*Advances in Brief*

# Menin, a Tumor Suppressor, Represses JunD-Mediated Transcriptional Activity by Association with an mSin3A-Histone Deacetylase Complex[1]

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## Abstract

**Menin, a gene product of multiple endocrine neoplasia type I (*MEN1*), is known to act as a tumor suppressor to repress JunD transcription factor. However, the mechanism by which Menin represses JunD transcriptional activity was still unclear. In this study, we found that Menin is a corepressor against JunD transcriptional activity via recruitment of histone deacetylases in an mSin3A-dependent manner. The amino acid search revealed that central domain of Menin includes a $\alpha$-helical mSin3-interacting domain [SID (371–387)]. The SID mutation of Menin (L381P/A385P) abolished the interaction between mSin3A and paired amphipathic helix 2 domain of Menin and reduced its ability to repress JunD transcriptional activity, implicating that SID of Menin is important for recruiting an mSin3A-histone deacetylase complex to repress JunD transcriptional activity.**

## Introduction

The AP-1[4] family of transcription factor is composed of dimers of Jun (c-Jun, JunB, and JunD) and Fos (c-Fos, FosB, Fra-1, and Fra-2) and mediates gene expression under a variety of stimulatory conditions (1). These stimuli can induce c-Jun expression, phosphorylate c-Jun through the activation of mitogen-activated protein kinase cascades, and increase c-Jun transcriptional activity (2). Unlike c-Jun and JunB, both of which behave as immediate early genes, JunD is constitutively expressed in all cell types (3–5) and acts as a negative regulator of ras-dependent cell growth and protects cells from p53-dependent senescence and apoptosis (6, 7).

Menin is a tumor suppressor protein encoded by MEN1, a causative gene associated with tumors of parathyroid, enteropancreatic neuroendocrine tissue, and anterior pituitary (8–10). Indeed, overexpression of Menin in ras-transformed cells results in decreased proliferation and tumor growth, supporting that Menin serves as a tumor suppressor (11).

It has been reported that Menin represses JunD transcriptional activity (12). Because JunD is constitutively expressed in cell, a repression mechanism is required to block the constitutive activation of JunD in quiescent cell. Menin has been shown specifically to interact with JunD but not with other members of Jun family (c-Jun and JunB) because their interactions are mediated through the far

NH$_2$-terminal region of JunD that is missing in c-Jun and JunB (12–14). In addition to JunD, Menin is also known to interact with other transcription factors, including NF-$\kappa$B, Smad3, p53, and Pem, implicating a general role of Menin in regulating transcription (15–18). Although Menin has been reported primarily as a nuclear protein, some part of Menin is present in cytosol and interacts with a tumor metastasis suppressor, nm23, and has low GTPase activity in the presence of nm23 (19). Menin is reported to interact with intermediate filaments, glial fibrillary acidic protein, and vimentin (20).

Menin is known to repress JunD-activated transcription. However, the detailed molecular mechanism by which Menin represses JunD is still unclear. Gobl *et al.* (13) showed that a HDAC inhibitor, TSA, reverses Menin-mediated JunD transcriptional repression, suggesting that HDACs may be involved in Menin-mediated transcriptional repression of JunD. In addition, certain tumor suppressors are known to have a capacity of recruiting HDACs (21–23). These facts attempted us to investigate if a tumor suppressor, Menin, is a component of HDAC complex.

## Materials and Methods

**Cell Culture and Transfections.** HEK293 and HeLa cells were grown with DMEM containing 10% fetal bovine serum and 50 units/ml streptomycin/penicillin. Jurkat cells were grown with RPMI 1640 containing 10% fetal bovine serum, 1 mM glutamate, and 50 units/ml streptomycin/penicillin. HEK293 cells were transfected with Lipofectamine reagent per manufacturer's protocol (Invitrogen).

**DNA Constructions.** Menin constructs (pcDNA3-Menin-myc and pCI-VSV-Menin) were kindly provided by Dr. Sunita K. Agarwal (NIH, Bethesda, MD) and Dr. Chang-Xian Zhang (Centre National de la Recherche Scientifique-Unite Mixte de Recherche, University of Lyon, Lyon, France), and pGEX-JunD (1-347) by Dr. Anders E. Gobl (Uppsala University Hospital, Uppsala, Sweden). Deletion mutants of Menin were made by subcloning of PCR fragments into pcDNA3-myc/His vector (Invitrogen). Mammalian expression vectors for mSin3A, HDAC1, and HDAC2 were described previously (24). For making mammalian expression vector for HA-tagged human JunD, PCR fragment of JunD (1-347) was inserted into pSG5-HA vector. Gal4-JunD(FL) and truncated mutants of Gal4-fused Menin are subcloned by insertion of PCR fragments into pM vector (Clontech).

**Coimmunoprecipitation.** Transiently transfected HEK293 cells were harvested after 24 h and lysed with a lysis buffer [20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5% NP40, and 1 mM phenylmethylsulfonyl fluoride]. Cell lysates were immunoprecipitated with suitable antibodies along with protein-A/G beads (Santa Cruz Biotechnology). Immunoprecipitates were separated by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with appropriate antibodies. Antibodies for Menin and mSin3A were purchased from Santa Cruz Biotechnology, and anti-c-myc and anti-HA monoclonal antibodies were purchased from Covance and antiflag (M2) monoclonal antibody from Sigma.

***In Vitro* Transcription/Translation.** [$^{35}$S]-labeled deletion mutants of Menin were prepared using STP3 (T7) *in vitro* transcription/translation kit (Novagen). HEK293 cell lysates containing c-myc-tagged mSin3A were added and incubated for 2 h in the presence of anti-mSin3A antibody (Santa Cruz

Biotechnology) and protein A/G beads. Immunoprecipitates were subjected to SDS-PAGE and then gel was dried and analyzed by autoradiography.

**HDAC Fluorescent Activity Assay.** Nuclear extracts prepared from Jurkat cells ($1 \times 10^8$) were incubated with anti-Menin polyclonal antibody for 2 h in the presence of protein-A/G beads. Immunoprecipitates were washed out three times with a washing buffer (PBS containing 0.1% NP40). To verify that Menin contains HDAC activity, immunoprecipitate was treated for 30 min with TSA (500 nM) before addition of fluogenic substrate. HDAC activity was measured using HDAC fluorescent assay kit by manufacturer's protocol (BioMol).

## Results

The Menin-mediated repression of JunD can be reversed by TSA (13), suggesting that Menin may be exerting its transcriptional repression effect on JunD by recruiting HDACs. However, it remains unclear how Menin is associated with HDACs. It is known that a number of transcriptional repressors indirectly recruit HDACs through mSin3, a general corepressor (25). We thus assessed this possibility by performing coimmunoprecipitation between Menin and [35S]-labeled mSin3A using *in vitro* transcription/translation. Menin specifically binds to mSin3A not to β-galactosidase as a control (Fig. 1A).

We then investigated the association of Menin with HDACs by transient transfection of cells with expression vectors for c-myc-tagged Menin and flag-tagged HDAC1, followed by coimmunoprecipitation. Immunoprecipitate with anti-c-myc contains flag-tagged HDAC1, indicating that Menin binds to HDAC1 (Fig. 1B). We then transfected cells with plasmids encoding c-myc-tagged Menin, c-myc-tagged mSin3A, and flag-tagged HDAC1 or HDAC2 and immunoprecipitated cell lysates with antiflag antibody. In the complementary experiment, both flag-tagged HDAC1 and HDAC2 show interaction with c-myc-tagged Menin even without overexpression of mSin3A (Fig. 1C, Lanes 3 and 4). When all of them were coexpressed, both of mSin3A and Menin were coimmunoprecipitated with flag-tagged HDAC1 antibody (Fig. 1C, Lane 2). To verify whether endogenous Menin contains HDAC activity, we immunoprecipitated nuclear extract of Jurkat cells with anti-Menin antibody and then measured its

Fig. 2. mSin3A-HDACs bind to central domain of Menin. A, mapping of the mSin3-binding domain and (B) HDAC1-binding domain in Menin. HEK293 cells were transfected with c-myc-tagged mSin3A or flag-HDAC1 with various c-myc-tagged Menin deletion mutants. For detecting their interactions, cell lysates were immunoprecipitated with anti-mSin3A (A) or antiflag(M2) antibody (B) and Western blotted with anti-c-myc antibody. [35S]-labeled deletion mutants of Menin (295–610 and 445–610) were mixed with mSin3A-overexpressing cell lysates and immunoprecipitated with anti-mSin3A antibody. Samples were loaded into SDS-PAGE, and their interactions were detected by autoradiography. C, diagram of deletion mutants of Menin. n.d., not determined.

HDAC activity. Menin immunoprecipitates show HDAC activity, whereas the treatment of TSA significantly reduces its HDAC enzymatic activity, indicating that endogenous Menin is a component of HDAC-containing complex (Fig. 1D).

We next mapped mSin3A-binding domain in Menin by coimmu-

Fig. 1. Menin is a component of mSin3A-HDAC complex. A, Menin associates with mSin3A. Menin-overexpressing cell lysates were incubated with [35S]-mSin3A for 2 h, and immunoprecipitation was performed with anti-Menin antibody. Immunoprecipitates were subjected to SDS-PAGE and analyzed by autoradiography. B, Menin associates with HDAC1. HEK293 cells were transfected with c-myc-tagged Menin (pcDNA-Menin-myc) and flag-tagged HDAC1 (pBJ5-flag-HDAC1). Cell lysates were immunoprecipitated with anti-c-myc antibody and probed with antiflag (M2) monoclonal antibody. C, Menin associates with HDAC1/2 through mSin3A. HEK293 cells were transfected with different combinations of c-myc-tagged Menin (pcDNA-Menin-myc), c-myc-tagged mSin3A (MT-mSin3A), flag-tagged HDAC1 and 2 (pBJ5-flag-HDAC1 and pBJ5-flag-HDAC2) as described in panel. Cell lysates were immunoprecipitated with antiflag antibody and immunoblotted with anti-c-myc monoclonal antibody. D, Menin has an HDAC activity. Immunoprecipitates of Menin from Jurkat cell lysates were incubated with fluogenic HDAC substrates and measured HDAC activities. (1, precipitation with control serum; 2, precipitation with anti-Menin antibody; and 3, precipitation with anti-Menin antibody followed by treatment of TSA.)
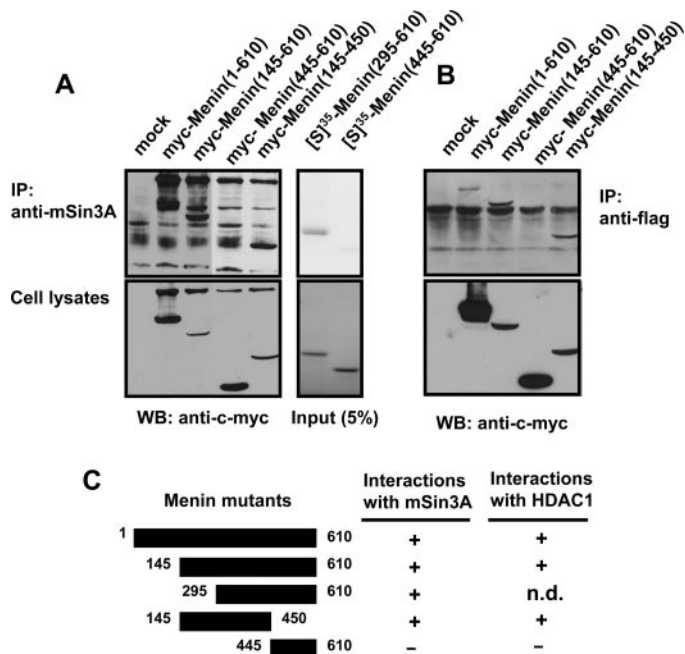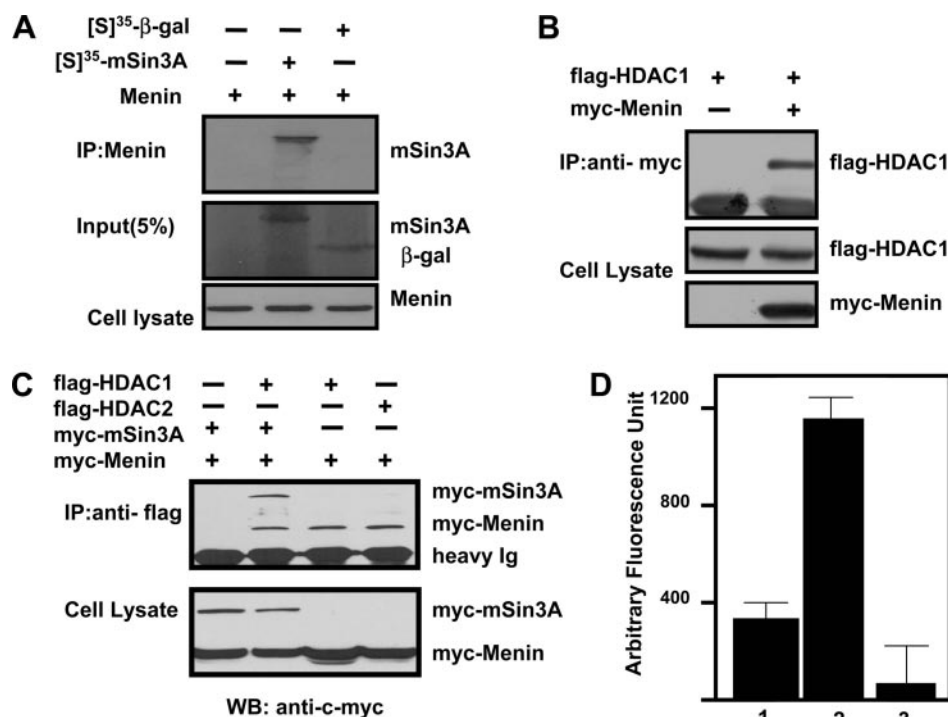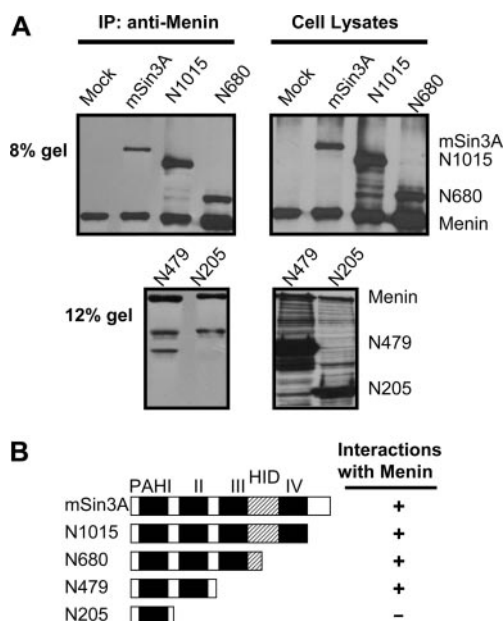
Fig. 3. Interacting domain of mSin3A with Menin. *A*, PAH2 domain binds to Menin. HEK293 cells were transfected with c-myc-Menin and various deletion mutants of mSin3A. Cell lysates were immunoprecipitated with anti-Menin antibody and Western blotted with anti-c-myc antibody. *B*, diagram of deletion mutants of mSin3A.

noprecipitation between mSin3A and various c-myc-tagged or [S][35]-labeled Menin deletion mutants (Fig. 2, *A* and *C*). Menin mutants lacking in $NH_2$-terminal region (145–610 and 295–610) and mutant containing central domain (145–450) have strong interactions with mSin3A, whereas Menin mutant containing COOH-terminal region (445–610) does not interact with mSin3A, indicating that central domain of Menin (295–450) is involved in recruitment of mSin3A (Fig. 2*B*). We then investigated HDAC1-binding domain in Menin (Fig. 2, *B* and *C*). Similarly, HDAC1 specifically binds to the central domain of Menin (145–450), implicating that Menin recruits HDAC1 through mSin3A. *Vice versa*, we mapped Menin-binding domain in mSin3A by coimmunoprecipitation between Menin and various mSin3A deletion mutants (Fig. 3, *A* and *B*). Deletion of PAH4, the HDAC-interacting domain, and PAH3 had no effect on the binding of mSin3A to Menin. However, deletion of the PAH2 (N205) abolished the binding of Menin to mSin3A, indicating that PAH2 domain is required for interaction between Menin and mSin3A (Fig. 3*B*).

We then searched amino acid sequence within central domain of Menin to figure out how Menin recruits mSin3A. Interestingly, Menin has a putative SID, similar to those of Mad1-SID and Pf1-SID for recruiting mSin3A-HDAC complex (Fig. 4*A*; Refs. 26, 27). Display of these residues (371–387) on a helical wheel shows that Menin-SID appears amphiphatic (data not shown). Most of hydrophobic residues are oriented to one face. In fact, the highly conserved hydrophobic
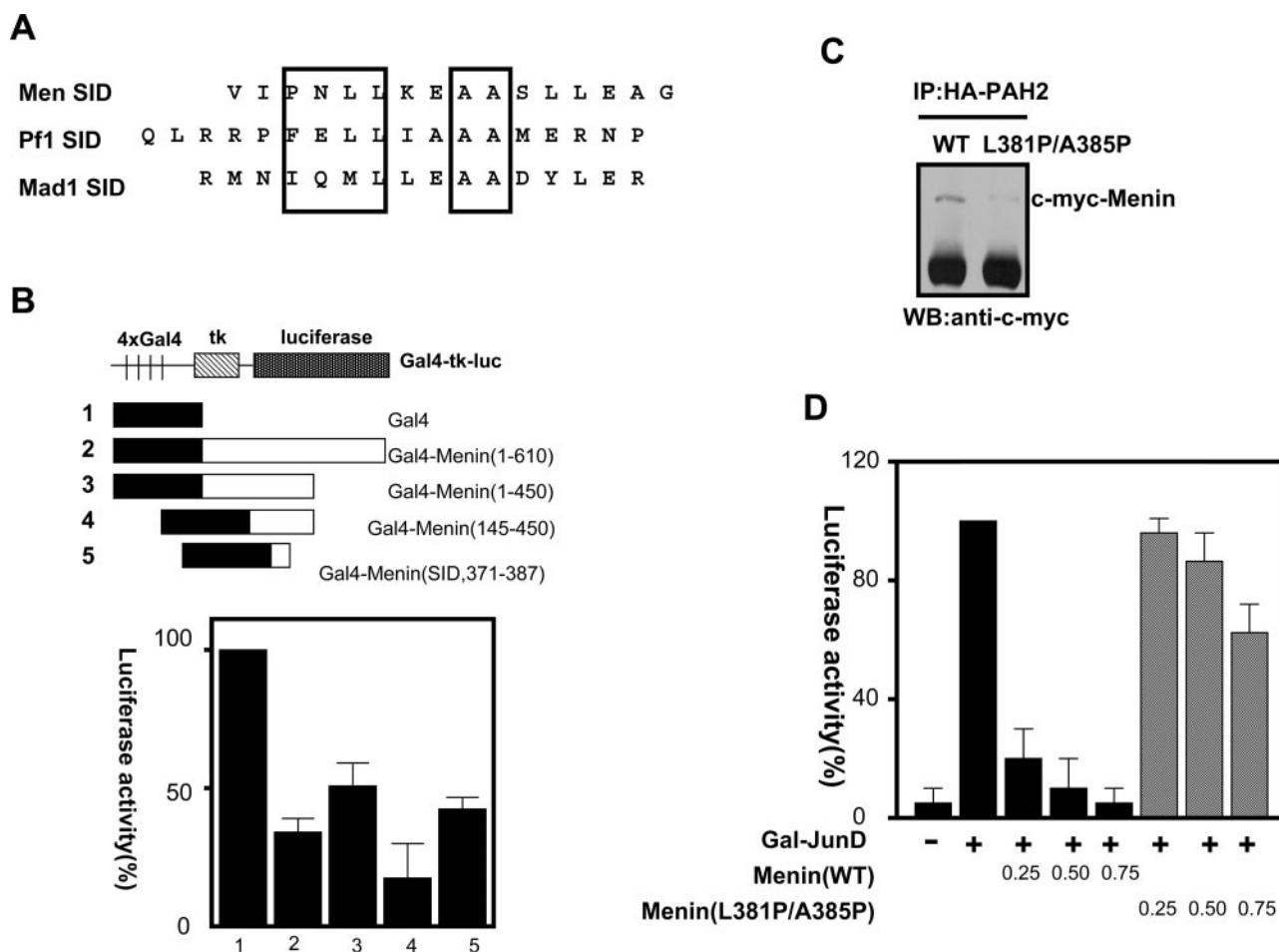


Fig. 4. An α-helical SID within central domain in Menin is sufficient to repress JunD transcriptional activity. *A*, Menin has a putative SID. Menin has a α-helical SID involved in mSin3A-binding. *B*, Gal4-Menin(SID) represses *tk*-driven luciferase reporter gene activity. HeLa cells were transfected with Gal4DB-fused Menin mutants along with 4xGal4-*tk*-luciferase reporter. Reporter gene activities were normalized with protein concentration. *C*, SID-mutant of Menin (L381P/A385P) cannot interact with mSin3A. HEK293 cells were transfected with either pcDNA-myc-Menin(WT) or pcDNA-myc-Menin(L381P/A385P) along with pSG5-HA-PAH2. Cell lysates were immunoprecipitated with anti-HA antibody, and probed with anti-c-myc antibody. *D*, SID of Menin is critical for repressing JunD transcriptional activity. HeLa cells were transfected with increasing amount of either pcDNA-myc-Menin(WT) or pcDNA-myc-Menin(L381P/A385P) along with Gal4-JunD (pM-JunD) under the Gal4-driven luciferase reporter gene (pG5-luc).

face of Mad1-SID is reported to be required for interaction of PAH2 domain of mSin3A (26, 27). As with Mad1-SID, a putative Menin-SID is likely to play a role in repressing transcription activity by recruiting mSin3-HDAC. To investigate whether SID located in middle region of Menin has an ability to repress transcription, we transiently transfected HeLa cells with truncated mutants of various Gal4DB-Menin, along with Gal4-*tk*-luciferase reporter gene. Both full-length and COOH-terminal truncated Menin significantly repress reporter gene activity. Furthermore, Gal4-Menin (SID) as well as Gal4-Menin (145–450) is sufficient to repress *tk*-luciferase reporter gene (Fig. 4*B*).

To address the biological role of SID of Menin in JunD transcriptional activity, we first made SID mutant of Menin (L381P/A385P) by converting leucine 381 to proline and alanine 385 to proline and transiently transfected HEK293 cells with either wild-type of Menin [pcDNA-myc-Menin(WT)] or SID mutant of Menin [pcDNA-Menin(L381P/A385P)] along with HA-tagged PAH2 of mSin3A (pSG5-HA-PAH2). Coimmunoprecipitation showed that PAH2 specifically interacts with wild type of Menin, although it cannot interact with Menin(L381P/A385P), indicating that this SID of Menin is important for interaction between Menin and mSin3A (Fig. 4*C*). We then examined the effect of SID mutation on JunD transcriptional activity by transient transfection of increasing amount of either Menin(WT) or Menin (L381P/A385P) under the Gal4-JunD-driven luciferase reporter gene. Menin(WT) significantly decreased JunD transcriptional activity; however, Menin (L381P/A385P) did not repress JunD transcriptional activity (Fig. 4*D*).

## Discussion

In this study, we provide direct evidence that Menin is a specific JunD repressor that recruits HDACs through association with mSin3A, a general transcriptional corepressor. Recently, it was reported that overexpression of Menin represses JunD transcriptional activity by uncoupling JunD phosphorylation from mitogen-activated protein kinase activation (28). However, it does not seem to be sufficient to explain repression mechanism of JunD transcriptional activity in quiescent state. Therefore, the previous findings that HDAC inhibitor TSA restores Menin-repressed JunD transcriptional activity (13), and JunD, unlike other AP-1 transcription factor (JunB and c-Jun), is constitutively expressed in quiescent state (3–5), raised the possibility that Menin serves as a repressor at the transcriptional level.

Histone acetylation and deacetylation is well known to play a pivotal role in chromatin remodeling and gene expression. The mechanism of transcriptional regulation by histone acetylation and deacetylation is mediated by two distinct types of enzymes, histone acetyltransferase, and HDAC. Coactivators such as p300 and CBP have intrinsic histone acetyltransferase domain and increase the accessibility of transcription factor to DNA promoter. On the other hand, corepressors recruit HDACs and repress transcriptional activity (25).

We demonstrate that Menin associates with HDACs through general corepressor mSin3A. mSin3A is shown to bind to HDAC through HDAC-interacting domain and contains four PAH domains, a subset of which has been shown to mediate interactions between mSin3A and other transcription factors. For example, PAH1 associates with N-CoR (29, 30), PAH3 interacts with SAP30 (31), and Mad protein and Cabin1 associate with PAH2 (24, 32, 33). It has been demonstrated that SID helix forms an amphipathic α-helix, mediating mSin3-interaction through a cluster of residues on hydrophobic faces (27). As with Mad proteins, Menin also has a SID helix within central domain, of which hydrophobic residues are oriented to one face. We

confirmed that Menin repressed JunD transcription activity through Menin-SID (Fig. 4*D*).

Although many efforts have been done to figure out function of Menin in tumor suppression, it is still unclear how Menin works as a tumor suppressor. Moreover, the findings of embryonic lethality of Menin-knockout mice and absence of strong homology with other proteins make it more complicated to elucidate mechanisms of its tumor suppressor activity (34).

Moreover, the other reason why it is difficult to understand the functional role of Menin in tumorigenesis is that Menin is likely to cooperate with other transcriptional factors for tumorigenesis. Basically, it was reported that c-myc seems to initiate tumorigenesis by cooperation with Menin mutation in pancreatic β-cell neoplasia and that Menin negatively regulates telomerase along with Mad1/c-myc pathway (35, 36).

Recently, Menin was found to associate with RPA2, $M_r$ 32,000 subunit of replication protein A required for DNA replication, recombination, and repair (37). This finding suggests that tumorigenicity of Menin is somehow related with DNA replication, recombination, and repair. Once in a while, RPA2 also links with transcription in term that it interacts with several transcription factors, signal transducers and activators of transcription 3, and Replication Protein Binding Trans-Activator (RBT1) (38, 39), and RPA is a component of RNA polymerase II complex (38, 39, 40), indicating that Menin still has potential to work as a tumor suppressor at transcriptional level.

Given that Menin binds to and represses different transcription factors, NF-κB, Smad3, as well as JunD, Smad3, and NF-κB associate with JunD and augment AP-1 transcriptional activity (41, 42), we postulate the possibility that JunD may integrate Smad3 and/or NF-κB transcriptional activity under certain circumstances, and Menin serves as a tumor suppressor to collectively repress these complexes of transcription factors by recruiting mSin3A-HDACs.

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