Menin and MLL cooperatively regulate expression of cyclin-dependent kinase inhibitors

Thomas A. Milne*, Christina M. Hughes†, Ricardo Lloyd‡, Zhaohai Yang*, Orit Rozenblatt-Rosen†, Yali Dou§, Matthew Meyerson‡, and Jay L. Hess*†

*Department of Pathology and Laboratory Medicine and Abramson Family Cancer Research Institute and Department of Cancer Biology, University of Pennsylvania School of Medicine, Philadelphia, PA 19104; †Department of Medical Oncology, Dana–Farber Cancer Institute and Department of Pathology, Harvard Medical School, Boston, MA 02115; ‡Department of Pathology, Mayo Clinic College of Medicine, Rochester, MN 55905; and §Laboratory of Biochemistry and Molecular Biology, The Rockefeller University, New York, NY 10021

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Mutations in the MEN1 gene are associated with the multiple endocrine neoplasia syndrome type 1 (MEN1), which is characterized by parathyroid hyperplasia and tumors of the pituitary and pancreatic islets. The mechanism by which MEN1 acts as a tumor suppressor is unclear. We have recently shown that menin, the protein product, interacts with mixed lineage leukemia (MLL) family proteins in a histone methyltransferase complex including Ash2L, Rbp5, and WDR5. Here, we show that menin directly regulates expression of the cyclin-dependent kinase inhibitors p27Kip1 and p18Ink4c. Menin activates transcription by means of a mechanism involving recruitment of MLL to the p27Kip1 and p18Ink4c promoters and coding regions. Loss of function of either MLL or menin results in down-regulation of p27Kip1 and p18Ink4c expression and deregulated cell growth. These findings suggest that regulation of cyclin-dependent kinase inhibitor transcription by cooperative interaction between menin and MLL plays a central role in menin’s activity as a tumor suppressor.

Abbreviations: MEN1, multiple endocrine neoplasia syndrome type 1; MLL, mixed lineage leukemia; CDK, cyclin-dependent kinase; ChIP, chromatin immunoprecipitation.

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complex by coimmunoprecipitation and that menin and MLL colocalize at target promoters in vivo. Interestingly, binding of MLL to transcriptional targets is highly dependent on menin, suggesting that transcriptional activation by menin involves MLL recruitment. Point mutations that occur in MEN1 patients were found to inhibit menin binding to target promoters. Finally, we found that expression of p27Kip1 is decreased in tumors from MEN1 patients compared with normal neuroendocrine tissues.

Materials and Methods

Cell Lines. Mll knockout and wild-type fibroblasts have been described (18). Menin knockout and wild-type fibroblasts were established in the laboratories of X.H. as described (11). For comparisons of growth and CDK inhibitor expression, particular care was given to seeding cells at comparable densities.

washed three times with buffer BC500 and eluted with BC100. The bound proteins were BC850. An aliquot of the BC500 fraction (inhibitor mixture (Roche Diagnostics) at 1
diluted against BC100 buffer [20 mM Tris, pH 7.8
20L f-WDR5 cells by a modified Dignam procedure (31) and
ing clone was expanded. Nuclear extracts were obtained from
analyzed for expression of Flag-WDR5, and the highest express-
selected by using 0.5 mg
epitope to the WDR5 N-terminal. The FLAG-WDR5 construct
WDR5 cDNA was subcloned into the pIRES-neo mammalian
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nos. A300-087A and A300-086A, respectively). Anti-MLL C-
terminal antibody was obtained from S. Korsmeyer (Dana–Farber Cancer Institute, Harvard Medical School, Boston). Anti-menin
was from Bethyl Laboratories (catalog no. C300-105A). Anti-p27
rabbit polyclonal antibody for immunohistochemistry is from Santa
Cruz Biotechnology (C-19; catalog no. sc-528). Anti-p27 antibody for Western blots is from BD Biosciences (no. 610241). Anti-
vinculin and β-actin antibodies are from Sigma (catalog nos. V9131
and A5316, respectively). Horseradish peroxidase (HRP)-
conjugated sheep anti-rabbit Ig(H+L) and HRP-conjugated sheep
anti-mouse antibodies (Roche Diagnostics) were used as secondary
reagents for Western blotting.

**Immunofluorescence Purification of MLL1-Containing Complex.** A human
WDR5 cDNA was subcloned into the pIRES-neo mammalian expression vector (Clontech/BD Biosciences), adding the FLAG
epitope to the WDR5 N-terminal. The FLAG-WDR5 construct was transfiltered into HeLa cells, and stable transformants were
selected by using 0.5 mg/ml G418. G418-resistant clones were
analyzed for expression of Flag-WDR5, and the highest expressing
clon was expanded. Nuclear extracts were obtained from 20L t-WDR5 cells by a modified Dignam procedure (31) and
dialized against BC100 buffer [20 mM Tris, pH 7.8/0.2 mM
EDTA/20% glycerol/1 mM DTT/0.2 mM PMSF/protease
inhibitor mixture (Roche Diagnostics) at 1× strength] before
loading onto a phosphocellulose column P11. Bound proteins were eluted sequentially with BC100, BC300, BC500, and
BC850. An aliquot of the BC500 fraction (~2 mg) was incubated with anti-FLAG M2 agarose (Sigma). The bound proteins were washed three times with buffer BC500 and eluted with BC100
containing 0.25 mg/ml Flag peptide (Sigma). The elution fraction was analyzed by immunoblotting.

**Luciferase Assays.** Sequences from −1100 to −27 of the human
p27Kip1 promoter were amplified by PCR, confirmed by sequenc-
ing, and cloned into the KpnI and HindIII sites of the luciferase
reporter pTAL-Luc (Clontech/BD Biosciences). The p18hakc
promoter-luciferase reporter shows menin activates transcription from the p27Kip1 or p18hakc promoters (green versus blue bar). The L22R (gray) and A242V (red) mutants show impaired ability to
activate transcription. (F) Binding of the menin mutants L22R (gray) and A242V (red) are reduced at the p27Kip1 and p18hakc loci compared with wild-
type menin (Men1−/− with reexpressed menin, green). (Inset) Western blot shows the L22R (gray lane 3) and A242V (red lane 4) mutants are ex-
pressed at similar levels to wild-type menin protein (green lane 2). Binding and menin expression in
Men1−/− cells with an empty vector are shown as controls.

**Immunohistochemical Staining.** Paraffin-embedded normal and
tumor tissues were obtained with institutional review board
(IRB) approval from the Mayo Clinic. Additional normal parathyroid and pancreas tissues were obtained with IRB approval from the Cooperative Human Tissue Network. Antigen retrieval was performed on sections to be stained for
menin and MLL by boiling in 10 mM sodium citrate (pH 6.0) for
10 min. Tissues were stained for p27Kip1 by using antigen retrieval as described (17).

**Results**

Menin Is Associated with an MLL-Containing Histone Methyltransferase Complex in Nonhematopoietic Cells. The association of menin with histone methyltransferase complexes has important implica-
tions for its ability to act as a transcriptional activator. However, some controversy has arisen regarding the composition of menin and MLL complexes. We initially reported that menin was associated with an evolutionary complex including MLL2 (13). Subsequent inspection of mass spectroscopy spectra revealed that MLL peptides were also present in the menin immunoprecipitation, and, consistent with this finding, the recently reported biochemical purification of MLL also revealed menin (17). However, other published complexes of either trx or MLL-associated proteins lacked menin (19, 32). We wished to confirm whether menin and MLL were associated in nonhematopoietic cells. These experiments showed that menin and the previously reported homologs of yeast Set1 complex proteins coimmunoprecipitate with WDR5. Proteins coimmunoprecipitating with WDR5 included the proteolytic cleavage products of MLL, MLLN and MLL3, HASH2, RBBP5, and hDPY30 (Fig. 1A and B). Additional experiments showed that the menin–MLL interaction is mediated by sequences within the N-terminal 260 aa of MLL. Reciprocal immunoprecipitations with antibodies directed against MLL and menin immunoprecipitated the other, confirming that MLL and menin interact at endogenous levels (data not shown). Histone methyltransferase activity specific for lysine 4 could be precipitated by anti-menin antibodies in MLL knockout cells (data not shown). This and previously published work (13, 17) suggest that MLL and MLL2 are associated with similar but distinct complexes.

Menin- and MLL-Deficient Cells Show Increased Cell Growth and Decreased p27kip1 and p18ink4c Expression. Despite global deficiencies in Hox gene expression, whose expression is generally associated with proliferation, both Men1 and MLL knockout fibroblasts grow faster than wild-type cells (Fig. 1C and D). Conversely, reexpression of menin or MLL in Men1− or MLL-null fibroblasts, respectively, slows growth rates to levels comparable with those of wild-type cells (Fig. 1C and D).

We therefore examined whether expression of p18ink4c and p27kip1 is altered in Mll and Men1 knockout fibroblast lines. Western blot analysis showed that both p18ink4c and p27kip1 were expressed at reduced levels in Mll knockout cells (Fig. 1C and data not shown) and that p27kip1 was expressed at reduced levels in menin knockout cells (Fig. 1D). Quantitative RT-PCR showed decreased expression of both p27kip1 and p18ink4c in Mll-null cells (Fig. 1E, blue versus green bars), which could be restored by MLL expression (Fig. 1E, red and gray bars). Expression of p27kip1 and p18ink4c was similarly reduced in menin null cells (Fig. 1F, blue versus green bars). Stable expression of menin (red bars), but not the patient derived menin point mutants L22R (black bars) or A242V (light blue) bars), could be restored by MLL expression (Fig. 1F, red and gray bars). Expression of both CDKs. The magnitude of these changes is likely to be significant because haploinsufficiency of either p27kip1 or p18ink4c results in either spontaneous or chemically induced tumorigenesis (25, 26).

Menin and MLL Bind Directly to the p27kip1 and p18ink4c Loci. Chromatin immunoprecipitation (ChIP) with anti-menin antibodies was then done to determine whether menin directly regulated p27kip1 and p18ink4c. These experiments showed menin bound directly to the coding regions of both genes (Fig. 2 C and D). Signal was abolished in Men1 knockout cells (red bars) and is restored by menin reexpression (light blue bars) confirming the specificity of the antibody (Fig. 2 C and D). No binding of menin was detected to Gapdh in either cell type, indicating that menin does not bind to all transcriptionally active loci. Only minimal differences in menin binding to the p27kip1 and p18ink4c loci were detected between Mll+/− and Mll−/− cells (Fig. 2 C and D, blue versus green bars), indicating that MLL is not required for menin binding. Luciferase reporter assays showed that menin reexpression in Men1-null cells activated transcription from both p27kip1 and p18ink4c promoters approximately 4- to 5-fold (Fig. 2E, green versus blue bars).

Importantly, the menin mutants L22R and A242V were impaired in their ability to activate both promoters (gray and red bars). ChIP studies suggested that this deficiency was the result of defective recruitment. Men1-null cells were established stably expressing comparable levels of either wild type or the L22R or A242V menin mutants (Fig. 2F Inset). ChIP with menin antibodies showed that the amount of the L22R or A242V menin mutants associated with the p27kip1 and p18ink4c loci was greatly decreased compared with wild-type menin (Fig. 2F, gray and red bars).

MLL Association with p27kip1 and p18ink4c Loci Is Menin-Dependent. MLL increases transcription and interacts with menin, suggesting that transcriptional activation by menin involves MLL recruitment to target promoters. To investigate this possibility, we performed ChIP to localize MLL binding in Mll and Men1 wild-type and knockout cells. Surprisingly, lower MLL protein levels were seen in Men1-null cell compared with wild-type cells (Fig. 3A). ChIP showed that MLL directly binds to the p27kip1 and p18ink4c loci (Fig. 3B and C) and that, in the absence of menin, a drastic reduction is seen in MLL binding (Fig. 3B and C, red versus gray bars). Menin reexpression had minimal effects on MLL protein levels
but resulted in increased binding at the p27Kip1 and p18Ink4c loci (Fig. 3B and C, light blue bars). Overall, the data suggest that transcriptional regulation by menin involves increasing MLL protein association with target loci.

**MLL Is Coexpressed with Menin in Endocrine Tissues.** We then examined normal tissues and tumors from MEN1 patients for menin, MLL, and CDK inhibitor expression. Pancreas, pancreatic adenomas, and normal and hyperplastic parathyroids were obtained from the files of the Mayo Clinic with additional normal tissues provided by the Cooperative Human Tissue Network. Immunohistochemical staining showed that both menin and MLL are expressed at high levels in neuroendocrine tissues, including pancreatic islets and parathyroid (Fig. 5, which is published as supporting information on the PNAS web site). Reduced expression of menin was seen in most islet cell tumors compared with normal islets in the same sections (Fig. 4A versus D). Reduced expression of MLL was also seen in the MEN1 tumors (Fig. 4B versus E). These results, along with the finding of less Mll expression in Men−/− cells (Fig. 3A), suggest that MLL expression could be either directly or indirectly regulated by menin. However, ChIP experiments to date have not shown menin binding to Mll coding regions.

**MEN1 Tumors Show Decreased p27Kip1 Expression Compared with Normal Endocrine Tissues.** We focused on assessing p27Kip1 expression because minimal nuclear staining for p18Ink4c was seen in human neuroendocrine tissues. Nonneoplastic islets from seven MEN1 patients showed strong expression of p27Kip1 (Fig. 4C). Variable but reduced intensity of expression was seen in all islet cell tumors in the same sections, which showed an increase in the proportion of nonstaining cells (20.00% versus 5.43%, paired t test, P = 0.01) (Fig. 4F and G). Similar results were seen in parathyroids from normal patients (n = 5) compared with hyperplastic parathyroids from MEN1 patients (Fig. 6, which is published as supporting information on the PNAS web site); however, the levels were heterogeneous and of lower statistical significance (34.4% versus 20.00%, unpaired t test, P = 0.11, n = 5).

**Discussion**

Although the majority of attention has focused on menin as a transcriptional repressor, our work suggests that menin functions as a tumor suppressor through transcriptional activation of CDK inhibitors. Menin has been shown to bind nonspecifically to DNA; however, the mechanisms responsible for its recruitment to specific chromosomal sites are not known. One possibility is that menin binds with higher affinity to yet-to-be-identified DNA sequences. In addition, menin interacts with the serine 5 phosphorylated C-terminal domain of RNA polymerase II (13) as well as a number of transcription factors, including activator protein-1 (AP-1) family members JunD and c-Jun, NF-κB, and mothers against decapentaplegic (SMAD) family members (10). These findings raise the possibility that menin is targeted to transcriptionally active promoters through interactions with promoter-bound RNA polymer-
Menin interacts with MLL as part of an evolutionarily conserved Polycomb methylase complex, possibly explaining the ability of menin to activate transcription. Furthermore, both menin and MLL colocalize at target genes in vivo. Menin binding to transcriptional targets is apparently not dependent on MLL. However, menin dramatically increases the amount of MLL bound at the p27Kip1 and p18ink4c loci, suggesting that it either directly or indirectly promotes MLL recruitment to these targets. Once recruited, MLL could enhance transcription by a number of mechanisms. One possibility is that transcription is enhanced by histone H3 lysine 4 methylation mediated by the SET domain of MLL. The other role is highly reminiscent of Bmi1, a mammalian Polycomb group protein that antagonizes MLL function and negatively regulates Hox gene expression. The abilities of Bmi1 to promote neural and hematopoietic stem cell renewal, as well as to cooperate with Myc in oncogenesis, are both linked to its negative regulation of another CDK, p18ink4c (36).

The N-terminal MLL sequences required for interaction with menin are retained in leukemogenic MLL fusion proteins (17), raising the question of whether MLL fusion proteins up-regulate CDK inhibitors in hematopoietic cells and, if so, how this regulatory pathway is bypassed to allow for leukemogenesis. One possible explanation is that the targets depends on the cell type, which affects what targets are expressed. In early hematopoietic progenitor cells, where a cluster of Hox genes are expressed at high levels (37) but p27Kip1 is either not expressed or expressed at low levels (38), MLL fusion proteins predominantly up-regulate Hox gene expression, resulting in leukemia (16). In keeping with this finding, we have found that activation of a tamoxifen-inducible form of MLL-ENL only transiently up-regulates p27Kip1 but persistently up-regulates the leukemogenic targets Hoxa9 and Meis1 (T.A.M. and J.L.H., unpublished results). In pancreatic islet cells, these leukemogenic target genes are minimally expressed (39), but p27Kip1 is abundant, suggesting that the predominant effect of alterations in the menin–MLL axis is decreased CDK expression resulting in deregulated cell growth (Fig. 4H).

Finally, this work raises the surprising possibility that loss-of-function MLL plays a role in neoplasia. Deletions of the 11q23.3 locus spanning the MLL gene are extremely common in a variety of tumors, including pituitary and follicular adenomas, neuroblastoma, and malignant melanoma, and, in some cases, of mantle cell lymphoma and acute lymphoblastic leukemia (40–42). MLL does not have inactivating mutations in these tumors, and the predominant effect of alterations in the menin–MLL axis is loss of tumors, including pituitary and follicular adenomas, neuroblastoma, and malignant melanoma, and, in some cases, of mantle cell lymphoma and acute lymphoblastic leukemia (40–42). MLL does not have inactivating mutations in these tumors, and the predominant effect of alterations in the menin–MLL axis is embryonic lethal and Mll heterozygous mice do not show a higher rate of tumors. Nonetheless, MLL suppresses growth of a variety of cell types and interacts with at least two tumor suppressors, menin and INI1 (34), which is mutated in rhabdoid and other primitive neuroectodermal tumors, warranting additional studies to assess whether MLL is a “tumor susceptibility and resistance gene” (43).

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