Helical ultrastructure of the oncogenic metalloprotease meprin α in complex with a small molecule hydroxamate inhibitor.

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The zinc-dependent metalloprotease meprin α is predominantly expressed in the brush border membrane of proximal tubules in the kidney and enterocytes in the small intestine and colon. In normal tissue homeostasis meprin α performs key roles in inflammation, immunity, and extracellular matrix remodelling. The latter activity is furthermore important for driving aggressive metastasis in the context of certain cancers such as colorectal carcinoma. Accordingly, meprin α is the target of drug discovery programs. In contrast to meprin β, meprin α is secreted into the extracellular space, whereupon it oligomerises to form giant assemblies and is the largest extracellular protease identified to date (∼6 MDa). Here, using cryo-electron microscopy, we determine the high-resolution structure of the zymogen and mature form of meprin α, as well as the structure of the active form in complex with a prototype small molecule inhibitor and human fetuin-B. Our data reveal that meprin α forms a giant, flexible, left-handed helical assembly of roughly 22 nm in diameter. We find that oligomerisation improves proteolytic and thermal stability but does not impact substrate specificity or enzymatic activity. Furthermore, structural comparison with meprin β reveal unique features of the active site of meprin α and helical assembly more broadly.

Metalloprotease | Meprin α | Structural biology | Immunity | Cryo-electron microscopy

Introduction

Proteases critically underpin numerous processes on various levels via post-translational modification of proteins; these include cellular functions such as cell proliferation and differentiation (1, 2), necrosis (3) and apoptosis (4), angiogenesis (5), migration (6, 7) and more. Resultantly, proteases represent a fundamentally crucial component of normal cellular function and as such abnormal expression or dysregulation can be linked to various diseases. For these reasons, proteases are the attention of drug discovery programs and are exploited as diagnostic markers (8).

Astacin proteases, a subfamily of metzincin superfamily, are found in a variety of different species of vertebrates, invertebrates and bacteria. Meprin α, together with the evolutionary related meprin β, represent a subgroup of astacins found in vertebrates only (9). As with all astacins, the primary structure of the active site is characterized by two conserved motifs: the zinc-binding motif (HExxHxxGxx-HExxRxDRD) and the methionine-turn SxMHY (10, 11).

Both meprin α and β are highly expressed in epithelial cells of kidney and intestine and they have been demonstrated to a minor extent in intestinal leukocytes, skin, lung, brain, and certain cancer cells (10, 12–17). Within these tissues, numerous potential substrates are present including bioactive proteins and peptides, growth factors, adhesion molecules, and components of the extracellular matrix (ECM) (18). Differential regulation and localisation of meprins provides access to these substrates, enabling specific functions in pro- and anti-inflammatory responses, ECM assembly and remodelling, cytokine activation and signalling, as well as cell-cell adhesion (19–26).

A prominent example is the pro-collagenase function. Both meprin α and β are N- and C-pro-collagenases of pro-collagen I and III and are, as such, important for collagen assembly and tensile strength (19, 21, 27). Likewise, the pro-inflammatory IL1β is activated by both enzymes, although the product resulting from processing by meprin α is two-
Fig. 1. The cryo-EM reconstruction of helical meprin α. a. A representative particle of meprin α is roughly 100–150 nm in length (vertical scale bar, ∼50 nm) and corresponding two-dimensional class averages of segmented meprin α particles. Dashed box shows the magnified view of the reconstructed segment. Homodimeric meprin α subunits are coloured alternating in black and grey. A central dimeric subunit is shown in purple and pink. b. A single meprin α subunit illustrating the three globular domains (M12A [red], MAM [blue] and MATH [orange]) and the helical pro-domain (dark grey). c. Corresponding atomic model of a single meprin α subunit and domain schematic coloured as in (b). d. View along the filament axis of the meprin α helix showing a segment of four meprin α subunits. The catalytic M12A domain (red) is found on the inside of the helical tube (lumen), while the MAM and MATH domains (both light grey) are located on the periphery of the helix. e. The meprin α helix interface is defined by two head-to-tail dimers of the MAM and MATH domains which join adjacent meprin α homodimers. In both (d) and (e) a dashed line outlines a single meprin α covalent homodimer.

To achieve their function, both meprins cleave a specific but distinct motif. In meprin β this is characterised by a striking preference for negatively charged amino acids in the P1' position unlike most other extracellular proteases (33). In contrast, meprin α favours neutral aliphatic and aromatic residues with less pronounced preference for acidic amino acids (33). As such, meprin α and β partially discriminate between substrates, resulting in distinct activity profiles. These differences are thought to drive distinct functional properties in vivo. Nevertheless, as exemplified above, some overlap in substrates also results in redundant physiological roles.

Of note, meprin α has been suggested to drive aggressive metastatic colorectal cancer (34–36). Epithelial cells of the normal human colon secret meprin α into the lumen, whereas in a colon carcinoma cell line meprin α is secreted in a non-polarized fashion increasing substrate accessibility. Moreover, high concentrations of meprin α activity in primary tumour stroma promotes tumour progression and metastasis. This is thought to occur due to meprin α pro-migratory and pro-angiogenic activity by way of ECM remodelling and transactivation of the EGF/EGFR/MAPK signalling pathway by shedding the ligands EGF and TGFα (37). Interestingly, in-
hibitation of meprin α by actinin, a naturally occurring inhibitor, was able to abrogate these effects in vitro, suggesting meprin α is a promising target for therapeutics (37). Importantly, meprin α dysregulation does not necessarily impact meprin β, whose important regulatory and physiological functions are maintained. For example, meprin β shows a protective effect in IBD and promotes mucus turnover by cleavage of MUC2, preventing bacterial overgrowth in intestinal mucosa (31, 38, 39).

Thus, non-specific inhibition of meprins may have either beneficial or deleterious effects on health depending on the context. For these reasons, specific inhibitors of meprin α have been sought to mitigate its function in disease progression and to investigate the pathophysiological role of meprins in further detail. In general, specific inhibitors for meprin α and meprin β separately might be useful to treat certain diseases, such as progressive cancers, without disrupting the physiological function of the corresponding homologue. In order to provide controlled inhibition of meprin α functions while maintaining the physiological function of meprin β, and vice versa, significant effort has been directed to develop protease specific compounds (40–42). However, owing to high conservation between the two enzymes this has been non-trivial and thus off-target inhibition of proteolytic activity is a current limitation of existing candidates.

Both meprin α and β are expressed as glycosylated zymogens. The domain architecture of both proteases comprises an N-terminal protease domain (M12A), a MAM (meprin, A-5 protein, and receptor protein-tyrosine phosphatase μ) domain, a MATH (Meprin and TNF receptor-associated factor [TRAF] homology) domain, an EGF-like domain, and, lastly, a short transmembrane and cytosolic region (Supp Figure S1a, b). Both proteases are enzymatically processed to remove the autoinhibitory N-terminal prodomain by trypsin-like activities (for example by the fibrinolytic protease, plasmin) in order to form the active protease (43).

A key difference between meprin α and meprin β is that the former includes a furin cleavage site between the MATH and EGF-like domain. This permits furin-mediated shedding of meprin α within the secretory pathway and, subsequently, secretion into the extracellular milieu (44, 45). In contrast, meprin β remains predominantly membrane associated, with rare shedding events mediated via ADAM10/17 proteolytic cleavage of the sequence immediately preceding the transmembrane domain (17, 18, 46, 47). Shedding of meprin β has been observed to drive distinct substrate specificity and function suggesting localisation affects accessibility to certain substrates (48). Furthermore, expression of meprin α is localised in the stratum basale of the epidermis, while meprin β is found in the stratum granulosum (15). As such, some functions of meprin α and meprin β may be dependent on their localisation and tissue distribution.

In regard to quaternary structure, both enzymes form co-valently (disulphide) linked homo- and heterodimers (10). The quaternary complex is critical for the localisation and oligomerization of meprin α. Heterodimeric meprin αβ remains tethered at the plasma membrane via membrane bound meprin β (44). Such heterodimers can further interact to form heterotetramers (48–50). In contrast, furin-mediated release of meprin α homodimers results in the formation of meprin α oligomeric assemblies, as has been shown for recombinant rat meprin α (50). The non-covalent interface that mediates this homo-oligomerisation was mapped by cross-linking mass spectrometry to the MAM domain (51). In contrast, meprin β homodimers do not oligomerise when tethered to or upon shedding from the membrane (10).

In addition to low stoichiometric assemblies, the homodimer of meprin α is capable of forming large non-covalently associated soluble oligomers with a reported size of up to 6 MDa (50). Meprin α is, therefore, the largest known secreted protease complex. The oligomers have been characterized by means of SEC, EM and light scattering to be comprised of a heterogeneous population of ring, circle, spiral, and tube-like structures (50). While the crystal structure of meprin β has been solved in the active, inactive and inhibitor bound states (53, 54), the structure of meprin α remains to be determined.

The heterogeneity of meprin α oligomers precludes structure determination by X-ray crystallography, accordingly, we used cryo-EM to determine its structure. Our data show that both thezymogen and active form of the meprin α ectodomain (i.e., the region released through furin cleavage) is capable of self-association to form a flexible, giant helical assembly. Using a single particle like approach we determined the high-resolution reconstructions of meprin α in its zymogen and active state. Additionally, we determined structures of meprin α in the presence of a prototype selective inhibitor and the native inhibitor human fetuin-B. This structure represents a valuable tool for rational drug design and provides a basis for structural comparisons between the homologous enzymes. Lastly, we propose possible mechanisms for further investigation that may implicate meprin α polymerisation as an important component in correct function and regulation.

**Results**

Meprin α forms a giant flexible left-handed helical filament. It has previously been observed that meprin α function requires two proteolytic events. Firstly, the zymogen is proteolytically shed from the membrane via furin proteases, thus releasing these into the extracellular milieu. After shedding from the membrane, meprin α dimers associate non-covalently to form oligomers in the MDa range. Secondly, the zymogen is converted to the active state by proteolytic removal of the pro-peptide. To characterise the structural and biochemical properties of meprin α, we expressed a truncated recombinant construct in S2 cells that lacked both the EGF-like and transmembrane regions (Supp Figure S1a, b). The secreted wild type meprin α was purified from the conditioned media via hydrophobic interaction followed by affinity chromatography (Supp Figure S1c, d). Since size exclusion chromatography regularly resulted in loss of protein and activity this method was avoided. Activation of the zymogen was accomplished by way of magnetic trypsin beads. These enabled the cleavage of the pro-peptide and facilitated subse-
quent removal of trypsin without further dilution of the protein sample by additional purification steps.

Inspection of the recombinant material by electron microscopy revealed meprin \( \alpha \) forms extraordinarily large and flexible helical filaments (Figure 1a, Supp Movie 1, Supp Table 1). To obtain the structure of these filaments, we resorted to a single particle-like analysis to overcome the intrinsic flexibility and symmetry breaking in silico (Supp Figure S2). In this approach, \( \sim 20 \) nm segments of the filament were reconstructed without symmetry generating a 10–12 Å resolution reconstruction, which suffered from errors in alignment due to global flexibility. This reconstruction enabled non-overlapping sub-regions consisting of two meprin \( \alpha \) dimers to be localised within the original extracted particles with roughly nanometre precision. High resolution refinement of these sub-regions (to 2.4–3.4 Å resolution) was achieved by treating these as independent single particles after masking and subtracting the signal of neighbouring segments, thereby overcoming the prohibitive alignment errors resulting from continuous conformational heterogeneity of the filament.

The meprin \( \alpha \) oligomer resembles a spring of roughly 22 by 200 nm in dimensions (Figure 1a, Supp Figure S3), with some filaments observed to be greater than 500 nm long (\( \sim 27 \) MDa). We note that filaments of actomyosin (55) and the microtubule (56) possess extensive contacts between neighbouring turns. In contrast, the meprin \( \alpha \) filament lacks such contacts, forming a highly spacious helical groove of roughly \( \sim 7 \) nm and a hollow core of \( \sim 15 \) nm (Figure 1a). Resultantly, meprin \( \alpha \) is intrinsically flexible, observed to expand, contract, and bend along the axis of the helix (Supp Figure S3a; Supp Movie 2).

Each meprin \( \alpha \) subunit strongly resembles the homologous protein meprin \( \beta \). The subunit is a compact triad comprising the globular M12A protease, MAM and MATH domains (Figure 1b, c). In a single asymmetric unit of the helix, two meprin \( \alpha \) subunits homodimerise into a C2 arrangement via the M12A and MATH domains. The meprin \( \alpha \) M12A domain is typical, containing the catalytic zinc ion within the active site. Each M12A domain is positioned on the inner surface of the helix, jutting inwards forming a triangular arrangement (Figure 1d). The MAM and MATH domains buttress the protease domain, forming the outer structure of the helix interacting via a head-to-tail arrangement (Figure 1e), while at the dimer interface a conserved disulphide bond stabilises MAM/MAM interactions. This arrangement of dimers forms an indefinite filament which coils into a left-handed helical ultrastructure.

Cisplatin treatment is known to cause an increase of meprin \( \alpha \) in urine by inducing damage to epithelial cells in the kidney (57). To determine whether higher-order oligomers could be found within native source material, we conducted an analytical size-exclusion chromatography anal-

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**Fig. 2. Meprin \( \alpha \) helices are defined by two key interfaces.**

a. Segment of meprin \( \alpha \) helix showing a single helical interface (blue, orange) defined by the MAM and MATH domains. High magnification view of this region is shown (right) as a cartoon model.

b. Boxed region of the MAM/MATH interface illustrating key residue contacts forming at the helical interface. Visible is R372 which forms key salt bridges with adjacent aspartic acids.

c. Local sequence alignment of MAM/MATH domains between meprin \( \alpha \) and meprin \( \beta \) showing multiple charge swap and charge loss mutations.

d. Meprin \( \alpha \) homodimer interface showing covalent C308 disulphide bridge.

e. Frequently occurring mutation associated with cancers (curated from the COSMIC database (52) are shown as green spheres. These residues map to the MAM/MATH domains which define the helical interface.
ysis of mouse urine after cisplatin treatment. Indeed, we observed evidence that large meprin α oligomers do exist in the urine of mice treated with cisplatin, albeit these were smaller than recombinant wild type meprin α (Supp Figure S1e).

**Analysis of meprin α oligomisation.** The assembly of meprin α is apparently governed by two unique interfaces (Figure 2a, b). The dimer interface is most extensive (1374 Å²) and highly conserved between both meprin α and meprin β (Figure 2c, Supp Figure S4, S5). This interface is defined by the M12A protease and MAM domains, which are dependent on a conserved MAM/MAM disulphide bond to form the covalently linked homodimer (Figure 2d). Two identical MAM/MATH interfaces (610 Å²) each provided by two homodimers together drive the head-to-tail formation of the non-covalent helical assembly of multiple homodimers (Figure 2a, b). This latter interface is defined by extensive charge complementarity and salt bridges between the MAM and MATH domains and has a calculated cumulative solvation free energy (∆G) of −5.2 kcal mol⁻¹.

In contrast to meprin α, shed meprin β does not form higher-order oligomeric species. Superposition of MAM/MATH domains of meprin β and meprin α reveal the helical interface is abolished in meprin β due to multiple charge swap and loss-of-charge mutations (Figure 2c). Furthermore, multiple non-conserved residues within the MAM groove result in steric effects that likely further hinder formation of a stable interface (Figure 2c). In this regard, we note that frequently occurring cancer-associated mutations cluster to the MAM/MATH domains and, therefore, the interface of meprin α helices (Figure 2e, Extended Data 1) (52). In contrast, meprin β cancer-associated mutations more frequently cluster within the M12A protease domain (Extended Data 1) (52).

To assess the impact of these interfaces, we produced three mutants of meprin α by abolishing a salt bridge found at the helix interface (R372T and R372A) and the intermolecular disulphide bridge that stabilises the homodimer interface (C308A). No discernible differences could be observed between wild-type, R372T, and R372A meprin α by reducing and non-reducing SDS-PAGE, whereas mutant C308A appeared to be monomeric in all conditions (Supp Figure S1c, d). MALDI-TOF mass spectrometry (MS) determined the molecular mass of meprin α C308A to be exclusively monomeric (single charged ion peak at 75 kDa), while an additional disulphide bridged dimeric species (∼130 kDa) was observed for both wild-type and R372T meprin α (Supp Figure S6a). For all variants, thezymogen form was roughly 6 kDa higher in mass than the activated counterpart consistent with the removal of the propeptide. Further, all forms had a molecular mass that exceeded the calculated mass by ∼8 kDa, most likely corresponding to extensive glycosylations as observed in the cryo-EM reconstruction.

Further analysis by multi angle dynamic light scattering (MDALS) revealed different hydrodynamic radii for meprin α C308A and R372T variants, indicating the covalent and non-covalent interfaces were disrupted by mutagenesis (Supp Figure S6b). Wild type meprin α itself is polydisperse (hy-
Fig. 3. Meprin α oligomers are proteolytically and thermally stable compared to lower-stoichiometry variants. 

a. Periodic arrangement of the active site and pro-domain are highlighted as yellow in the surface representation of an idealised meprin α helical segment. 

b. Example curve representing a series of first-order rates (determined by fitting the linear region of fluorescence versus time) at a given meprin concentration. The $k_{\text{cat}}/K_m$ is determined from this graph for several meprin concentrations and in triplicate. 

c. First order rate constants ($k_{\text{cat}}/K_m$) of meprin α and variants for small fluorogenic peptide cleavage. Oligomeric state does not appear to affect rate of cleavage of small molecule substrate. 

d. Inhibitory constant ($IC_{50}$) of meprin α inhibitors. Determined by fitting and normalising the linear region of fluorescence versus time, in the presence of varying amount of inhibitor at a fixed meprin α concentration. 

e. Globular proteinaceous inhibitor, murine fetuin-B, and small molecule compound 10d were unaffected by oligomeric state. 

f. Proteolytic stability of meprin α and variants against trypsin and plasmin. Meprin α oligomers were more stable compared to lower-stoichiometric variants. 

g. Meprin α thermal stability measured by nanoDSF. Meprin α possess superior thermal stability compared to variants that lack either the disulphide bridge (most drastic) or helical interface interactions. All measurements are statistically significant to all others (p<0.0001, ****) unless otherwise indicated.
α (Figure 3g). Therefore, oligomerisation may play a role in protein regulation by way of increasing half-life in vivo.

Unlike small molecule substrates and inhibitors, which are not sterically occluded by the helical packing, we investigated the inhibition of meprin α in vitro by murine fetuin-B, a 42 kDa globular protein. Remarkably, we observed no significant difference in the inhibitory capacity of fetuin-B between oligomeric and monomeric variants of meprin α indicating the oligomer does not interfere with binding (Figure 3d, e). Molecular docking analysis of fetuin-B to the meprin α oligomer instead suggests a compact, intercalated packing is possible that is not impacted by the helical rise of meprin α (Supp Figure S8a-d). Further, the interfaces predicted to form this meprin α/fetuin-B oligomer are conserved (58, 59) (Supp Figure S8f). This intercalated packing of fetuin-B is suggestive of possible higher order inhibitory oligomers. We therefore imaged meprin α in complex with human fetuin-B by cryo-EM and generated a 3.7 Å resolution reconstruction (Supp Figure S8g, Supp Figure S9). Indeed, this structure confirms the intricate packing suggested by the molecular docking analysis, albeit with some degree of flexibility. The presence of a conserved interface suggests the oligomeric nature of meprin α confers a selection pressure on fetuin-B and supports the notion that meprin α oligomers exist in vivo.

Inhibition of meprin α. Comparison between the cryo-EM reconstructions of the zymogen and activated states reveal subtle conformational changes about the M12A catalytic domain (Figure 4a-c, Supp Table 1, Supp Figure S9). The pro-peptide forms a small helical cork which plugs the active core and thus sterically occludes the active site (Figure 4d). Cleavage of an exposed flexible loop, defined by residues L61-L67 located distal to the active site, releases the pro-peptide and exposes the catalytic core of the active site. Accordingly, the steric occlusion of the active site represents the major autoinhibitory mechanism. Upon activation, the two lobes of the M12A catalytic domain collapse around the active site (RMSD 3.3 Å), permitting the formation of a more tightly folded active site core (Figure 4e).

The majority of known meprin inhibitors possess a hydroxamic acid moiety that targets the central zinc atom of the active site (60). Thus previous generations of inhibitors, e.g. actinonin or sulphonamid-based compounds, exhibit poor specificity and can lead to off-target effects by inhibiting other metalloproteases. In particular, there is a need for specific inhibitors of meprin proteases that target either meprin α or meprin β. Our group has previously developed a small molecule inhibitor of meprin α, compound 10d (3-[bis(1,3-benzodioxol-5-ylmethyl)amino]propanehydroxamic acid) (42). The compound also binds to the zinc via a hydroamate moiety, but is built on a modified scaffold, i.e. a tertiary amine functionalised with two 1,3-benzodioxole groups. This modified scaffold leads to a high selectivity over other zinc-
metalloproteases, i.e. MPPS and ADAMs. Compound 10d shows promising inhibitory activity and furthermore is specific to meprin α with an IC₅₀ of 160 nM versus 2950 nM for meprin β.

To characterise the structural basis of binding and mechanism of inhibition by the small molecule inhibitor we determined the structure of meprin α after co-incubation with compound 10d. Inspection of the active site revealed new cryo-EM density positioned deeply within the groove of the active site (Figure 4c, f). Chemical docking simulations were used to flexibly fit the ligand structure to the density, revealing interactions between the hydroxamic acid group and putative zinc cation core of the catalytic triad as expected (Figure 4f, g). The two 1,3-benzodioxole groups provide further supporting interactions, predominantly from hydrophobic interactions. One benzodioxole group is deeply buried within the S1’ pocket forming an anchor point, while the other sits flush against the central helix of the M12A active site. Notably, due to the large width of the M12A groove, which accommodates the entire pro-domain α-helix, relatively few contacts are present between the M12A lobes and compound 10d. As such, we observe some degree of inhibitor mobility as assessed by loss of ordered cryo-EM density. Nevertheless, in this position both the catalytic zinc and binding pocket are sterically occluded by compound 10d, thereby inhibiting the proteolytic function of meprin α (Figure 4g).

Despite the selectivity of compound 10d for meprin α most contacts of the interaction are strongly conserved between meprin α and meprin β (Supp Figure S10). Notably, however, R238 of meprin β (R242 in meprin α) adopts a distinct rotamer position relative to meprin α, due to charge repulsion with R146 (Y149 in meprin α). In this position, R238 of meprin β sterically occludes the binding of compound 10d (Supp Figure S10). Further modification of compound 10d to exploit this charged residue and to increase contact surface area with the M12A catalytic groove may be beneficial in terms of improving affinity and specificity to meprin α.

Discussion

Proteases are crucial players in fundamental cellular processes in human health such as proliferation, differentiation, inflammation, and ECM homeostasis, which drive various pathophysiological states when dysregulated. For example, degradation of the ECM is a typical hallmark of aggressive metastatic cancers by enabling cell migration. Meprin proteases function in the epithelium to regulate ECM homeostasis while having both anti- and pro-inflammatory roles in cell signalling (26). Indeed, meprin α has been implicated as an oncogenic factor which, when highly expressed, is correlated with more metastatic and aggressive forms of certain colorectal cancers. Further elevated levels of meprin α are observed in connection with nephritis (61–63).

Current available drugs to meprin α bind with high affinity but lack high specificity. They also inhibit the functions of meprin β. Therefore, efforts to engineer more selective and potent meprin α inhibitors are ongoing. While the structure of meprin β has been described previously, the structure of meprin α was unavailable. Structures of meprin β have provided mechanistic insights and supported rational design efforts to develop specific inhibitors to this homologue. Conversely, since the structural differences of these proteases underpin the selectivity of compounds, the absence of a meprin α structure has obfuscated drug discovery programs.

Previous studies report meprin α forms large, heterogeneous oligomeric species. We therefore used an electron microscopy approach to elucidate the structure. The high-resolution structures of the meprin α oligomer were determined in four key states. The zymogen form of meprin α is plugged by an α-helical pro-peptide that sterically occludes the active site. Upon proteolytic cleavage of the propeptide the active is exposed and the M12A protease domain undergoes a conformational relaxation. The exposed active site defines a deep pocket that positions the catalytic glutamic acid in close proximity to the substrate peptide as described for meprin β (53). Structural studies of meprin α in complex with a prototype selective inhibitor revealed the mode of inhibition. Furthermore, these data reveal subtle differences between meprin α and meprin β that may underpin inhibitor specificity. As expected, the prototype inhibitor was observed to competitively bind to the active site thereby blocking substrate engagement. These data provide long sought-after details of the meprin α active site for drug discovery programs and offer insight into the unusually large oligomeric form of meprin α.

Intriguingly, meprin α dimers associate to form giant left-handed helical filaments of seemingly no restriction to length in vitro. Protein oligomerisation drives many biological processes such as allosteric control of activity, regulation of protein activity by spatial sequestration, control of local concentrations, increased stability against denaturation (64, 65), and more. As such, formation of oligomers offers functional, genetic, and physicochemical advantages over monomeric counterparts. A prominent example of this includes oligomeric tripeptidyl peptidase II (TPPII), a cytosolic dimeric enzyme classified as the largest peptidase complex so far (66). But, while TPPII forms a spindle-like rigid structure, the meprin α helix is flexible with no further contacts between neighbouring turns. In TPPII this rigid structure results in restricted access to the active site and the peptidase acts only on smaller und unfolded substrates and was shown to have 10-fold increased activity after oligomerization. Overall, in vitro no such correlation could be found for oligomeric meprin α compared with lower-stoichiometric variants indicating that the functional role of the oligomerisation, if any, may only be apparent in vivo. In contrast, stability assays indicate that meprin α oligomers are less susceptible to proteolytic degradation and have improved thermal stability when compared to lower stoichiometry forms. The increased stability against proteolytic degradation seems to be meaningful considering the secretion of meprin α into compartments with high proteolytic potential such as the lumens of intestine and proximal kidney tubuli.

Lastly, it has been established that differential recognition and cleavage of substrates is dependent on enzyme lo-
calisation, such as membrane bound versus soluble meprin β. As is the case with TPPII, the formation of a helix could also function to sequester and concentrate activity to a localised region (67, 68). The effect of localised activity would not be captured in our in vitro assays, however in vivo it may be important for the catabolic function in the lumen of the intestine and proximal kidney tubuli, and additionally, for the controlled degradation of the extracellular matrix. Intriguingly, in some tissue contexts such as the epidermis, meprin α expression is observed to be specifically localised in particular subregions (e.g. the stratum basale). Owing to their size, meprin α filaments may have limited capacity to diffuse into neighbouring tissue areas thereby restricting the ECM remodelling activity to appropriate regions.

Considering these findings and our structures, we mapped frequently mutated residues that are associated with disease to the domains that control meprin α oligomerisation. This localisation suggests that these mutations may drive disease by destabilising the oligomeric form. For example, by the release of higher proportions of freely diffusing meprin α dimers that exert function in a dysregulated manner (Figure 5). The restricted helical arrangement further suggests that some activators, substrates, and inhibitors may have limited accessibility to the meprin α pro-peptide and active site. However, we were unable to detect any significant differences between meprin α oligomeric forms when assessing small molecule and globular substrates, nor small molecule and globular inhibitors. Indeed, structural studies of human fetuin-B in complex with meprin α are consistent with crystal structures of murine fetuin-B and meprin β (96). Unlike meprin β, these inhibitory complexes were observed to form supramolecular helical packing as a result of the intercalated arrangement of meprin α and numerous fetuin-B molecules.

Taken together, our findings suggest that differences between stoichiometric forms of meprin α may depend entirely on their localisation which, consequently, dictates their distinct activity profiles (15, 48). Ultimately the impact of these effects on biological function, if any, remains to be shown. It is currently unclear what role meprin α oligomerisation mediates at a cellular level. Previous studies suggest the tissue and cellular localisation of meprins underpins both correct function and regulates activities of these key proteases (15, 48). Therefore, we suggest meprin α oligomerisation may be a regulatory mechanism that functions to either stabilise, sequester and/or locally amplify proteolytic activity (Figure 5).

Further studies in these regards will be informative in understanding the role of meprin α in cancers and other human diseases. For example, studies in vivo to assess the physiological effects of meprin α depolymerisation and the importance of meprin α oligomerisation in normal ECM homeostasis.

Material and methods

Expression, purification, activation. Expression of pro-meprin α (and variants) was achieved in Schneider-2 Drosophila cells (S2 cells). Briefly, the sequence of pro-meprin α (V22-S600; uniprot Q16819) containing an N-terminal Strep-Tag was cloned into pMT/BiP/V5, enabling stable cell lines to be produced. The pro-meprin α variants C308A, R372T and R372A were produced by site-directed mutagenesis. To induce the production of pro-meprin α, S2 cells were grown in Schneider’s Drosophila Medium (Biowest) supplemented with 1 mM copper sulphate and 0.05% PluronicTM F-68 at 28 °C and 80 rpm for two days. The supernatant was harvested by centrifugation and immediately purified by hydrophobic interaction chromatography applying expanded bed adsorption (HIC-EBA, equilibration buffer 30 mM Tris-HCl pH 7.4, 1.5 M ammonium sulphate; elution buffer 30 mM Tris-HCl pH 7.4, 100 mM NaCl). The eluate of HIC-EBA was subjected to affinity chromatography using
Strep-Tactin® column (5 ml cartridge, GE Healthcare Life Science; equilibration buffer 30 mM Tris-HCl pH 7.4, 100 mM NaCl; elution buffer 30 mM Tris-HCl pH 7.4, 100 mM NaCl, 2.5 mM desthiobiotin). Finally, pro-meprin α was activated by trypsin cleavage applying immobilized trypsin on magnetic beads (PT3957-1, Takara, buffer 30 mM Tris-HCl pH 7.4, 100 mM NaCl, 5 mM CaCl₂).

Animal work. Mouse urine was collected in metabolic cages for 24 h. The animal experiment was approved by the responsible animal ethics committee of the state of Saxony-Anhalt, Germany (Landesverwaltungsamt Sachsen-Anhalt, Department of Consumer Protection and Veterinary Affairs, Halle (Saale), Saxony-Anhalt, Germany) under the following approval number: 42502-2-1473 MLU.

Kinetic analysis. The determination of enzymatic activity was based on the cleavage of the fluorescent peptide substrate Abz-YVADPK(Dnp)G-OH. Measurement of kinetic parameters was performed in 384 well black plates in a volume of 60 µl (assay buffer 50 mM HEPES, 150 mM NaCl, pH 7.4, 0.05% Brij). Enzyme solution in buffer was applied and subsequently substituted with inhibitor/DMSO normalisation (Digital Dispenser D330e, Tecan, Switzerland) and preincubated at 30 °C for 20 min. After addition of substrate reaction was measured at excitation/emission wavelength 340/420 nm on a plate reader (Clariostar, BMG Labtech, Germany). For estimation of first order rate constants of kcat/Km substrate concentrations from 0.8 to 83 µM were used. Concentration of active enzyme was determined to be 3–5 nM by titration of the active site using a tight binding inhibitor. The IC₅₀ were estimated at a substrate concentration of 10 µM varying inhibitor from 10 µM to 5 nM (compound 10d) and 200 nM to 0.03 nM (fetuin-B). Kinetic parameters were determined at least in triplicates in separate experiments and evaluated with GraphPad Prism software (Graphpad Software, Inc., USA). Mouse fetuin-B was a generous gift from Hagen Körschgen and Walter Stöcker (University Mainz).

Turnover of protein substrates. Different enzyme-substrate ratios of meprin α wild type and meprin α C308A were incubated with human tropoelastin, human fibronectin and rat collagen I. In the case of tropoelastin (kindly gifted by Mathias Mende, group of Prof. Pietzsch, Martin-Luther University Halle-Wittenberg), the reaction was carried out in 50 mM Tris-HCl pH 7.4 buffer at 37 °C and molar ratios of enzyme to substrate of 1:10⁴ and 1:10⁵. Samples (10 µg) were removed every 30 min for 3 h. The cleavage of fibronectin (ab209886, Abcam) was assessed in 50 mM HEPES pH 7.4 buffer containing 150 mM NaCl. Enzyme to substrate ratios of 1:10⁴ and 1:10⁵ were tested. Reaction conditions were as described above. Collagen I from rat tail was prepared according to Gorisse et al (69) and the reaction was carried out in 50 mM HEPES pH 7.4 at 37 °C at a ratio of 1:10⁴. All cleavage products were evaluated by reducing SDS-PAGE, followed by Coomassie-staining.

Proteolytic stability A total of 9 µl of enzyme (2 µM) were supplemented with 1 µl Trypsin/Plasmin (2 µM) or buffer and incubated at room temperature. After 20 min, reactions were stopped by addition of 2.5 µl 5-fold reducing sample buffer. Samples were heated and subjected to analysis by 10% SDS-PAGE.

NanoDSF. NanoDSF measurements were conducted on a Prometheus NT.48 (NanoTemper) instrument. Each meprin α variant was standardised to a protein concentration of 0.1–0.3 mg ml⁻¹ in 30 mM Tris-HCl, 100 mM NaCl, pH 7.4. Thermal unfolding was monitored by the intrinsic tryptophan fluorescence at emission wavelengths 350/330 nm in 1 °C min⁻¹ increments from 20 °C to 95 °C. The apparent melting temperature (Tₘ) was determined as the maximum of the first derivative of the 350/330 nm ratio. Each variant was measured in three independent experiments.

MDLDS/SEC-MALS. Multiangle dynamic light scattering analyses of meprin α was performed using a Zetasizer Ultra (Malvern Panalytical Ltd., UK) and He-Ne laser at 632.8 nm and constant power of 10 mW at 30 °C. Concentration of protein was 0.3 mg ml⁻¹ in 30 mM Tris-HCl, 100 mM NaCl, pH 7.4. Detector angle for polydispersity index (PDI) was 173°. The results are presented as the average value of three to five experiments. For size exclusion-multiangle dynamic light scattering, meprin α C308A (100 µl at 0.74 mg ml⁻¹) and meprin α R372T (100 µl at 0.44 mg ml⁻¹) were applied onto an AdvanceBio SEC column (300 Å, 7.8×300 mm, Agilent Technologies). The data was evaluated using ASTRA® 6 (Wyatt Technology).

MALDI-TOF-MS. For MALDI-TOF mass spectrometry all samples were purified using C4 ZipTip® pipette tips (Merck) before analysis as described in the supplied manual. The purified sample (1 µl) was mixed with 1 µl of DHAP-matrix (7 mg 2,5-dihydroxyacetophenone, 375 µl ethanol, 125 µl of 16 mg ml⁻¹ di-ammonium hydrogen citrate) and 1 µl of 0.1% TFA and applied onto a metal target plate. The samples were analyzed in linear positive mode (LP_30-210 kDa according to Bruker Daltonics) using an AutoFlexTM speed MALDI-TOF/TOF device (Bruker Daltonics). Protein Calibration Standard I and II (Bruker Daltonics) were applied for calibration of the device.

Cryo-EM and cryo-ET sample preparation and data collection. For tomography samples, Quantifoil Cu R 2/2 grids were glow-discharged for 30 s in a Pelco EasyGlowlow, and 3 µL of meprin α (0.5 mg ml⁻¹ in 30 mM Tris-HCl buffer, 100 mM NaCl, pH 7.4) was mixed with 10 nm gold nanoparticles and was applied to the glow discharged surface and blotted at 4 °C and 100% relative humidity for 3 s and a blot force of −3 using the Vitrobot IV System (Thermo Fisher Scientific). Grids were plunged in 100% liquid ethane. The grids were stored under liquid nitrogen until TEM data collection. Tilt series were acquired at 300 kV on a FEI Titan Krios G1 and digitised on a postGIF K2 Summit Direct Electron Detector. A dose-symmetric tilt acquisition scheme was used with 3°
increments, an electron dose of 2.6 e⁻/Å² per tilt. Micrographs were acquired in dose fractionation mode with 0.5 e⁻/Å² frame⁻¹ at a pixel size of 0.186 nm × 0.186 nm.

Similarly for single-particle cryo-EM, initial grid freezing conditions were tested and screened on a Tecnai T12 electron microscope (Thermo Fisher Scientific). Blotting was carried out as above, with the following modifications, a blot force of 2.5 s, blot force of ~5 and drain time of 1 s were used. The grids were stored under liquid nitrogen until TEM data collection. Briefly, dose fractionated movies were collected on a Titan Krios (Thermo Fisher Scientific), equipped with a Quantum energy filter (Gatan) and Summit K2 (Gatan) or K3 (Gatan) direct electron detector. Data acquisition was performed using either SerialEM (70) or EPU (Thermo Fisher Scientific). Meprin α/fetuin-B complex was made immediately prior to freezing, by mixing stoichiometric quantities (final concentration 1 mg ml⁻¹) and allowing to incubate for 3 minutes at room temperature. His-tagged recombinant human fetuin-B (11834-H08H) was purchased from SinoBiological. Similarly, compound 10d was added in excess (~8 fold Kₜ) and allowed to incubate for 1 minute, prior to freezing.

Cryo-EM and cryo-ET image analysis. Dose fractionated movies were firstly compressed to LZW TIFF format with IMOD (71) to save disk space. Correction of beam induced motion and radiation damage was performed with MotionCor2 (v1.3.0) (72). Corrected frames were dose-weighted and averaged for all further processing. Tilt series were aligned and backprojected using IMOD (71, 73, 74), and CTF corrected using NovaCTF (75). Subtomogram averaging was performed using Dynamo (76). Subtomograms were extracted following a cylindrical geometry and the asymmetric unit used for alignment corresponded to 2 protein dimers. Subtomogram averages were later used as initial volumes for single particle analysis.

Single-particle data was analysed as follows. Initial estimates of CTF were performed in CTFFIND (4.1.13) (77) or Warp (1.0.7) (78). A combination of manual and automated filament particle picking was performed by hand or with cYOLO (1.8.1) (79, 80) operating in filament mode. A small, hand-picked subset of images were used to train cYOLO. Particles were extracted within 400-pixel boxes and normalised within RELION (2.1-3.1) (81–83). Initial rounds of 2D classification in cryoSPARC (84, 85) and RELION were used to discard malformed particles and poor-quality images. Asymmetric refinements were performed in RELION. Flexibility resulted in large-scale, continuous conformational heterogeneity between the images. This was apparent in 2D class averages which displayed clear secondary features at the centre of the averages, with diffuse signal and poor coherency that worsened moving away from the centre along the helix lengthwise. Three-dimensional variability analysis in cryoSPARC revealed numerous modes of flexibility (86). This also prevented symmetry determination by layer line analysis. Resultantly, all the refinements of the full helical assembly that were attempted, gave rise to severely limited reconstructions with resolution on the order of nanometres.

The best reconstructions of helical complexes were used to guide the placement of meprin β dimer modes (PDB-4GWN) (53). A mask of approximately two dimers was used to carve ~6 regions of interest from the reconstruction. A small space was left between regions as a buffer to accommodate some variation in the placement of models. These regions of interest were individually removed from the reconstruction, to generate masks of the remainder of the helix. These masks were subsequently used for partial signal subtraction and localised particle extraction within a modified version of localised_reconstruction.py (87) or directly within RELION.

A carved segment of the original helix was re-boxed and used an initial volume. Localised reconstruction of these portions of helix gave rise to a notably more homogeneous volume that displayed secondary structures. Subsequent 2D and 3D classifications of these segments were performed in RELION or by heterogeneous refinement in cryoSPARC. Homogeneous refinements in cryoSPARC were performed to obtain optimised alignment and offset parameters. Peripheral regions of the localised reconstruction corresponding to the boundaries of the subtracted regions were noisy and diffuse. Therefore, these areas were subtracted and further rounds of local refinement in cryoSPARC were performed. CTF parameters were refined and corrected in cryoSPARC or RELION, including anisotropic magnification, beam tilt, trefoil, tetrafoil and astigmatism (83). Finally, non-uniform refinement was employed to account for variation in resolution across the reconstruction that gave rise to minor errors in alignments (85).

For reconstructions of fetuin-B/meprin α, RELION was unable to solve the global alignment problem. Therefore, asymmetric reconstructions of the whole helix were performed in cryoSPARC. Localised sub-particle extraction was performed in RELION and global alignment of the extracted subregions was performed in RELION (here cryoSPARC failed to solve the global alignment problem). Finally, local non-uniform refinement in cryoSPARC with gaussian prior yielded the final reconstruction. These final particles and optimised metadata were exported from cryoSPARC for 3D classification within RELION. Low resolution signal dominated and therefore the CTF was ignored until the first zero giving rise to markedly improved classification.

For all maps local resolution was estimated in RELION with a windowed FSC. Map sharpening was performed in cryoSPARC, RELION or deepEMhancer (88). Various amplitude corrected maps were employed for model building. Conversion between cryoSPARC and RELION were performed with pyem (89).

Model building. An initial model of full length pro-meprin α were generated via homology with meprin β (PDB-4GWN) (53) using the SWISS-MODEL server (90) and rigid body fit into the cryo-EM reconstruction with UCSF Chimera (v1.13) (91). Subsequently, the model was flexibly fit and interactively refined in ISOLDE (92), ChimeraX (v1.3) (93) and Coot (94, 95). Where possible, N-linked glycosylations were
modelled in Coot. Models of the active form and inhibitor-bound form were generated similarly, using the model of pro-meprin α as a starting template. Lastly, molecular docking analysis was carried out using the fetuin-B/meprin β crystal structure (PDB-7AUW) (96) superimposed onto the meprin α helix. Subsequently, a model of the meprin α/fetuin-B complex was generated from the model of active meprin α and the AlphaFold model of human fetuin-B (97, 98) (AF-Q9UGM5 v2). These were rigid body fit into the cryo-EM reconstruction and were subsequently flexibly fit and interactively refined in ISOLDE/ChimeraX using secondary structure and atomic coordinates available from the RCSB Protein Data Bank under 7UAB. Pro-meprin models were subject to real space refinement and validation as in Coot. Models of the active form and inhibitor-bound form were generated similarly, using the model of pro-meprin α bound with a non-polarized secretion of human meprin α. C.J. (1). These models were subsequently flexibly fit and interactively re-fined in ISOLDE/ChimeraX using secondary structure and atomic coordinates available from the RCSB Protein Data Bank under the following accession codes. Pro-meprin α (tetramer), EMD-26419 and PDB-7UB. Pro-meprin α (single subunit), EMD-26420 and PDB-7UAC. Full meprin α helix in the active state (C1 reconstruction). EMD-26421. Meprin α in the active state (single subunit), EMD-26422 and PDB-7UBA. Meprin α in complex with small molecule inhibitor (tetramer), EMD-26423 and PDB-7UAF. Meprin α in complex with the native fetuin-B inhibitor (tetramer), EMD-26426 and PDB-7UAE. Full meprin α helix in complex with the native fetuin-B inhibitor (C1 reconstruction), EMD-26424. Additional information and data are made available upon reasonable request from the corresponding author.

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AUTHOR CONTRIBUTIONS

D.S., C.F., M.W. expressed, purified, and analysed protein. D.S., C.F., C.J.L. prepared and screened cryo-electron microscopy samples. C.B.J., C.J.L., D.S. and C.F. processed and analysed cryo-electron microscopy data. C.B.J., C.J.L. and C.F. built and analysed model proteins. C.B.J. wrote the first draft. C.B.J., D.S., C.J.L., J.C.W. co-wrote, revised, and edited the manuscript. C.B.J., D.S., C.J.L., produced figures. H.V., A.D.M., C.J.L. collected cryo-electron microscopy data. D.R. and C.J.L. performed inhibitor docking and structural analysis. C.B.J., D.S., C.J.L. and C.F. produced figures. D.S., S.S., and J.C.W. acquired funding and provided supervision. All authors contributed to the discussion and interpretation of results.

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**Fig. S1. Meprin α expression and purification.**

a. Domain schematic of native meprin α consisting of a signal peptide (SP), pro-domain (PD), proteolytic (M12A protease) domain, meprin, A-5 protein, and receptor protein-tyrosine phosphatase γ (MAM) domain, TNF receptor-associated factor (TRAF or MATH) domain, epidermal growth factor-like (EGF) domain and a transmembrane region (TM). Arrows indicate sites of proteolysis during normal meprin α maturation.

b. Recombinant construct employed herein. Dashed boxes represent regions that are not genetically encoded in the recombinant construct.

c. Reducing 10% SDS-PAGE analysis of purified recombinant meprin α and variants.

d. As in (b) with non-reducing conditions.

e. Comparison of retention volume between recombinant meprin α (wild type, C308A and R372T) to native source meprin α isolated from mouse urine. Large oligomeric species were observed from the mouse urine after cisplatin treatment. These were, however, smaller than recombinant oligomeric meprin α.
Fig. S2. Summary diagram of cryo-EM image analysis strategy and reconstruction method. a. Helical segments are broken into 20-30 nm fragments and classified independently. An initial volume was created by sub-tomogram averaging. Refinement of a full helix was performed by single-particle analysis. b. Coordinates of meprin α tetramers were defined based on the final reconstruction of the full helix and used to reproject 2D coordinates onto the original extracted particles. c. Sub-regions of meprin α tetramers were re-extracted from the original particles within a smaller area and subsequently treated as independent single particles. d. Assumption of single particle behaviour enabled reconstruction of a tetramer localised reconstruction by performing global searches with C1 "symmetry".
Fig. S3. Summary figure of cryo-EM key statistics and analysis outcomes (compound 10d map). a. Representative micrograph of meprin α helices and, b, corresponding Fourier transform with high quality Thon rings. c. Class averages in 2D of meprin α helices and of meprin α tetramer subparticles after localised extraction. d. The final meprin α tetramer reconstruction coloured by local resolution. e. Corresponding per-voxel resolution frequency distribution. f. Composite map of the meprin α helix and corresponding directional resolution analysis. Maximum anisotropy is observed from the z-direction due to the tendency of helices to lie flat within the ice. g. Fourier shell correlations including tight, loose and no mask curves, phase randomised half-maps, mask corrected FSC and histogram of directional 3DFSC voxels (frequency). h. Angular distribution and orientation assignment of observed particles after refinement.
Fig. S4. Sequence alignment of meprin α and meprin β. Secondary structure elements are shown above based on the structure reported herein, arrows represent β-strands and tubes represent α-helices. Green circles represent N-linked glycosylations. The active site triad of histidine residues are marked in bold font and red. The catalytic glutamic acid is black bold font emphasised.
Fig. S5. ConSurf analysis of meprin α dimer and oligomer interfaces. 

a. Monomer of meprin α with conserved surface rendering showing highly conserved dimeric interface (right). 

b. Focus of helical interface as conserved surface rendering (with adjacent monomer in transparent cartoon), showing conservation to some degree.

c. Focus of dimer interface (with cartoon model of adjacent monomer in transparent), showing strong conservation corresponding to the binding site of the adjacent monomer.

d. Single monomer of meprin α shown in context of the helical oligomer.
Fig. S6. Biophysical characterisation of wild type meprin α and variants. a. MALDI-TOF analysis of wild type meprin α, C308A and R372T, as both zymogen and mature form. Peaks of about 75 kDa (zymogen) and 69 kDa (mature form) equal [M+H]$^+$ or [M+2H]$^+$, peaks of about 38 kDa (zymogen) and 35 kDa (mature form) equal [M+2H]$^{2+}$. b. Comparative analysis of hydrodynamic radius by MALDS of meprin α and mutants C308A and R372T with the Zetasizer Ultra. Overlay of intensity particle size distribution (left). Overlay of volume particle size distributions results are presented as the average value of three to five experiments (right). c. SEC-MALS analysis of Pro-Meprin α variants C308A and R372T. Zymogenic meprin α C308A and R372T form dimeric structures, whereas for C308A a homogenous peak was observed, for R372T a heterogenous peak was detected, including two forms of dimeric pro-meprin α R372T. d. Cryo-TEM (120 kV) of meprin α and β. Mutant variants of meprin α form dimers and small oligomers.
Fig. S7. Meprin α activation, substrate degradation and activity. Oligomeric state does not appear to drastically affect substrate specificity or rate of degradation. 

a. Cleavage of rat tail procollagen by wild type meprin α and variant C308A. Specific cleavage of procollagen by both meprin α variants result in same cleavage pattern. Samples analysed using reducing 10% (w/v) SDS-PAGE, visualized by Coomassie-staining.

b. Cleavage of human fibronectin by wild type meprin α and variant C308A. Specific cleavage of Fibronectin by both meprin α variants result in same cleavage pattern.

c. Cleavage of human tropoelastin by wild type meprin α and variant C308A. Complete degradation of tropoelastin by wild type meprin α within 3 h (molar ratio of 1:10⁴) and by meprin α C308A within 30 min (molar ratio of 1:10⁵). Samples analysed using reducing SDS-PAGE (4-20% (w/v) gradient gel), visualized by Coomassie-staining. In each sample 10 µg of substrate were applied, as well as a control of substrate only (S; substrate incubated for 3 h at 37 °C in the absence of meprin α).
Fig. S8. Molecular docking and 3.7 Å cryo-EM reconstruction of fetuin-B inhibitor bound to meprin α.

a. Rigid body fit of murine fetuin-B/meprin β crystal structure (PDB 7AUW) to helical structure of meprin α. Arrangement of fetuin-B reveal monomers may pack into a slanted intercalated state that is not significantly prohibited by steric clashes.

b. View of a tetramer of fetuin-B based on meprin α docking reveals potential interactions to form a higher-order inhibitory filamentous complex are possible.

c. Side view of single fetuin-B dimer forms a “horseshoe” where putative interactions between inter-subunit fetuin-B domains may occur shown in (d), and (e). Models are not refined, rigid body fitting results in some minor clashes.

d, e. The predicted oligomeric interface corresponds to an evolutionarily conserved interface revealed by ConSurf analysis.

g. Side and top views of the cryo-EM reconstruction of human fetuin-B (red) in complex with meprin α (grey, black) at 3.7 Å resolution. Fetuin-B is observed to pack intimately within the meprin α active groove and intercalate as a secondary helix.
Fig. S9. Summary figure of cryo-EM key statistics and analysis outcomes (zymogen, active and fetuin-B maps). a., e., i., m. Fourier shell correlations including tight, loose and no mask curves, phase randomised half-maps, mask corrected FSC, map-to-model FSC and histogram of directional 3DFSC voxels (frequency). b., f., j., n. Final reconstruction coloured by local resolution. c., g., k., o. Corresponding per-voxel resolution frequency distribution. d., h., l., p. Angular distribution and orientation assignment of observed particles after refinement.
Fig. S9. cont. Summary figure of cryo-EM key statistics and analysis outcomes (full helical active and fetuin-B maps). q., u., Fourier shell correlations including tight, loose and no mask curves, phase randomised half-maps, mask corrected FSC and histogram of directional 3DFSC voxels (frequency). r., v. Final reconstruction coloured by local resolution. t., x. Corresponding per-voxel resolution frequency distribution. s., w. Angular distribution and orientation assignment of observed particles after refinement.
Fig. S10. Structural comparison of meprin α and meprin β within the active site. a. Superposition of meprin α (red) and meprin β (purple) with select conserved side chains (grey) and divergent side chains (coloured) visible. b. Sequence alignment of meprin α and meprin β with secondary structure elements shown above (β-strands as arrows or α-helices as tubes). The catalytic glutamic acid is bold font and underlined. The active site tirade of histidines is shown in red bold font. Meprin α residue Y149 (meprin β R146) is non-conserved therefore presenting either as tyrosine or arginine in meprin α and meprin β respectively. This single residue appears to drive charge repulsion of R238 in meprin β (R242 in meprin α) causing a different rotamer position to be adopted relative to meprin α. This alternative rotamer conformation sterically occludes compound 10d (green; density shown as isosurface) suggesting how the drug remains selective despite major contacts of the drug and meprin α being conserved across both homologs. Conversely, in meprin α R242 is not repelled by Y149 and therefore adopts a conformation that does not interfere with drug binding.