The tripartite architecture of the eukaryotic integral membrane protein zinc metalloprotease Ste24

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Abstract
Ste24 enzymes, a family of eukaryotic integral membrane proteins, are zinc metalloproteases (ZMPs) originally characterized as "CAAX proteases" targeting prenylated substrates, including α-factor mating pheromone in yeast and prelamin A in humans. Recently, Ste24 was shown to also cleave nonprenylated substrates. Reduced activity of the human ortholog, HsSte24, is linked to multiple disease states (laminopathies), including progerias and lipid disorders. Ste24 possesses a unique "α-barrel" structure consisting of seven transmembrane (TM) α-helices encircling a large intramembranous cavity (~14 000 Å³). The catalytic zinc, coordinated via a HExxH...E/H motif characteristic of gluzincin ZMPs, is positioned at one of the cavity's bases. The interrelationship between Ste24 as a gluzincin, a long-studied class of soluble ZMPs, and as a novel cavity-containing integral membrane protein protease has been minimally explored to date. Informed by homology to well-characterized soluble, gluzincin ZMPs, we develop a model of Ste24 that provides a conceptual framework for this enzyme family, suitable for development and interpretation of structure/function studies. The model consists of an interfacial, zinc-containing "ZMP Core" module surrounded by a "ZMP Accessory" module, both capped by a TM helical "α-barrel" module of as yet unknown function. Multiple sequence alignment of 58 Ste24 orthologs revealed 38 absolutely conserved residues, apportioned unequally among the ZMP Core (18), ZMP Accessory (13), and α-barrel (7) modules. This Tripartite Architecture representation of Ste24 provides a unified image of this enzyme family.

KEYWORDS
computational biology, endopeptidase, lipodystrophy, membrane proteins, metalloprotease, progeria, zinc

INTRODUCTION

Proteases, abundant enzymes across all forms of life, conduct a remarkably broad range of biological functions.1 Approximately 2% of genome-encoded mammalian proteins are proteases.2 Due to their abundance and copious biological roles, protease dysfunction can lead to deleterious health consequences.1 Proteases are targeted to virtually all cellular compartments from cytosolic/luminal space to the membranes of organelles and the cell exterior. Because of their "gatekeeping" status, peripheral and integral membrane protein proteases play further specialized and vital roles in metabolic and signaling pathways.3–5

Ste24 enzymes, a family of integral membrane proteins, function as zinc metalloproteases (ZMPs) and are found in all eukaryotic
organisms.6 The first Ste24 family member, Ste24p (where "p" is an abbreviation for protein), was discovered and characterized in the yeast Saccharomyces cerevisiae, where it is involved in posttranslational modification of the peptide-derived a-factor mating pheromone.7,7 Nascent a-factor processing involves three proteolytic cleavages, two of which Ste24p is competent to perform.6 One Ste24p cleavage occurs between a farnesylated Cys and the sequence Val-Ile-Ala, which form a C-terminal "CAAX box" motif (C is Cys; A is typically an aliphatic amino acid; and X is one of several amino acids less restricted in characteristics).10 Ste24p is capable of, though not required for, carrying out the CAAX box cleavage,9 and uniquely required for one of the two other nonprenylated site cleavages.8 The ZMP protease Axlp, or its homolog Ste23p, is required for ultimately completing a-factor maturation via cleavage of the remaining site.11

The human HsSte24 enzyme, also known as FACE-1 and ZMPSTE24, is localized to the endoplasmic reticulum and the inner nuclear membrane.6,12 The earliest functional characterization of HsSte24 demonstrated its role in the processing of prelamin A to mature intermediate filament protein lamin A.13,14 Lamins provide mechanical stability to the nuclear envelope and function as scaffolds for DNA repair and replication complexes.15,16 Prelamin A is a CAAX box-containing substrate that undergoes two proteolytic cleavages, at the CAAX box and an N-terminal site.17 Both cleavages can be performed by HsSte24, with a unique requirement for prelamin A cleavage of the nonprenylated site (analogous to Ste24p).17-20 The Ras Converting CAAX Endopeptidase 1 (Rce1), an unrelated cysteine protease, can also perform CAAX box cleavage of both a-factor7 and prelamin A.17

Deficiencies in HsSte24 activity, whether by mutation18,21,22 or inhibition,23,24 restrict prelamin A maturation and lead to disease states known as laminopathies, which range from progeria (premature aging syndrome) to lipodystrophies.25,26 Inhibition of HsSte24 has particular clinical relevance, as AIDS patients receiving viral protease inhibitors as part of their drug regimen develop lipodystrophies because of off-target interaction of HIV protease inhibitor drugs with HsSte24.24,27 The cellular role for Ste24 family enzymes has expanded recently, beyond its role in prenylated protein processing. Both fungal (yeast) and human Ste24 have been shown to clear a downstream effector.31,32 Strikingly, overexpression of HsSte24 in IfitmDel cells (all ifitm genes deleted) yielded the same reduced viral infection phenotype as seen in IFITM-expressing cells, suggesting that HsSte24 is the antiviral "causative agent."31 Also, catalytically inactive HsSte24 in these experiments produced the same results, indicating that proteolytic function is not required.

Yeast30,33 and human23,30,34-37 Ste24 orthologs have been the most rigorously characterized Ste24 family members both functionally and structurally. Additionally, functional complementation between human and yeast Ste24 orthologs has been established in vivo where HsSte24 rescues defects in a-factor biogenesis associated with Ste24p knockout yeast (ste24A).9 Crystal structures of yeast (Saccharomyces mikatae; SmSte24)33 and human Ste24 (HsSte24) reveal these two Ste24 structures to be highly similar (RMSD of Cα atoms ~1.7 Å).33,35,37 The structure of Ste24, as exemplified by SmSte24 (PDB: 4IL3), possesses a striking seven-transmembrane (TM) helical "α-barrel" structure encapsulating a voluminous reaction cavity (~14 000 Å3; Figure 1A). The zinc-coordinated active site is localized at one "end" of the approximately cylindrical reaction cavity (Figure 1A,B). Ste24 is associated with the gluzincin subclan of ZincMPs (MEROPS designation MA[E]), which use a glutamate, Glu390 in SmSte24, or histidine residue as the third ligand coordinating the zinc atom (ie, HExxH...E/H) (Figure 1B,C).29,41 Despite the status of the Ste24 family as a gluzincin, structural homology to and sequence conservation with gluzincins (or particular subsets of gluzincin families) has been scanty investigated. Such an analysis is especially important for identifying conserved structural elements and catalytically essential residues, in addition to the HExxH...E/H gluzincin motif, which form the complete Ste24 active-site. Furthermore, the description of Ste24 as a gluzincin describes only a portion of this large, multidomain, membrane-inserted enzyme.

In this study, we develop a conceptual framework for Ste24 that encompasses its identities as both a gluzincin and a complex, cavity-containing integral membrane protein protease. Rather than a structural model, we develop a representational architecture informed by both functional and structural homology data. Through a combination of bioinformatics and structural comparison to homologous, soluble gluzincins, we have developed a Tripartite Architecture representation of the Ste24 enzyme family. We believe this model provides a better framework for understanding Ste24 mechanism, and will be conducive to better rationalize future functional characterization of Ste24 enzymes.

2 | MATERIALS AND METHODS

2.1 | Identification of soluble gluzincin ZMPs homologs

The Dali server42 was used to identify structurally homologous ZMP structures. A query model of Ste24 was developed containing elements only from the Loop 5 Domain (L5D), C-terminal Domain (CTD), and the portions of TM α-helices VI and VII containing the 297HExxH301...E390 gluzincin motif. For analysis and comparison, structures of homologous gluzincin ZMPs were aligned with the SmSte24 structure (PDB 4IL3) by use of the Pymol program SUPER.43 The four conserved gluzincin residues, HExxH...E, of the orthologs were used to seed the structural alignment algorithm.
2.2 | Generation of the Ste24 enzyme multiple sequence alignment

Homologous sequences to \textit{S. mikatae} Ste24 (SmSte24) were identified through iterative searching using the blastp suite available on the NCBI web server (https://blast.ncbi.nlm.nih.gov/Blast.cgi). In total, 58 homologous sequences were identified from fungi, plant, animal, and protist kingdoms. The program Clustal Omega (EMBL) was used to construct a multiple sequence alignment (MSA) from these identified sequences, which identified 38 absolutely conserved residues. Sequence identity and similarity scores between each sequence and SmSte24 are reported in Table S1, and were calculated using the EMBoss Needle global sequence alignment tool available from EMBL-EBI (http://www.ebi.ac.uk/Tools/psa/emboss_needle/). The MSA was graphically represented using ESPript 3.0 (http://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi).

3 | RESULTS

3.1 | The Ste24 active-site pocket: homology to soluble gluzincins

Inspection of the zinc-coordinating residues of Ste24 reveals a characteristic HExxH motif of the Zincin family of ZMPs (Figure 1B). Zincins are further subdivided by the identity of the third amino acid ligand to the zinc (ie, beyond the two histidine residues of the HExxH motif). In gluzincins, a glutamate residue most often provides the third ligand; therefore, all gluzincins share the consensus motif "HExxH…E/H." Invariantly, all residues of the gluzincin motif originate from a neighboring pair of \(\alpha\)-helices. Among Ste24 enzymes, the base of the two TM \(\alpha\)-helices VI and VII contribute these four residues (Figure 1B). SmSte24 residues His297, His301, and Glu390 are the zinc ligands and Glu298 acts as a catalytic base. Gluzincin ZMPs also possess a region of \(\beta\)-sheet orthogonal to these \(\alpha\)-helices, where a \(\beta\)-strand contributes catalytically essential residues. I nt h ec a s eo fS t e 2 4e n z y m e s ,a CTD composed of a series of loop-connected \(\alpha\)-helices located between TM \(\alpha\)-helices I and VII is positioned in such a fashion (Figure 1C).

Currently, 29 distinguishable gluzincin members have been assigned in the MEROPS database, with Ste24 designated as MA(E)/M48A. Therefore, a structure-based search of the PDB database using the DALI server was used to identify those gluzincin families most homologous to Ste24. Currently available structures of Ste24 comprise fungal (SmSte24) and human (HsSte24) orthologs. While SmSte24 and HsSte24 structures are highly similar, Ste24 mammalian orthologs contain a variable length insert between the \(\alpha\)-helix of the LSD and TM \(\alpha\)-helix VI (37 residues in HsSte24) (Figure S1); this insert is disordered in all HsSte24 crystal structures. Therefore, the SmSte24 fungal ortholog structure (PDB 4IL3) was used as a representative query for structural ZMP homologs.

\begin{figure}
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\includegraphics[width=\textwidth]{figure1.png}
\caption{Structural summary of the Ste24 family. A, Ribbon representation of the structure of Ste24 from the fungus (yeast) \textit{S. mikatae} (PDB 4IL3). A total of seven transmembrane (TM) \(\alpha\)-helices span the membrane (indicated by parallel black lines). The zinc ion (shown as a small gold sphere) is positioned at the "lower" membrane interface and ligated by residues from two of the TM helices. B, Stereo view of the Ste24 gluzincin structural motif. The image is generated from the left-hand orientation of the Ste24 molecule as seen in panel A. Ste24 possesses the gluzincin \(297^HExxH301…E390\) consensus sequence motif located at the base of TM helices VI and VII. Residues His297, His301, and Glu390 coordinate the zinc ion, with Glu298 acting as the general base during turnover. C, Topology diagram of Ste24. The color-coding of the ribbon diagram in panels A and B is maintained. TM \(\alpha\)-helices are labeled I-VII. Two, large membrane interfacial regions flank the coordinated zinc ion: the mixed \(\alpha\)-helix-\(\beta\)-sheet Loop 5 Domain (L5D) located between helix V and helix VI, and the \(\alpha\)-helical C-terminal Domain (CTD).}
\end{figure}
Use of the entire SmSte24 monomer (Figure 1A) as a search model for structurally homologous soluble gluzincins failed because the majority of the DALI-identified structures recognized the TM α-helices of SmSte24 as opposed to the region immediately surrounding the gluzincin motif. Therefore, a truncated SmSte24 ‘gluzincin-only’ search model, including the LSD, the CTD, and portions of TM α-helices VI (residues 292–301) and VII (residues 390–408) was constructed and input to the DALI server (http://ekhidna2.biocenter.helsinki.fi/dali/). The query identified soluble homologs belonging to constructed and input to the DALI server (http://ekhidna2.biocenter.helsinki.fi/dali/). The query identified soluble homologs belonging to three gluzincin families: thermolysin (M4), neprilysin (M13), and an uncharacterized ZMP from Geobacter sulfurreducens (M48) (Figure 2A), whose active sites all possess homology with Ste24 (Figure 2B). Structural homology of Ste24 to M13 gluzincins has not been previously reported, while homology to M4 and M48 gluzincins has previously been established.33,37 (The ZMP from G sulfurreducens is in the M48B subfamily, MA(E)/M48B, and is the only full-length structure of a non-M48A subfamily member.)

Protease active-site residues can be categorized into two interdependent functional classes: (a) residues interacting with peptide backbone atoms and/or promoting cleavage of the scissile bond (ie, catalytic “C” residues) or (b) residues responsible for recognizing specific peptide side-chain identities or chemical properties (ie, specificity “S” residues) with those C-terminal to the substrate scissile bond designated with the prime (’) symbol.52 Alignment of the active-sites of the three soluble gluzincin homologs (Figure 2A, right-hand panels) to SmSte24 (Figure 2B; right-hand panel) reveals an arrangement of opposing C and S residues that is typical for a protease active site.52 The dependence of Ste24 activity on identified C residues (Asn263/Ala264 on the β3-strand of LSD, and His434/Arg440 on the α7-helix of CTD) has been previously documented (Figure 2B).33,37 The S residues identified for Ste24 (Leu282 on α3-helix of LSD and Leu410/Leu413 of α5-helix of CTD) are predicted to form the S1’ surface that interacts with the substrate residue side chain immediately C-terminal to the scissile bond (ie, the P1’ residue).53,54 For all four structural homologs, S1’ forms a hydrophobic pocket (Figure 2). This observation is consistent with the nonpolar or hydrophobic character of the non-CAAX box P1’ cleavage sites in α-factor and prelamin A, for which Ste24 is essential.14,55 In vitro cleavage of nonprenylated biologically derived peptides also occurs at nonpolar or hydrophobic sites.30

3.2 | The tripartite architecture of Ste24

The position of the SmSte24 molecule relative to the membrane bilayer was determined via the Orientation of Membrane Protein server.56 The structural elements of the active site are external to or at the membrane interface (Figure 1A). Positioning of the Ste24 active site at the membrane surface, vs. within the bilayer interior, is consistent with identification of soluble, gluzincin ZMPs as Ste24 homologs. In contrast, the active sites of intramembrane ZMPs, such as Site-2 Protease, are located within the most hydrophobic portion of the membrane interior.57 Comparison of Ste24 enzymes to homologous, soluble gluzincins indicates that residues essential for catalysis arise from TM α-helices VI and VII, the LSD (β3-strand and α3-helix), and the CTD (α5- and α7-helices) (Figure 2B). For the purposes of this study, we refer to this grouping as the “ZMP Core” module (Figure 3A). The designation of a “ZMP Core” module, shared by Ste24 with soluble ZMPs, is further supported by our recent structure of HsSte24 complexed with the classical ZMP inhibitor phosphoramidon (Figure 3B).35 Phosphoramidon is a competitive inhibitor and transition-state analog of several soluble ZMPs, particularly the gluzincins thermolysin (M4)58 and neprilysin (M13).39 Phosphoramidon also inhibits HsSte24 proteolysis and its binding mode, localized solely within the “ZMP Core” module, is highly conserved among these three families (M4, M13, and M48) of ZMPs.35

Among soluble gluzincins, the ZMP Core module is only a portion of the entire structure (Figure 2A, left-hand panel). Accessory structural elements are responsible for positioning the ZMP Core module.

We refer to this grouping of elements as the “ZMP Accessory” module. In the case of Ste24 enzymes, the termini of TM α-helices I–V and the remaining elements from the LSD and CTD buttress the ZMP Core module and form the ZMP Accessory module (Figure 1A.C). Additionally, the ZMP Core module may be further “capped” to restrict substrate access to the ZMP Core. The ZMP Core module of Ste24 is capped by the large, intramembranous cavity formed by TM α-helices I–VII (Figure 2B), though the role for this cavity in Ste24 catalysis is currently unknown. In neprilysin (M13) and G sulfurreducens ZMP (M48), their ZMP Core modules are capped by extended (soluble) protein domains (Figure 2A). The ZMP Core module of thermolysin (M4) does not possess this structural “hat” (Figure 2A).

Based upon these observations, we propose a modular Tripartite Architecture for the Ste24 family of integral membrane protein ZMPs consisting of: (a) a largely cytoplasmic active-site “ZMP Core” module, (b) a mixed soluble/membrane-interfacial “ZMP Accessory” module (in close apposition to the ZMP Core module), and (c) a TM heptahelical “α-barrel” module (of currently undetermined/unknown function) adjacent to the ZMP Core and ZMP Accessory modules (Figure 3C.D).

3.3 | The Ste24 family MSA

Fifty-eight eukaryotic sequences homologous to SmSte24 were identified by querying the NCBI nonredundant protein sequence database. Homologs were identified in multicellular and unicellular organisms (Table S1). The pair-wise identity between SmSte24 and each ortholog was greater than 30%. A multiple sequence alignment (MSA) revealed 38 absolutely conserved residues (8% of the SmSte24 sequence), including the HExxH...E/H gluzincin motif (Figure S1). This set includes three progeria-associated mutations of HsSte24 that map to SmSte24 (Pro246, Asn263, and Leu437).18,21,60 The ZMP Core, ZMP Accessory, and α-barrel modules of SmSte24 contain 79, 135 and 247 residues, respectively (Figure 3D). The percentages of absolutely conserved residues in each module
Identification of essential residues for Ste24 protease activity outside the HEExH...E/H gluzincin motif. A. Gluzincin ZMPs homologous to Ste24. The structures are sorted from least similar (top) to most similar (bottom) based on the Dali similarity or Z score between the Ste24 search model and the identified structures. Inspection of output from the Dali server\textsuperscript{42} revealed thermolysin (PDB 1LNF, Dali Z score 3.6), neprilysin (PDB 1DMT, Dali Z score 4.9), and the ZMP from \textit{G. sulfurreducens} (PDB 3C37, Dali Z score 6.3) as soluble, gluzincin structures most homologous to Ste24. The functional annotations for the selected gluzincins thermolysin (M4), neprilysin (M13), and the \textit{G. sulfurreducens} ZMP (M48) are based on MEROPS\textsuperscript{38} database assignments. Conserved $\alpha$-helical (containing the HEExXE...E motif residues shown as sticks) and $\beta$-strand elements are highlighted and represent a canonical gluzincin structure motif. Note the neprilysin and \textit{G. sulfurreducens} ZMP structures carry an extra domain situated above this motif. Closer inspection of each individual core region identifies residues (shown as sticks) essential for optimal substrate cleavage (indicated by C) and specificity for the side chain of the amino acid immediately C-terminal to the scissile bond (indicated by S$1'$) where the prime (') designation indicates enzyme specificity sites for amino acid side chain(s) C-terminal to the scissile bond.\textsuperscript{65} ZMP residues participating in peptide bond cleavage (ie, catalytic) are absolutely conserved and positioned among the three gluzincins. ZMP residues involved in substrate recognition (ie, side-chain specificity) are chemically conserved and positioned orthogonally to the catalytic ZMP residues forming a sandwich-like motif. (B) Putative catalytic and specificity sites within Ste24. Ste24 TM $\alpha$-helices V, VI and the $\beta$-3-strand of the LSD form the equivalent canonical gluzincin structure motif found in the homologous gluzincin structures. As in the neprilysin and \textit{G. sulfurreducens} ZMP structures, Ste24 possesses an extra domain, in this case a transmembrane (TM) heptahelical "$\alpha$-barrel," situated above the gluzincin motif. Investigation of this region in Ste24 implicates additional residues of the $\alpha$3 element of the LSD and the $\alpha$6/$\alpha$7-helices of the CTD as important for catalytic and specificity roles. Note that the four catalytic residues Asn263, Ala264, His434, and Arg440 (SmSte24 numbering) are absolutely conserved and identically positioned among all four gluzincins
scale with our current level of understanding of the functions of these domains: 23%, 10% and 3%, respectively. Inspection of SmSte24 and HsSte24 structures alongside those of soluble, homologous gluzincins allows assignment of putative roles for these 38 absolutely conserved amino acid residues (Table 1), which are

| Module       | Residue     | Attribution            |
|--------------|-------------|------------------------|
| ZMP Core     | His297      | Zinc Ligand            |
| ZMP Core     | His301      | Zinc Ligand            |
| ZMP Core     | Glu390      | Zinc Ligand            |
| ZMP Core     | Glu298      | Active site Base       |
| ZMP Core     | Asn263      | Catalytic              |
| ZMP Core     | Ala264      | Catalytic              |
| ZMP Core     | His434      | Catalytic              |
| ZMP Core     | Arg440      | Catalytic              |
| ZMP Core     | Leu282      | S1 pocket              |
| ZMP Core     | Leu410      | S1 pocket              |
| ZMP Core     | Asp394      | Second Sphere Ligand   |
| ZMP Core     | His306      | Second Sphere Ligand   |
| ZMP Core     | Gly300      | Structural: Core Only  |
| ZMP Core     | Pro435      | Structural: Core Only  |
| ZMP Core     | Asp280      | Structural: Accessory Interface |
| ZMP Core     | Thr281      | Structural: Accessory Interface |
| ZMP Core     | Arg387      | Structural: Accessory Interface |
| ZMP Core     | Leu437      | Structural: Core & Accessory Interface |
| ZMP Accessory | Arg36       | Structural: Accessory Only |
| ZMP Accessory | Gln37       | Structural: Accessory Only |
| ZMP Accessory | Tyr65       | Structural: Accessory Only |
| ZMP Accessory | Gly152      | Structural: Accessory Only |
| ZMP Accessory | Asn154      | Structural: Accessory Only |
| ZMP Accessory | Asp424      | Structural: Accessory Only |
| ZMP Accessory | Phe145      | Membrane Interface     |
| ZMP Accessory | Phe221      | Membrane Interface     |
| ZMP Accessory | Pro47       | Structural: Core Interface |
| ZMP Accessory | Pro426      | Structural: Core Interface |
| ZMP Accessory | Ser256      | Structural: Core Interface |
| ZMP Accessory | Gly268      | Structural: Accessory & Core Interface |
| ZMP Accessory | Val277      | Structural: Accessory & Core Interface |
| ZMP Accessory | Pro219      | Structural: α-barrel Only |
| α-barrel     | Pro138      | Structural: α-barrel Only |
| α-barrel     | Pro373      | Structural: α-barrel Only |
| α-barrel     | Tyr142      | Membrane Interfacing   |
| α-barrel     | Asp164      | Structural: Accessory/ α-barrel Interface |

Note: A total of 38 absolutely conserved residues are assigned to their respective module (ZMP Core, ZMP Accessory, or α-barrel) within the Tripartite Architecture representation. Those positions, whose functionality can be assigned via comparisons to structural and/or functional characterization of thermolysin (M4), neprilysin (M13), and the G sulfurreducens ZMP (M48), or a subset of the three, are indicated with bolded font. Based on their uniqueness among Ste24 family enzymes, as opposed to those positions with overlap among well-characterized, soluble ZMPs, several positions are simply labeled as “Structural” or “Membrane Interfacial” based solely on inspection of available Ste24 family crystal structures. In several instances, a residue participates in interfacial interactions between two specific modules: the ZMP Core and Accessory or ZMP Accessory and α-barrel modules. None of the 38 residues form structural interactions between the ZMP Core and α-barrel modules.

4 | DISCUSSION

4.1 | Bioinformatic and structural analyses yield a functionally insightful modular Tripartite Architecture of the Ste24 enzyme family

A structure-based search was used to identify gluzincin ZMPs homologous to the Ste24 family. The search identified thermolysin (M4), neprilysin (M13), and the G sulfurreducens ZMP (M48). These results enabled us to develop a modular Tripartite Architecture model for Ste24 (Figure 3). We posit that one essential role of the ZMP Accessory and α-barrel modules is to ensure that the ZMP Core is properly formed and situated to proteolyze substrates. These modules differentiate the Ste24 family from its soluble gluzincin counterparts, as the zinc-centered active-site sits at the membrane interface and requires molecular interactions from the extra-membrane buttress (i.e., ZMP Accessory) and membrane-spanning, α-barrel “hat” (i.e., α-barrel module) (Figures 1 and 3). As expected, structural homology to soluble
gluzincins is scant in the ZMP Accessory module and completely absent in the α-barrel module (Figure 4 and Table 1).

4.2 The ZMP Core module S1′ specificity pocket

Among M4 and M13 gluzincins, the S1′ specificity residues form a pocket that is the chief determinant of substrate specificity. In thermolysin (M4) and neprilysin (M13) X-ray crystal structures, S1′ specificity pockets are composed of aromatic and hydrophobic residues favoring substrate P1′ side chains that possess complimentary hydrophobic character. Our analysis reveals an analogous hydrophobic pocket in structures of G. sulfurreducens ZMP (M48) and SmSte24 (Figure 2). In Ste24, this pocket is composed of Leu282 and Leu410, both absolutely conserved, and Leu413, conserved in 57 of 58 orthologs (Figures 2 and 3, Table 1). Consistent with this
observation, SmSte24 has been shown to have preference for cleaving nonprenylated substrates at the hydrophobic P1 position.40

### 4.3 TM α-helices VI & VII and the α6/α7 CTD loop contribute additional residues to the ZMP Core

The ZMP Core module contains 18 absolutely conserved residues of which 15 can be readily rationalized by direct comparison to all three gluzincin structural homologs of Ste24: thermolysin (M4), nephrilysin (M13), and the G *sulfurreducens* ZMP (M48) (Figure 2 and Table 1).

Residues Gly300, His306, Arg387, Pro435, and Leu437 derive from four separate elements within the ZMP Core module. Residues His306 and Arg387 lie above the zinc center and derive from TM α-helices VI (His306) and VII (Arg387), whereas Gly300, located in TM α-helix VI, is positioned between the zinc ligands His297 and His301 (Figure 4). The Pro435 residue is in a short loop between helices α6 and α7, and is positioned approximately across from the zinc center in proximity to catalytic residue His434 (Figures 2B and 3B). Leu437 is situated at the N-terminus of the α7-helix, preceding the catalytic residue Arg440. Gly300, His306, Pro435, and Leu437 all facilitate the positioning of residues crucial for proteolysis (Table 1). Pro435 and Leu437 share structural analogy to nephrilysin (M13) and G *sulfurreducens* ZMP (M48), whereas His306 shares analogy only with the G *sulfurreducens* ZMP (M48).

Gly300, with no structural analogies in thermolysin (M4), nephrilysin (M13), or G *sulfurreducens* ZMP (M48), is in the zinc HEExxH motif, preceding the C-terminal histidine residue (Figure 4). The identity of the residues located between the catalytic glutamate base and histidine zinc ligand can be highly variable among gluzincins with the only restriction being that neither position is occupied by an amino acid with a charged side chain.40,51 Absolute conservation of this glycine is not unique to the Ste24 family, however, as the M2 gluzincin family, which includes angiotensin-converting enzyme (ACE), does contain glycine at the equivalent position.64

The His306 imidazole forms a second-sphere hydrogen bond with the His301 imidazole, which itself is a direct ligand to the zinc. Among gluzincins, the identity of this second sphere ligand to the C-terminal, HEExxH histidine is often variable, but the presence of a second sphere ligand is maintained. This variation is in contrast to the other absolutely conserved second sphere ligand, an aspartate, identical in all four proteases and forming absolutely conserved hydrogen bond networks; in SmSte24, Asp394 interacts with the zinc ligand His297 and the catalytic residue Arg440 (Figure 2 and Table 1). Arg387 is the only MSA-identified residue of TM α-helix VII that has an interfacial structural role (Table 1) and, among the four proteases, is unique to Ste24. The catalytic His434 residue is located in the α6/α7-loop. The Pro435 residue breaks the α7-helix at its N-terminus, and is the final residue in a loop connecting the α6- and α7-helices (Figure 4). A similar proline-containing structural motif is observed in nephrilysin (M13) and G *sulfurreducens* ZMP (M48). The α7-helix contains the catalytic Arg440 of Ste24.

The Leu437 side chain is part of a hydrophobic, leucine-zipper motif between ZMP Core module helices α5 and α7 of the CTD. The use of a hydrophobic, leucine-zipper like motif is also conserved in nephrilysin (M13) and G *sulfurreducens* ZMP (M48) structures. Ste24 and nephrilysin share an additional conserved feature in this region. In Ste24, a loop element between TM α-helices I and II contributes further hydrophobic residues near the α5/α7 interface, with the absolutely conserved Pro47 residing within this loop; in nephrilysin (M13), a perpendicular α-helical element contributes residues fulfilling the same role.

### 4.4 Residues in the ZMP Core and ZMP Accessory module coordinate the L5D β3-strand and surrounding turns

A β-strand positioned on a side of the zinc-centered active-site is a common structural feature among ZMPs and donates catalytically essential residues (both side-chain and main-chain moieties) for proteolysis40,50 (Figure 2). In Ste24, the L5D β3-strand of the ZMP Core module fulfills this role, and is “catalytically aligned” by the ZMP Core module (residues of the α3-helix of the L5D, Figure 3C,D) and by the ZMP Accessory module (residues of the β3/4-turn and β4-strand of the L5D β-sheet, TM α-helix III/IV loop, and the TM α-helix II (Figure 3D).

The β2/β3-turn of the L5D is a nexus of coordinated molecular interfaces from multiple structural elements; interacting absolutely conserved residues include Asp280 and Thr281 (α3-helix of the L5D) of the ZMP Core module, and Tyr65 (TM α-helix II), Gly152 and Asn154 (TM α-helix III/IV loop) of the ZMP Accessory module (Figure 4). Residues Asp280 and Thr281, in the L5D α3-helix of the ZMP Core module, form hydrogen bonds with side-chain and main-chain residues of the β2/β3-turn, respectively. Asp280 forms a hydrogen bond with the Ser256 hydroxyl-bearing side chain. His261, conserved in 56 of 58 orthologs (Figure S1), forms two hydrogen bonds with absolutely conserved Ste24 residues: via its backbone carboxyl to Thr281 (ZMP Core Module) and via its sidechain to Tyr65 (ZMP Accessory module). (The remaining two orthologs have a glutamate at this position, suggesting that a hydrogen bond at this location may be conserved in the Ste24 family [- Figure S1]). The TM α-helix III/IV loop contacts the β2/β3-turn, forming a short β-sheet with hydrogen bonds occurring between the two elements. Residues Gly152 and Asn154 are located in the loop and turn elements, and appear to facilitate their positioning. The Gly152 residue sits at the terminus of the TM α-helix III, while the Asn154 side-chain amide forms a hydrogen bond with the carbonyl of the TM α-helix IV terminus residue Thr157, conserved in 56 of 58 orthologs (Figure S1).

The β3/β4-turn and β4-strand of the L5D present the other significant interface to the ZMP Core β3-strand. Among MSA-identified residues, Gly268 preempts the β3/β4-turn, and the Val277 side chain forms the base of a hydrophobic pocket localized approximately at the center of the β4-strand and surrounded by aromatic and hydrophobic residues from the neighboring β3- and β2-strands of the L5D.
β-sheet. Furthermore, the presence of an analogous glycine and valine-centered hydrophobic interface is also present on equivalent elements of thermolysin (M4), neprilysin (M13), and the G. sulfurreducens ZMP (M48) structures, suggesting a common structural motif among these closely related gluzincins.

4.5 | ZMP Accessory module residues from TM α-helix I and the CTD α6-helix complete a unique Ste24 family structural motif

A significant portion of the Ste24 enzyme family ZMP Core module is formed by the CTD, including the antiparallel α5- and α7-helices and the α6/α7-loop (Figures 3 and 4). The α6-helix of the CTD, associated with the ZMP Accessory module, is positioned at the membrane interface, approximately perpendicular to the α5- and α7-helices of the ZMP Core module. Residues of the ZMP Core and Accessory modules form structural interactions with the N- and C-termini of this α-helix, respectively. The C-terminal end of the α6-helix forms a salt-bridge with Arg387, in a portion of TM α-helix VII within the ZMP Core module. The N-terminus, via Asp424, forms a second salt-bridge with Arg36 of the neighboring TM α-helix I of the ZMP Accessory module. The Gln37 residue of the TM α-helix I is optimally positioned to form hydrogen bond interactions with the peptide backbones of residues forming the α5/α6-loop. The structures of SmSte24 and HsSte24 contain large gaps ("fenestrations") between TM α-helices, and these fenestrations were posited to function as entry (TM α-helices I/VII)3,37 and exit (TM α-helices I/VII)3,37 portals to the active site, as it is encircled by TM α-helices and located within the membrane interior (Figure 1A).

4.6 | The Ste24 α-barrel module

A TM heptahelical "α-barrel" module, absent in soluble gluzincins and unique to the Ste24 enzyme family, contacts the ZMP Core and ZMP Accessory modules (Figures 3C and 4). The α-barrel module of Ste24, composed of amphipathic α-helices, forms a voluminous cavity (~14 000 Å³ in SmSte24)33 within the membrane interior. The vast majority of TM α-helices in integral membrane proteins are hydrophobic; therefore, use of the SmSte24 α-barrel module in a Dali search yielded both soluble and membrane proteins with structural similarity. The highest-scoring result (Z score of 17.3, with the next highest of Z score less than six) was HsSte24, reinforcing the complete structural novelty of the α-barrel module. Of the top 100 "hits," approximately two thirds were to soluble protein complexes, reflecting the amphipathic nature of the helices in the α-barrel search model. Absolutely conserved Ste24 residues Trp97, Pro138, Tyr142, Asp164, Pro219, Phe340, and Pro373 reside in the α-barrel module (Figure 4). Of these seven residues, Pro138, Asp164, and Pro373 are located within the membrane. Trp97 and Phe340 are predicted to be located at the membrane interface opposite to that of the ZMP Core and Accessory modules, while Tyr142 and Pro219 share the same membrane boundary as the ZMP Core and Accessory modules (Table 1).
Residues Tyr142 and Asp164 participate in significant membrane-interfacial interactions with the ZMP Accessory module. Tyr142 and Phe145, both located within TM β-helix III, are positioned at the membrane interface between the α-barrel and ZMP Accessory modules, respectively (Table 1). The Asp164 residue forms an interfacial salt bridge with an Arg258 located at the j2/j3-turn of the L5D in the ZMP Accessory module. The three absolutely conserved prolines of the α-barrel module are all located within TM α-helices. Pro138 introduces a small kink in TM α-helix III; its location approximately one turn away from the membrane interfacial residue Tyr142 may mitigate its effect upon proteolytic function. In contrast, Pro373 introduces a large kink and realignment (~90° with respect to the membrane normal) of TM α-helix VII, which contains the zinc ligand Glu390 and second-sphere ligand Asp394. The site of this kink is ~30 Å from the zinc-center. The Pro219 residue is positioned near the terminus of TM α-helix V and is a half turn away from the ZMP Accessory module residue Phe221, which is predicted to rest at the membrane. Residues Trp97 and Phe340 are furthest removed from the zinc center, and are positioned on the opposing membrane leaflet. Trp97 is located in the α1-helix and rests at the center of an interfacial, hydrophobic pocket formed from residues of TM α-helices III and VII, and Phe340 of helix α4. Although functions of absolutely conserved residues “distal” to the Ste24 active site are currently unknown, recent studies implicating HsSte24 as a “translocon unclager” indicate the presence of functional “hot spots” far from the active site. Roles for absolutely conserved residues of the α-barrel module in essential “nonenzymatic” functions may also exist; these could include residues essential for biogenesis or protein stability. Also, residues conserved more narrowly, within animals rather than also in fungi, plants and protists (Table S1), may provide insight into the ability of HsSte24 to function (nonenzymatically) as a broad spectrum viral restriction factor.

5 | CONCLUSION

A detailed bioinformatic and structural analysis of the Ste24 family of ZMPS, in the context of well-characterized soluble gruzinc orthologs, enabled development of the Tripartite Architecture representation of this integral membrane protein ZMP. The ZMP Core and Accessory modules are capped by a membrane-spanning α-barrel module, so named because of our current lack of understanding of its functional role. In light of the ever-growing literature of Ste24 family orthologs and emergent clinically relevant roles of HsSte24, the Tripartite Architecture will aid in the interpretation of disparate functional data. Furthermore, our proposed Tripartite Architecture can act as a platform to design experiments to better understand both the functional overlap and individual uniqueness of Ste24 family enzymes among different family orthologs (eg, fungal/mammalian/plant/protist). Additionally, the Tripartite Architecture will be of utility in the overall context of ZMPS, and may also be applicable to other integral membrane protein enzymes whose active sites reside at a membrane interface.

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Additional supporting information may be found online in the Supporting Information section at the end of this article.

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