CHAPTER 3

Menin:
The Protein Behind the MEN1 Syndrome

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Abstract

The cloning of the *MEN1* gene in 1997 led to the characterization of menin, the protein behind the multiple endocrine neoplasia Type 1 syndrome. Menin, a novel nuclear protein with no homology to other gene products, is expressed ubiquitously. *MEN1* missense mutations are dispersed along the coding region of the gene but are more common in the most conserved regions. Likewise, domains of protein interaction often correspond to the more conserved segments of menin. These protein interactions are generally facilitated by multiple domains or encompass a large portion of menin. The exception to this rule is a small stretch of amino acids mediating the interaction of menin with the mSin3A corepressor and histone deacetylase complexes. The C-terminal region of menin harbors several nuclear localization signals that play redundant functions in the localization of menin to the nuclear compartment. The nuclear localization signals are also important for the interaction of menin with the nuclear matrix. Menin is the target of several kinases and a candidate substrate of the ATM/ATR kinases, implying a role for this tumor suppressor in the DNA damage response. Menin is highly conserved from *Drosophila* to human but is absent in the nematode and in yeast.

Introduction

Cloning of the gene for multiple endocrine neoplasia Type 1 (*MEN1*) in 1997 led to the identification of menin, the protein encoded by *MEN1*.[1,2] The 67 kDa menin is translated from a 2.8 kb transcript expressed ubiquitously and throughout mammalian development.[1,3-5] The *MEN1* gene is composed of 10 exons spanning a 9.0 kb region of genomic DNA (Fig. 1A).[1] Exon 1 is noncoding and accounts for most of the 5′ UTR of the menin transcript. Exons 2 to 10 code for the 610 amino acid menin protein (id: AAC51229). A variant of 615 amino acids (id: AAC51230) was also identified in clones from a human leukocyte cDNA library and in a few EST clones.[1] The 615 amino acid variant, translated from an alternatively spliced transcript, contains an in-frame insertion of five amino acids at position 149 of the menin open reading frame. Several transcripts with different 5′ UTR sequences have also been reported but the structure of the menin protein derived from these variants is not altered.[6] Little is known about the expression of the 615 amino acid menin and 5′ UTR variants of the menin mRNA. One of the important conclusions of early molecular studies is that menin is a novel protein showing no homology to other gene products.

Menin Is a Nuclear Protein—Role of the C-Terminal Region

The nuclear localization of menin was confirmed by immunofluorescence, epitope tagging and western blotting analyses of subcellular fractions.[7] Deletion analysis identified the C-terminus

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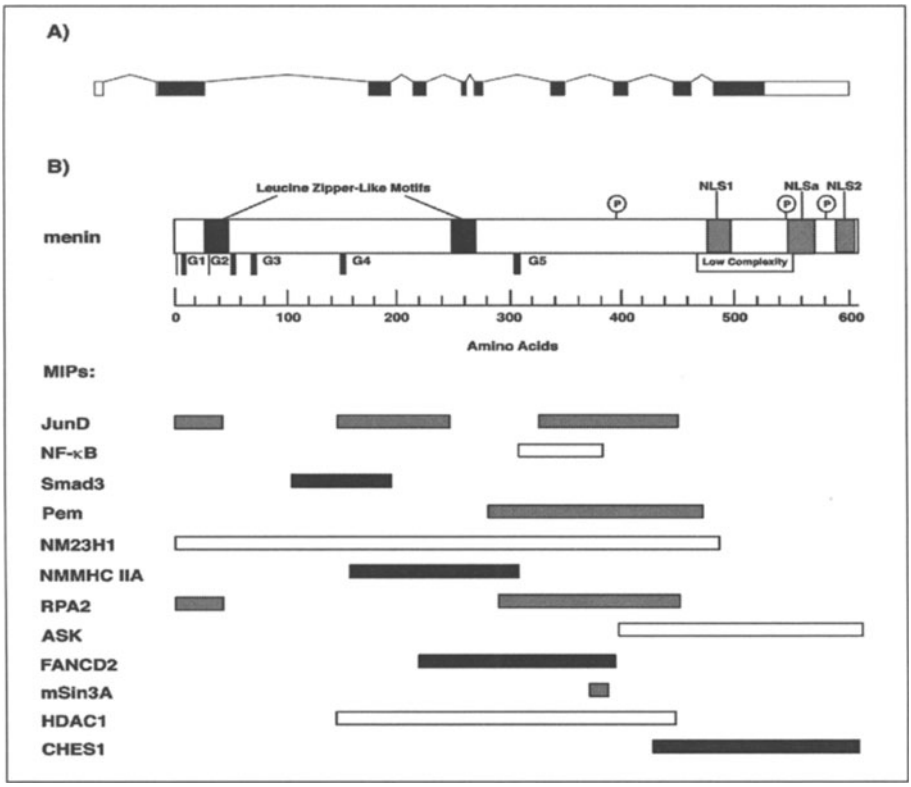

Figure 1. A) Exon-intron structure of the human *MEN1* gene. B) Schematic representation of functional domains of menin and binding sites of menin-interacting proteins (MIPs). Menin contains two leucine zipper-like motifs, two nuclear localization signals (NLS1 and NLS2) and an accessory NLS (NLSa). Five GTPase motifs (G1 to G5) are present in menin. A putative low complexity region spans amino acids 465 to 551. Menin is phosphorylated on two serine residues, Ser543 and Ser583, in 293T-cells. Phosphorylation of Ser394 in 293T-cells and M059K glioblastoma cells was observed in response to γ-irradiation and UV treatment, respectively. The regions of the menin sequence that have been implicated in the binding to different interacting proteins are indicated by black, white or gray bars under a schematic representation of the structure of menin.[12,20,25-27,45-50]

as the determinant of menin nuclear localization. Within this region, two independent nuclear localization signals (NLS1 and NLS2) are sufficient to target GFP or EGFP to the nuclear compartment.[7,8] Mutation analysis of NLS1 (amino acids 479-497) and NLS2 (amino acids 588-608) indicated that they play a redundant role in the nuclear localization of menin. More recently, La and coworkers reported that a third cluster of basic residues, located between amino acids 546 and 572, contributes to the nuclear localization of menin but is not sufficient to target GFP to the nucleus.[9] This region appears to function as an accessory signal (NLSa) in the nuclear localization of menin by NLS1 and NLS2 (Fig. 1B).

The localization of menin is consistent with the observation that several menin-interacting proteins are transcription factors, epigenetic regulators or proteins involved in DNA synthesis or repair (Chapter 5).[10] Menin associates with chromatin in vivo and binds dsDNA in vitro.[11,12] However, point mutations in any of the three NLSs abolish in vitro DNA binding without affecting the nuclear localization or association of menin with chromatin.[9] The same mutants are

also deficient in repression of the *IGFBP2* promoter and inhibition of cell proliferation, implying an additional role for the NLSs apart from the nuclear localization of menin. Chromatin immunoprecipitation (ChIP) assays revealed that menin is recruited to the promoter region of the *IGFBP2* gene and that intact NLSs are required for this activity.[9] Menin also interacts with the 5′ UTR region of the *caspase-8* locus in vivo and is capable of inducing the expression of this gene in mouse fibroblasts. Mutations in a single NLS also abolish the activation of the *caspase-8* gene by menin. Currently, there is no evidence that the DNA binding activity of menin is critical for recruitment to target loci. Menin binds DNA in a sequence-independent manner and, thus far, attempts at identifying specific DNA binding sites for menin have been unsuccessful.[13] Global genome analysis by ChIP assays indicates that menin is widely distributed in the genome, consistent with the notion that menin interacts with the phosphorylated carboxy terminal domain (CTD) of the RNA polymerase II large subunit, several transcription factors and epigenetic regulators.[14,15,16] It is possible that the recruitment of menin depends primarily on the interaction with a nuclear protein but that sequence-independent DNA binding by the positively charged NLSs plays a secondary role in this process.[9]

The importance of the C-terminal region of menin is highlighted by the fact that approximately 70% of the *MEN1* mutations are frameshift or nonsense mutations.[17,18] These mutations would disrupt the nuclear localization of menin and possibly other functions dependent on the presence of intact NLSs. Germline or somatic missense mutations have not been identified in NLS1 or NLS2 and only a few target the accessory region NLSa. This may be expected if the NLSs play a redundant role in the nuclear localization of menin but raises some questions regarding the significance of other activities dependent on the presence of intact NLSs.

The localization of menin to discrete nuclear foci has also been reported by separate groups in NIH 3T3 and HeLa cells.[12,19] Others, however, failed to observe this pattern of sub-nuclear localization using different cell types and antibodies, or a different approach such as GFP-tagging.[7] The association of menin with telomeres was described at meiotic prophase in mouse spermatocytes.[19] However, this pattern of menin localization was not observed in somatic cells and thus appears to be restricted to meiotic cells.

Leucine-Rich Domains in Menin

Two major approaches, yeast two-hybrid screening and proteomic analysis of co-immunoprecipitated proteins, have been employed to identify menin-interacting proteins and complexes (Chapter 5). The molecular analysis of these interactions indicated that large and/or multiple domains of menin are often required for association with its binding partners (Fig. 1B). The exception to this rule is a short stretch of centrally located amino acids (371-387) mediating the interaction of menin with the mSin3A corepressor and component of histone deacetylase complexes. The sixteen amino acid region, which includes tandemly repeated leucine residues, is predicted to form an amphipathic α-helix resembling the mSin3A-interacting domain (SID) of the Mad1 and Pf1 transcriptional repressors.[20-22] Point mutations within the SID region of menin abolish the interaction with mSin3A and the capacity of menin to repress the trans-activation function of JunD.[20]

Other leucine-rich regions have been the subject of investigation. Dreijerinck and collaborators showed recently that menin acts as a coactivator for nuclear receptor responsive genes such as *TFF1*.[23] In MCF7 breast carcinoma cells, menin was important for H3K4 trimethylation and ligand-dependent activation of this gene by the estrogen receptor α (ERα). Nuclear receptors interact with a leucine-rich motif in several coactivators (LXXLL). Since menin bound directly to the AF2 trans-activation domain of ERα in vitro these authors investigated the role of a conserved LLWLL motif (amino acids 263-267) in this process. Proteins encoded by clinically relevant *MEN1* mutations affecting the leucine-rich motif (L264P, L267P) were indeed deficient in the interaction with ERα and activation of the *TFF1* promoter. However, mutations in several residues outside the LLWLL motif also impaired this activity and thus menin may depend on a larger domain for the control of ERα-dependent gene expression.

The LLWLL motif is also part of a putative leucine zipper, one of two such candidate dimerization regions identified in menin (Fig. 1B).[24] However, the evidence of a role for these domains in protein dimerization, including menin homodimerization, is presently lacking.[4,24] Interestingly, menin does interact with the JunD bzip transcription factor but binds to the N-terminus and not the leucine zipper domain of JunD. Menin does not interact with other bzip members of the AP-1 family.[24]

GTPase Signature Motif

Menin associates with nm23H1/nucleoside diphosphate (NDP) kinase A, a multi-functional protein and candidate suppressor of tumor metastasis.[25] Results reported by Yamaguchi and co-investigators suggest that nm23H1 stimulates a latent GTPase activity in menin that is dependent on the interaction with nm23H1.[26] Menin was shown to bind GTP with low affinity but to hydrolyze GTP efficiently in association with nm23H1. A signature motif, conserved in GTPases and consisting of short stretches of a few amino acids grouped in five regions, was also identified in the N-terminal portion of menin. All regions (G1 to G5) are well conserved in mammals but not in more divergent species such as the zebrafish or *Drosophila melanogaster* (Fig. 2). Germline or somatic missense mutations in menin have been reported for the G4 region but are absent in other regions of the GTPase signature (Fig. 2). A subset of these *MEN1* missense mutants, analyzed by Yamaguchi and co-investigators, retained the GTPase activity.[26] Further studies are required to assess the significance of this GTPase activity and its relationship to the function of menin as a tumor suppressor.

Post-Translational Modification in Response to DNA Damage

Mouse embryo fibroblasts (MEFs) mutant for the *Men1* gene are hypersensitive to ionizing radiation and are deficient for a DNA damage-activated checkpoint.[27] Similar defects in cell cycle arrest were observed in *Drosophila* strains mutant for *Mnn1*, the *MEN1* homologue in the fruit fly, implying a conserved function for menin in the DNA damage response.[27] In agreement with this notion, two recent studies identified menin as a putative target of the ATM/ATR kinases.[28,29] A similar approach was employed in these studies wherein proteins phosphorylated in response to DNA damage were identified by immunoaffinity phosphopeptide isolation followed by sequence analysis using mass spectrometry. Using this approach, phosphorylation of Ser394 was detected in γ-irradiated 293T and UV-treated M059K glioblastoma cells.* Ser394 is located in a putative region of disordered secondary structure and is not conserved in other species (Figs. 2 and 3). Menin is also phosphorylated on Ser543 and Ser583 in 293T-cells but the kinase(s) responsible for phosphorylation of these residues and their function are presently unknown.[30] Ser583 is highly conserved and located in proximity of NLS2; Ser543 is present in mammalian menin but is not found in the zebrafish and *Drosophila* orthologues (Fig. 2). The association of menin with chromatin or the nuclear matrix is enhanced following UV treatment or γ-irradiation, suggesting that menin sub-nuclear localization or recruitment is regulated in response to DNA damage.[12,31] The treatment of UV-treated HEK293 cells with caffeine, an inhibitor of the ATR kinase, decreased menin localization to chromatin while overexpression of constitutively active CHK1 enhanced the association with chromatin. These results suggest that menin localization is regulated by an ATR-CHK1-dependent pathway in UV-treated cells.[31] The study of menin post-translational regulation is just beginning and additional sites of phosphorylation and modification are likely to be uncovered in the near future.

Conservation of Menin Structure, Protein Interactions and Function

MEN1 orthologues have been identified in vertebrates, sea urchin, snail and insects but are missing in the nematode and in yeast.[4] In vertebrates, the degree of protein identity to human menin ranges from 67% in the zebrafish to over 96% in mammalian species such as the mouse and rat (Fig. 2). Vertebrate species also share a similar exon/intron and overall structure of the *MEN1* gene, which consists of 10 exons with exon 1 being noncoding (Fig. 3A). Transcript variants with

different 5′ UTRs, generated from alternative splicing of intron 1, have also been described in the mouse and rat.[5,32,33] The *Drosophila MEN1* gene (designated *Mnn1*) contains fewer exons but encodes a larger 763 amino acid protein 83 kDa in molecular weight.[34,35] Long stretches of amino acids located in the C-terminal portion of the protein and missing in vertebrate menin account for the larger size of *Drosophila* menin. Translation initiation is also predicted to occur at a different methionine codon, extending the N-terminus of *Drosophila* menin by 12 amino acids.[34] Cerrato and coworkers described the nuclear localization of menin in tissues of the third instar larva.[36] Putative nuclear localization signals are present at the C-terminus of *Drosophila* menin. A protein variant of 530 amino acids, lacking the C-terminal region of the 83 kDa protein, is predicted by the analysis of *Drosophila* cDNAs (id: NP 723252). We also described the expression of a 70 kDa heat shock-inducible form of menin in early embryos.[37] Little is known about the function of these menin variants in *Drosophila*.

Sequence divergence is found principally at the C-terminus of the protein (amino acids 465 to 551) in a proline-rich region of low sequence complexity that also accounts for much of the size difference in *Drosophila* menin (Fig. 2-3B). In mammals, the C-terminal region of menin is required for interaction with several proteins but fine mapping of this region is generally lacking (Fig. 1). Significantly, the majority (88%) of missense mutations identified to date are located in the N-terminal and "core" regions of menin (amino acids 1-447), i.e. in regions of greatest conservation (Figs. 2-3B). The overall identity of human and *Drosophila* menin is 46%. However, 65% of the 116 *MEN1* missense mutations identified thus far correspond to amino acids conserved in *Drosophila* menin.[18] This conservation reaches 82% in zebrafish, highlighting the importance of these amino acids in menin function. Prediction of secondary structure by the Russell/Linding definition (GlobProt)[38] identifies several putative regions of disordered structure, including the C-terminal region of low sequence complexity. *MEN1* missense mutations are lacking in regions predicted to be disordered (underlined in Fig. 2).

Unlike *Men1* in mammals, *Mnn1* function is not required for development in *Drosophila* and *Mnn1* mutant flies are fertile.[36,37,39-41] However, two different responses are affected by the absence of menin in the fruit fly. Defects in S-phase arrest have been described in response to ionizing radiation.[27] Mouse embryo fibroblasts mutant for *Men1* showed similar defects, implying a conserved function for menin in this response to DNA damage. Cell cycle arrest and viability were recovered in γ-irradiated flies overexpressing the forkhead family member Ches1. The human homologue, CHES1 (FOXN3), binds to human menin in vitro and co-immunoprecipitates in vivo, indicating a direct interaction between these proteins.[27] Whether or not this is also true for the *Drosophila* counterparts was not addressed in these studies.

We reported that *Mnn1* mutant strains are unable to mount a proper stress response. Developmental arrest and increased lethality were observed in response to heat shock, hypoxia, hyper-osmolarity and oxidative stress.[37] The expression of several heat shock proteins (HSPs) was impaired by the absence or over-expression of menin. *Mnn1* loss-of-function mutants expressed normal levels of HSP70 in the first 15 min of the heat shock response but were unable to sustain this expression beyond that point. In contrast, embryos over-expressing menin failed to down-regulate the expression of HSP70 upon return to the normal temperature. Thus, menin plays a role in the maintenance of HSP expression. In *Drosophila*, HSP70 expression depends on the H3K4 histone methyltransferase Trithorax (Trx) and components of the TAC1 chromatin modifying complex.[42] In mouse embryos and cells, menin interacts with histone methyltransferase (HMTase) complexes containing the Trithorax group proteins MLL2 or Ash2L. The action of these HMTases in the maintenance of *Hox* gene expression depends on their association with menin.[16,43,44] Recently, we observed that menin and Trithorax co-immunoprecipitate in protein lysates of *Drosophila* S2 cells, indicating that the interaction of menin with histone methyltransferase complexes has been conserved during evolution (our unpublished results[16,43]).

Menin interacts genetically with *Drosophila* Jun and Fos, components of the AP-1 transcription factor, but does not appear to bind directly to these proteins.[34,36] This contrasts with the direct interaction of menin and JunD described in human cells.[45] In general, the interaction of menin

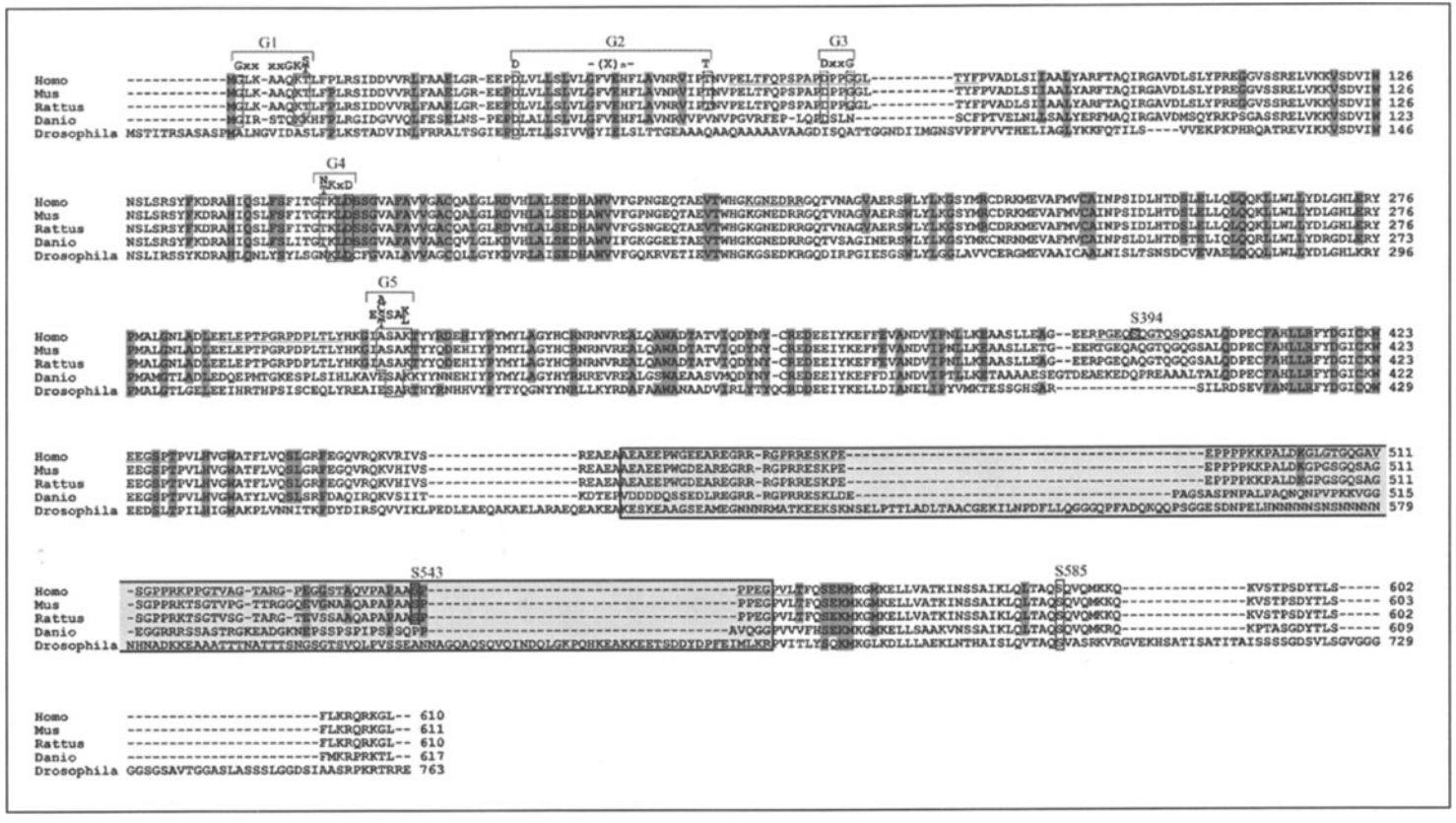

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Figure 2, viewed on previous page. Conservation of amino acids corresponding to missense point mutations in *MEN1*. Darkly shaded boxes indicate conserved residues in relation to known germline and somatic point mutations in human.[18] Underlined sequences show putative disordered secondary structure in the human sequence as predicted by GlobProt using the Russell/Linding definition.[38] Light grey box indicates a core low-complexity region as determined by multiple alignment of vertebrate sequences and predicted by SEG (our unpublished results).[51] Dashed-box regions show conserved putative GTPase motifs (G1-G5) determined by Yaguchi et al.[26] Consensus amino acid sequences are shown above. Solid boxes indicate serine phosphorylation sites Ser543 and Ser583 and the putative ATM and ATR phosphorylation site Ser394.[28-30,52] *Homo* indicates *Homo sapiens*; *Mus*, *Mus musculus*; *Rattus*, *Rattus norvegicus*; *Danio*, *Danio rerio*; and *Drosophila*, *Drosophila melanogaster*. A color version of this image is available at www.landesbioscience.com/curie

with known binding partners, identified in mammals, has not been investigated in *Drosophila* and other nonmammalian species.

Conclusion

Missense mutations have been identified along much of the menin open reading frame and do not cluster in "hot spots" pointing to domains of greater importance in the MEN1 syndrome. Regions of protein interaction are generally large or composed of multiple domains (Fig. 1B). Finer mapping of these regions will likely be important for the characterization of menin structure and function. Little is known about the dynamic of menin interaction with its binding partners.

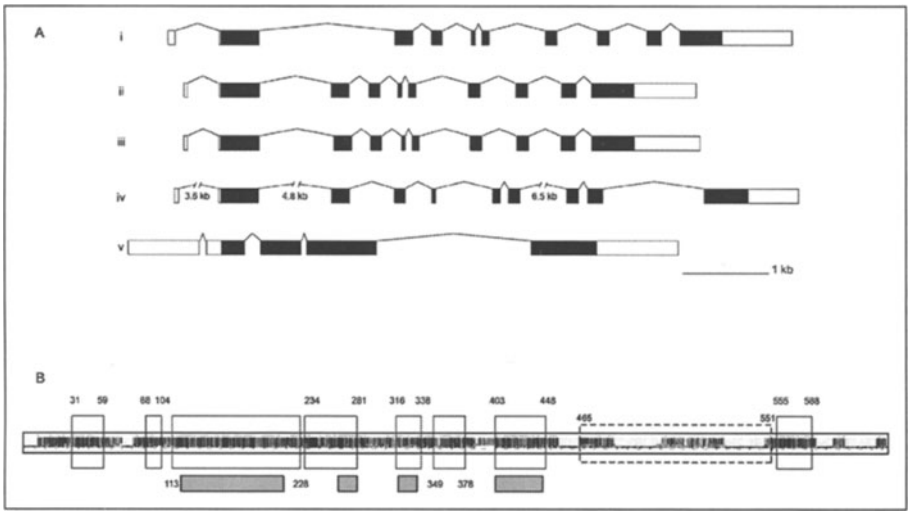

Figure 3. Panel A shows conservation of the exon-intron organization of the *MEN1* gene in (i) *Homo sapiens* (NC_000011.8) (ii) *Mus musculus* (NC_000085.5, AF109390) (iii) *Rattus norvegicus* (NC_005100.2, AB023400) (iv) *Danio rerio* (NC_007118.2) and (v) *Drosophila melanogaster* (NT_033779.4). Coding exons are indicated by black boxes and noncoding exons by white boxes. Introns are depicted as lines and approximate lengths are specified only for those introns too large to be drawn to scale. Panel B shows a schematic representation of the most highly conserved protein regions among five species (human, mouse, rat, zebrafish and fruit fly) menin homologues. Large boxes represent aligned regions with greatest degree of conservation in vertebrate species (calculated as the largest continuous aligned segments with lowest entropy). Gray boxes represent regions most conserved with *Drosophila* menin. Short functional motifs such as nuclear localization signals falling outside larger conserved regions are not indicated. The hatched box represents a putative core low complexity region in vertebrate sequences. Amino acid numbers refer to the human sequence.

The study of post-translational modifications of menin may shed some light on the mechanisms governing the interaction of menin with other proteins. A more extensive characterization of the menin-containing complexes will lead to the identification of additional binding partners and a better understanding of the role of menin in tumor suppression. The understanding of this role will continue to depend on multiple experimental approaches including the study of model systems, the characterization of domains of conserved protein interactions and the determination of the tri-dimensional structure of menin.

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