established the role of menin as an epigenetic co-factor in the context of a few epigenetic modifications: H3K4me3, H3K9me3 and H4R3me2s, and DNA methylation. Given that DNA methylation and multiple histone modifications are modulated by menin raises the question whether epigenetic changes in MEN1-associated tumors would encompass multiple epigenetic marks. Future studies directed towards determining the changes in multiple histone modifications and/or DNA methylation in tumors (epigenome signatures) compared to corresponding normal cells could be valuable for understanding the tumorigenesis process and potential treatment opportunities. The challenges for conducting such studies for MEN1-associated tumors are the non-availability of ‘corresponding’ specific normal cell types, and the lack of relevant cell lines from human tumors. Perhaps generating mouse xenografts of human tumors could prove to be useful in establishing cell lines. A recent study has shown that in the context of human PNETs using a human hepatocyte growth factor (HGF) ligand facilitated the successful generation of xenografts in mice because PNETs express high levels of the HGF receptor c-MET (Krampitz et al. 2016). Similar methods could be used to establish cell lines from MEN1 and sporadic tumors. Such tumor models and cell lines will facilitate a deeper understanding of epigenetic changes during the tumorigenesis process to exploit epigenetic therapy as a treatment option for the tumors of MEN1-associated endocrine cell types.

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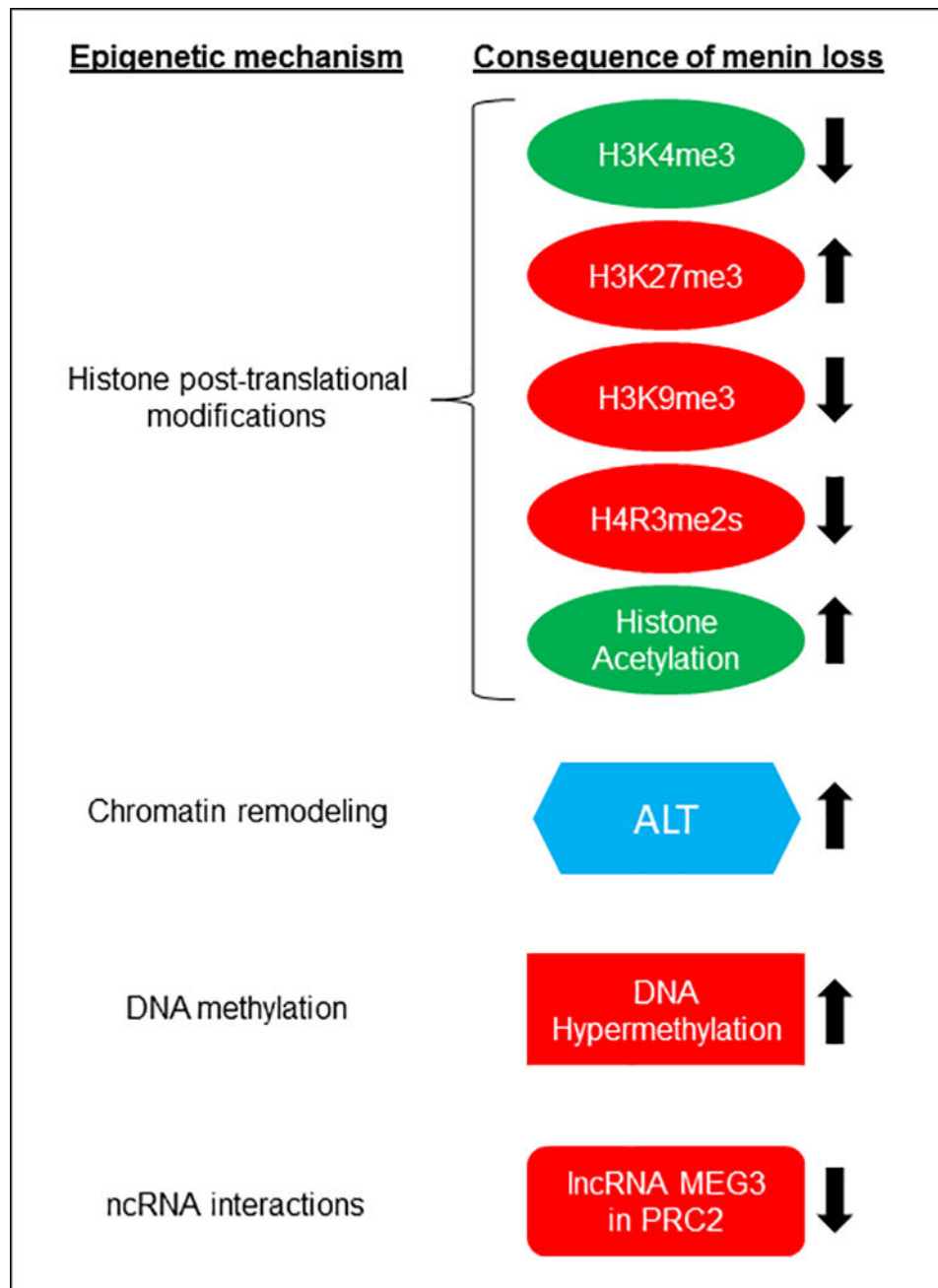
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**Figure 1. Overview of menin-dependent epigenetic regulation in MEN1-associated endocrine cell types**

Epigenetic mechanisms, and the observed or predicted consequence of menin loss are shown that were investigated in various studies of human or mouse endocrine tumors, or studies conducted in endocrine/endocrine-related cell lines. Green or Red, indicates the normal effect of the epigenetic event resulting in the activation or repression of gene expression, respectively. Up or Down arrow, indicates increase or decrease, respectively, of the epigenetic event in a cell line or tumor with menin-deficiency. ALT (alternative lengthening

of telomeres) is shown in blue (and not in red or green) because it is an event associated with chromatin structure at telomeres.

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