Menin Directly Represses Gli1 Expression Independent of Canonical Hedgehog Signaling

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Abstract

Multiple endocrine neoplasia type 1 (MEN-1), is an inherited tumor syndrome resulting from mutations in the tumor suppressor gene menin (MENI). Menin plays an essential role in both repressing and activating gene expression. However, it is not well understood how menin represses expression of multiple genes. Upon MEN1 excision, the transcription factor Gli1 and its target genes, including Pchb1 and c-Myc, were shown to be elevated in the absence of an apparent Hedgehog pathway-activating ligand or when Smoothened (SMO), a key component of the pathway, is inhibited. Menin binds to the GLI1 promoter and recruits PRMT5, a histone arginine methyltransferase associated with transcriptional repression. Both PRMT5 binding and histone H4 arginine 3 methylation (H4R3m2s) are decreased at the GLI1 promoter in MEN1-excised cells. Moreover, MEN1 ablation resulted in increased binding of transcriptionally active Gli1 at the GLI1 promoter in a manner not influenced by the canonical Hedgehog signaling pathway. Inhibition of Gli1 by the small-molecule inhibitor GANT-61 led to decreased expression of Gli1 and its target genes in MENI-depleted cells. Furthermore, GANT-61 potently suppressed proliferation of MENI-excised cells as compared with control cells. These findings uncover a novel epigenetic link whereby menin directly represses Gli1 expression, independent of the canonical Hedgehog signaling pathway, via PRMT5 and its repressive H4R3m2s mark.

Implications: Inhibition of GLI1 suppresses neuroendocrine tumors harboring mutations in the MENI gene. Mol Cancer Res; 11(10); 1215–22. ©2013 AACR.

Introduction

Multiple endocrine neoplasia type 1 (MEN-1) is an inherited tumor syndrome, with development of tumors in several endocrine organs including pancreatic islets (1–4). The gene mutated in this syndrome, MEN1, encodes a protein of 610 amino acid residues, menin (5, 6). Menin interacts with a variety of proteins including the transcription factors JUND (7–9), NF-kb (10) and SMAD3 (11), and represses the transcriptional activity of JUND and NF-kB. The Hedgehog (Hh) pathway has previously been implicated in insulin production and secretion in INS1 cells (12), and it regulates insulin production in adult mice (13). The Hh signaling pathway regulates diverse biological processes ranging from embryonic development to cell cycle and tumorigenesis (14). Canonical Hh signaling is triggered by the binding of Hh ligands (Sonic, Indian, or Desert Hh) to its receptor Patched 1 (PTCH1), resulting in the release of PTCH1-mediated repression of the 7 trans-membrane protein smoothened (SMO) (15). Activated SMO triggers the dissociation of GLI proteins from the SUFU-Fü-COS2 complex (16), resulting in the nuclear translocation of GLI1 and GLI2, degradation of the repressor GLI3, and subsequent increase in the transcription of GLI1 target genes including Pchb1 and Gli1 itself (14, 15). Constitutive activation of this signaling pathway, either through Pchb1 inactivating mutations or SMO activating mutations, has been reported in several malignancies including basal cell carcinoma and medulloblastoma (17, 18).

GLI1, the main effector of the Hh signaling pathway, can also be regulated independent of the canonical Hh signaling pathway (19, 20). The TGF-β signaling pathway activates Gli1 even in the presence of cyclopamine, an SMO antagonist (20), the Ewing’s sarcoma-Friend’s leukemia insertion activates Gli1 directly (21) and GLI1 mediates the survival and transformation of pancreatic ductal adenocarcinoma (PDAC) cells induced by activated K-RAS (19).

Acetylation of proteins is a dynamic process involving HATs and HDACs, and regulates many diverse functions including DNA recognition, protein–protein interaction, and protein stability (22). HDACs play a role in transcriptional
regulation including suppression of several tumor suppressors including JunB, Prs1, and Plagl1 (23). On the contrary, both HDAC activity and recruitment are required for transcriptional activation of genes including Gja1, Irf1, and Ghp2 (23). Similarly, GLI1 physically interacts with HDAC, and HDAC-mediated deacetylation of GLI1 results in the transcriptionally active, deacetylated-GLI1 species (24).

Recently, we have shown that menin interacts with protein arginine methyltransferase 5 (PRMT5), a repressive histone methyltransferase, and epigenetically represses canonical Hh signaling via regulation of GAS1 (25), a crucial cofactor required for binding of Hh ligand to the PTCH1 receptor (26–28). However, it is unclear whether the menin/PRMT5 axis also regulates other steps of the Hh signaling cascade. Here, we show that menin binds to the Gli1 promoter, and recruits PRMT5 and promotes repressive histone arginine methylation, H4R3m2s, at the promoter region, resulting in decreased Gli1 expression. Furthermore, binding of menin to the Gli1 promoter leads to reduced binding of GLI1 and HDAC1 to the Gli1 promoter. Pharmacologic inhibition of GLI1 resulted in decreased expression of Gli1, PTCH1, and C-MYC.

Materials and Methods

Plasmids and cell culture

Retroviral plasmids expressing Flag-tagged or mutant menin have been described elsewhere (29). Men1-null MEF cells complemented with wild-type (WT) or mutant menin, and 293 cells were cultured in Dulbecco’s modified Eagle’s Medium medium supplemented with 10% FBS and 1% Pen/Strep (29).

RNA extraction and quantitative real-time PCR

Total RNA was extracted from cultured cells with Trizol and an RNEasy extraction kit from Qiagen. Details and primer sequences can be found in Supplementary Table S1. The Ct values for the target genes were normalized to Gapdh Ct values, and analysis was done using the relative quantification method according to instructions from ABI.

Antibodies and Western blotting

Whole cell lysates were prepared using RIPA buffer (Sigma) supplemented with mammalian protease inhibitor cocktail (Sigma), and subjected to Western blotting as previously described (29). The primary antibodies used were anti-menin (Bethyl, A300-105A), anti-PRMT5 (Abcam, ab31751), anti-GLI1 (Rockland, 100-401-223), anti-Histone H4 Symmetric Di-Methyl R3 (Abcam, ab5823), and anti-Histone H3 (Abcam, ab1791).

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assay was conducted as previously described using a Quick ChIP kit from Imgenex (30). Briefly, cells were fixed with 1% formaldehyde, lysed in ChIP lysis buffer supplemented with a protease inhibitor cocktail, and the genomic DNA was sheared into 200 to 1000 bp fragments by sonication with a Bioruptor sonicator (Diagenode), followed by incubation with either control anti-immunoglobulin G (IgG) antibody or a specific primary antibody at 4°C overnight. The immunoprecipitated antibody–protein–DNA complex was collected using salmon sperm DNA-coated Protein G agarose beads. The eluted protein–DNA complex was reverse cross-linked at 65°C overnight. q-PCR was conducted on the precipitated DNA, and normalized to percent input of genomic DNA. Binding was quantitated as 2ΔΔCt where ΔΔCt = Ct (input) – Ct (ChIP). Primer sequences are listed in Supplementary Material. Antibodies used for ChIP were anti-menin (Bethyl, A300-105A), anti-PRMT5 (Abcam, ab31751), anti-GLI1 (Rockland, 100-401-223), anti-Histone H4 Symmetric Di-Methyl R3 (Abcam, ab5823), and anti-Histone H3 (Abcam, ab1791).

Luciferase assay

MEF cells were transfected with the indicated plasmids using Fugene (Roche). Dual-luciferase reporter assay (Promega) kits were used according to the manufacturer’s instructions. The GLI-binding site-driven luciferase reporter plasmid was kindly provided by Sasaki (31). Both firefly and renilla luciferase activities were measured by Luminoskan Ascent (Thermo Fisher). Results were representative of 3 independent experiments.

Statistical analyses

Statistical analysis was conducted using Graphpad Prism (v 5.0, Graphpad Software). The data are presented as the mean ± SD of n determinations unless noted otherwise. A two-tailed Student t test was used for measuring statistical differences.

Results

Menin represses GLI1 and its target genes independent of Hh ligand-mediated signaling

Our previous studies show that menin dampens canonical Hh signaling by epigenetically repressing GAS1 (25), a crucial cofactor, along with CDO and BOC (26, 28, 32), promoting Hh ligand binding to its receptor PTCH1 (14). Interestingly, in the absence of Hh ligand, and hence canonical signaling, Gli1 mRNA levels were reduced in Men1-null MEFs complemented with WT menin as compared to control cells (Fig. 1A). To determine whether certain MEN1 disease-related point mutations affect Gli1 expression, we complemented menin-null MEFs with disease-related mutant menin, A242V, and showed that the mutant lost its ability to repress Gli1 (Supplementary Fig. S1), possibly because of the diminished interaction with PRMT5 as we showed earlier (25). Furthermore, PTCH1, a direct GLI1 target, was reduced upon complementing Men1-null cells with menin (Fig. 1B). To further determine whether excision of endogenous Men1 yields similar results, we treated Men1fl/fl;CreER MEFs with either dimethyl sulfoxide (DMSO) or 4-hydroxy tamoxifen (4-OHT) and showed that Men1 excision results in 6-fold increase of Gli1 (Supplementary Fig. S2). Notably, ectopic menin expression substantially repressed smoothened agonist (SAG)-induced Gli1 expression (Fig. 1C), suggesting that menin further
represses Gli1 expression downstream of GAS1 and SMO, which is the canonical Hh ligand-induced signaling pathway. As such, it is plausible that menin-mediated suppression of GLI1 can be attributed to suppression of Gli1 expression independent of the canonical GAS1/PTCH1/SMO-mediated Hh signaling.

We found that PIME1 cells, a mouse pancreatic islet derived cell line in which floxed Men1 (Men1f/f) can be conditionally excised by adding 4-OHT (ref. 33; Fig. 2A), were not responsive to either cyclopamine, a chemical SMO inhibitor (Fig. 2B) or SAG (Fig. 2C), a SMO activator, based on unaltered Gli1 mRNA levels. These results indicate that PIME1 cells are not responsive to canonical Hh signaling. Many immortalized or tumor-derived cell lines have been shown to lose their response to Hh ligand stimulation due to perturbations in the Hh signaling pathway (34). In this regard, PIME1 cells serve as an ideal tool for studying the potential role of menin in regulating Gli1 expression independent of the canonical Hh signaling pathway. Men1 excision in PIME1 cells resulted in significant upregulation of Gli1 mRNA (Fig. 2B and C). To examine whether ectopic menin expression in Men1-excised PIME1 cells represses Gli1 expression, we complemented the Men1-excised cells with either control vector or retroviruses expressing menin. Quantitative real-time PCR (qRT-PCR) showed that menin expression in the cells partially restored the repression of Gli1 mRNA levels (Fig. 2D). Consistent with these results, we found that ectopic expression of menin in BON cells, a human neuroendocrine carcinoid cell line that expresses low levels of endogenous menin (35) and are unresponsive to Hh signaling (data not shown), also reduced Gli1 mRNA levels (Supplementary Fig. S3). These findings show menin's role in repressing Gli1 expression independent of the canonical Hh signaling pathway.

Menin represses Gli1 via PRMT5-mediated histone methylation

To determine how menin represses Gli1 expression in an Hh signaling-independent manner, downstream of SMO, we conducted a ChIP assay to evaluate whether menin directly binds the Gli1 promoter to regulate its expression. ChIP assay in PIME1 cells showed that menin bound to the Gli1 promoter, and Men1-excised resulted in increased binding of GLI1 to the Gli1 promoter (Fig. 3A), suggesting partial overlap between menin and GLI1 binding regions at the Gli1 promoter. It has been reported that GLI1 can bind to its promoter and activate its own expression (36, 37). We have previously shown that menin directly interacts with and recruits PRMT5, a repressive
histone arginine methyltransferase, and its histone H4 arginine 3 methylation (H4R3m2s) mark to the Gas1 promoter and represses its expression (25). To determine whether menin recruits PRMT5 to the Gli1 promoter and represses its expression, we conducted a ChIP assay, and found that Men1 excision in PIME1 cells reduced PRMT5 binding and H4R3m2s at the Gli1 promoter (Fig. 3A). As a control, total H3 enrichment at the Gli1 promoter was not altered upon Men1 excision (Supplementary Fig. S4). These results indicate that Gli1 expression can be repressed epigenetically by PRMT5, likely via menin-mediated recruitment of PRMT5. Furthermore, in Hh-ligand-responsive MEFs, we observed that in the absence of Hh ligand, menin bound to the Gli1 promoter using ChIP assay, and consistently, ectopic menin expression led to an increase in PRMT5 binding and H4R3m2s at the Gli1 promoter (Fig. 3B). Together, these findings indicate that menin directly suppresses GLI1 partially by recruiting PRMT5 and its repressive histone methylation mark to the Gli1 promoter in both Hh signaling-responsive MEFs and nonresponsive PIME1 cells.

As the Gli1 gene can be activated by binding of GLI1 to its own promoter (36, 37), we conducted luciferase reporter assays with a GLI1-binding site-driven luciferase reporter gene (31), and found that ectopic expression of either menin or PRMT5 in menin-null cells repressed expression of the reporter gene (Fig. 4A). Notably, a catalytically inactive PRMT5 mutant, R368A (38), not only failed to suppress reporter gene activity in the presence or absence of menin, but even modestly increased the expression of the reporter (Fig. 4A, lane 7 vs. lane 3). These results suggest that the PRMT5 catalytic mutant may exert a dominant negative effect on the endogenous WT PRMT5 by blocking its binding to menin. As a control, both WT and mutant PRMT5 were expressed at comparable levels (Fig. 4B). These findings are consistent with the notion that menin directly represses Gli1 in an SMO or Hh ligand-independent manner via interacting with PRMT5.

Menin affects binding of the active GLI1–HDAC complex to its target genes

GLI1 is an acetylated protein, interacts physically with HDAC1 and HDAC2, and its deacetylation results in transcriptional activation of target genes including Gli1 and Pech1 (24). Similarly, menin interacts with HDAC1 and HDAC2 via mSin3A, a general transcription corepressor (39). To determine whether menin affects binding of the active, deacetylated GLI1–HDAC1 complex to the Gli1 promoter, we conducted a ChIP assay, and found that Men1 excision in PIME1 cells resulted in increased Gli1 binding (Fig. 5A). Similarly, HDAC1 binding at the Gli1 promoter was also increased upon Men1 excision, indicating that menin negatively affects binding of the active GLI1–HDAC1 complex to the Gli1 promoter.
by recruiting PRMT5 and its histone acetyltransferase activity to GLI1 (19). Furthermore, the protein levels of PTCH1, a direct target of GLI1, were unaltered in control cells, treatment of Men1-excision PIME1 cells with GANT-61 resulted in a larger decrease of cell proliferation in Men1-excision PIME1 cells as compared to control WT cells at similar concentrations (Fig. 6C). Although the decrease in cell proliferation upon GANT-61 treatment is moderate, it is likely that other factors besides increase in GLI1 account for menin-excision-induced increase in cell proliferation. Nonetheless, our findings suggest that elevated expression of GLI1 along with its target genes upon Men1 excision in PIME cells at least partially accounts for increased cell proliferation.

**Discussion**

Here, we identify the role of menin in repressing GLI1 independent of the canonical Hh signaling pathway. First, we show that menin recruits PRMT5 and its repressive histone H4R3 symmetric dimethylation (H4R3m2) mark to the GLI1 promoter (Fig. 3A). Second, menin negatively affects binding of the active GLI1–HDAC1 complex to the promoter of its target genes including GLI1 (Fig. 4A). Epigenetic regulation of Hh signaling has previously been reported including hyper-methylation of components of the Hh signaling pathway in tumors (42), and epigenetic repression of GLI1 by SNF5, a core component of the SWI/SNF chromatin remodeling complex (43). Our results indicate that menin-PRMT5-mediated epigenetic suppression of GLI1 is crucial for regulating the pro-oncogenic GLI1 expression.

Noncanonical GLI1 activation, independent of mutations in Hh pathway components, has previously been reported (19–21). In PDACs, SMO is dispensable in the ductal epithelium, and GLI1 is regulated in a SMO-independent mechanism mediated partly by TGF-β and KRAS (19). However, we did not observe any changes in total K-RAS protein levels upon Men1 excision in both Hh ligand responsive MEFs and Hh ligand nonresponsive PIME1 cells (data not shown). Furthermore, menin is necessary for TGF-β-mediated repression of parathyroid cell proliferation (44), and it is thus unlikely that increased cell proliferation and GLI1 upregulation upon Men1 excision results from enhanced TGF-β signaling. We have shown that menin represses GLI1 directly by recruiting PRMT5 and its histone methylation mark to the GLI1 promoter (Fig. 3A). Importantly, ectopic expression of catalytically inactive PRMT5 mutant results in increased GLI1 transcriptional activity (Fig. 4A), showing that menin and PRMT5 act in concert to repress GLI1 at least partly via PRMT5-catalyzed histone arginine methylation. Previous reports have shown that the canonical Hh signaling pathway plays an important role in the development and maintenance of small-cell lung cancer (45) and in the pathophysiology of gastrointestinal neuroendocrine carcinomas (46). Thus, in addition to
activated canonical Hh signaling observed in certain neuroendocrine tumors as detailed earlier, we uncover here that direct activation of GLI1 in an Hh ligand-independent manner upon Men1 mutation may play a role in tumorigenesis of endocrine organs.

It was recently reported that SNF5 localizes to GLI1-regulated promoters, and loss of SNF5 results in aberrant activation of Gli1, a process that is insensitive to the SMO-antagonist NVP-LDE225 but sensitive to the GLI1-antagonist HPI-1 (43). Furthermore, subunits of the mSin3A/HDAC corepressor complex copurify with hSWI/SNF complexes (47), and PRMT5 associates with Brg1 and hBrm-based hSWI/SNF complexes (48). Thus, the menin–PRMT5 complex might synergize with the mSin3A-HDAC and the SWI/SNF complex to repress the expression of GLI1 target genes. Moreover, it has been reported that hSWI/SNF-associated PRMT5 methylates hypo-acetylated histones H3 and H4 more efficiently than hyper-acetylated histones H3 and H4, thus showing synergism between repressive histone deacetylation and PRMT5-mediated histone methylation. Similarly, it is possible that inactive, acetylated-GLI1 is modified by the menin–PRMT5 complex further reinforcing the repressed state, and decreasing the expression of GLI1 target genes. Men1 excision did not affect the global protein levels of HDAC1 (Supplementary Fig. S5). Thus, it is unlikely that the mode of GLI1 repression by menin occurs at the level of REN-Cul3 E3 ubiquitin ligase complex that targets HDAC for proteasomal degradation (24). Menin associates with both HDAC1 and HDAC2 through mSin3A (39), and it is possible that menin compromises the interaction between HDAC and GLI1. This would result in an increase of the transcriptionally inactive, acetylated-GLI1 species, resulting in a decrease of Gli1 target gene expression. Furthermore, menin might interfere with binding of the active GLI1–HDAC complex to the promoters of GLI1 target genes as shown by ChIP assays above (Fig. 5A), and partially supported by the observation that Gli1 mRNA levels in Men1-null cells are more sensitive to TSA-induced reduction than in control WT cells (Fig. 5B).

In conclusion, we show that menin epigenetically represses GLI1 by recruiting PRMT5 and its histone methylation mark to the Gli1 promoter, and interferes with binding of the active GLI1–HDAC complex to the Gli1 promoter (Fig. 6D). Our studies define a novel mechanism controlling the levels of GLI1 via a menin-mediated epigenetic pathway, and provide a rationale for directly inhibiting GLI1 for treating neuroendocrine tumors.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: B. Gurung, X. Hua

Development of methodology: B. Gurung, X. Hua

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): B. Gurung

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): B. Gurung, X. Hua

Writing, review, and/or revision of the manuscript: B. Gurung, X. Hua

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

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References


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