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RESEARCH

Alternative splicing generates isoform diversity in *MEN1*

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Abstract

Although the gene *MEN1* has a long-standing association with cancer, its mechanisms of action remain incompletely understood, acting both as a tumour suppressor in neuroendocrine tumours and as an oncogene in leukaemia. The best-characterised isoform of the encoded protein, MENIN, is the 610-amino acid MENIN isoform 2. We hypothesise that some of the complexity of *MEN1* biology can be attributed to a currently unappreciated contribution of different MENIN isoforms. Through *in silico* data mining, we show alternative splicing along the entire length of *MEN1*. Splice junction data suggest that the transcript encoding MENIN isoform 2 is the most abundant in all tissues examined, making a strong argument for this to be the reference transcript/protein isoform of *MEN1*. We also report novel splicing events, including a novel exon from within intron 7 that is relatively highly expressed in many tissues. These splicing events are predicted to contribute to MENIN diversity by generating isoforms with in-frame insertions, deletions or unique amino termini that, in turn, could have altered interactions with partner proteins. Finally, we have compiled 2574 unique genomic variants reported in *MEN1* within somatic and germline databases and have identified several variants that could impact individual MENIN isoforms. We have also collated studies pertinent to MENIN function in the literature and summarised the impact of *MEN1* variants on 74 biological variables. We propose a set of four *MEN1* variants, MENIN^{L22R}, MENIN^{H139D}, MENIN^{A242V} and MENIN^{W436R}, that represent a cohort with different biological properties, which should be investigated concurrently to better dissect MENIN function.

Keywords: MEN1; MENIN; isoforms; alternative splicing; mutation

Introduction

Mutations in the gene *MEN1*, which encodes the protein MENIN, are causal in multiple endocrine neoplasia 1 (MEN1) syndrome (OMIM: 131100). Nevertheless, *MEN1* remains enigmatic, almost 30 years after its cloning. MEN1 syndrome is notable for the development of neuroendocrine tumours (NETs), usually adenomas, with an autosomal dominant pattern of inheritance and high penetrance in the parathyroid, anterior pituitary,

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adrenal cortex and pancreatic islets, although other endocrine and neuroendocrine cell types can also be involved (Thakker 2010). With positional cloning to chromosome 11 (Larsson *et al.* 1988) and refinement of the locus to *MEN1* (Chandrasekharappa *et al.* 1997), it was immediately recognised that the causal gene likely encoded a tumour suppressor in NETs, with loss of heterozygosity of the wild-type allele unmasking a mutant allele that was non-functional, or only partially functional, in associated tumours. This has been borne out in larger MEN1 syndrome cohorts as well as in sporadic NETs, including gastrinomas, pancreatic neuroendocrine tumours (pNETs), pituitary and parathyroid tumours (Patel *et al.* 1990, Heppner *et al.* 1997, Zhuang *et al.* 1997*a*,*b*, Hessman *et al.* 1998, Wang *et al.* 1998). We, and others, have recently shown that the loss of *MEN1*, in the context of a defined copy number change signature, may identify a subset of pNETs with poorer prognosis (Scarpa *et al.* 2017, Lawrence *et al.* 2018).

The most widely studied isoform of the encoded protein, MENIN, is derived from a 10-exon transcript (representative transcript NM_001370259.2) with an open reading frame (ORF) that translates to a 610-amino acid protein (MENIN isoform 2) (Chandrasekharappa *et al.* 1997). The first characterised function of MENIN was its physical association with the transcription factor JUND, along with concomitant repression of JUND-dependent reporter gene expression (Agarwal *et al.* 1999). MENIN can also immunoprecipitate with the Set1-like lysine methyl transferases KMT2A (also known as mixed lineage leukaemia 1 (MLL1)) and KMT2B (Yokoyama *et al.* 2004, 2005, Milne *et al.* 2005). Part of the COMPASS-like protein complexes, KMT2A/B are responsible for the trimethylation of histone 3 on lysine 4 (H3K4me3) at a subset of genomic loci, thereby facilitating transcription by promoting DNA accessibility (Cenik and Shilatifard 2021). Rearrangements involving *KMT2A* are observed in a subset of acute and mixed lineage leukaemia, including over 70% of childhood leukaemia (Milan *et al.* 2022). Critically, balanced translocations create KMT2A fusion proteins that have lost the methyltransferase domain of KMT2A but retain the MENIN binding motif. The central role of MENIN in establishing the oncogenic functions of KMT2A fusion proteins is evident from the observation that it is the only COMPASS component that is indispensable for KMT2A-driven *HOX* gene expression (Yokoyama *et al.* 2004) and leukaemogenesis (Yokoyama *et al.* 2005).

The role of MENIN as a tumour suppressor, while equally important, is less clearly defined. Although homozygous deletion of *Men1* in mice is embryonic lethal (Crabtree *et al.* 2001), heterozygous *Men1*$^{+/-}$ mice develop tumours in a range of tissues, including the pituitary, adrenal cortex, lung, parathyroid and pancreas (Crabtree *et al.* 2001, Bertolino *et al.* 2003*a*, Loffler *et al.* 2007, Harding *et al.* 2009), phenocopying MEN1 syndrome in man. Furthermore, targeted deletion of *Men1* in the parathyroid (Libutti *et al.* 2003) and in various cell types of the pancreas (Crabtree *et al.* 2003, Bertolino *et al.* 2003*b*, Biondi *et al.* 2004, Lu *et al.* 2010, Shen *et al.* 2009, 2010, Li *et al.* 2015) results in hyperproliferation and adenomas in these tissues. Mechanistically, deregulated cell cycle control has been implicated in the increased proliferation of these tumours – the cell cycle inhibitors (CDKis) *Cdkn1b* and *Cdkn2c* are transcriptional targets of KMT2A and MENIN, and the expression of the CDKis *Cdkn1b*, *Cdkn2c*, *Cdkn1a* and *Cdkn2b* is decreased in pancreatic tumours (Milne *et al.* 2005, Karnik *et al.* 2005, Schnepp *et al.* 2006). In addition, the deletion of *Cdk4* completely prevents islet and anterior pituitary hyperplasia in *Men1*$^{+/-}$ mice (Gillam *et al.* 2015). However, altered cell cycle control is unlikely to be the sole contributor to tumourigenesis, as changes in CDKi expression occur after the onset of pancreatic islet hyperplasia following acute deletion of *Men1* in mice (Schnepp *et al.* 2006, Yang *et al.* 2010) . In addition, targeted deletion of *Men1* in the liver leads to decreased expression of *Cdkn1b* and *Cdkn2c* but not tumour formation in this organ (Scacheri *et al.* 2004, 2006). Finally, the propensity for islet β-cell tumour development, regardless of the pancreatic cell type in which *Men1* is deleted, suggests an unresolved role for MENIN in cell identity (Shen *et al.* 2009, 2010, Lu *et al.* 2010).

How diverse *MEN1* variants in MEN1 syndrome and cancer generate different structural and functional alterations in MENIN, yet converge on its tumour-suppressive role in NETs, remains unknown. In addition, although MENIN interacts with many cellular proteins, it does not possess any discernible functional domains apart from nuclear localisation (Guru *et al.* 1998, La *et al.* 2006) and export (Cao *et al.* 2009) signals. Such fundamental knowledge gaps and ambiguities have led to an incomplete understanding of the basic biology of *MEN1*, compounding the difficulty in reconciling MENIN's roles as both an oncogene and tumour suppressor in cancer. Here, we have used *in silico* data mining, gene expression analysis and literature searches to investigate the role of *MEN1* isoforms in cell function.

Materials and methods

MEN1 expression and splicing from GTEx

The Genotype-Tissue Expression (GTEx) portal (GTEx Analysis Release V8 (dbGaP Accession phs000424.v8.p2)) was interrogated with the search term 'MEN1' as the gene ID. Organ expression profile and exon junction expression data of *MEN1* were downloaded on 3 July 2023, with four tissues (brain – cerebellar hemisphere, brain – frontal cortex (BA9), cells – EBV-transformed lymphocytes and cells – cultured fibroblasts) excluded. Biological sex-specific expression data was downloaded on 18 July 2023. The GTEx Project was supported by

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the Common Fund of the Office of the Director of the National Institutes of Health, and by NCI, NHGRI, NHLBI, NIDA, NIMH and NINDS. *MEN1* reference sequences were obtained from GenBank and Ensembl (build 109) on 7 July 2023. Splice junctions of reference transcripts were determined by alignment to the *MEN1* reference gene (LRG_509; NG_008929.1) using BLAST. Expressed sequence tags (ESTs) were inspected in the University of California Santa Cruz (UCSC) Genome Browser.

MENIN isoform prediction

Putative ORFs in *MEN1* were identified in inferred full-length *MEN1* transcripts using Open Reading Frame Finder (National Library of Medicine).

Curation of MEN1 variants

cBioPortal, COSMIC, LOVD and ClinVar were queried between 23rd June 2023 and 27th June 2023 for this study. In cBioPortal, the gene '*MEN1*' was queried in all available studies ($n = 379$), equating to 190,354 samples from 181,665 patients, and all resultant data were downloaded. In COSMIC, the gene 'MEN1' was queried in all available studies, equating to 23,245 samples, and all resultant data were downloaded. From LOVD and ClinVar, all variants pertaining to the gene 'MEN1' were downloaded. For data from cBioPortal, COSMIC and LOVD, the data were consolidated to ensure there were no duplicate entries of the same variant within each dataset (Supplementary Fig. 4A). For ClinVar, only variants with the following clinical significance were retained – conflicting interpretations of pathogenicity, liikely pathogenic, pathogenic, pathogenic/likely pathogenic and uncertain significance. Variant Validator (Freeman *et al.* 2018) was used to obtain HGVS-compliant variant descriptions pertinent to the following references – human genome 38 (hg38), NM_001370259.2, NP_001357188.2, NM_000244.3, NP_000235.2, NM_001370251.2 and NP_001357180.2. Finally, Variant Validator output data across all three databases were consolidated to ensure there were no duplicate entries for the same variant, resulting in 2575 unique *MEN1* variants (Supplementary Fig. 4A). Genomic coordinates were then uploaded into Variant Effect Predictor (Martin *et al.* 2023) to predict the effect on the gene (e.g. 5'UTR variant, frameshift variant).

Review of the literature

Literature searches were performed in PubMed on 14th August 2023. First, the search term 'MEN1' was used to capture literature relevant to *MEN1*, and returned 4051 papers. Next, the search term 'MENIN NOT MEN1' was used to capture literature pertinent to MENIN, excluding those that would already have been captured with the search term 'MEN1'; this returned 521 papers. Thus, a total of 4572 papers were retrieved, relevant to *MEN1* and its encoded protein, MENIN. The abstracts of all papers were evaluated for mentions of MENIN-interacting proteins and/or MENIN mutants and biological function. Forty-seven papers were reviewed in full to determine the methodologies used for protein–protein interactions and the effects of *MEN1* variants on protein function.

RNA extraction and reverse transcription

HEK293T cells (150,000 cells per well of a 6-well plate) were plated in 2 mL of complete culture media (DMEM (Thermo Fisher Scientific) supplemented with 10% fetal calf serum (Thermo Fisher Scientific) and 1% penicillin/streptomycin (Thermo Fisher Scientific)) for 48 h. RNA was extracted from the cells using Trizol (Ambion/Thermo Fisher Scientific) as per the manufacturer's instructions. Five hundred ng of RNA was reverse transcribed to cDNA with AffinityScript Reverse Transcriptase (Agilent) as per the manufacturer's instructions. Amplification of the region spanning exons 7 and 8 of *MEN1* was performed with the following primers: hMEN1_Ex7_8_screen_F: GCCAAGACCTACTATCGGGA, hMEN1_Ex7_8_screen_R: AGCAGGTTGGGGATGACATC. PCR was performed with Taq polymerase (Qiagen) with an annealing temperature of 54°C for 35 cycles on a Veriti Thermocycler (Applied Biosystems). PCR products were separated by electrophoresis on a 2% agarose gel. Bands were gel extracted and cloned into pGEM-T Easy (Promega) for Sanger sequencing. Sequences were then aligned to LRG_509 to deduce splice junctions.

Results

MEN1 is widely expressed across human tissues

To profile the expression of *MEN1* across human tissues, we explored its expression in GTEx, where gene expression data in 50 tissues from 983 individuals obtained at autopsy has been deposited (Consortium, 2013). *MEN1* had broad tissue expression across both endocrine and non-endocrine tissues, with the highest expression seen in the brain cerebellum and the lowest in the left ventricle of the heart (Fig. 1A). Expression in tissues was similar between biological male and female tissue samples (Supplementary Fig. 1A).

MEN1 undergoes alternative splicing along the entire gene

Mapping of *MEN1* reference transcripts from GenBank and Ensembl to the *MEN1* reference gene revealed significant alternative splicing of *MEN1* along the entire length of the gene (Supplementary Table 1).

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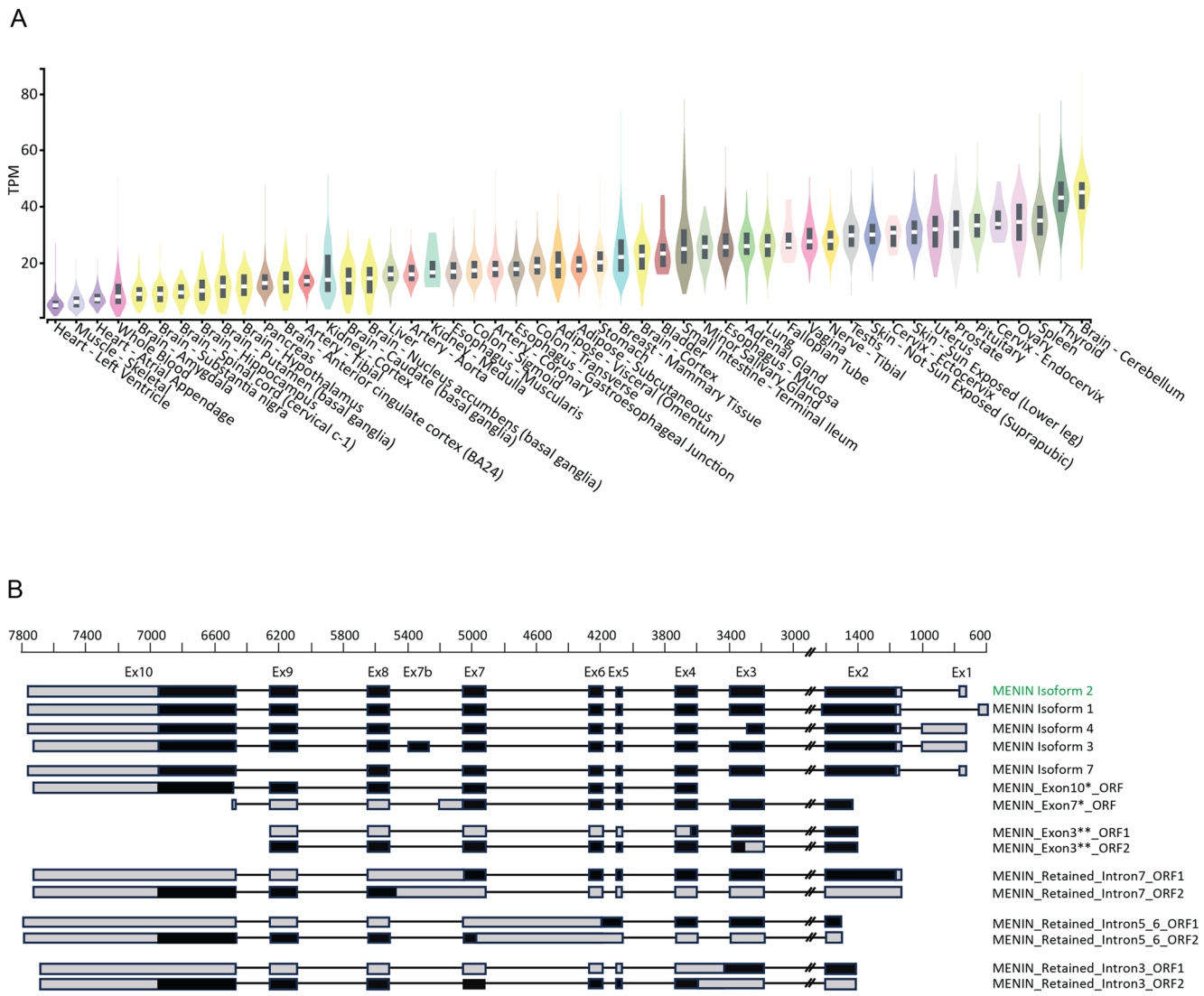

Figure 1

Expression and alternative isoforms of human *MEN1*. (A) Expression of *MEN1* across 50 human tissues, displayed as transcripts per million (TPM) and ranked by abundance. (B) Predicted MENIN ORFs (black) that generate different MENIN isoforms in representative *MEN1* transcripts. Green 'MENIN Isoform 2': encoded by the MANE select transcript.

The choice of reference transcript for any gene, including *MEN1*, can be contentious (Nelakurti *et al.* 2020, Perner *et al.* 2023), with possibilities including the longest transcript, longest ORF, most abundant transcript, or most clinically relevant variant (Morales *et al.* 2022). For *MEN1*, the 610-amino acid MENIN protein is the most extensively studied isoform (MENIN isoform 2) and is encoded by the most abundant transcript (see below). Thus, we chose to use the MANE select transcript NM_001370259.2, corresponding to this protein isoform, as the comparator in this work. The *MEN1* MANE select transcript contained ten exons, with translation predicted to initiate in exon 2 (Fig. 1B); the approximately 1.2 kilobase (kb) region upstream of the translation start site underwent extensive splicing to generate 5' untranslated regions (UTRs) of variable length and sequence in different reference transcripts (Supplementary Fig. 1B, Supplementary Table 1).

When present in transcripts, exons 4, 5, 6, 8 and 9 were invariant. All other exons underwent alternative splicing events (Fig. 1B), with one event for exon 2 (exon 2*, representative transcript NM_000244.4), two events for exon 3 (exon 3*, representative transcript NM_001370262.2 and exon 3**, representative transcript ENST00000394374.7) and one event for exons 7 (exon 7*, representative transcript ENST00000672079.1) and 10 (exon 10*, representative transcript ENST00000394376.6) observed (Supplementary Table 1). In addition, *MEN1* reference transcripts indicated the inclusion of an additional exon from within exon 7 (exon 7b, representative transcript NM_001407143.1),

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the skipping of exon 9 (representative transcript NM_001407152.1) and the retention of introns 3, 5–6 and 7 (Supplementary Table 1).

Alternative splicing of MEN1 occurs in vivo

It is imperative to understand whether alternative splicing of *MEN1*, as predicted by reference transcripts, occurs *in vivo* in cells and tissues, as they would potentially result in MENIN isoforms with different biological functions. Thus, we searched the UCSC Genome Browser, Genbank and GTEx for further support of alternative splicing of *MEN1*. The Genbank entry U93236.1 represents the first full-length ORF of *MEN1* cloned from a leucocyte cDNA library (Chandrasekharappa *et al.* 1997). This transcript has an identical ORF to the MANE select transcript (Supplementary Table 1). The use of exon 3* was also supported by a full-length ORF (BC002544.2) cloned from a choriocarcinoma sample, as well as by ESTs. The use of exon 2* and retention of introns 3, 5–6 and 7 were supported by ESTs (Supplementary Table 1) as well as short-read sequencing, as visualised in Integrated Genomics Viewer (IGV) tracks (Fig. 2A and B).

The use of exon 7b was supported by a partial EST (BE772061) (Supplementary Table 1) obtained from a prostate tumour. While this EST captured the splicing of exon 7b to exon 8, it lacked the 5' end of the exon (data not shown). To confirm the expression of this additional exon and to map its 5' boundary, we probed cDNA from HEK293T cells. PCR performed using primers designed in exons 7 and 8 revealed the expression of products of varying lengths (Fig. 2C). Sanger sequencing of the approximately 210 bp fragment revealed it to represent the canonical splicing of exons 7 and 8 (data not shown). On the other hand, the approximately 320 bp fragment demonstrated splicing of exons 7, 7b and 8 (Fig. 2D and E respectively), confirming the use of exon 7b *in vivo*. To the best of our knowledge, the alternative splicing of exon 3**, exon 7*, exon 10* and the skipping of exon 9 are not currently supported by cloned ORFs, ESTs or short reads.

Splice junction use suggests variable expression of MEN1 transcript variants

To further understand the alternative splicing of exons and infer relative isoform abundance, we analysed GTEx for short reads supporting splice junction use in *MEN1*. Junctions 12, 9, 8, 7, 6, 5, 3 and 1 were the most abundant, and when used together, would give rise to the MANE select transcript (Supplementary Fig. 2). Junction 4, which supports the splicing of exon 7b to exon 8, was also broadly and relatively highly expressed across all tissues examined. Junction 11, spanning exon 2* and 3, showed a more restricted tissue distribution and lower expression compared to junction 12, where exon 2 is spliced to exon 3. The lower expression of exon 2* was also evident in IGV, where the read coverage of the extra 15 nucleotides of this alternative exon was much lower than that of the rest of the exon (Fig. 2B). Finally, junction 10, which spans exons 3* and 4, showed the most restricted tissue distribution and lowest expression (Supplementary Fig. 2) but was nevertheless clearly detectable. Alternative splicing generating exon 3**, exon 7*, exon 10* and the skipping of exon 9 are not supported by splice junction reads in GTEx at present.

Alternative splicing could generate significant diversity in MENIN isoforms

To determine the consequences of *MEN1* alternative splicing on protein isoforms, we performed *in silico* translation and sequence alignment. The MANE select transcript is predicted to encode a single ORF, resulting in a 610 amino acid protein, MENIN isoform 2 (Supplementary Table 1, Fig. 1B and Supplementary Fig. 3). Relative to the MANE select transcript, the use of exons 2*, 3*, 7b and 10*, along with the skipping of exon 9, are all predicted to be in-frame, giving rise to protein isoforms of 615 (MENIN isoform 1), 575 (MENIN isoform 4), 652 (MENIN isoform 3), 607 (MENIN_Exon10*_Predicted_ORF) and 555 (MENIN isoform 7) amino acids, respectively (Supplementary Table 1, Fig. 1B and Supplementary Fig. 3).

Several *MEN1* reference transcripts are predicted to contain multiple ORFs or encode atypical isoforms. The first ORF, when exon 3** is used, when introns 3, 5–6 or 7 are retained, or the ORF when exon 7* is used, would result in a truncated protein with unique carboxy (C) termini (Fig. 1B and Supplementary Fig. 3). However, downstream methionine residues, whether in-frame (MENIN_Retained_Intron5_6_ORF2), from within introns (MENIN_Retained_Intron3_Predicted_ORF2, MENIN_Retained_Intron7_Predicted_ORF2), or from a different reading frame (MENIN_Exon3**_Predicted_ORF2), would also result in the translation of the C-terminal of MENIN but with unique amino (N) termini (Fig. 1B and Supplementary Fig. 3).

MENIN associates with interacting partners across its entire length

Focussing on full-length isoforms supported by cloned ORFs or ESTs, we mapped the insertions and deletions in MENIN isoforms 1, 4 and 3 relative to isoform 2. The solved crystal structure of MENIN isoform 2 shows it to resemble a curved left hand composed of α-helical folds and β-sheets (Murai *et al.* 2011, Huang *et al.* 2012, Shi *et al.* 2012). The five-amino-acid insertion in isoform 1 is predicted to occur immediately downstream of helix α6 in the thumb domain of MENIN, while the 35-amino-acid deletion in isoform 4 would lead to the loss of the β5 and α8 in the thumb domain of

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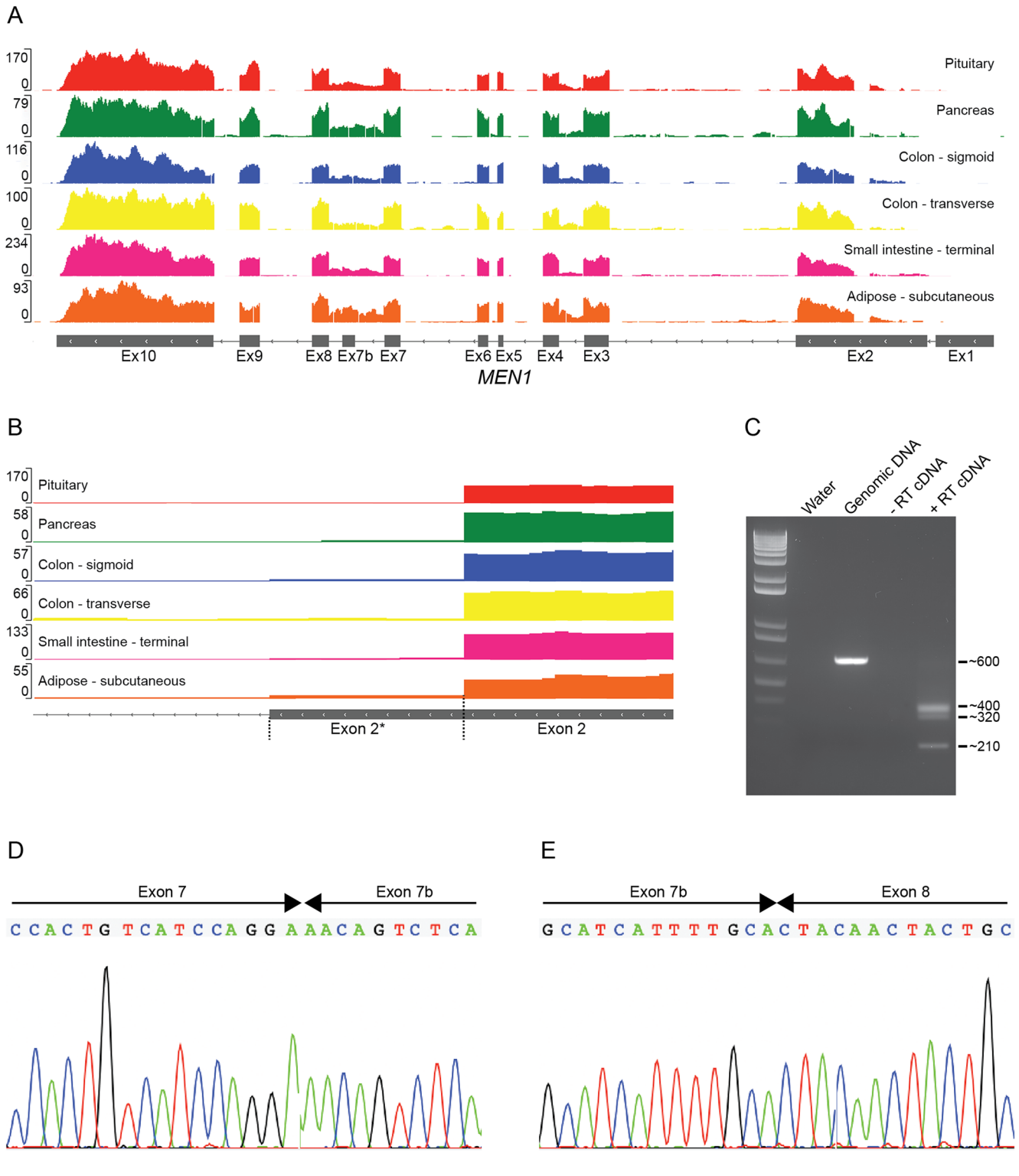

Figure 2

Expression of alternatively spliced exons in *MEN1*. (A) IGV screenshot of RNA-seq read coverage of *MEN1* in selected human tissues. (B) IGV screenshot of RNA-seq read coverage of the 3' end of exon 2 in selected human tissues. Boundaries of exon 2 and 2* are shown in the schematic below. (C) PCR amplification by primers spanning exons 7 and 8 of *MEN1* from cDNA synthesised from HEK293T RNA (+RT cDNA); Water (negative control), Genomic DNA (positive control), -RT cDNA (genomic DNA contamination of RNA). (D and E) Electropherogram traces of the 5' and 3' boundaries of exon 7b spliced to exons 7 and 8 respectively. RT – reverse transcriptase.

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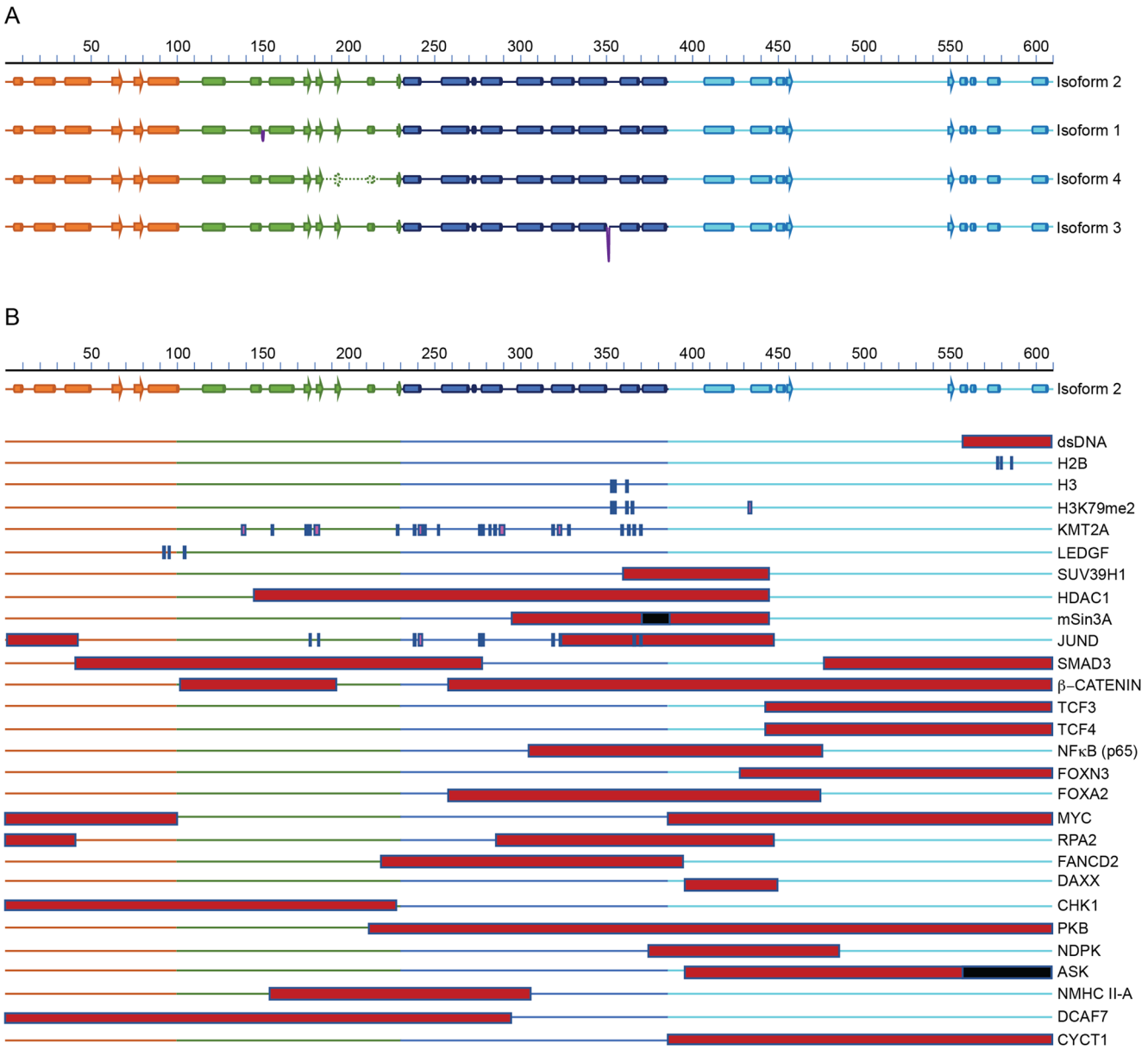

Figure 3

Schematic of MENIN. (A) Top – crystal structure domains of MENIN isoform 2, indicating regions that contribute to the heel (orange), thumb (green), palm (dark blue) and fingers (light blue). Bottom – superimposed MENIN isoforms 1, 4 and 3. Solid purple lines – amino acids inserted in specific MENIN isoforms, dashed line – amino acids lost in specific MENIN isoforms. Note that amino acids 460-519 remain unresolved in the crystal structure of MENIN. (B) Domains of MENIN required for interaction with partner proteins. Red – domains identified by deletion mapping, pink blue bound boxes – residues identified from crystallography experiments, black (mSin3A) – putative mSin3A interacting domain identified by sequence homology and black (ASK) – interacting domain mapped from patient-derived truncated MENIN variants.

MENIN (Fig. 3A). In addition, the 42-amino-acid insertion in isoform 3 is predicted to occur just downstream of α14 in the palm domain of MENIN (Fig. 3A).

Publications in PubMed from database inception to June 2023 were reviewed to map interacting domains on MENIN for other proteins/biomolecules. MENIN interacts with a wide spectrum of cellular partners (Supplementary Table 2) ranging from DNA, histones and chromatin modifiers to kinases, ubiquitin ligases and cytoskeletal proteins, with binding domains mapping along the entire length of MENIN (Fig. 3B). Given the protein diversity predicted by alternative splicing, MENIN isoforms 1, 3 and 4 could have altered protein–protein interactions relative to MENIN isoform 2. For instance, MENIN isoform 4 lacks a

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portion of the protein that interacts with partners including SMAD3 (Fig. 3B).

Most MEN1 variants are missense or lead to premature protein termination

To understand the spectrum of genomic variants in *MEN1*, we examined the public databases cBioPortal, COSMIC (predominantly somatic), LOVD and Clinvar (predominantly germline). About 2574 unique variants were identified across all databases post filtering (Supplementary Fig. 4 and Supplementary Table 3). Information on the origin of the allele (somatic and/or germline) was present for 2347 variants; 62% of variants were found only in the germline, 25% were somatic and 13% were found in both (Fig. 4A).

We next examined the types of genomic variants identified in *MEN1*. The majority of variants were either missense (approximately 50%) or led to frameshifts/premature protein termination (31%), with variants in intronic regions, UTRs, affecting splicing, resulting in in-frame insertions/deletions or synonymous changes varying in frequency from 1.6% to 6.6% (Fig. 4B). The proportion of missense to frameshift variants was roughly 2:1 for variants found exclusively in the germline genome and 1:1 for exclusively somatic variants (Supplementary Fig. 4B). Synonymous changes account for almost 10% of variants found only in the somatic genome and 2% of variants in the germline genome, post filtering (Supplementary Fig. 4B).

MEN1 variants could impact specific MENIN isoforms

Genomic variants in *MEN1* were observed along the entire ORF, beginning at the initiator methionine and including the stop codon (Supplementary Table 3). We noted specific variants that could impact individual isoforms of MENIN. Several variants in the 15-nucleotide extension of exon 2* could result in missense changes or the acquisition of a stop codon in MENIN isoform 1 (Fig. 4C and Supplementary Table 3). In addition, variants affecting the splice donor site of exon 2 (e.g. NC_000011.10:g.64809665-64809663, Fig. 4C) and exon 3 (e.g. NC_000011.10:g.64807890-64807889, Fig. 4D) could destroy the canonical splice donor sites and force the use of exon 2* and exon 3* respectively, resulting in MENIN isoforms 1 and 4. Finally, we examined exon 7b and identified two deletions and one single nucleotide variant that could impact MENIN isoform 3 (Fig. 4E and Supplementary Table 3).

MEN1 variants have disparate functional effects

The role of MENIN as both a tumour suppressor and an oncogene in tumourigenesis underscores the importance of understanding the biological consequences of variants identified in *MEN1*. We collated published studies on the biological effects of *MEN1* mutants to serve as a resource to guide future research. In total, we have curated 74 biological variables, including but not limited to promoter binding, transactivation potential and binding to other proteins (Supplementary Table 3). The best-characterised biological effect of MENIN variants is protein stability, having been investigated for 49 variants across multiple studies; about one-third of variants investigated ($n = 14$) were stable, with the remaining showing decreased protein stability.

Binding to JUND and subsequent repression of JUND transactivation potential and cooperativity with KMT2A have also been investigated (Supplementary Table 3). Roughly half of the variants investigated in the literature demonstrated strong binding to JUND (10/21) and KMT2A (4/7), with the remainder showing little interaction with the respective partners (Supplementary Table 2 and Table 1). Loss of binding correlated with loss of functional regulation by MENIN, but the reverse was not always true. For instance, both MENIN^{L22R} and MENIN^{W436R} (unstable variants) bound JUND with high affinity, but only the latter retained the ability to inhibit JUND-dependent reporter gene expression (Table 1). In addition, MENIN^{L22R} bound KMT2A but not the obligate COMPASS partner LEDGF (Yokoyama & Cleary 2008), yet demonstrated high methyl transferase activity on recombinant H3 (Table 1). Unsurprisingly, MENIN variants with no methyl transferase activity (e.g. MENIN^{H139D} and MENIN^{A242V}) could not catalyse H3K4me3 of the *Hoxc8* promoter or induce *Hoxc8* expression despite binding the cognate promoter (Table 1).

Men1 variants in the general population could impact biological properties of MENIN

We noted that about 13% of variants catalogued (329/2574) were found in general population databases (gnomADg AF and gnomADe AF), with the majority (314/329) present at a frequency of less than 0.1%. Intriguingly, most of these variants were also identified in germline databases (LOVD and ClinVar) that report variants associated with MEN1 syndrome, and around a third (110/329) were reported as somatic variants (Supplementary Table 3). Of note, two variants reported in the general population, MENIN^{R171Q} and MENIN^{R415Ter}, are functionally impaired in their ability to suppress foci formation by LCT-10 cells compared to wildtype MENIN (Supplementary Table 3). We also note that MENIN^{T344M}, recently reported as a resistance mutation to MENIN-KMT2A inhibitors (Perner *et al.* 2023), is observed at low frequency in the general population (Supplementary Table 3).

Discussion

Although one of the earliest cancer-associated genes identified, the biology of *MEN1* remains poorly understood. Herein, we highlight the role for alternative

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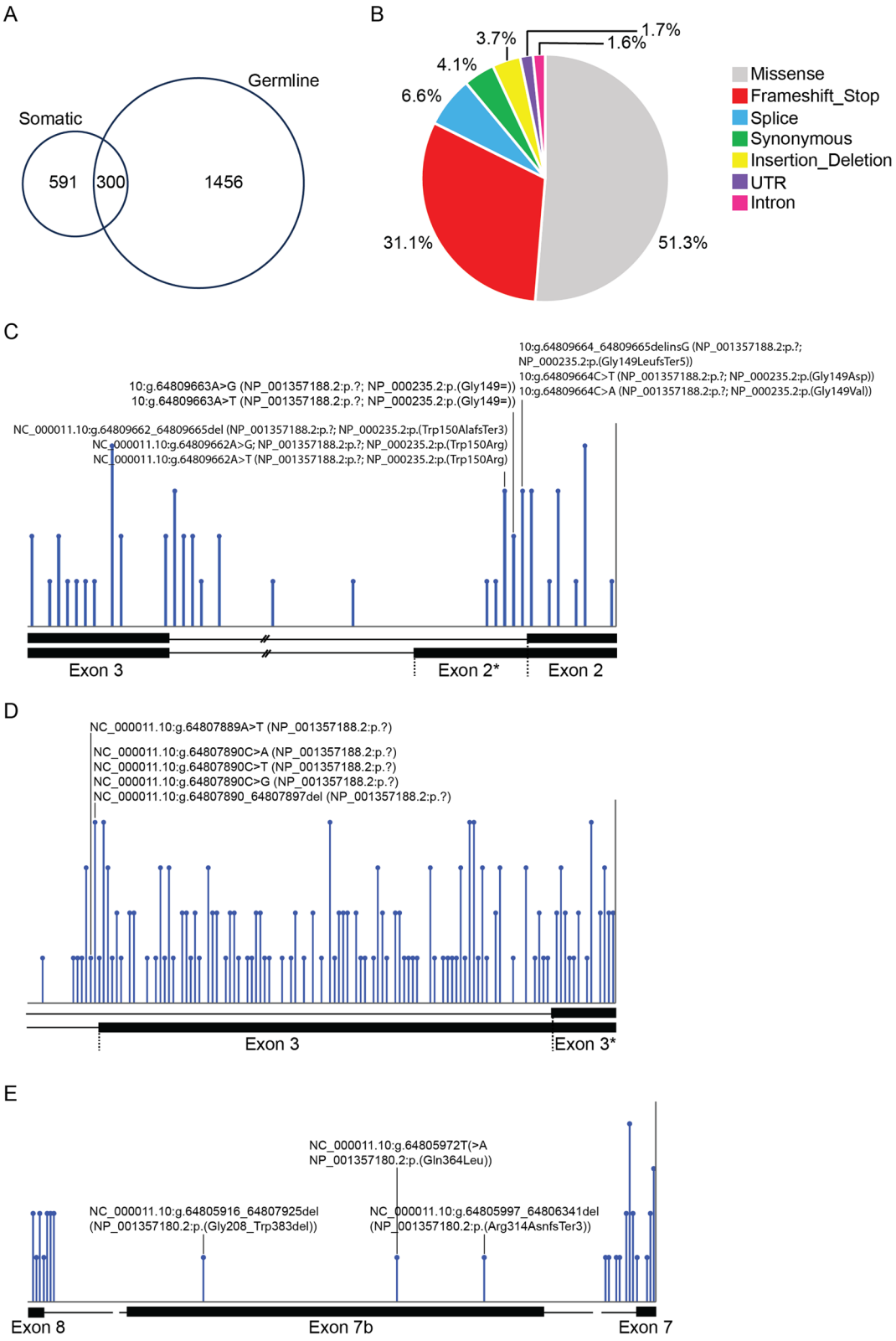

Figure 4

Variants identified in *MEN1*. (A) Source of *MEN1* variant alleles reported in public databases. (B) Functional consequences of variants identified in *MEN1*. (C–E) Frequency distribution of variants in the genome of *MEN1* around (C) exons 2 (3′) and 3 (5′), (D) exon 3 (3′) and (E) exons 7 (3′) and 8 (5′). NP_001357188.2 – MENIN isoform 2, NP_000235.2 – MENIN isoform 1, NP_001357180.2 – MENIN isoform 3.

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Table 1 Summary of selected biological features for specific *MEN1* variants reported in the literature. Derived and summarised from Supplementary Table 3.

Variant	Stability	JUND binding	Inhibiting JUND trans-activation	KMT2A binding	LEDGF binding	NMHC II-A binding	H3 methyl transferase activity (*in vitro*)	***Hoxc8*** promoter binding	***Hoxc8*** promoter H3K4me3	Induction of ***Hoxc8*** expression	H4 methylation
WT	Stable	High	Yes	High	High	High	High	High	High	High	High
L22R	Unstable	High	No	High	Low	None	High	Mod	–	No	None
H139D	Unstable	None	No	None	None	None	None	Mod	Low	No	–
A242V	Stable	None	No	Mod	Low	High	None	Mod	Low	No	None
W436R	Unstable	High	Yes	High	High	High	Mod	–	–	–	–

–, no data available.

splicing in generating MENIN isoform diversity that could be functionally relevant both in normal tissues and during oncogenesis. With emerging recognition of the importance of noncanonical ORFs in health and disease (Chen *et al.* 2020), further characterisation of MENIN isoforms is warranted. We also highlight several mutants that we propose should be investigated in parallel to better clarify MENIN function.

MEN1 is broadly expressed and undergoes dynamic alternative splicing

Previous studies demonstrated broad *MEN1* expression in embryonic and adult tissues in man (Lemmens *et al.* 1997, Wautot *et al.* 2000) and mice (Stewart *et al.* 1998, Bassett *et al.* 1999, Guru *et al.* 1999), with transcripts of variable lengths noted in human, mouse, rat and *Drosophila* (Lemmens *et al.* 1997, Stewart *et al.* 1998, Bassett *et al.* 1999, Guru *et al.* 1999, Karges *et al.* 1999, Guru *et al.* 2001). Expanding these observations, we have demonstrated that *MEN1* is expressed in all human tissues examined in GETEx and have identified three sources of transcript diversity – alternative transcription start sites as previously reported (Fromaget *et al.* 2003), significant intron retention and alternative splicing of exons. Intron retention is seen in over 80% of protein-coding genes (Middleton *et al.* 2017) and affects several introns of *MEN1*. Given the mapped size of the *MEN1* gene (approximately 6.8 kb), it is likely that intron retention accounts for some of the longer transcripts previously observed by Northern blot analysis (Lemmens *et al.* 1997).

We noted extensive alternative splicing of exons along the entire length of *MEN1*, including previously reported alternative splicing of 5' UTR exons (Karges *et al.* 1999, Khodaei-O'Brien *et al.* 2000, Forsberg *et al.* 2001) and the use of exons 2* (Chandrasekharappa and Teh 2003) and 3* (see below). In addition, we also provide multiple lines of evidence to support the use of a novel exon, exon 7b. The broad tissue expression supports a physiological role for *MEN1* in almost all tissues and is consistent with the fact that conventional deletion of *Men1* is embryonic lethal in mice (Crabtree *et al.* 2001), but it leaves open the question of why the pathological effects of *MEN1* in cancer manifest in a more restricted set of tissues. We suggest that alternative isoform expression could play a role in this tissue bias. In support of this, the variable read depth of sequences spanning *MEN1* splice junctions in GTEx suggests dynamic *MEN1* isoform expression across tissues. We deduce that the most abundant transcript encodes MENIN isoform 2, the 610 amino acid protein. However, we also infer that the use of exon 7b is relatively high in tissues such as the small intestine, liver and thyroid, while other alternative splicing events were lower but still detectable. Furthermore, there remain uncharacterised *MEN1* variants *in vivo*, given that we amplified an uncharacterised 400 bp fragment

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across exons 7 and 8. We support MENIN isoform 2 being regarded as the reference isoform for this gene because of its broad and abundant tissue expression and the wealth of literature pertinent to this isoform.

Alternative splicing of MEN1 may generate protein isoforms with altered function

The consequences of altered 5' exon use and intron retention for *MEN1* remain to be functionally characterised, with effects on ribosome recruitment and translation (Chen *et al.* 2017, Hollerer *et al.* 2019) possible. However, we predict that alternative splicing of protein coding exons and intervening introns could also be an unexpected source of isoform diversity. While the inclusion of introns can result in nonsense-mediated mRNA decay or altered splicing rates (Monteuuis *et al.* 2019), they could also provide alternative initiator methionine for the translation of the C-terminal of MENIN but with unique N-termini, as has been reported in yeast (Hossain *et al.* 2016).

We predict that alternative splicing of protein-coding exons generates remarkable diversity in MENIN with diverse protein functions *in vivo*. Isoforms 1 and 4 would have alterations in the thumb domain, while isoform 3 has an insertion in the palm domain of MENIN. Given that protein interaction interfaces occur along the entire length of the protein, it is likely that the different isoforms have different biological functions. In support of this, isoform 4, which lacks a portion of MENIN that includes the SMAD3 binding interface (Fig. 3), cannot bind SMAD3 or support SMAD3-dependent reporter gene expression (Canaff *et al.* 2012).

Genomic variants in MEN1 in cancer have altered protein function and could impact MENIN isoform expression

We have catalogued 2574 unique *MEN1* variants in public databases up to June 2023. The majority were reported in germline compared to somatic genomes, similar to previous studies (Lemos & Thakker 2008, Nelakurti *et al.* 2020). The reason for this bias is unknown but raises the possibility of negative selection of mutations in *MEN1* in somatic cancers and hints at an oncogenic role for MENIN there, in contrast to its tumour-suppressive role in MEN1 syndrome. We note that pan-cancer analyses indicate preferential accumulation of synonymous mutations in oncogenes over tumour suppressor genes (Supek *et al.* 2014) at frequencies similar to what we observe for *MEN1* in somatic samples here.

Reported variants in *MEN1* occur along the entire length of the gene, including in exons 2* and 7b that are unique to MENIN isoforms 1 and 3 respectively. A significant proportion of patients presenting with MEN1 syndrome currently do not have germline mutations identified (Lemos & Thakker 2008). Although clinical overlap with other cancer syndromes could account for a proportion of these, variants in currently unscreened genomic regions of *MEN1* could explain other cases. One third of the variants identified in *MEN1* would result in the introduction of a premature stop codon in MENIN. While such truncating variants would intuitively be expected to result in the loss of portions of the carboxy terminal and thus affect protein function (Ikeo *et al.* 2004, Duan *et al.* 2023), protein instability and degradation by the proteasome is also a common outcome in MENIN (Shimazu *et al.* 2011).

The functional consequences of missense variants, which make up 50% of variants in *MEN1*, are more varied. A comprehensive review of the literature identified 74 biological variables for MENIN, with the most studied variables being protein stability and interactions with JUND and KMT2A. MENIN missense variants display a combination of responses to these variables (Supplementary Table 3). Such complexity highlights both the gaps and challenges of understanding MENIN biology in cancer. While the sheer number of variants in *MEN1* can make addressing this challenge a daunting prospect, we believe that reducing this to a smaller cohort with complementary biological outcomes is a reasonable strategy to prioritise variants for future research. Reflecting the diversity of biological outcomes in protein stability, protein–protein interactions, and histone methylation (Table 1), we propose that $MENIN^{L22R}$, $MENIN^{H139D}$, $MENIN^{A242V}$ and $MENIN^{W436R}$ could form a limited set of pathogenic *MEN1* variants that are investigated together to dissect a wider array of functional effects of MENIN.

We believe that aberrant exon use, and subsequent isoform expression, is a key contributor to altered *MEN1* function in cancer. Almost 7% of *MEN1* variants in our study are predicted to alter splicing. Variants that disrupt canonical splice sites can promote alternative exon use, and indeed, mutations affecting the splice donor site of intron 3 of *MEN1* are known to favour the use of exon 3* in cells (Hai *et al.* 2000, Canaff *et al.* 2012, Karges *et al.* 2000). We have curated several variants that may similarly promote the use of exons 2* and 3*. Of importance, 10% of exonic variants in the Human Gene Mutation Database promote alternative exon splicing (Soemedi *et al.* 2017), suggesting that alternative splicing of *MEN1* in oncogenesis may be more widespread than appreciated.

It is generally accepted that the penetrance of *MEN1* variants in MEN1 syndrome is high, with over 90% of individuals reporting some manifestation of the syndrome (Brandi *et al.* 2021). Thus, it is intriguing that a small, but significant, number of variants curated in this study were present in the general population database gnomAD at low frequencies. Limited functional data exist for these variants, but two have compromised biological activity. It remains to be resolved whether time and/or modifiers are required

for oncogenesis for potentially low penetrance *MEN1* variants in the general population. Given that at least one of these variants confers resistance to small molecule inhibitors, pharmacogenetics of *MEN1* may be of clinical importance in cancer care.

One limitation of our current work is that it has involved the analysis of short-read sequencing data. While informative, the identity of *MEN1* isoforms can only be inferred at this stage; long-read sequencing is needed to accurately identify isoform expression *in vivo*.

Supplementary materials

This is linked to the online version of the paper at https://doi.org/10.1530/EO-24-0014.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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