Role of Zmpste24 in Prelamin A Maturation.

Douglas Paul Corrigan
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Role of Zmpste24 in Prelamin A Maturation

A dissertation presented to the faculty of the Department of Biochemistry and Molecular Biology East Tennessee State University

In partial fulfillment of the requirements for the degree Doctor of Philosophy in Biomedical Sciences

by
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August 2005

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ABSTRACT

Role of Zmpste24 in Prelamin A Maturation

by

Douglas P. Corrigan

The nuclear lamins form a karyoskeleton providing structural rigidity to the nucleus. One member of the lamin family, lamin A, is first synthesized as a 74 kDa precursor, prelamin A. Following the endopeptidase and methylation reactions which occur after farnesylation of the CAAX-box cysteine, there is a second endoproteolysis that occurs 15 amino acids upstream from the C-terminal farnesylated cysteine residue. Studies with knockout mice have implicated the enzyme Zmpste24 as a candidate to carry out one or both of these proteolytic reactions. In this body of work, the CAAX endopeptidase activity of recombinant, membrane reconstituted, Zmpste24 is demonstrated using a prelamin A farnesylated tetrapeptide as substrate. To monitor the second upstream endoproteolytic cleavage a 33 kDa prelamin A carboxyl terminal tail of prelamin A was expressed in insect cells. This purified substrate possesses a fully processed CAAX box, and, therefore, constitutes a valid substrate for assaying the second endoproteolytic step in lamin A maturation. In vitro reactions with this substrate and insect cell membranes bearing recombinant Zmpste24 demonstrate that Zmpste24 may possess the ability to directly catalyze the second endoproteolytic cleavage. Previous studies on nuclear envelope fractions have ascribed this second activity to a chymotrypsin like protease. However, Zmpste24 contains the canonical HEXXXH domain, a common characteristic of zinc
Experiments on Zmpste24 in this work demonstrate that inactivating the HEXXH domain by site directed mutagenesis results in a loss of the first endoproteolysis reaction, while the second endoproteolytic activity is retained. Supporting these data is the observation that a truncated mutant of Zmpste24 (residues: Met$^1$ - Pro$^{230}$) that does not contain the HEXXH motif, loses the first endoproteolytic activity while retaining the second. Furthermore, this second activity is not sensitive to the metalloproteinase inhibitors EDTA and 1,10-orthophenanthroline but is sensitive to the chymotrypsin inhibitor TPCK and its fluorescent analogue, FFCK. The fact that Zmpste24 can be affinity labeled with FFCK suggests that this second activity is directly caused by a second, yet unidentified, active site with a chymotrypsin-like catalytic mechanism.
ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

AEBSF  aminoethylbenzenesulfonylfluoride
BAF    barrier to autointegration factor
BSA    bovine serum albumin
CHO-K1 Chinese-hamster ovary-K1 cells;
CMT    Charcot-Marie-Tooth disorder
CMV    cytomegalovirus
CPM    counts per minute
DCM    dilated cardiomyopathy
DFP    diisopropylfluorophosphonate
DTT    dithiothrietol
E-64   trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane
EDMD   Emery Dreifuss muscular dystrophy
EDTA   ethylenediaminetetraacetic acid
FBS    fetal bovine serum
FFCK   5(6)-carboxyfluoresceinyl-L-phenylalanine chloromethyl ketone
FPLD   familial partial lypodystrophy
FPP    farnesyl pyrophosphate
GCL    germ cell less
HGGS   Hutchison-Gilford progeria syndrome
HPLC   high pressure liquid chromatography
ICMT   isoprenyl cysteine methly transferase
INM    inner nuclear membrane
Lap's  lamina associated polypeptides
LBR    lamin B Receptor
LGMD   limb girdle muscular dystrophy
MAD    mandibuloacraldysplasia
MALDI-TOF matrix-assisted laser-desorption ionization–time-of-flight
NPC    nuclear pore complex
OG     octyl glucoside
ONM    Outer nuclear membrane
Opa    1,10 orthophenanthroline
PBS    phosphate buffered saline
PCR    polymerized chain reaction
PM     pore membrane
PMSF   phenylmethylsulfonylfluoride
PrelaminAct prelamin A – Carboxyl terminus
psi    pounds per square inch
PSSM   position sensitive scoring matrix
PVDF   polyvinylidifluoride
Rb     retinoblastoma
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>RCE1</td>
<td>ras converting enzyme 1</td>
</tr>
<tr>
<td>RD</td>
<td>restrictive dermopathy</td>
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<tr>
<td>Rf</td>
<td>retention factor</td>
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<tr>
<td>RPS</td>
<td>reverse position specific</td>
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<tr>
<td>RT-PCR</td>
<td>reverse transcription PCR</td>
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<tr>
<td>SAM</td>
<td>S-adenosyl methionine</td>
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<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
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<tr>
<td>Sf21</td>
<td>spodidoptera frugiperda</td>
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<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
</tr>
<tr>
<td>SREBP1</td>
<td>sterol regulatory element binding protein 1</td>
</tr>
<tr>
<td>TBST</td>
<td>tris buffered saline tween 20</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
</tr>
<tr>
<td>TPCK</td>
<td>N-tosyl-L-phenylalanine chloromethyl ketone</td>
</tr>
<tr>
<td>WS</td>
<td>Werner's syndrome</td>
</tr>
<tr>
<td>Zmpste24</td>
<td>zinc metallo proteinase Ste24</td>
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CHAPTER 1
INTRODUCTION

The Nuclear Envelope

At the center of the eukaryotic cell is the complex and dynamic structure of the nucleus, a multifunctional organelle that: 1) stores genetic information in the form of chromatin; 2) regulates gene expression, and 3) controls the overall dynamics of the mitotic process (Lamond and Earnshaw 1998; Mizuno 1999). Separation of the contents of the nucleus from the rest of the cell is governed by the structure of the nuclear envelope, a dual membranous structure containing both phospholipids and proteins (Dessev 1992). Not only does the nuclear envelope provide a static structural role for defining the boundaries of the nucleus, it also plays a dynamic role in gene regulation by providing attachment points for chromatin and other genetic regulatory factors that influence the transcription of genes (Pfaller and Newport 1995; Marshall and others 1996). In addition to these roles, the nuclear envelope contains channels known as nuclear pores that govern the regulated passage of mRNA and other signaling molecules that transmit information from the cytosol to the nucleus (Macara 2001). Complex signaling pathways that originate at the periphery of the cell via protein receptors and other signaling events usually cascade in a manner to direct that signal to the nucleus (Leonard and others 1999; Roberts 1999). The convening of these signaling pathways at the nucleus will then trigger the appropriate response such as modulating the timing of mitosis, changing the pattern of gene expression, and/or initiating the apoptotic program, leading to cell death. It is evident, then, that the nucleus is akin to the workings of a microprocessor at the heart of a computer, and that the nuclear envelope plays a critical role in defining the nuclear structural boundaries, and regulating the dynamics of complex cellular responses.
Overall Structure of The Nuclear Envelope

During interphase, the dual membranous structure of the nuclear envelope surrounds the nucleus and is comprised of an inner (INM) and outer (ONM) nuclear membrane, the outer being contiguous with the endoplasmic reticulum (Worman and Courvalin 2000). The pore membranes (PM), where the nuclear pore complex (NPC) reside, form a membranous connection between the INM and the ONM (Fig 1) (Ostlund and Worman 2003).

Figure 1 Schematic of nuclear envelope structure. (Adapted from: Ostlund C. Nuclear envelope proteins and neuromuscular diseases. Muscle Nerve 2003;27:393-406)
Nuclear membranes: Dark Blue; Nuclear Pore Complexes: Yellow; Lamina: Red; Chromatin: Light Blue.
At the inner nuclear membrane periphery is an underlying network of intermediate filament family proteins known collectively as the lamins, which provides a structural meshwork for attachment of various transmembrane proteins, chromatin, and transcription machinery (Fawcett 1966; Gerace and Burke 1988; Stuurman and others 1998). Examples of the most common proteins that interact at the inner nuclear membrane periphery are the lamins, chromatin, lamina-associated polypeptides (LAP’s), Lamin B Receptor (LBR), Otefin, MAN1, Emerin, Nesprin, barrier-to-autointegration factor (BAF), and the transcription factors retinoblastoma (Rb), sterol regulatory element binding protein (SREBP-1), Germ-cell-less (Gcl), and Oct-1 (Goldman and others 2002; Goldman and others 2005) (Fig 2). The complexity of interactions that occur between the scaffolding proteins, chromatin, transcription factors, and other proteins at the inner surface of the inner nuclear membrane have profound implications on the overall structural integrity of the cell, cell-cycle control, DNA replication, and regulation of gene expression (Hutchison and Worman 2004). This complexity is governed by an intricate control and regulatory system within the cell to control the disassembly and reassembly of the entire nucleus during the mitotic program of higher eukaryotes (Ostlund and Worman 2003).
The Structure and Nature of the Lamina

Like the plasma membrane, the structure and stability of the inner nuclear membrane is responsive to a complex mesh of intermediate filament proteins (Fawcett 1966; Aebi and others 1986; Fisher and others 1986). Specific to the nuclear envelope, this underlying intermediate filament meshwork is comprised predominantly of the structural proteins known as the lamins (Stuurman and others 1998). The intermediate filament nature of the lamins make them similar to the structure of desmin, neurofilaments, keratins, and vimentin, which are cytoplasmic intermediate filament proteins (Coulombe
and others 2001). The general structure of the lamins consists of four conserved $\alpha$-helical coiled-coil rod domains characterized by heptad repeats of apolar residues (denoted 1A, 1B, 2A, and 2B), surrounded by head and tail globular domains (Hutchison and Worman 2004) (Fig 3).

The lamins polymerize by forming obligate parallel dimers which interact at the coiled-coil interface, which then assemble in an antiparallel geometry to form higher-order intermediate filaments having a diameter of 10-13 nm (McKeon and others 1986). These filaments become laterally arranged into a two-dimensional matrix that underlies the INM and interconnects the nuclear pore complexes (Aebi and others 1986).

The nuclear lamina tethers the nuclear face of the inner nuclear membrane to the underlying chromatin maintaining overall nuclear architecture (Gotzmann and Foisner 1999) and has become known as a tensegrity element that maintains overall nuclear stability by resisting tensile and mechanical deformation (Hutchison and Worman 2004).
A growing number of proteins have been shown to directly interact with the lamins, linking the function of the lamins to much more dynamic and broader physiological processes than that of the more traditional static structural role (Goldman and others 2002). The inner nuclear membrane scaffold has been shown to mediate chromatin organization and condensation (Paddy and others 1990), nuclear assembly and disassembly (Ulitzur and others 1992), DNA synthesis (Spann and others 1997), transcriptional control (Dreuillet and others 2002; Kumaran and others 2002; Lloyd and others 2002; Mancini and others 1994), and apoptosis (Rao and others 1996; Zhivotovsky and others 1997). This wide spectrum of lamina associated events in vivo demonstrates that the lamina plays a much larger role than predicted by the static structural model of lamin function.

The lamins are classified as either type A or type B depending on their behavior during mitosis, physiochemical properties, and their tissue expression specificity (Gerace and Blobel 1980; Krohne and Benavente 1986; Broers and others 1997). More specifically, the A-type lamins are expressed mainly in highly differentiated cell types, are neutral, and become soluble during the disassembly of the nuclear envelope during mitotic division. In contrast, the B-type lamins are expressed ubiquitously in all cell types, are acidic, and remain associated with membranous structures during lamina depolymerization and mitotic breakdown of the nucleus. The A-type lamins A, AΔ10, C, and C₂ are all differentially spliced products of the LMNA gene (Fisher and others 1986; Furukawa and others 1994; McKeon and others 1986). The lamins classified as B-type are encoded by two separate genes: Lamin B1 is the sole product of the LMNB1 locus, while Lamin B2 and Lamin B3 are products of the LMNB2 gene (Pollard and others 1990; Biamonti and others 1992; Furukawa and Hotta 1993). The tissue expression pattern of the LMNA
locus is primarily driven by the developmental program controlling embryogenesis and organ differentiation (Rober and others 1989) and is found mainly in terminally differentiated mammalian cell types. Lamin A is generally not found in nascent embryonic cells, and does not become significantly expressed until after birth (Stewart and Burke 1987). In the adult, lamin A expression is completely absent in haematopoietic, immune, and epithelial stem cells. However, in progeny of these stem cells, prelamin A expression is observed after complete differentiation (Rober and others 1990). Lamin B1 and Lamin B2, in contrast, are needed for cell survival and, consequently, are widely expressed in all cell types, including adult stem cells (Harborth and others 2001; Steen and Collas 2001). These observations along with the fact that the Lamin C2 and Lamin B3 isoforms are found solely in germ-line cells (Furukawa and Hotta 1993; Furukawa and others 1994), suggest that the lamins have a broad spectrum of tissue specific effects, the implications of which are not fully understood yet. It has been hypothesized that the tissue specific differential expression pattern of lamin A and C influences chromatin organization as to maintain the differentiated state of the cell (Rober and others 1989).

**Laminopathies**

There are many disease phenotypes associated with altered nuclear envelope function, collectively referred to as laminopathies (Burke and Stewart 2002; Mounkes and others 2003). Most of the laminopathies discovered to date have been mapped to the LMNA locus and, therefore, a great deal of research effort has focused on lamin A. Mutations in LMNA can affect cardiac, muscular, skeletal, neural, or adipocyte tissues, or any combination of these (Hutchison and others 2001). More specifically, mutations in the
LMNA gene are associated with at least 9 different human diseases including the autosomal dominant form of Emery-Dreifuss muscular dystrophy (EDMD) (Bonne and others 1999), dilated cardiomyopathy type 1A (DCM1A) with conduction system defects (Fatkin and others 1999), limb-girdle muscular dystrophy 1B with atrioventricular conduction disturbances (LGMD1B) (Muchir and others 2000), Dunnigan-type familial partial lipodystrophy (FPLD) (Shackleton and others 2000), Charcot-Marie-Tooth disorder type 2B1 (CMT2B1) (De Sandre-Giovannoli and others 2002), mandibuloacral dysplasia (MAD) (Novelli and others 2002), restrictive dermopathy (RD) (Navarro and others 2004), atypical Werner's syndrome (Chen and others 2003), and Hutchison-Gilford progeria syndrome (HGPS), a premature aging disorder that affects all tissues and results in premature death (Eriksson and others 2003; Goldman and others 2004). The first genetic model studied for laminopathies was LMNA null mice which die early at 8 weeks of age and exhibit growth retardation, skeletal dysplasia, and cardiomyopathy, resembling EDMD and DCM disorders in humans (Sullivan and others 1999). The first human occurrence of a complete lamin A disruption resulted in death after premature birth (Muchir and others 2003). This infant exhibited muscular dystrophy and severe joint contractures and, thus, resembled the phenotype in LMNA^−/− mice. Therefore, lamin A is absolutely required for postnatal development and its absence is lethal. The laminopathies associated with the less severe laminopathies are primarily point mutations and premature truncations leading to altered nuclear function and morphology and are found scattered across the entire length of the lamin A molecule (Burke and Stewart 2002) (Fig 4). An exception to this rule is the case of HGPS, in which a point mutation G608G(GGC > GGT) within exon 11 causes a cryptic splice site to be activated, resulting
in the loss of 50 amino acids near the carboxy terminus (Eriksson and others 2003). Interestingly, this 50 amino acid deletion removes the canonical endoproteolytic cut site between Tyr$^{646}$ and Leu$^{647}$ but does not affect the presence of the CAAX box. Presumably, this laminAΔ50 mutant would possess a prenylated and carboxymethylated carboxyl-terminal cysteine, identical to that of prelamin A. However, this has not been experimentally verified to date.

Figure 4 Most common lamin A mutations resulting in disease. (Adapted from: Burke B. Life at the Edge: The Nuclear Envelope and Disease. Nat Rev Mol Cell Biol 2002;3:575-585)

It is not immediately evident how mutations in a protein that is more or less ubiquitously expressed in most terminally differentiated cells can give rise to phenotypes.
that affect such a wide array of different tissues. Currently, there are three major hypotheses that attempt to explain the mechanisms responsible for lamin A associated diseases: nuclear fragility; perturbations in cytoskeletal-nuclear interactions; and global and/or specific gene expression changes (Burke and Stewart 2002; Mounkes and others 2003). A model in which lamin A malfunction causes nuclear fragility is an attractive explanation for phenotypes associated with skeletal muscle and heart tissue disorders, such as EDMD, LGMD, and DCM. In this model, shear stress due to mechanical use of the muscle would eventually damage the nucleus and lead to cell death. This mechanical fragility model is supported by the fact that more than 50% of lamin A mutations associated with myopathy cause abnormal nuclear architecture when expressed in myoblasts and fibroblasts (Ostlund and others 2001; Raharjo and others 2001). However, this shear stress model fails to explain the tissue pathology of FPLD, a phenotype that affects the function of adipocytes which is a tissue that does not experience near the same amount of repetitive mechanical stress as muscle.

A more plausible model to explain the variety of tissue specific pathologies associated with mutations in one protein is the gene-expression model. Lamin A has been found to directly interact with a variety of gene-regulatory factors, including SREBP1 (Lloyd and others 2002), MOK2 (Dreuillet and others 2002), Rb (Mancini and others 1994), and BAF (Gotzmann and Foisner 1999). As a specific example of this hypothesis, SREBP1 is a transcription factor that activates genes involved in cholesterol biosynthesis, adipocyte differentiation, and lipogenesis (Kim and Spiegelman 1996), and has been shown to directly interact with the globulin like tail of lamin A (Lloyd and others 2002). Mutations in Lamin A that cause FPLD bind SREBP1 with 25-40% less affinity (Lloyd and others
2002). Therefore, it becomes attractive to link FPLD to changes in adipocyte function due to genes affected by altered SREBP1 function.

In addition to binding directly to specific gene regulatory factors, lamin A interacts directly with naked DNA (Luderus and others 1994), histones (Taniura and others 1995), and organizes heterochromatin (Kourouli and others 2000). Gross disruptions in the organization of chromatin, which is found in cells from EDMD patients, has the potential to change global expression pattern of hundreds, or possibly, thousands of genes (Ognibene and others 1999). In light of this, unraveling the fundamental causes for each specific lamin A mutation linked to a laminopathy may only be possible through experiments that measure global gene expression patterns using cDNA chip technology.
The Processing Pathway of Prelamin A

Out of the 7 isoforms of lamin molecules, Lamin A exhibits a unique set of post-translational modifications prior to assembly into the nuclear lamina (Sinensky and others 1994b). Lamin A is first synthesized as a precursor, prelamin A, before fully maturing and assembling into the nuclear lamina (Gerace and others 1984; Dagenais and others 1985; Lehner and others 1986; Beck and others 1990). Similar to Lamin B, lamin A possesses a canonical CAAX box (where C=cysteine, A = aliphatic amino acid, X = S, M, C, A, Q, L) at the carboxyl-terminus of the molecule that undergoes three sequential post-translational modifications (Vorburger and others 1989; Sinensky and others 1994b). More precisely, the series of post-translational modifications at the CAAX box result in the prenylation of the cysteine sulfhydryl via a prenyltransferase, removal of the AAX tripeptide by a CAAX endopeptidase, and finally, carboxymethylation of the newly exposed cysteinyl carboxy group via an isoprenylcysteine methyl transferase (ICMT) (Sinensky and others 1994b) (Fig 5). Lamin B and Lamin A proceed through the same first set of three modifications. However, unique to prelamin A, the last 15 carboxyl-terminal residues including the farnesylated and carboxymethylated cysteine of prelamin A are removed in a second endoproteolytic step (Weber and others 1989; Beck and others 1990; Lutz and others 1992). This final endoproteolytic removal of the 2 kDa prenylated peptide generates mature lamin A, which then is able to assemble into the filamentous meshwork of the lamina underlying the nuclear envelope (Lutz and others 1992).
Figure 5 The processing pathway of prelamin A. Prelamin A undergoes a unique series of post translational modifications at the designated CAAX box, CSIM, including farnesylation, AAX endoproteolysis, carboxymethylation at the farnesylated cysteine, and a second endoproteolytic cleavage between tyrosine\(^{646}\) and leucine\(^{647}\), 15 residues upstream from the farnesylated cysteine residue. This results in the production of mature lamin A, along with the release of a ~2 kDa prenylated peptide.

In general, proteins with a CAAX box at the carboxy terminus can undergo an isoprenoid lipid modification by either a farnesyl isoprenoid (15 carbon) when the X amino acid is S, M, C, A, Q, or by a geranylgeranyl group (20 carbon) when the X amino acid is leucine (Moores and others 1991; Seabra and others 1992; Armstrong and others 1995).
The function, localization, and binding characteristics of a large variety of cellular proteins can be modulated via a post-translational lipid modification at the c-terminus with a prenyl moiety (Hennekes and Nigg 1994; Fu and Casey 1999). Examples of proteins that are modified by isoprenoid substituents are the nuclear lamins, fungal mating factors, protein kinases, the Ras superfamily of GTPase proteins such as p21ras, phosphodiesterase subunits, and heterotrimeric G proteins involved in receptor signaling (Glomset and others 1990; Zhang and Casey 1996). The isoprenyl and subsequent carboxymethyl modifications made to CAAX box proteins confers the ability to associate with membranes either by a purely hydrophobic interaction with the phospholipid bilayer (Black 1992; Rando 1996), or a prenyl-protein interaction with a membrane receptor protein (Marshall 1993; Kisselev and others 1995). Examples of the latter mechanism is the interaction of farnesylated lamin B with its inner nuclear membrane anchor, the lamin B receptor (LBR) (Ye and Worman 1994), and the prenyl specific interaction of prelamin A with NARF (Barton and Worman 1999).

Unlike lamin B, the lamin A precursor, prelamin A, is a unique example of a prenylated protein in mammalian cells that undergoes an additional proteolytic step subsequent to the canonical CAAX box modifications described above (Beck and others 1990). In the process of maturation of prelamin A to lamin A, the last 18 carboxy terminal residues (15 residues upstream from the farnesylated cysteine) are removed in two endoproteolytic steps, the second being an endoproteolytic cleavage between Tyr646 and Leu647 (Weber and others 1989). This endoproteolytic cleavage removes a ~2 kDa farnesylated peptide and allows the mature lamin A to correctly assemble into the nuclear lamina. Removal of this prenylated 2 kDa peptide is absolutely essential for assembly into the nuclear lamina.
envelope. Evidence supporting this includes; incubating cells with lovastatin, which inhibits isoprenoid biosynthesis, causes nonprenylated prelamin A to accumulate into nucleoplasmic aggregates; incubating these cells with mevalonate causes this aggregated prelamin A to convert to lamin A and properly assemble into the nuclear envelope; and a lamin A construct missing this carboxyl-terminal farnesylated peptide, when heterologously expressed in mammalian cells, properly assembles into the lamina at the nuclear periphery (Lutz and others 1992). While it is necessary for prelamin A to be farnesylated and carboxymethylated before the second endoproteolytic reaction can occur to convert prelamin A to lamin A (Kilic and others 1997), the presence of this prenylated peptide acts to block the polymerization of Lamin A into the intermediate filament network.

Several lines of in vivo and in vitro evidence have demonstrated that the ability of the second endoproteolytic reaction to occur depends on a completely modified CAAX box. For example, when cells are incubated with mevinolin, a compound that specifically reduces endogenous isoprenoid pools by inhibiting of isoprenoid biosynthesis, the conversion of prelamin A to lamin A is abolished resulting in the accumulation of prelamin A into nucleoplasmic aggregates (Beck and others 1990; Lutz and others 1992). Experiments in which the CAAX box cysteine is mutated to abolish isoprenyl attachment have also shown that prelamin A accumulates in nucleoplasmic aggregates, and that conversion to lamin A is blocked (Holtz and others 1989; Hennekes and Nigg 1994). Obviously, from these observations, it can be concluded that a prenylated substrate is required for endoproteolytic processing of prelamin A. However, results from experiments conducted in vitro with partially purified nuclear envelope fractions and a synthetic
substrate mimicking the last 18 residues of prelamin A, are consistent with the conclusion that prelamin A must not only be farnesylated, but also AAXed and carboxymethylated before endoproteolysis can take place (Kilic and others 1997). In agreement with this is the observation that ICMT⁻/⁻ mice, which presumably should not be able to carboxymethylate the prelamin A substrate, accumulate prelamin A in their cells (Bergo and others 2002). Based on these observations, the substrate recognition constraints of the endoprotease catalyzing the second reaction of prelamin A represents a novel and highly specific interaction. Previous attempts in our lab to purify the endoprotease catalyzing the second clip have been partially successful (Kilic and others 1999), but the exact genetic identity of this enzyme has remained elusive. However, endoproteolytic activity has been measured in nuclear envelope extracts using a model S-farnesyl, cysteiny1 methyl ester radioiodinated peptide corresponding to the carboxyl-terminal 18 amino acid residues of human prelamin A (Kilic and others 1997). Evidence that the activity measured in a crude nuclear envelope fraction was due to the bona fide prelamin A endoprotease included: activity was present in the nuclear envelope; endoproteolysis occurred between Tyr⁶⁴⁶ and Leu⁶⁴⁷; substrate needed a farnesylated and carboxymethylated cysteine for reactivity; and the activity was non competitively inhibited by N-Acetyl farnesyl methyl cysteine (Kilic and others 1997; Kilic and others 1999).

Evidence for the Role of Zmpste24 in Prelamin A Maturation

The gene for the prelamin A endoprotease remained a mystery until genetic data obtained from both yeast and mice provided specific information regarding its identity. The first evidence was observed in the yeast with respect to the protein Ste24p, which
was found to be the primary zinc-dependent metalloprotease responsible for the endoproteolytically processing of the prenylated mating factor precursor, α-factor (Fujimura-Kamada and others 1997). The proteolytic maturation of α-factor parallels that of prelamin A in that it is first processed at the CAAX box by farnesylation, AAXing, and carboxymethylation and then undergoes endoproteolysis 26 residues upstream from the prenylated cysteine between a threonine and an alanine (Anderegg and others 1988; Chen and others 1997). However, unlike prelamin A, α-factor undergoes a third endoproteolytic step catalyzed by the Axl1p protease that cleaves 12 residues upstream from the prenylated cysteine, producing the mature farnesylated dodecapeptide (Fig 6). Ste24p catalyzes both the AAXing reaction and first endoproteolytic cleavage 26 residues amino terminal to the prenylated cysteine, apparently mediated by a canonical zinc-metalloprotease HEXXH active site (Fujimura-Kamada and others 1997; Boyartchuk and Rine 1998; Tam and others 1998; Tam and others 2001).
Figure 6 Processing pathway of the yeast pheromone, α-factor. (Adapted from: Burke B. Life at the Edge: The Nuclear Envelope and Disease. J Cell Biol 1998;142:635-649)
The similarities between the processing of prelamin A in mammals and a-factor in yeast suggested the possibility that the human orthologue to Ste24p could play a role in prelamin A maturation. The cloned and expressed human orthologue, Zmpste24 (Face-1), was shown to fully complement yeast Ste24p by possessing the ability to process the prenylated a-factor at both the AAXing step and the first endoproteolytic cleavage 26 residues amino terminal to the prenylated cysteine (Tam and others 1998; Schmidt and others 2000). Although the function of a-factor in yeast is entirely different from the function or prelamin A in mammals, the substrate processing similarities between a-factor and prelamin A (three CAAX box modifications followed by an upstream second endoproteolytic clip) suggests that the human orthologue to yeast Ste24p may be the prelamin endoprotease.

Ste24 is a member of a larger group of zinc metalloproteinases of the M48 peptidase family (MEROPS Database). This family of proteinases contains homologues from bacteria (*E.Coli, H. influenzae*), fungi, higher eukaryotic multicellular organisms, and mammals. (MEROPS Database). When comparing the yeast and mammalian forms of Zmpste24, the human form of Zmpste24 is 36% identical and 51% similar to the *S. cerevisiae* form (Tam and others 1998). They both share the common characteristics of the canonical HEXXH zinc metalloproteinase domain, and 7 potential transmembrane domains. Topologically, Ste24p has been shown to be localized to ER membrane in the yeast, and is retained within the ER via a dilysine ER retention signal at the carboxy terminus (Tam and others 1998). In contrast, human Zmpste24 does not contain an identical form of this ER retention motif, but may possess a degenerate ER retention motif.
lysines at positions 3 and 6 residues from the carboxy terminus). Mouse Zmpste24 has been demonstrated to localize at the ER and nuclear envelope (Pendas and others 2002).

To elucidate the \textit{in vivo} function of mammalian Zmpste24, various mice Zmpste24\textsuperscript{-/-} knockout strains have been generated and phenotypically characterized (Leung and others 2001; Bergo and others 2002; Pendas and others 2002). It was observed that mice deficient in Zmpste24 exhibited skeletal abnormalities, muscle weakness, growth retardation associated with premature death, dilated cardiomyopathy, and lipodystrophy, which are all phenotypes associated with defects in lamin A that cause human laminopathies (Bergo and others 2002; Pendas and others 2002). Upon evaluating the fate of prelamin A in fibroblasts derived from Zmpste24\textsuperscript{-/-} mice, it was observed that prelamin A accumulates in these cells without conversion to mature lamin A. Furthermore, membranes from these cells were inactive, compared to wild-type, in carrying out the N-terminal processing of yeast a-factor, suggesting that Zmpste24, like Ste24p, possesses a farnesylation dependent endoproteolytic activity (Leung and others 2001). The results from the Zmpste24 knockout experiments in conjunction with the yeast data concerning a-factor led to the obvious hypothesis that prelamin A is the physiological substrate for human Zmpste24.

Assigning Zmpste24 as a prelamin A endoprotease had one major inconsistency with previous data from \textit{in vitro} experiments on crude nuclear envelope fractions. More specifically, the enzymatic activity in the nuclear envelope catalyzing the second endoproteolytic cleavage could be abolished by serine protease inhibitors (Kilic and others 1999). However, all endoproteolytic activity of Ste24 and human Zmpste24 on the a-factor substrate could be inhibited by the zinc metalloprotease inhibitor,
1,10-orthophenanthroline (Schmidt and others 2000; Leung and others 2001; Tam and others 2001). Both yeast Ste24 and human Zmpste24 contain a canonical HEXXH (X = any amino acid) domain, which is a characteristic of the gluzincin family of zinc metalloproteases (Hooper 1994). Fundamentally, serine proteases function by a completely different catalytic mechanism than zinc metalloproteases. In the superfamily of serine proteases, the three residues that form the catalytic triad (Ser, His, and Asp), form a charge relay system to hydrolyze the peptide bond (Rawlings and Barrett 1994; Barrett and Rawlings 1995). Hydrolysis in a zinc metalloproteinase is catalyzed by a Zn$^{+2}$ divalent cation coordinated by the histidine and glutamic acid in the HEXXH motif (Hooper 1994). Because endoproteolytic activity on a crude nuclear envelope fraction is sensitive to the serine protease inhibitors aprotinin, PMSF and isocoumarin, and yeast Ste24p is sensitive to the Zn$^{+2}$ chelator, Opa, it didn’t seem likely that Zmpste24 could be the endoprotease catalyzing the second reaction. However, it could be argued from the genetic data that the sole responsibility of Zmpste24 is to catalyze the first AAXing reaction, and that some other serine like endoprotease performs the second reaction. This possibility could not be ruled out from the Zmpste24$^{-/-}$ genetic data from mice because the accumulation of prelamin A in mouse fibroblasts could simply be due to the failure of the endoprotease to convert prelamin A to lamin A because of an unfully processed CAAX box. In other words, if the function of Zmpste24 is solely to AAX prelamin A, then a lack of Zmpste24 activity in the cell would necessarily cause the accumulation of a non-AAXed and, thus, non carboxymethylated prelamin A molecule. The experimental observations that ICMT$^{-/-}$ mice accumulate prelamin A (Bergo and others 2002) along with in vitro data using model prelamin A peptides (Kilic and others 2002)
conclusively demonstrate that a fully processed CAAX box is needed for the second endoproteolytic activity. Therefore, the phenotypic manifestations of Zmpste24<sup>−/−</sup> mice could simply be due to a lack of the first endoproteolytic AAXing reaction on prelamin A.

**Diseases Associated with Zmpste24**

The first phenotypic observations associated with impaired Zmpste24 function came from the generation of Zmpste24 knockout mice. Interestingly, the first Zmpste24<sup>−/−</sup> mice were reported to exhibit no phenotype and were not recognizably different from wild type control mice (Leung and others 2001). This first observation was shortly thereafter supplanted by a second group who published a paper demonstrating that Zmpste24<sup>−/−</sup> mice exhibit phenotypes akin to the manifestations seen in laminopathies (Pendas and others 2002). Five months later, the group that published the first Zmpste24<sup>−/−</sup> paper that showed no phenotype, reported in a second revised publication that Zmpste24<sup>−/−</sup> mice did, indeed, have a laminopathy related phenotype, but that they were reluctant to report this in their first publication due to the possibility that the neomycin cassette used in Zmpste24 gene disruption was altering the transcription of neighboring genes (Bergo and others 2002). The common set of phenotypes from both groups show that Zmpste24<sup>−/−</sup> mice exhibit retarded growth curves, premature death at 20 weeks, alopecia, muscle weakness in the limbs, low plasma glucose levels, and abnormal gait, and kyphosis (exaggerated outward curvature of the spine). However, Pendas also reported that Zmpste24<sup>−/−</sup> mice manifest other pathologies such as dilated cardiomyopathy, ventricular thinning, secondary liver disease, growth plate dysplasia in the femur and tibia, and a
partial lypodystrophy characterized by loss of subcutaneous fat, phenotypes that were not seen in the Zmpste24−/− mice studied by Bergo. In contrast, the mice generated by Bergo and others showed reduced cortical and trabecular bone volumes in conjunction with spontaneous bone fractures similar to those seen in osteogenesis imperfecta. This skeletal myopathy was not reported for the Zmpste24−/− mice investigated by Pendas. Because these phenotypes were similar to those seen in human and mice lamin disorders, Pendas studied the structure of the nucleus using confocal fluorescent microscopy using typical nuclear envelope markers (Lamin A/C, Lamin B1, and emerin). While wild type cells showed a typical ovoid appearance, cells from Zmpste24−/− mice showed abnormal nuclear morphological perturbations at the nuclear envelope resembling membranous herniations at the nuclear wall. The fact that the set of abnormalities seen in these mice resemble those of traditional laminopathic disorders prompted Pendas to study the processing of prelamin A in fibroblasts from these mice. Western blots with prelamin A and lamin A/C antibodies on cell lysates derived from Zmpste24−/− mice demonstrated that the premature 74 kDa prelamin A form accumulates at the expense of the presence of the 72 kDa mature form. Accumulation of prelamin A at the nuclear periphery in Zmpste24−/− cells was also verified by indirect immunofluorescence using prelamin A specific antibodies. As a final demonstration that prelamin A was the in vivo substrate for Zmpste24, fibroblasts from Zmpste24−/− mice were transfected either with prelamin A, or both prelamin A and Zmpste24. Cotransfection with Zmpste24 restored endoproteolytic processing of prelamin A to the lamin A mature form. The results from these experiments were the first that generated the hypothesis that prelamin A is the in vivo substrate for Zmpste24. It is important to note that this hypothesis
was not tested in vitro, nor was the accumulated precursor prelamin A protein characterized to determine if farnesylation, AAXing, or carboxymethylation took place at the CAAX box. Because these experiments were not carried out, it was not possible at that time to assign Zmpste24 as responsible for the first, second, or both endoproteolytic reactions. There also remained the possibility that Zmpste24 was not either of these endoproteases but was rather a membrane anchor or an upstream activator of other proteases involved in the maturation of prelamin A.

In humans, Zmpste24 mutations have been linked to a number of lethal and non lethal disorders. The first to be discovered was linked to a rare autosomal recessive disorder known as mandibuloacral dysplasia (MAD: OMIM 608612 and OMIM 248370), which was previously only linked to a Arg527His mutation in the LMNA gene (Agarwal and others 2003). Agarwal demonstrated that patients with MAD could also possess compound heterozygous mutations in the gene for Zmpste24 in which Zmpste24 from one allele is prematurely truncated due to a Phe361fsX379 mutation, and the other allele carries a Trp340Arg substitution adjacent to the HEXXH motif. MAD is characterized by underdevelopment of skeletal features, abnormally small lower jaw, degeneration of bone (acro-osteolysis), atrophy of the skin, scarce brittle hair, mottled skin pigmentation, type B lipodystrophy, and, in some cases, progeroid appearance (Schrander-Stumpel and others 1992; Agarwal and others 2003). From the fact that mutations in both Zmpste24 and Lamin A could manifest the same phenotypic characteristics, Agarwal hypothesized that MAD associated with mutations in Zmpste24 was caused by abnormal processing of prelamin A. Although the homozygous Arg527His mutation in lamin A has been shown to lead to the accumulation of the prelamin A precursor (Capanni and others
2005), accumulation of prelamin A from patients with Zmpste24 associated MAD has not been experimentally tested. Interestingly, each Zmpste24 mutation was tested for processing of a-factor in yeast mutants incapable of producing mature a-factor. The results showed that the prematurely truncated form of Zmpste24 was incapable of complementing this defect, while the Trp340Arg mutant was able to restore processing of a-factor less efficiently than wild type Zmpste24. Therefore, if these results can be correlated to the processing of prelamin A, then it can be expected that patients harboring both of these Zmpste24 mutations would be able to process prelamin A to some extent. However, the rate of conversion may be so inefficient that some amount of prelamin A accumulation may occur in these cells.

Zmpste24 mutations have also been linked to a lethal disorder known as restrictive dermopathy (RD: OMIM 275210). RD is also known as lethal tight skin contracture syndrome and is characterized by slow growth during gestation, tight translucent skin, severe contractures of the joints, craniofacial abnormalities, epidermal hyperkeratosis, and death following birth due to pulmonary hypoplasia and restricted movement (Navarro and others 2004). RD has been linked to both heterozygous mutations in prelamin A and homozygous loss of function mutations in Zmpste24 (Navarro and others 2004; Navarro and others 2005). More precisely, patients have been observed to carry a heterozygous de novo point mutation in lamin A that results in the loss of exon 11. Patients carrying this type of mutation show the accumulation of a truncated form of prelamin A. Patients with RD have also been found to carry a homozygous 1085dupT mutation (encoding Phe361fsX379, identical to the MAD mutation) in Zmpste24, leading to a prematurely terminated inactive form of Zmpste24. Cells from RD patients with this Zmpste24
mutation accumulate prelamin A and show abnormal nuclear morphology along with mislocalization of lamin associated proteins (Navarro and others 2004). Therefore, the correlation between prelamin A maturation and Zmpste24 in RD patients argues that Zmpste24 is involved in the processing of prelamin A.

Both RD and MAD can arise from mutations in lamin A or Zmpste24. In fact, the same premature Zmpste24 truncation mutation is observed in both of these disorders. This raises a puzzling question: how does the same mutation in one gene give rise to different phenotypes? This peculiarity is compounded by the fact that RD is lethal, but MAD is not. The most probable explanation is that MAD patients have only been identified with compound heterozygous mutations for Zmpste24, with one allele being partially functional. However, in RD patients, both alleles for Zmpste24 are truncated, resulting from a recessive inheritance. Therefore, if prelamin A is indeed the substrate for Zmpste24, then the major difference between RD and MAD cells should be the level of unprocessed prelamin A present. More specifically, in cells derived from patients with RD where both alleles of Zmpste24 are disfunctional, a relatively high level of prelamin A should be observed, and this is, indeed, what is observed (Navarro and others 2005). In contrast, in cells derived from MAD patients a lower accumulation of prelamin A should result because one partially functional copy of Zmpste24 is present. This line of reasoning leads to the hypothesis that the precursor protein, prelamin A, is relatively toxic to cells in a dose dependent manner, because RD patients with the highest level of prelamin A die shortly after birth, while patients with MAD live a normal life span. This hypothesis is supported by experiments that compared the phenotype of Zmpste24−/− mice and Zmpste24+/− LMNA+/- mice that produce half the normal level of lamin A (Fong and others
The results from this experiment revealed that by reducing the amount of prelamin A that accumulates due to loss of Zmpste24 function by a factor of 2 results in a total loss of the Zmpste24−/− induced phenotype.

The effects of siRNA induced Zmpste24 knockdown in cell culture has been studied in HeLa cells and has supported the hypothesis that prelamin A accumulation is toxic (Gruber and others 2005). More specifically, upon induction of Zmpste24 knockdown, HeLa cells abruptly stop cell division and can 1) directly proceed through apoptosis or 2) form dysmorphic hyperlobulated nuclei that accumulate unprocessed prelamin A at the periphery of the nuclear envelope. In addition, if lamin A is knocked down prior to Zmpste24 knockdown, or if the experiments are repeated in HL60 cells that lack endogenous lamin A, the apoptotic or dysmorphic phenotype is completely absent. This is in agreement with the experiments performed in Zmpste24−/− LMNA+/− mice that show that the toxicity of prelamin A is dose dependent.

This prelamin A toxicity hypothesis is supported by the fact the prelamin A also accumulates in cells from patients bearing certain mutations in the lamin A protein itself. Prelamin A has been observed to accumulate in patients that possess the following lamin A mutations: R527H resulting in MAD; R482L resulting in FPLD; and S143F resulting in atypical WS (S143F) (Capanni and others 2005). Also, internally truncated forms of prelamin A that still possess the CAAX box have been shown to accumulate in HGPS (Goldman and others 2004) and RD (Navarro and others 2004). Interestingly, in all of these disorders lipodystrophy is a common phenotype, and experiments have shown that the accumulation of prelamin A, but not lamin A, in MAD, FPLD, and WS cells sequesters the adipogenic transcription factor SREBP to the nuclear envelope and down regulates
PPARγ, a transcription factor activated downstream by SREBP (Capanni and others 2005). This same in vivo data regarding the function and localization of SREBP can be reproduced in wild type cells that were induced to accumulate prelamin A by treating them with mevinolin, an inhibitor of isoprenoid biosynthesis. Therefore, in addition to high doses of prelamin A being toxic to cells, it has been demonstrated that defective prelamin A processing can alter the activity of specific transcription factors. Therefore, it would be expected that pathologies associated with mutations in the endoprotease that processes prelamin A would phenocopy laminopathies linked with the accumulation of prelamin A. This prediction is supported by the fact that RD (Navarro and others 2005), MAD (Agarwal and others 2003), along with progeroid like symptoms (Shackleton and others 2005) have now all been linked not only to mutations in LMNA but also mutations in Zmpste24.

Overall, this phenotypic data argues that prelamin A is the in vivo substrate for Zmpste24, and that the farnesylated 2 kDa peptide representing the last 15 carboxyl-terminal residues of prelamin A is somehow toxic to cells. In light of this, it may be possible to account for the wide ranging differences in phenotypic manifestations among most of the known laminopathies by considering the fact that the precise expression level of prelamin A in each cell type varies in the population due to inherent genetic background differences. Therefore, the pathology of lamin A disorders is expected to exhibit individual variation in that the manner in which the disease manifests itself is based primarily on two factors: the rate at which prelamin A is being expressed in each tissue; and the rate at which other modifications take place, including endoproteolysis, to convert prelamin A to lamin A. The balance of these two rates...
ultimately determines the amount of prelamin A that accumulates in any given cell, and
the amount of prelamin A present in the cell, in turn, controls the severity and/or type of
phenotypic manifestation. A dose dependent hypothesis of prelamin A function is
attractive, for it offers an explanation towards the multifaceted array of tissue specific
disorders associated with disfunction of a single protein, prelamin A. However, the exact
biochemical mechanism(s) behind the downstream effects of prelamin A accumulation is
inherently complex due to the many functions, interactions, and binding partners
presently known for lamin A.

**Specific Aims**

Although the mouse knockout data and the observation that human diseases
associated with Zmpste24 malfunction resemble laminopathy like phenotypes build a
strong case for Zmpste24 being the first, second, or both endoproteases involved in
prelamin A maturation, this can not be conclusively demonstrated unless Zmpste24 is
tested against the prelamin A substrate *in vitro*. In all of the background experiments
reported, an *in vitro* experiment to test the hypothesis that Zmpste24 is the prelamin
endoprotease has not been conducted. Therefore, the major aim of this body of work was
to demonstrate the precise role of Zmpste24 in prelamin A endoproteolytic processing. To
complete this goal, two different substrates were employed to monitor each
endoproteolytic reaction in the processing pathway of prelamin A. To assay AAXing, we
synthesized a farnesylated tetrapeptide that modeled the carboxyl-terminal end of
prelamin A, and followed its conversion upon incubation with recombinant Zmpste24. We
also recombinantly expressed a fully processed (farnesylated, AAXed, and methylated)
carboxyl-terminal fragment of prelamin A and employed this as a substrate to monitor the second endoproteolytic cleavage. The generation of recombinant active Zmpste24, which contains 7 transmembrane domains, and the substrate prelamin A, which possesses a complex series of post translational modifications, is obviously not trivial, and this may explain the lack of such an experiment in the literature to date.
CHAPTER 2
MATERIALS AND METHODS

Materials

The BacPak8 baculovirus protein expression system was from Clontech and employed Sf21 (Spodoptera frugiperda) insect cells from Invitrogen. Sf21 cells were grown in Grace’s Insect Medium (Gibco) containing 10% heat-inactivated FBS (Gibco), 0.1% Pluronic F-68 (Cellgro), 10 mg/ml Gentamicin (Gibco), supplemented with 500 mg/L CaCl₂, 2800 mg/L KCl, L-glutamine, 3330 mg/L lactalbumin hydrolysate, and 3330 mg/L yeastolate. CHO-K1 cells were cultured in Ham’s F-12 medium containing 5% fetal calf serum, 100 units/ml penicillin, and 100 µg/ml streptomycin (F12FC5). Antibodies used in this study included: mouse monoclonal anti-Zmpste24 (recognizing residues 56-76) a gift from S. Young (Gladstone Institute); rabbit polyclonal anti-Zmpste24 (recognizing residues 440-455) (Abgent); mouse monoclonal anti-(His)₆ (BD Biosciences); goat-anti-mouse IgG-HRP (Pierce); goat-anti-rabbit IgG-HRP (Santa Cruz Biotechnology); donkey-anti-goat IgG-HRP (Santa Cruz Biotechnology); mouse monoclonal anti-farnesyl (Sigma), goat polyclonal anti-lamin A (Santa Cruz Biotechnology), rabbit polyclonal anti-prelamin A (Sinensky and others 1994a) and anti–mouse IgG Texas red-conjugated antibody (Biomeda). Radioactive isotopes included: [³⁵S]-methionine (specific activity 1175 Ci/mmol; ICN Biomedicals); 5-R, S-[³H] mevalonate (specific activity 60 Ci/mmol; American Radiolabeled Chemicals, St. Louis), [³H]-methyl-S-adenosylmethionine (specific activity 66.8 Ci/mmol; ICN Biomedicals), [³H]-FPP (specific activity 20 Ci/mmol; American Radiolabeled Chemicals, St. Louis).
Cloning of Prelamin A, Zmpste24, and ERp57

The template construct for prelamin A was a kind gift from H.J. Worman (Columbia University) and included the C-terminal residues 389-664 of human prelamin A (prelaminAct) cloned into the vector pBFT4 (Barton and Worman 1999). We performed PCR directed cloning of this insert fused to a (His)₆ tag at the N-terminus using two separate PCR reactions: The first stage used the forward primer 5′-ATGGCTCATCATCATCATCATCATCTGTCCCCCAGCCCTACCTC-3′ and the reverse primer 5′-GCGAATTCTTACATGATGCTGCAGTTCT-3′. The product from this PCR was then amplified with forward primer 5′-TAGGATCCACCATGGCTCATCATCATCATCATCATCTGT-3′ and the same reverse primer as in the first reaction. This product was directionally cloned into the (5′)-BamH1 (3′)-EcoR1 restriction sites of the pBacpak8 baculovirus expression vector (Clontech). Translation of this sequence in the Sf21 baculovirus system resulted in the following protein sequence containing an N-terminal (His)₆ tag fused to residues 389-664 of prelamin A:

MAHHHHHHHLSPSPTSRGRASSHSSQTQGGGSVTKKRKLESTERSSSFSQHART
SGRVAVEEVDEEGKFVRLRNSNEDQSMGNWQIKRQNGDDPLMTYRFPFKFTLKAG
QVVTIVAAGAGATHSPPTDLVWKAPQTWGCNLSRTALINSTGEEVAMRKLVRSVTV
VEDDEDEDDDLHHHHHSCHCSSSDPAEYNLRSRTVLCGTCQPADKASASSGSA
QVGGPISSGSSASSVTVTRSYRSVGGSGGGSFQDNLVTRESLLPQNSQNC
IM
To make the RSY RLG mutant \( (Y^{647} \rightarrow R^{647}) \) of prelamin A, we introduced a point mutation using the Quickchange Kit (Stratagene) into this prelaminAct/pBacpak8 construct with the primer 5'-GTCACCGCTCCTACCGCCTGGGCAACTCCAG-3' along with its complimentary sequence.

Zmpste24 was cloned into the (5')-Nhe1 (3')-BamH1 site of the pcDNA3.1 construct using RT-PCR on HeLa RNA (Stratagene). The forward primer was 5'-GCGGCTAGCATGGGGATGTCGCTCCGGA-3' and the reverse primer was 5'-GCGGGATCCGGACATCTCAGTGTTGCTTCATAG-3'. Zmpste24 was then subcloned using this as the template into pBacpak8 with the first set of primers being 5'-CATCATCATCATCATCATATGTCGCTCCGGA-3' (Forward) and 5'-GTGAGCGCCCGGTCAGTGTTGCTTCATAGATTATTCAAAGC-3' (reverse) and the second set being 5'-GCTGGCTCGAGACCATGGGCCATCATCATCATCATCATATGTCGCTCCGGA-3' (forward) and the same reverse primer as the first reaction. The PCR product was directionally cloned into pBacpak8 using the (5')-Xho1 (3')-Xma1 restriction sites. Translation of this sequence in the Sf21 baculovirus system resulted in the following protein sequence containing an N-terminal (His)_6 tag fused to residues 1-475 of Zmpste24:

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MGHHHHHHHMWASLDALWEMPAEKRIFGAVLLFSWTVYLVWETFLAQRRQRIYKTTTHV
PPELGQIMDSETFEKSRQLYQDKSTFSWGSGLYSETEGLTILLFGGIPYLWRLSGRFGC
YAGFGPEYEITQSVLLATLFSALTGLPWSLYNFTVESIKHGFNQQTGLFMMKDAIKK
FVVTQICLLPVSSLILYIKIGGYFFIAYWLFTLVVSSLVLVTIYADYIAPLFDKFTPLPEGK
LKEEIEVMASKIDFPLTKVYYVEGSKRSSHNSAYFYGGFKRNLVRLDFDTLLEEVSVLNKDI
QEDSGMMEPRNEEEGNSEEIKAKVKNKKQGCKNEEVLAVLGHHELGHWKLGHTVKNIIIS
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The ERp57 served as a mock infected control to emulate protein expression changes due to viral infection. ERp57 containing a (His)$_6$ tag at the N-terminus was cloned similarly using the first set of primers:

5'-ATGGCTCATCATCATCATCATCGCCTCCGCGCTAGC-3' (forward) and
5'-GCACGCGAATTCTACTGCTTTAGAGATCCTCCTG-3' (reverse).

The second amplification used:

5'-GCTACTGGATCCCATGGCTCATCATCATCAGCTCGCTCCGCTAGC-3' (forward) and the same reverse primer as the first reaction. This was cloned into the (5')-BamH1 (3')-EcoR1 site of pBacPak8. Each construct was verified for accuracy by sequencing. Recombinant high-titer baculovirus stock was produced for prelaminAct, Zmpste24, and ERp57 according to the manufacturer’s protocol (Clontech) in the Sf21 baculovirus expression system.

**Cloning of Zmpste24 Mutants and Truncations**

The (His)$_6$-WT Zmpste24 in pBacpak8 was used as the template plasmid in the QuickChange Site Directed Mutagenesis Kit (Stratagene). Six mutants were generated from this wild type plasmid and are designated: ΔHEXXH, H335A, Y379Stop, L290Stop, P230Stop, and C109Stop. Primers were designed to introduce these mutations into the
WT sequence and were used in the QuickChange PCR reaction according to the manufacturer’s protocol. The primers used to introduce these mutations are as follows:

△HEXXH  5’-CTCGCTGTACTAGGGTGAGTTGGGACATAC-3’
H335A   5’-CTCGCTGTACTAGGGCCGACTGGGGCAGATCG-3’
Y379Stop 5’-TTTGCTCGATTTGTTTTGAGTAGCGCCACCACCTA-3’
L290Stop 5’-ACTAGAAGAGTACTCTGTATAGAACATCCAGGAGG-3’
P230Stop 5’-TGACAAATTTCACACCTCTGTAGGGAAAGGACTTAAGAAG-3’
C109Stop 5’-GACTTTCTGGACCGTCTTGGTTATGCTGGGCTTTTG-3’

For each of these sense primers, anti-sense primers were designed as well, which correspond to the reverse complement of these sequences. Each of these mutant plasmids was introduced into the Sf21 baculovirus system for protein expression using the same protocol as the wild type sequence.

Baculovirus Expression Protocol

Spodoptera frugiperda (Sf21) (Invitrogen) cells diluted to 1 x 10⁶ cells/ml, were grown in log phase growth in Grace’s Insect Medium (Gibco) containing (10% FBS-heat inactivated, 0.1% Pluronic F-68, 10 mg/ml Gentamicin), were infected with high-titer recombinant baculovirus expressing either human Zmpste24, PrelaminAct or ERp57 (mock transfected control) at a multiplicity of infection of 10. Subsequently, 72 hours post-infection, cells were pelleted and stored at -80°C. These Sf21 cell pellets were then used for producing enzyme enriched membranes or purified substrate.
Preparation of Enzyme Enriched Membranes

Sf21 cells expressing either Zmpste24 or Erp57 (mock transfected control) were resuspended in 50 mM Tris-HCl pH 7.0 and disrupted by either sonication or a French press at 1000 psi. Nuclei and debris were removed by centrifugation at 500Xg for 5 min and membranes were then pelleted by centrifugation at 200,000Xg for 1.5 h. The membrane pellet was then washed with 50 mM Tris-HCl and centrifuged multiple times to remove all cytosolic components and possible sources of protease activity from the cytosolic fraction. Membranes were resuspended in 50 mM Tris-HCl and stored frozen at −80°C in multiple aliquots (Otto and others 1999). Protein content was determined according to the method of Lowry using the DC Protein Assay Reagent Kit (Biorad).

Immunoblotting

Protein samples were separated by SDS/PAGE and then electroblotted onto a PVDF membrane (Immobilon-P Millipore). The membranes were saturated with 5% (wt./vol.) nonfat dry milk in TBST [50 mM Tris·HCl (pH 8.0), 138 mM NaCl, 2.7 mM KCl, 0.05% Tween 20] (Sigma), then incubated 1 hr. with the primary antibody of interest. The membranes then were washed with TBST and incubated for 1 hr. with either goat-anti-mouse IgG-HRP (with anti-Zmpste24 (residues 56-76) or anti-(His)$_6$ as primary), goat-anti-rabbit IgG-HRP (with anti-prelamin A or anti-Zmpste24 (residues 440-455) as primary) or anti-goat IgG-HRP (with anti-lamin A as primary). Detection was performed with the Enhanced Chemiluminescence (ECL) kit (Pierce).
**Metabolic Labeling of PrelaminAct**

After infection with baculovirus (60 hours post-infection), Sf21 cells were labeled either 4 hours with $[^{35}\text{S}]$-methionine (100 mCi/ml, specific activity 1175 Ci/mmol) or 16 hours in growth medium containing 5-R, S-$[^{3}\text{H}]$-mevalonate (125 mCi/ml, specific activity 60 Ci/mmol). During mevalonate labeling, insect cells were treated with lovastatin (10 mg/ml) (Tocris). After labeling the Sf21 cells were harvested, sonicated in lysis buffer (50 mM NaH$_2$PO$_4$, 300 mM NaCl, 10 mM imidazole, pH 8.0), separated into membrane and cytosolic components, and purified using Ni-NTA beads (Qiagen). The $[^{3}\text{H}]$-mevalonate and $[^{35}\text{S}]$-methionine labeled prelaminAct fractions were separated on a 4-12% gradient (MES) and 10% (MOPS) SDS-PAGE gel system (Invitrogen), respectively. Dried gels were visualized by either fluorography or phosphorimager.

**Purification of (His)$_6$ Tagged Prelamin A and Zmpste24**

Sf21 cells, expressing recombinant protein, were subject to lysis by sonication in a lysis buffer (50 mM NaH$_2$PO$_4$, 300 mM NaCl, 10 mM imidazole, pH 8.0, 1mg/ml E-64). Membrane and cytosol fractions were separated by centrifugation (100,000 X g for 1 hour). Membrane bound proteins were solubilized in 1% NP-40 (prelamin A) or 1.5% octyl glucoside (Zmpste24), homogenized, and insoluble material removed by centrifugation at 100,000Xg. Cytosolic and membrane fractions were incubated for 2h at room temperature with Ni-NTA beads in lysis buffer. After incubation, the beads were washed with 8 vol. of lysis buffer containing 20 mM imidazole and then eluted with 1 volume of lysis buffer containing 250 mM imidazole for 15 minutes. Samples were analyzed by SDS-PAGE and immunoblot.
**Immunoprecipitation with anti-Farnesyl**

Ni-purified \[^{35}\text{S}^\]\text{-Met labeled prelamin Act was immunoprecipitated by incubation with an anti-farnesyl antibody in 30 mM HEPES (pH 7.5), 10 mM NaCl, 5 mM MgCl\(_2\), 25 mM NaF, 1 mM EDTA and 5% Nonidet P-40 (vol./vol.) for 16h at 4 °C. The complexes were isolated on protein A-Sepharose beads. The beads were washed, the immunoprecipitate was resuspended in 0.1N NaOH and incubated for 30 min at 30 °C to release precipitated protein. The pH of the eluted sample was neutralized by the addition of 0.1 M HEPES free acid. The products were separated on 10% SDS-PAGE (Invitrogen), transferred to PVDF membrane, and then visualized with a FUJIFILM FLA-5000 Phosphorimager (Fujifilm Medical Systems USA, Inc.).

**MALDI-TOF Mass Spectrometry**

Proteins were purified by SDS-PAGE, visualized by Coomassie staining and the appropriate bands excised. The isolated proteins were treated with iodoacetamide and digested with trypsin. MALDI-TOF was with a PerSeptive Voyager DE-RP mass spectrometer in the linear or reflector mode. This was performed by Mary Ann Gawinowicz at the Protein Chemistry Core Facility at Columbia University.

**Mammalian Cell Culture and Transfection**

For transient transfections, CHO-K1 cells were seeded at 3 X 10\(^5\) cells/well in a 6-well culture plate and transfected with the Zmpste24/pcDNA3.1 construct using the Stratagene GeneJammer transfection reagent according to the manufacture's protocol.
Indirect Immunofluorescence

Transiently transfected CHO-K1 cells on glass coverslips were briefly rinsed in phosphate buffered saline (PBS) then fixed with 4% formaldehyde in PBS (pH 7.4) for 15 min at 20 °C. Following three washes in PBS, the cells were permeabilized with 0.2% Triton X-100 in PBS for 5 min on ice, quickly washed, and blocked with 10% BSA in PBS for 5 min on ice. The coverslips were incubated for 1h at room temperature with mouse monoclonal anti Zmpste24 antibody (diluted 1/500) developed against residues 56-76. Cells were then washed and incubated at room temperature for 1h with anti–mouse IgG Texas red-conjugated antibody (diluted 1/500). Images were obtained by digital deconvolution of 10-slice stacks acquired on a Nikon Diaphot 200 microscope equipped with a Photometrics Sensys cooled CCD digital camera or Nikon D100 Oncor Z-Drive using Oncor Image Software.

Farnesylation of CSIM Tetrapeptide

Each reaction mixture contained, in a total volume of 100 µl, 50 mM Tris chloride (pH 7.5), 50 µM ZnCl₂, 3 mM MgCl₂, 20 mM KCl, 1 mM DTT, 0.2% (v/v) octyl β-D-glucoside with 10 µM farnesyl pyrophosphate 100 µM Ac-CSIM-OH (Sigma Genosys), and 0.3 µg of farnesyltransferase (Sigma) (Goldstein and others 1991). When radiolabeled substrate was prepared, [³H]-FPP (20Ci/mmol) was substituted for cold FPP in the reaction above (final concentration of 1µM). After 1h incubation at 37 °C, the reaction was terminated by the addition of 0.1 µl of concentrated HCl. Ac-(farnesyl)CSIM-OH was extracted with ethyl acetate (2 x 100 µl), dried under N₂ and dissolved in ethyl acetate. To confirm purity,
reaction products were separated by TLC on plastic-backed Silica Gel G thin layer sheets (20 x 20 cm, Whatman) developed with chloroform:acetone:methanol:acetic acid (70:15:8:2). The farnesylated product was visualized by brief staining with iodine vapor (cold substrate), or by fluorography ([³H]-FPP labeled substrate). Fluorography was accomplished by spraying the TLC plates with Enhance (Perkin Elmer) and exposing the film to Kodak-AR film in the presence of an intensifying screen.

**Base Release Assay for AAXing Activity**

The AAXing proteolysis mixtures were conducted in a total volume of 20 µl in buffer [250 µM zinc acetate, 200 mM NaCl, and 200 mM HEPES (pH 7.5)] with either 2 µM farnesylated a-factor or 2 mM Ac-(farnesyl)CSIM-OH (see above) and 30 µg of a membrane preparation from insect cells expressing either Zmpste24 or Erp57. After incubation at 30 °C for 25 min, reactions were terminated by heating to 95 °C for 1 min. Methylation reactions were then initiated by the addition of premixed 100 µg E. coli lipid and 0.13 µg purified Ste14p (ICMT) and [³H]-methyl-S-adenosylmethionine (55 µCi/ml, specific activity 66.8 Ci/mmol) to the reaction mixtures (Hrycyna and others 1995). After incubating 60 min at 30 °C, the reactions were stopped by the addition of 1M NaOH/1% SDS, which initiated base hydrolysis of the incorporated [³H]-methyl group. After base hydrolysis, 50 µl of each mixture was immediately spotted onto a heavy filter paper lodged into the neck of a scintillation vial containing 10 ml of scintillation fluid (ScintiSafe Econo 2, Fisher Scientific). After 2-3 hrs the filters were removed, and the radioactivity released determined by liquid scintillation counting.

Zmpste24 was expressed in insect cells and Ni-purified as described above. The activity of purified Zmpste24 was reconstituted by diluting the purified Zmpste24 5X into heat inactivated insect membranes in 50 mM Tris, pH 7.0, and then forming vesicles by sonication for 3 minutes. CAAX proteolysis mixtures contained, in a total volume of 20 µl, Ac-*fCSIM-OH (20,000 CPM), 30 µg of either reconstituted Zmpste24 or heat inactivated membranes, and buffer containing 200 mM NaCl, 200 mM HEPES (pH 7.5), 30 mM zinc acetate. In the samples preincubated for 15 min at 30°C with 2 mM 1,10 orthophenanthroline (a Zn$^{+2}$ chelator), zinc acetate was not used in the buffer. After incubation at 37 °C for 20 min, reactions were terminated by the addition of 100 µl of chloroform-methanol (1:1) followed by 100 µl of 1 M citric acid to achieve phase separation. The (lower) organic layer was collected, dried under N$_2$, dissolved in ethyl acetate, and spotted onto plastic TLC plates which were developed in chloroform:acetone:methanol:acetic acid. (70:15:8:2). Substrate (Rf 0.3) and product (Rf 0.58) were localized by autoradiography and counted using a liquid scintillation counter. For scintillation counting, the spots were cut and counted in 10 ml of scintillation fluid (Fisher Scientific).

Cotransfection of Zmpste24 and PrelaminAct into Sf21 Cells

The recipient cells used for cotransfection were Sf21. Eight hours after plating, cells were infected (baculovirus) with either prelamin Act or both prelamin Act and Zmpste24 in the presence or absence of lovastatin (10 mg/ml) (Tocris). Sixty-four hours post-infection cells were collected by scraping and centrifugation at 1,000Xg for 5 min. The cells were
washed with PBS, resuspended in lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, pH 8.0), and prelamin Act purified with Ni-NTA beads. The purified products were analyzed by SDS-PAGE and immunoblot with anti-lamin A or anti-prelamin A.

**In vitro Endoprotease assay on [³H]-Mevalonate Labeled PrelaminAct**

PrelaminAct purified from insect cells metabolically labeled with [³H]-mevalonate (see above) was incubated with membranes from insect cells expressing either Zmpste24 or Erp57 (mock transfected). The reaction mixture was separated on SDS-PAGE (4-12% MES) and visualized by fluorography. Reactions were also performed with [³H]-mevalonate labeled prelamin A immobilized on Ni-NTA beads. [³H]-mevalonate labeled material released was determined at various times by liquid scintillation counting.

**In vitro Assay using [³⁵S]-Met labeled PrelaminAct as Substrate**

Purified [³⁵S]-Met labeled prelaminAct was incubated with 30 ug of insect membranes in 50 mM Tris-HCl pH 7.0 in a final volume of 20 ml at 37°C for various periods of time. Each reaction contained the thiol protease inhibitor E-64 (Roche Molecular Biochemicals) to reduce background protease activity from the Sf21 insect cells. A prelaminActΔCAAX-pBFT4 construct (gift from H.J. Worman; Columbia University) which contained residues 389-660 (a stop codon after the terminal cysteine) was in vitro translated in the presence of [³⁵S]-Met with the Quick TnT system from Promega. The product from this reaction was diluted 400:1 and incubated with membranes as above. Products were separated on 10% MOPS SDS-PAGE gels then visualized using a
FUJIFILM FLA-5000 Phosphorimager. Densitometry was performed using the provided software.

**Labeling of Zmpste24 with FFCK**

Membranes from Zmpste24 or mock transfected cells were incubated in the presence of 20 uM FFCK (Immunochemistry Technologies, Bloomington, MN) for 30 min at 37°C. Where noted, membranes were either first heat inactivated or preincubated in the presence of TPCK. After incubation, labeled products were purified using Ni-NTA chromatography and separated on 10% MOPS SDS-PAGE gels. Fluorescence of FFCK was visualized using a FUJIFILM FLA-5000 Phosphorimager with excitation of 473 nm and a blue filter. Gels were then transferred to PVDF membranes and western blot performed with anti-(HIS)_6. Western blots were aligned with the fluorescent image with reference marks. The appropriately labeled 54 kDA bands for FFCK labeled and control samples were cut out and MALDI-TOF was performed as described above.
CHAPTER 3
RESULTS

Insect Cell Expressed and Purified Prelamin ACT is Fully Processed

A number of factors were taken into consideration in designing a suitable substrate for assaying endoproteolytic activity. It is necessary for the substrate to be fully post-translationally modified at the CAAX motif in order for the prelamin A endoprotease to cleave the prelamin A substrate (Kilic and others 1997). In addition, because these assays were to be conducted \textit{in vitro}, the substrate needed to be soluble. Therefore, the N-terminal coiled-coiled domain of lamin A was omitted. In light of these considerations, a C-terminal fragment of prelamin A (residues 389-664 including the CAAX motif, CSIM) tagged with \((\text{His})_6\) at the N-terminus, was expressed using a baculovirus expression system in insect cells and then purified from isolated membranes using Ni-NTA chromatography. This C-terminal fragment of prelamin A, prelaminAct, has previously been demonstrated to be water soluble and, therefore, is suitable for \textit{in vitro} studies (Barton and Worman 1999). The state of post-translational modification at the CAAX box of the purified prelaminAct substrate was then monitored using a variety of techniques (described below).

The purified substrate is recognized in immunoblots by a prelamin A specific antibody (Fig 7). The prelamin A antibody was raised against the 14 amino acid sequence of prelamin A (LLGNSSPRTQSPQN), residues 647-660 (Sinensky and others 1994a) which is removed during endoproteolytic maturation (Figure 1). Immunoblots with an anti-lamin A specific antibody were also positive (data not shown). The lamin A antibody recognizes
a portion of lamin A excluding the prelamin A specific C-terminal 15 residues. These results indicate that the recombinantly expressed substrate contains both a prelamin A specific sequence at the C-terminal end and a lamin A specific sequence.

Figure 7 PrelaminAct expressed in insect cells contains carboxy terminal sequence. The C-terminal half of prelamin A (residues 389-664) fused to (His)$_6$ at the N-terminus was cloned and expressed in insect cells. The expressed prelaminAct is solubilized from membranes and purified by binding and elution from Ni-agarose beads as described in Chapter 2. The elutions were resolved using SDS-PAGE and immunoblot was performed with anti-prelamin A. Cytosolic fraction (lane 1), membrane fraction (lane 2), and mock transfected purification (lane 3).

In order to confirm that the prelamin A substrate is prenylated, prelaminAct expressed in insect cells was metabolically labeled with $[^3H]$-mevalonate, a precursor in the biosynthetic pathway of isoprenoids. After labeling, the prelaminAct substrate was affinity purified, separated by SDS-PAGE, and visualized using fluorography (Fig 8). The single $[^3H]$-labeled band at 33 kDa indicates that the insect cell host is competent for prenylating the specific CAAX box sequence associated with prelamin A, CSIM.
Figure 8 PrelaminAct expressed in insect cells contains a prenyl moiety. PrelaminAct was expressed in insect cells in the presence of 5-R, S-[3H]-mevalonate and purified using Ni-NTA chromatography. The purified elution was resolved by SDS-PAGE, and visualized by fluorography.

It is possible for CAAX box proteins to be modified with a geranylgeranyl moiety rather than a farnesyl. The mevalonate labeling experiment above did not distinguish between these two potential forms of modification. To verify that the substrate is farnesylated, prelaminAct was expressed in the presence of [35S]-Methionine. After metabolic labeling, prelaminAct was separated into membrane associated or cytosolic fractions and then immunoprecipitated with an anti-farnesyl cysteine specific antibody (Fig 9). The membrane associated prelaminAct was precipitated by this antibody, consistent with the conclusion that prelaminAct is properly farnesylated. In contrast, only a small percentage of the cytosolic fraction of prelaminAct was recognized by this antibody (Fig 9, lanes 3 and 4) consistent with the conclusion that the cytosolically localized protein is not farnesylated. It has been observed that expression of farnesylated proteins, such as Ras,
in insect cells results in only a portion of the expressed proteins being lipidated. This is due to limitations on the capacity of insect cells for isoprenoid biosynthesis (Khosravi-Far and Der 1995). Therefore, this result is in agreement with the observation that prenylation targets proteins to membranes. Based on these observations, the membrane fraction from insect cells expressing prelaminAct was used as the source of prenylated substrate.

Figure 9 PrelaminAct substrate contains a farnesylated cysteine. Immunoprecipitation of purified cytosolic and membrane associated [³⁵S]-methionine labeled prelaminAct, with an anti-farnesyl antibody was performed. Immunoprecipitates (Lanes 1 and 3) and flow through (FT) (Lanes 2 and 4) of membrane (Lanes 1 and 2) and cytosolic fractions (Lanes 3 and 4) were analyzed by SDS-PAGE and visualized by fluorography.

Finally, to directly demonstrate that the substrate is farnesylated, AAXed and carboxyl-methylated, MALDI-TOF mass spectrometry on trypsin digested, membrane associated, prelaminAct isolated as a band from a SDS-PAGE gel, was performed. The C-terminal farnesylated, AAXed, and carboxyl-methylated peptide, TQSPQNC, (MW =995.13 Da) was observed (Fig 10). Furthermore, incompletely processed intermediates, as well as the nascent precursor peaks, were absent from the spectra. These data confirm that the membrane-bound purified substrate is completely modified with the
correct post-translational modifications at the CAAX box and, therefore, can be employed as a valid substrate for assaying the second upstream endoproteolytic conversion in the processing of prelamin A.

Figure 10 The prelaminAct substrate is fully processed at the CAAX box. MALDI-TOF analysis on Ni-agarose and SDS-PAGE purified membrane associated prelaminAct was performed. The 33 kDa band was excised from the gel and digested with trypsin. The C-terminal peptide (depicted in inset) modified by AAXing, farnesylation, and carboxyl-methylation (MW 995.13 Da) is observed.
Zmpste24 Localization in CHO-K1 Cells

Cloning of the full length cDNA for human Zmpste24 was accomplished via RT-PCR from a HeLa cDNA library. This cDNA for human Zmpste24 was then cloned into the pCDNA3.1+ construct to verify expression as well as subcellular localization within mammalian cells. Previous reports have demonstrated that human Zmpste24 is localized to the ER and the golgi (Kumagai and others 1999). However, unlike yeast Ste24, Zmpste24 does not contain a canonical c-terminal ER retention motif (Schmidt and others 2000), giving rise to the possibility that Zmpste24 may be found at other locations within the cell other than the ER. It is to be noted that because Zmpste24 contains 7 transmembrane domains, it is topologically constrained to membranous structures. To assess the subcellular localization of human Zmpste24 in mammalian cells, Zmpste24 under the control of the constitutive CMV promoter was transfected into CHO-K1 cells and indirect immunofluorescence was performed. The results (Fig 11) are consistent with Zmpste24 localization to the ER (reticular staining throughout the cell) and to the nuclear envelope (perinuclear staining). The nuclear localization of Zmpste24 as well as its membrane topology are in agreement with data from previous work which demonstrates that purified nuclear envelope fractions contain endoproteolytic activity (Kilic and others 1999), and that the endoproteolytic conversion of prelamin A to lamin A can be accomplished solely in the nucleus (Lutz and others 1992).
Figure 11 Subcellular localization of Zmpste24 expressed in mammalian cells. Zmpste24 was cloned from a HeLa cDNA library into the mammalian expression vector pcDNA3.1 and transiently transfected into CHO-K1 cells. Subcellular localization was determined by indirect immunofluorescence with anti-Zmpste24 (residues 56-76). Note both the cytosolic and perinuclear distribution of Zmpste24.

**Expression of Zmpste24 in Insect Cells**

To produce recombinant enzyme for use in the intended *in vitro* substrate assays, human Zmpste24 was N-terminally fused to a (His)$_6$ tag and was expressed in the baculovirus Sf21 insect system. Zmpste24 was expressed for 72 hours post-infection, and then purified from detergent solubilized membranes using Ni-bead chromatography.
Elutions were separated via SDS-PAGE, and then immunoblotted with a Zmpste24 specific antibody (Fig 12). This resulted in a diffuse band with an apparent MW of ~54 kDa, the predicted molecular mass of the protein. From the immunoblot it is evident that Zmpste24 appears to undergo proteolysis during expression and/or purification. Because Zmpste24 was purified using Ni-beads, and because the (His)$_6$ tag is located at the N-terminus, the amino terminus of Zmpste24 is intact. The presence of an intact N-terminus is further confirmed by immunoblot of the purified material with anti-Zmpste24 (residues 56-76) and anti-(His)$_6$ (Fig 12, lanes 3 and 5, respectively). MALDI-TOF mass spectral analysis of a tryptic digest of the 54 kDa band indicated sequences in this protein predicted to be found in Zmpste24 with 34% sequence coverage spanning residues 50-481 (data not shown). This included a sequence corresponding to the predicted carboxyl-terminal peptide of Zmpste24. A band appearing at 54 kDa in the immunoblot with anti-Zmpste24 (residues 440-455), is also consistent with the purified Zmpste24 having an intact C-terminus (Fig 12, lane 4). The data obtained from both the western blots and the mass spectral analysis confirm that full length human Zmpste24 is being expressed in the insect system.
Figure 12 Expression of Zmpste24 in insect cells. Zmpste24 was expressed in Sf21 cells for 72 hours post-infection with high-titer recombinant virus. Cells were lysed using a microtip sonicator. Total cell lysate was fractionated into membranes which were solubilized in 1.5% Octylglucoside. Ni-bead chromatography was performed and the 250 mM imidazole elution was separated using SDS-PAGE. Immunoblot with anti-Zmpste24 (residues 56-76) (Lanes 1-3), anti-Zmpste24 (residues 440-455) (Lane 4), or anti-(His)_6 (Lane 5) was performed. Lane 1: Ni-agarose purified elution of mock transfected cells. Lane 2: Total cell extract before purification. Lanes 3-5: Ni-agarose purified elutions of Zmpste24.

**AAXing Activity of Zmpste24 on Yeast a-factor**

The above experiments show that the insect system is appropriate for expressing full length Zmpste24, but these experiments do not show that the enzyme being made is active. Tam and coworkers have reported that the human enzyme Zmpste24, like its
yeast homologue Ste24p, possesses endopeptidase activity towards the farnesylated yeast a-factor CAAX box, resulting in removal of the c-terminal tripeptide adjacent to the prenylated cysteine, i.e., AAXing (Tam and others 1998). Therefore, to ascertain if insect cell expressed Zmpste24 is enzymatically active, membranes from insect cells expressing Zmpste24 were assayed for AAXing activity using farnesylated a-factor as substrate with the standard base release assay. Briefly, in this assay, removal of the tripeptide adjacent to the farnesylated cysteine is measured indirectly by the ability of isoprenylcysteinemethyltransferase (ICMT) to transfer a carboxymethyl group from the methyl donor S-adenosylmethionine (SAM) to the newly exposed cysteiny1-carboxy group (Hrycyna and others 1995). Membranes from cells expressing Zmpste24 showed an approximate 9-fold increase in activity when compared to membranes from mock infected cells (Fig 13). These results demonstrate that Zmpste24 expressed in insect cells possesses CAAX endopeptidase activity towards the a-factor substrate. Demonstrating that the Zmpste24 expressed protein from insect cells contains proteolytic activity against yeast a-factor is critical to using this system in any further proteolytic studies on prelamin A.
Figure 13 AAXing activity of Zmpste24 on a-factor substrate. Membranes derived from insect cells expressing either human Zmpste24 or Erp57 (Mock) were incubated with the prenylated a-factor surrogate substrate (gift from Susan Michaelis). Reactions were performed for 20 min and then stopped by boiling. Recombinant ICMT and the methyl donor [3H-Methyl]-S-adenosylmethionine were then added to carboxymethylate any AAXed substrate. Incorporated counts were then released with 1M NaOH and counted. Reactions were repeated in triplicate.
AAXing Activity of Zmpste24 on Prelamin A

Although the AAXing activity of human Zmpste24 on the yeast a-factor substrate has previously been shown in the literature, its ability to AAX prelamin A has not, to this date, been reported. One of the primary goals of this present body of work is to test the hypothesis that Zmpste24 is the physiologic AAXing enzyme for prelamin A. To test this hypothesis, a farnesylated tetrapeptide prelamin A specific CAAX sequence, Cys-(farnesyl)-Ser-Ile-Met, was tested as a substrate in the base release assay. The substrate was constructed by synthesis of the tetrapeptide, N-(Ac)-Cys-Ser-Ile-Met, and then farnesylation of the cysteine using [3H]-farnesyl pyrophosphate (FPP) and purified farnesyltransferase. This substrate was purified via preparative thin layer chromatography and then used in a base release assay with Zmpste24 enriched insect membranes. The data show an approximate 7-fold increase in the activity of membranes from cells expressing Zmpste24 relative to the activity of membranes from mock infected cells (Fig 14). Therefore, Zmpste24 has CAAX endopeptidase activity towards the prelamin A specific CAAX box sequence, N(Ac)-Cys(farnesyl)-Ser-Ile-Met in vitro.
Figure 14 AAXing activity of Zmpste24 on a prelamin A tetrapeptide. Membranes derived from insect cells expressing either human Zmpste24 or Erp57 (Mock) were incubated with the substrate: N(Ac)-Cys(farnesyl)-Ser-Ile-Met. The same procedure was used for base release as in Fig 8. Reactions were repeated in triplicate.

**AAXing Activity of Purified and Membrane Reconstituted Zmpste24**

Because the above experiments were conducted on crude membrane preparations, they do not verify definitively that Zmpste24 is the enzyme directly catalyzing the proteolytic conversion. To directly verify that this activity is solely based on hydrolysis due to Zmpste24, the enzyme was purified from detergent solubilized membranes using Ni-chromatography and reconstituted into heat inactivated insect membranes. The membrane reconstituted enzyme was tested against the
N(Ac)-Cys(farnesyl*)-Ser-Ile-Met labeled substrate using a TLC based assay for measuring the formation of product. This TLC assay has increased sensitivity and accuracy over the base-release assay used in the previous experiments. Results from this reconstitution experiment demonstrate that purified Zmpste24 has the ability to AAX the prelamin A model substrate, farnesylated CSIM (Fig 15). An eight-fold increase in activity with Zmpste24 infected cells over mock infected is observed as well as the inhibition of activity upon the addition of Zn$^{+2}$ chelator 1,10 orthophenanthroline (Opa). Although the activity does not appear to be inhibited 100%, the level of this activity is not statistically distinguishable from the level observed in the mock control. Inhibition by Opa is consistent with the AAXing activity of Zmpste24 being due to a Zn$^{+2}$ dependent active site, which is a typical characteristic of metalloproteinases. Also consistent with the activity being due to a metalloproteinase is the observation that this AAXing activity is not inhibited by the chymotrypsin like protease inhibitor, TPCK.
Figure 15 AAXing activity of purified and reconstituted Zmpste24 is Zn$^{+2}$ sensitive.

Zmpste24 was purified using Ni-agarose chromatography and reconstituted into heat inactivated insect membranes. N(Ac)-Cys(farnesyl*)-Ser-Ile-Met was incubated with either mock-infected membranes (lane 1) or reconstituted Zmpste24 preincubated with no inhibitor (lane 2), 2 mM 1,10-orthophenanthroline (lane 3), or 100 ug/ml TPCK (lane 4). Products were extracted, separated on TLC, and visualized by fluorography. The radiolabeled product N(Ac)-S-farnesyl*-L-cysteine was identified by comigration with a bona fide commercial standard.
Second Endoproteolytic Processing Activity of Zmpste24

Motivation for these next set of experiments comes from the fact that the experiments described above confirm that Zmpste24 does indeed possess the first endoproteolytic AAXing activity towards the substrate prelamin A, but do not answer the question regarding the role, if any, that Zmpste24 plays in the second endoproteolytic processing step. In addition to the first processing event, it is also reasonable to hypothesize that Zmpste24 may catalyze the second endoproteolytic cleavage converting prelamin A to the mature lamin A. Justification for this reasoning comes from the observation that when the mouse orthologue to Zmpste24 is knocked out in mice, Zmpste24 -/- cells lose the ability to process prelamin A to mature lamin A (Bergo and others 2002; Pendas and others 2002). If the first AAXing reaction is blocked in the processing pathway of prelamin A to Lamin A, then it follows that the subsequent carboxymethylation and second endoproteolytic events will not occur (Kilic and others 1997; Bergo and others 2002). However, if Zmpste24 AAXing activity is abolished, it is feasible that RCE1 may be able to complement this AAXing defect, as in the case of a-factor in yeast (Boyartchuk and Rine 1998; Tam and others 1998). Therefore, the experimental observations that prelamin A to Lamin A conversion is blocked in Zmpste24 -/- cells may not be due to the loss in the AAXing event (due to RCE1 complementation) but may be entirely due to the loss in the second endoproteolytic reaction. In support of the hypothesis that human Zmpste24 may have the ability to perform both reactions is the experimental observation that the yeast orthologue to Zmpste24, Ste24, has the ability to process a-factor at the first and second proteolytic sites (Tam and others 1998; Tam and others 2001). This leads to the
hypotheses that Zmpste24 may be performing the first, second, or both endoproteolytic steps during the processing of mammalian substrate, prelamin A.

In order to test whether Zmpste24 can process prelamin A to lamin A in vivo, prelaminAct, or both prelaminAct and Zmpste24, was expressed in insect cells, and then the conversion of prelamin A to lamin A was followed via immunoblot. Coexpression of Zmpste24 and prelamin A results in a product, 2 kDa smaller in MW than prelaminAct, that is recognized by a lamin A antibody, indicating that conversion of prelamin A to lamin A is being mediated by Zmpste24 expression (Fig 16). This conversion to lamin A is blocked by incubation of the cells with lovastatin, an inhibitor of isoprenoid biosynthesis, which previously has been demonstrated to block prelamin A maturation in mammalian cells by blocking prenylation (Beck and others 1990; Sinensky and others 1990). It was observed that the prelamin A antibody recognizes the putative 33 kDa prelaminAct product only (data not shown), while the lamin A antibody recognizes both the unprocessed form of prelaminAct (upper band) and the processed form, lamin A (lower band). This result demonstrates that the change in migration is due to a cleavage that removes the C-terminal prelamin A sequence.
Figure 16 Zmpste24 processing of prelaminAct \textit{in vivo}. Zmpste24 was virally transfected into insect cells expressing N-terminally (His)$^6$ tagged prelaminAct or prelaminAct mutated at the endoproteolytic cleavage site ($L^{647} \rightarrow R$). The cells were then incubated for 72h in the presence or absence ofLovastatin. PrelaminAct and laminAct were purified using Ni-agarose chromatography, separated by SDS-PAGE and visualized by immunoblot with anti-lamin A.

To determine the specific cleavage site at the C-terminus of prelamin A, this coexpression experiment was repeated with a prelaminAct construct containing the hexapeptide sequence RSY-RLG bearing a point mutation ($L^{647} \rightarrow R$) at the p1 site of the wild type sequence RSY LLG. Other \textit{in vivo} experiments have shown that this mutation of prelamin A cannot be processed to lamin A when expressed in mammalian cells (Hennekes and Nigg 1994). Zmpste24 coexpression along with this uncleavable mutant of prelaminAct resulted in no conversion, as expected (Fig 16, lane 5). This \textit{in vivo} data
supports the conclusion that Zmpste24 is hydrolyzing the peptide bond of prelamin A between Tyrosine$^{646}$ and Leucine$^{647}$, as would be expected for the bona fide prelamin A endoprotease. The overall results of these in vivo experiments along with the observed substrate specificity is particularly indicative that processing is governed by the expression of Zmpste24, considering the absence of lamin A in insect cells, and, therefore, the probable absence of similar endogenous protease activities.

It is important to point out at this point that attempts to purify Zmpste24 and reconstitute the second endoproteolytic activity were not successful. The results from these trials suggested that Zmpste24 loses this second activity upon extraction from the membranes. Various detergents were used to carry out membrane extraction and solubilization of the enzyme including octylglucoside, NP-40, TX-100, and deoxycholate. From these trials, octylglucoside performed the best in extraction and in further downstream purification steps (Ni-chromatography). Various aliquots along the entire process of extraction, purification, and reconstitution, process were tested for activity. The only fractions that possessed any type of activity associated with the second endoproteolytic step were membranes before extraction. Reconstitution was attempted on the purified Zmpste24 fractions by mixing with membrane extracted lipids from either insect cells, HeLa cells, or E.coli cells and dialyzing against various reconstitution buffers overnight to form vesicles. These reconstituted vesicles all gave negative results when tested for endoproteolytic activity. Although the first AAXing endoproteolytic activity was able to be reconstituted with ease, the second activity was only evident from Zmpste24 enriched membranes before any further processing steps. One possible explanation for this loss of activity is that some other membrane associated co-factor is needed for the
second endoproteolytic activity. The significance of these observations will be discussed in detail within Chapter 5 - Discussion. For these reasons, all further experiments demonstrating the second endoproteolytic activity of Zmpste24 on prelamin A are conducted on Zmpste24 enriched membranes produced from insect cells.

The above in vivo experiments provide a physiological framework for the role of Zmpste24 in the processing of prelamin A, but do not definitively demonstrate that this proteolytic activity is uniquely due to Zmpste24 mediated catalysis. To determine if the second prelamin A endoproteolytic cleavage can be reconstituted in vitro, a prelaminAct substrate was constructed which was metabolically labeled with [³H]-mevalonate, a biosynthetic precursor in the isoprenoid pathway. A substrate bearing a radiolabeled farnesyl moiety allowed the endoproteolytic reaction to be followed simply by measuring the counts associated with the ~2 kDa C-terminal farnesylated fragment. When incubated in vitro with Zmpste24 enriched membranes, this substrate released a ~ 2 kDa [³H]-labeled fragment, the approximate size of the last 15 residues of prelamin A, as the sole labeled product (Fig 17). The negative control, mock control membranes, showed no apparent activity towards this substrate.
Figure 17 Zmpste24 catalyzed cleavage of a 2 kDa fragment from prelaminAct.

PrelaminAct was metabolically labeled with [3H]-mevalonate for 16 hours, 48 hours after infecting with the prelaminAct construct. PrelaminAct was then purified with Ni-agarose. Products formed after incubation with either mock transfected (lane 1) or Zmpste24 transfected (lane 2) membranes were separated by SDS-PAGE and visualized by fluorography.

To evaluate the kinetics of the release of this 2 kDa prenylated fragment, an assay was designed in which the [3H]-mevalonate labeled prelaminAct substrate was bound to Ni-agarose beads. The bound and labeled prelaminAct was washed extensively but was not eluted from the beads. The immobilized prelaminAct then served as the substrate in the kinetic assays. After incubating the immobilized prelaminAct with Zmpste24 membranes, the tritium counts in the supernatant were monitored at various time points.
From analysis of the gel fluorogram (Fig 17), it is evident that the major product after incubation with Zmpste24 membranes is a ~2 kDa fragment. Therefore, measuring any releasable counts from prelaminAct immobilized to beads upon incubation with Zmpste24 enriched membranes can be safely interpreted as this 2 kDa product. The results of this kinetic bead assay show the that the formation of product increases with time and goes to completion between 1 and 2 hours (Fig 18). Notably, membranes from mock infected cells did not give rise to measurable product, consistent with the conclusion that product formation arises from the expressed Zmpste24 rather than an endogenous activity of the insect cell membranes.
Figure 18 Kinetics of release of ~2 kDa product from prelaminAct. Purified $[^3H]$-mevalonate labeled prelaminAct was immobilized on Ni-agarose beads and washed extensively. The beads were incubated with membranes from Zmpste24 or mock transfected cells, or just buffer. At various times, aliquots of the supernatant were counted to determine release of the farnesylated C-terminus of prelamin A ($\bullet$ = Zmpste24 membranes, ■ = mock infected membranes, ▲ = blank control (just buffer)).

The results from the above two experiments definitively show that a ~2 kDa prenylated C-terminal fragment of prelaminAct is being released upon incubation with Zmpste24 enriched membranes. To complement these results and to determine if this cleavage reflects the physiological processing pathway of prelamin A, it was important to establish whether or not the parent 33 kDa substrate was being converted to a 31 kDa...
product. To accomplish this, it was necessary to design a substrate whose N-terminally released product could be followed as a readout of the processing event. This was carried out by expressing and purifying a \([^{35}S]-\text{Met metabolically labeled 33 kDa prelaminAct,}

and then monitored the processing of this substrate upon incubation with membranes from cells expressing Zmpste24. The results reveal that the 33 kDa prelaminAct band was converted to a 31 kDa form in a time-dependent fashion (Fig 19A). In the controls with membranes from mock transfected cells incubated with wild type prelaminAct substrate, we observed little or no conversion of the parent prelaminAct substrate (Fig 19B). However, a non-specific proteolytic activity degraded the 31 kDa formed product over time, which is evident from inspection of the lower band (Fig 19A). It was determined through trial and error that preincubation of membranes with the thiol protease inhibitor E-64 reduced this non-specific background degradation of prelamin A and allowed the measurement of enriched Zmpste24 activity but did not completely eliminate this background degradation of the formed product. This degradation could be due to any number of non-specific carboxy, amino, or endopeptidases which are endogenously expressed in the Sf21 cell line. It is important to note that the only way to eliminate any endogenous proteolytic background is to purify and reconstitute the activity of Zmpste24, which was attempted but was unsuccessful. Incubating the membranes with any type of protease inhibitors in an attempt to eliminate this background could potentially eliminate the activity of Zmpste24 and, therefore, would be unfruitful. The protease inhibitor E-64 is a viable means to reduce some of this background because it is specifically designed to inhibit thiol proteases. Nonetheless, these results show that membranes from Zmpste24 expressing cells have the ability to convert prelaminAct in a time dependent fashion to a
form that is approximately 2 kDa lower in MW, consistent with the characteristics associated with the prelamin A endoprotease. These results also demonstrate that the overall design of the assay for monitoring this type of activity is robust and sensitive.

Figure 19 Zmpste24 can endoproteolytically process prelaminAct to laminAct.

PrelaminAct was metabolically labeled with [35S]-methionine in insect cells and purified with Ni-agarose. The purified substrate was incubated with either Zmpste24 or mock transfected membranes in the presence of E-64 for various times. The products were separated by SDS-PAGE, the gels were dried, and visualization by phosphorimager analysis was performed. A) PrelaminAct incubated with membranes derived from Zmpste24 expressing insect cells. B) PrelaminAct incubated with membranes derived from Erp57 expressing insect cells (mock).

The results presented in the figure above do not conclusively demonstrate the site of cleavage. To demonstrate the site of cleavage, a [35S]-Met metabolically labeled 33 kDa
prelaminAct bearing a point mutation at the p1 site of cleavage (L^{647}R) was expressed and purified. This mutation, as shown in the results of the *in vivo* experiments (Fig 16), is unable to be converted to fully mature laminAct. Using this uncleavable mutant with this endoproteolytic assay resulted in little or no conversion when compared to the processing of wild type prelaminAct (Fig 20). The extent to which endoproteolytic conversion occurred was quantified by densitometry and expressed as % conversion to the 31 kDa form. It is clear that the expression of Zmpste24 is resulting in proteolytic activity that is consistent with the expected substrate specificity of the prelamin A endoprotease.
Figure 20 Zmpste24 can process prelaminAct at the sequence RSY-LLG. PrelaminAct or its RSY-RLG (L^{647} \rightarrow R) mutant were metabolically labeled with [^{35}S]-methionine in insect cells and purified with Ni-agarose. The labeled substrates were incubated with Zmpste24 membranes for various times. The products were separated by SDS-PAGE and analyzed by phosphorimager analysis and quantitative densitometry. From the densitometry of the parent 33 kDa band and the lower 31 kDa band, the relative formation of the 31 kDa product (\([31 \text{ kDa Counts}] / [33 \text{ kDa + 31 \text{ kDa Counts}}]\)) was calculated as a function of time. (-■- = Zmpste24 membranes with WT prelaminAct, -▲- = Zmpste24 membranes with RSY-RLG mutant prelaminAct)
The 33 kDa band is recognized by prelamin A antibody, while both the 33 kDa and 31 kDa bands are recognized by the lamin A antibody (data independently verified on unlabeled substrate and not shown). Confirmation of the identity of these two bands by mass spectral analysis revealed that the 31 kDa band is missing the C-terminal peak corresponding to the peptide SYLLGNSSPR (MW 1094.21 Da). This is consistent with the conclusion that Zmpste24 cleaves between Tyrosine$^{646}$ and Lysine$^{647}$, as would be the case for lamin A maturation in whole mammalian cells (Fig 21).
Figure 21 Mass spectral identification of prelaminAct to laminAct conversion. Zmpste24 membranes were incubated with the purified prelaminAct substrate and the reaction was repurified using Ni-agarose beads. The beads were boiled and run on SDS-PAGE to separate the 33 kDa parent substrate from the 31 kDa product. Each band was excised and tryptic digested. MALDI-TOF analysis of the tryptic digested 33 kDa (top spectrum) and 31 kDa (bottom spectra) gel excised bands was performed. Notice the disappearance of the peak corresponding to the peptide SYLLGNSSPR (MW 1094.21 Da) in the 31 kDa band. The peak appearing at 920.06 corresponds to the predicted peptide from the prelaminAct sequence between residues 428-435.
Endoproteolytic Processing of PrelaminAct Depends on Processing at the CAAX Box

As mentioned earlier, the membrane bound substrate is fully processed in that it is farnesylated, AAXed, and carboxyl-methylated. Previous studies assaying the activity of nuclear envelope fractions on a model peptide substrate have shown that it is necessary for prelamin A to be fully processed at the CAAX box before it can act as a substrate for the endoprotease (Kilic and others 1997). In order to evaluate if the dependence on processing of prelamin A by Zmpste24 is contingent upon full processing at the CAAX box, an in vitro translated [35S]-Met labeled prelaminAct construct containing a stop codon after the cysteine, designated prelaminAct ΔAAX, was expressed (Barton and Worman 1999). Due to the lack of the tripeptide adjacent to the CAAX box cysteine residue (i.e., the AAX sequence), this substrate contains a cysteine at the carboxyl terminus that is neither farnesylated or carboxymethylated. In the absence of a farnesylated and carboxyl-methylated cysteine, prelaminAct substrate is not cleaved by Zmpste24 to any measurable extent (Fig 22). Comparing this to the results when a fully processed substrate was used (Fig 19A), leads to the conclusion that cleavage of prelamin A to lamin A by the endoprotease is, indeed, dependent upon a cysteine residue that is prenylated and carboxymethylated. Although this experiment did not distinguish between the enzyme-substrate specificity of a prenylated non-carboxymethylated substrate or a carboxymethylated non-prenylated substrate, it does, nonetheless, show that a non modified cysteine residue confers a lack of substrate processing. This also corroborates the findings of the cotransfection experiment which demonstrated that lovastatin treatment abolishes conversion of prelamin A to lamin A (Fig 16). Taken together, these results demonstrate the specific cleavage of prelaminAct in vitro by Zmpste24, and that
Zmpste24 behaves in a consistent manner to what is already known regarding the specificity and nature of the prelamin A endoprotease.

Figure 22 Endoproteolytic processing of prelamin A depends on CAAX box. A prelaminAct ΔAAX construct in the pBFT4 vector was *in vitro* translated using a rabbit reticulocyte transcription/translation system in the presence of $[^{35}S]$-Met. This substrate was incubated with Zmpste24 membranes for up to 4 hours. The products of this reaction were resolved with SDS-PAGE and imaged with a phosphorimager.

**The Catalytic Mechanism Responsible for the Second Endoproteolytic Conversion**

The above experiments demonstrated conclusively that Zmpste24 has the ability to mediate both endoproteolytic events involved in the maturation pathway of lamin A. From what is presently known about Zmpste24, it is apparent that this protein contains the highly conserved Zn$^{+2}$ dependent metalloprotease motif HEXXH and, that AAXing activity associated with Zmpste24 is Zn$^{+2}$ dependent (Schmidt and others 2000). However, prior *in vitro* studies from our laboratory are not consistent with the second endoproteolytic activity being due to a metalloprotease. Rather, these studies, performed with various protease inhibitors, were consistent with the prelamin A endoprotease being a chymotrypsin like enzyme with a serine active site (Kilic and others 1999). In order to address this apparent discrepancy, the effect of various protease inhibitors on Zmpste24
catalyzed conversion of prelaminA to laminA was investigated using the same type of assay used in Fig 19. Zmpste24 enriched membranes were preincubated with various protease inhibitors, and substrate conversion reactions were performed in triplicate for 90 minutes. After phosphorimager analysis, the % conversion from 33 kDa to 31 kDa was quantititated. The results indicate that the conversion of prelamin A to lamin A is blocked by the serine protease inhibitors AEBSF and N-tosyl-L-phenylalanine chloromethyl ketone (TPCK) but is not inhibited to any appreciable extent with a mixture of Zn^{2+} chelators EDTA and 1,10-orthophenanthroline (Fig 23). Given the specificities of TPCK and AEBSF, these results suggest that the endoproteolysis of prelamin A by Zmpste24 is mediated by a chymotrypsin-like active site and not a Zn^{2+} dependent site.
Figure 23 Zmpste24 endoproteolysis of prelamin A is sensitive to chymotrypsin inhibitors. Endoproteolysis reactions on [35S]-methionine prelaminAct were performed as in Fig 14 for 90 minutes in the presence or absence of various serine and zinc metalloprotease inhibitors (1,10 orthophenanthroline 2 mM, EDTA 10 mM, AEBSF 1mg/ml, TPCK 100 ug/ml, FFCK 20 uM). Reactions were performed in triplicate and the relative formation of the 31 kDa product (% of total prelaminAct) was quantified at 90 minutes using densitometry.

In order to confirm that a chymotrypsin-like active site exists within Zmpste24, we attempted to affinity tag the putative active site histidine with FFCK
(5(6)-carboxyfluoresceiny-L-phenylalanine chloromethyl ketone), a fluorescent analogue of TPCK (Grabarek and others 2002a; Grabarek and others 2002b). FFCK specifically inhibits chymotrypsin-like proteases by the same mechanism as TPCK, namely by alkylation of the active-site histidine (Ong and others 1965; Shaw and Ruscica 1971). In this manner, both FFCK and TPCK affinity label active sites that function by a chymotrypsin like catalytic mechanism. Our results show that FFCK, like TPCK, was able to inhibit Zmpste24 endoprotease activity in vitro (Fig 23). To confirm that FFCK is directly interacting with Zmpste24, membranes from insect cells expressing Zmpste24, were incubated with FFCK followed by purification of Zmpste24 using nickel-affinity chromatography. The eluted fractions containing Zmpste24 were resolved by SDS-PAGE, and the fluorescent protein visualized (Fig 24). The fluorescently labeled purified protein runs at approximately 54 kDa and comigrates with Zmpste24 visualized by western blot with anti-(His)$_6$. Preincubation of Zmpste24 with TPCK blocks incorporation of FFCK, confirming that FFCK and TPCK inhibit Zmpste24 by a similar mechanism, presumably alkylation of the active site histidine.
Figure 24 Labeling of Zmpste24 with FFCK. Membranes from insect cells expressing (His)_6-Zmpste24 were incubated with the fluorescently labeled TPCK analogue, FFCK. After incubation with FFCK, Zmpste24 was purified using Ni-agarose chromatography and resolved by SDS-PAGE. Fluorescent imaging was performed using an excitation of 475 nm with a blue filter on a FUJIFILM-FLA 5000. Lane 1: Zmpste24 membranes incubated with FFCK. Lane 2: Zmpste24 membranes with no FFCK. Lane 3: Mock membranes incubated with FFCK. Lane 4: Zmpste24 membranes preincubated with TPCK before incubation with FFCK. After imaging, the proteins on this gel were transferred to a PVDF membrane and the identity of the labeled band confirmed by immunoblotting with anti-(His)_6 and alignment of the images using predefined reference points.

To confirm that the FFCK labeled Zmpste24, both the FFCK labeled and non-labeled Zmpste24 bands were isolated and subjected to tryptic digestion followed by MALDI-TOF mass spectral analysis. All peaks identified in the MALDI-TOF analysis mass fingerprinted the band as Zmpste24, with approximate 47% sequence coverage. No
peaks were obvious in the spectra that would represent the theoretical shift in MW upon FFCK derivitization (MW = 557 Da). Such a peak may not fly in the MALDI-TOF machine or may block tryptic digestion of a nearby tryptic cleavage site. The negative result obtained from the mock membrane control (Fig 24, lane 3), in addition with the immunoblot and mass spectral analysis, lead to the conclusion that FFCK is able to affinity tag Zmpste24.

As a final demonstration that the second endoproteolytic activity of Zmpste24 is not due to the Zn^{2+} dependent catalytic mechanism, the conserved HEXXH motif in Zmpste24 was deleted using site directed mutagenesis. In yeast, deletion of the HEXXH domain of Ste24 completely abolishes the proteolytic processing of a-factor. This ΔHEXXH mutant was constructed and expressed in the insect cell system. This mutant was compared to WT Zmpste24 for its ability to perform the first and second endoproteolytic reaction. The results from this experiment show that deleting the HEXXH motif in Zmpste24 abolishes its ability to AAX a-factor (Fig 25A). However, the ability of the ΔHEXXH mutant of Zmpste24 to endoproteolytically process prelaminAct to laminAct is retained and is comparable to the activity observed for WT Zmpste24 (Fig 25B). The results from this experiment were identical to the results obtained from assaying a His^{335} →Ala mutation of Zmpste24 (data not shown). This particular point mutation abolishes the critical zinc coordinating histidine in the HEXXH motif, resulting in a motif containing AEXXH. This control is significant for it shows that the loss of AAXing activity is due to a loss of the catalytic mechanism associated with the Zn^{2+} coordinating Histidine and is not due to an overall gross steric change induced by deleting the HEXXH motif entirely. This
result clearly demonstrates that the AAXing hydrolysis and second endoproteolytic reaction on prelaminAct are not mediated by the same catalytic mechanism.

Figure 25 Deletion of HEXXH motif abolishes AAXing, not endoproteolysis. A HEXXH deletion mutant of Zmpste24 was expressed in insect cells. Membranes from these insect cells were used in either a A) Base release AAXing assay on a-factor (as in Fig 13) or B) Endoproteolysis reaction on [35S]-Met labeled prelaminAct (as in Fig 19). In each experiment, a WT Zmpste24 control was used for comparison.
Attempts to Locate Second Active Site within Zmpste24

The data obtained from the protease inhibition profiles, affinity labeling by the chymotrypsin inhibitor, FFCK, and the HEXXH deletion mutant all lead to the same conclusion: The second endoproteolytic reaction is being catalyzed by an active site different from the canonical HEXXH zinc metalloproteinase motif. Presumably, this active site would be homologous to the endoproteinase sites contained in the serine family and, more particularly, to the chymotrypsin family \textit{s1}. In this family of proteases, a catalytic triad (His, Asp, Ser) forms a charge relay system to catalyze the hydrolysis of the amide peptide linkage (Barrett and Rawlings 1995). The most obvious experiment to conduct to determine if Zmpste24 contains such an active site is \textit{in silico} through use of the various bioinformatic tools available through the NCBI and the EMBL-EBI. A number of blast algorithms were performed on Zmpste24 against the non-redundant protein databases at these websites, and the results showed that Zmpste24 has no significant homology to any of the known protease families, other than the metalloproteinase family, M48. To increase the sensitivity of these blast algorithms to find distant homology matches that are not detected in a first attempt, the PSI (Position Specific Iterative) blast algorithm was used with 4 iterations. In this repetitive algorithm, a Position Sensitive Scoring Matrix is obtained (PSSM) for each iteration that tabulates in a matrix form the amino acid substitution frequencies each individual residue along the protein. This PSSM matrix that is formed on each iteration is then used in the next iteration of a RPS (Reverse Position Specific) blast algorithm. On each iteration, new proteins are identified with remote homology to the query sequence and continues until the algorithm converges (no new members are identified in any additional iterations). The results of this analysis of
Zmpste24 were also negative in that they did not detect any homologous protein family members within the serine protease family across all databases organisms.

In the course of the blast attempts at discovering a second serine like active site, it became apparent that there is an insertional sequence in human Zmpste24 when compared to orthologues in lower organisms (such as yeast) between residues 290 and 327. This insertional sequence, as predicted through topology modeling, is most likely within a cytosolic loop between transmembrane segments 5 and 6 (the same loop that bears the HEXXH motif). Within this loop is the tripeptide DSG, which is a conserved sequence in the active sites of many serine proteases. A ClustalW alignment is shown for this region of Zmpste24 alongside the corresponding regions surrounding the serine active residue of representative serine family proteases (Fig 26). This putative serine site was attractive because it was in a region missing from yeast and other lower organisms, which presumably do not convert prelamin A to lamin A as in the mammalian cell.
Human and mouse Zmpste24 were aligned in a 12 amino acid stretch surrounding the putative serine active site with other representative members of the serine protease family (ClustalW algorithm). Black background represents an identical match, light gray represents a similar substitution.

Therefore, our first attempt at identifying the serine active site was to mutate this serine to an alanine (Ser$^{298}$ → Ala), and to assay this for endoproteolytic activity. However, when the second endoproteolytic activity of this mutant was compared to wild type against the $[^{35}S]$-Met labeled prelaminAct substrate, the activities were virtually identical (data not shown).

Analysis of Zmpste24 C-Terminal Truncations

Because there are 33 other serine residues within Zmpste24, it was not efficient to mutate each one in the insect based system that was being used for the WT studies. To narrow down a region that may contain the active site, or a residue associated with the active site, 4 serial C-terminal truncations of Zmpste24 were constructed. This was
accomplished by site directed mutagenesis of the wild type construct to introduce premature stop codons at predetermined locations. The placement of the stop codons was chosen strategically within predicted cytosolic or lumenal loop regions outside the boundaries of transmembrane regions (Fig 27). Placement of the stop codons in these regions allowed for proper transmembrane insertion of predicted transmembrane helices n-terminal to the stop codon.

Figure 27 Placement of stop codons used in generation of Zmpste24 truncations. Shown is a predicted transmembrane topology map for Zmpste24, as predicted by the TMHMM Server v. 2.0 server (http://www.cbs.dtu.dk/services/TMHMM/). The positioning of the 4 amino acids mutated into stop codons (C109, P230, L290, and Y379) are shown.
The Y379Stop mutant was chosen because a similar truncation is observed in patients who have mandibuloacral dysplasia (MAD) or restrictive dermopathy (Agarwal and others 2003; Navarro and others 2004; Navarro and others 2005). This premature truncation in these patients occurs from a Phe361fsX379 mutation, which is a frameshift introduced from a spurious thymidine in the DNA ORF causing a premature stop to occur at residue 379 (Agarwal and others 2003). We wanted to assay this mutant for both AAXing and endoproteolytic activity to correlate the disease phenotype with a defective step in prelamin A processing, if any. Our results from this study indicate that a premature truncation of Zmpste24 introduced at residue 379 results in loss of AAXing activity with retention of the second proteolytic activity (Fig 28). This AAXing result correlates with the results found by Agarwal and others, who determined that the Phe361fsX379 mutant form of Zmpste24 could not complement a Ste24 deletion mutant of yeast defective in processing of the prenylated yeast substrate, a-factor (Agarwal and others 2003). The results of the endoproteolytic assay on prelaminAct are novel and demonstrate that the phenotype seen in MAD and RD patients is more than likely due to an inability of Zmpste24 to AAX the prelamin A substrate. Also worthwhile noting is the fact that the HEXXH motif (residues 335-339) is retained in this truncated form of Zmpste24, indicating that some other sequence C-terminal to the HEXXH motif is needed for AAXing to occur. Most likely, this is a putative upstream glutamate residue (Such as Glu402) needed to coordinate the Zn$^{+2}$ ion, a typical feature found in the zincin family of zinc metalloproteinases (Hooper 1994). Another explanation could be that residues C-terminal to Y379 are needed for substrate recognition and/or proper folding of Zmpste24.
Figure 28 Y379Stop truncation of Zmpste24 abolishes AAXing, not endoproteolysis. A Y379Stop mutant of Zmpste24 (resembling the Zmpste24 mutation found in MAD) was expressed in insect cells. Membranes from these insect cells were used in either A) base release AAXing assay on a-factor (as in Fig 13) or B) Endoproteolysis reaction on [35S]-Met labeled prelaminAct (as in Fig 19). In each experiment, a WT Zmpste24 control was used for comparison.
The overall results obtained from assaying the activity of the Zmpste24 truncations on the \[^{35}\text{S}^-\text{Met}\] prelaminAICT substrate showed that complete loss of endoproteolytic activity occurred for the C109Stop construct (Fig 29). From this data it can not be assumed that an active site serine residue is between residues 109 and 230. This is because a number of different explanations could explain this data, including: loss of one or all of the active site residues in the catalytic triad (His, Ser, or Asp); loss of substrate recognition; improper folding or topology of Zmpste24; or any combination of these factors.
Figure 29 Effect of C-terminal truncations of Zmpste24 on processing of prelamin A.

Endoproteolysis reactions on $[^{35}\text{S}]-\text{methionine prelaminAct}$ were performed as in Fig 19 for 45 minutes in the presence of membranes from cells expressing WT, Y379Stop, L290Stop, P230Stop, and C109Stop forms of Zmpste24. The relative formation of the 31 kDa product (% of total prelaminAct) was quantified at 45 minutes using densitometry.
The body of this work has explored the role that Zmpste24 plays in the processing pathway of the nuclear envelope scaffolding protein, prelamin A. Among the milieu of post-translationally modified mammalian proteins, prelamin A is distinctive in that it undergoes two distinct prenylation dependent endoproteolytic clips at the carboxy terminus. In the past, the exact identity of the enzyme responsible for the first AAXing reaction as well as the prelamin A endoprotease have remained elusive, but recent biological data moved Zmpste24 to the forefront of likely candidates. Such evidence includes the observation that Zmpste24−/− mice fail to process prelamin A to lamin A and present phenotypical defects associated with lamin A mutations (Leung and others 2001; Bergo and others 2002; Pendas and others 2002). Even more recently, it has been shown in humans that Zmpste24 is prematurely truncated in patients with restrictive dermopathy (Navarro and others 2004; Navarro and others 2005), mandibuloacral dysplasia (Agarwal and others 2003), and HGPS (Shackleton and others 2005), pathologies that are also caused by mutations in the LMNA gene. This genetic data in conjunction with the knowledge of the parallel processing pathways of a-factor in yeast and lamin A in mammals, generated the obvious hypothesis that Zmpste24 is involved in the proteolytic maturation of prelamin A. It was the goal of this current research to validate this in vivo data by assaying the function of Zmpste24 in vitro with respect to the substrate prelamin A.
Because of the exotic set of post-translational modifications made in the processing pathway of prelamin A and the direct link between these modifications and the two proteolytic processing steps, it was necessary to carefully design and construct substrates that mimic the physiologic processing events of prelamin A. Each endoproteolytic reaction occurs on substrates with unique molecular constraints, necessitating the design and construction of two different model substrates for monitoring each processing event. To follow the AAXing reaction, a tetrapeptide substrate mimicking the farnesylated CAAX box of prelamin A was synthesized, a straightforward biochemical method. However, designing a substrate for assaying the second endoproteolytic activity was more complicated in that it required a substantial portion of the c-terminus of the prelamin A molecule and needed to possess a farnesylated and carboxymethylated carboxyl terminal cysteine. The insect cell line was chosen to express a 33 kDa soluble form of the c-terminus of prelamin A (prelaminAct) because of its known fidelity in faithfully reproducing post-translational modifications that typically occur in the mammalian cell. Indeed, this substrate was confirmed to possess a fully processed cysteine bearing a farnesyl moiety and carboxymethyl group through mass spectral analysis (Fig 10).

The results presented in the body of this work have shown that membranes derived from Zmpste24 expressing insect cells have the ability to process prelamin A model substrates at two steps in the endoproteolytic pathway, namely AAXing (Fig 14) and c-terminal endoproteolysis (Fig’s 17-19). Such a dual function for this human enzyme is of no surprise because the yeast form of this enzyme is also able to process the a-factor substrate in a similar fashion at two points along its maturation pathway.
(Fujimura-Kamada and others 1997; Tam and others 1998; Schmidt and others 2000; Tam and others 2001). More specifically, α-factor is processed in a parallel manner to prelamin A with respect to the first set of four post-translational modifications made at the c-terminus, namely farnesylation, AAXing, carboxymethylation, and endoproteolysis, and then diverges from the prelamin A pathway by undergoing a third endoproteolytic reaction (Anderegg and others 1988; Chen and others 1997). During α-factor maturation, yeast Ste24 is responsible for both the AAXing reaction and the first endoproteolytic hydrolysis 26 residues upstream from the prenylated cysteine between threonine and alanine. Tam and others and Schmidt and others have discovered that human Zmpste24, like yeast Ste24, is able to endoproteolyze α-factor at these same locations in vivo and in vitro (Tam and others 1998; Schmidt and others 2000). Such an observation is strictly artificial in nature, because α-factor biogenesis does not occur in humans. However, extrapolating this yeast data to the realm of the mammalian cell does bring to light the type of structural moieties and constraints that may be present in the surrogate mammalian substrate. It is of interest that a singular protease can perform two separate cleavages using two different substrate recognition specificities, namely one being a prenylated CAAX box, and the other being upstream from this prenylated cysteine. The distance between the two cleavage sites is not a conserved parameter when comparing the yeast and human forms of Zmpste24 (yeast cutting 26 and human cutting 15 residues upstream from this prenylated cysteine).

Along these lines, the results reported in this body of work elucidate the identity of the bona fide physiological substrate for Zmpste24 by showing that this protease is capable of both AAXing (removal of the tripeptide adjacent to the prenylated cysteine) and
endoproteolysis of prelamin A 15 amino acids upstream from this prenylated cysteine between tyrosine$^{646}$ and leucine$^{647}$. The cleavage site was identified by assaying a noncleavable prelaminAct point mutant (Leu$^{647}$ → Arg) and by mass spectral analysis of the 31 kDa formed product (Fig’s 20 and 21). Even though the conserved hexapeptide region surrounding the cut site of prelamin A is not identical to the region surrounding the cut site of a-factor (prelamin A: RSY LLG; a-factor: TAT AAP), an enzymatic similarity between human Zmpste24 and yeast Ste24 is to be expected, considering that they are 36% identical and 51% similar in sequence homology, share a 7 transmembrane topology, and contain a HEXXH active site (Tam and others 1998).

The in vitro data provided in this body of work show that Zmpste24 is capable of performing both endoproteolytic steps in the maturation of prelamin A. However, these data do not rule out the possibility that other enzymes in the cell may be able to carry out either AAXing or endoproteolysis in addition to Zmpste24. However, both endoproteolytic steps can not have more than one enzyme responsible for its catalysis. This limitation comes from the observation that Zmpste24 loss of function mutants fail to convert prelamin A to lamin A at the second endoproteolytic cut site, although the extent of processing of the CAAX box of prelamin A has not been experimentally determined in vivo. If Zmpste24 as well as another protease were responsible for performing both AAXing and endoproteolysis, then a Zmpste24 loss of function mutation would not cause an accumulation of a lamin A precursor because the enzyme with overlapping functionality would complement the deficiency due to Zmpste24. Therefore, the fact that prelamin A accumulates when Zmpste24 is knocked out argues that Zmpste24 is the sole enzyme mediating either the first or second endoproteolytic steps. In yeast, two distinct
enzymes are responsible and redundant for AAXing a-factor; RCE1 and Ste24 (Boyartchuk and others 1997; Boyartchuk and Rine 1998; Tam and others 1998). When both RCE1 and Ste24 are deficient, a-factor processing is abolished. In addition to the a-factor substrate, RCE1 and Ste24 can both AAX a variety of substrates in the yeast with both overlapping and differential specificity depending on the nature of the CAAX box (Trueblood and others 2000). It is still unclear whether or not there are two or more prelamin A AAXing enzymes present in the mammalian cell. However, the body of evidence presented in this work argues that Zmpste24 alone mediates the AAXing of prelamin A. This reasoning comes from the experiments using the Y379Stop Zmpste24 truncation, which was modeled after the mutation found in patients with MAD (Agarwal and others 2003). Our results indicate that this prematurely truncated form of Zmpste24 is incompetent for AAXing and fully active for the second endoproteolytic step (Fig 25). Correlating this in vitro data to the physiological in vivo results suggests that patients harboring the MAD Zmpste24 mutation would accumulate prelamin A only if another AAXing enzyme is not present to complement the defect. However, if RCE1 or some other protease were capable of AAXing prelamin A, then the full complement of post translational modifications would occur to produce fully mature lamin A because Zmpste24 would be competent for the second endoproteolytic step. Therefore, if there were a second complementing AAXing enzyme, this type of Zmpste24 mutation should result in no phenotype due to normal lamin function. MAD is a disease state with a pronounced phenotypic effect and, therefore, it follows that Zmpste24 is the only protease responsible for carrying out AAXing on prelamin A in the human cell. To prove this assertion, prelamin A should be isolated from MAD patients and mass spectrally
characterized to identify the exact state of post translational modification present at the CAAX box. If isolated prelamin A from MAD patients is only partially processed at the CAAX box by evidence of a prenylated cysteine adjacent to three carboxyl terminal CAAX box residues, then it can be concluded that Zmpste24 is the only prelamin A specific AAXing protease within the cell. These data do not argue that all lamins use Zmpste24 as their sole source of AAXing activity. As an example of this, the processing of Lamin B1, which has the CAAX box sequence CAIM, is completely abolished in RCE1 -/- mouse cells but is maintained in Zmpste24 -/- cells (Maske and others 2003). In contrast to the findings for Lamin B1, we conclude that RCE1 is not involved in the processing of prelamin A. This may be due to substrate specificity differences and/or lack of accessibility due to compartmentalization.

This current set of in vitro data suggest that both steps in the maturation of prelamin A can be catalyzed by Zmpste24. Because the first AAXing reaction is assumed to be solely governed by Zmpste24 in vivo, this leaves open the possibility that another protease in addition to Zmpste24 may be able to perform the second endoproteolytic conversion. In this scenario, the accumulation of unfully processed prelamin A in Zmpste24 -/- cells is not due to the loss of the second endoproteolytic activity of Zmpste24 because a complementing protease is able to carry out this processing. Rather, the accumulation of unprocessed prelamin A stems from the experimental observation that when the first set of post-translational modifications of AAXing and subsequent carboxymethylation are not completed at the C-terminus of prelamin A, the second endoproteolytic reaction does not and can not occur (Kilic and others 1997). This possibility of another protease being found in the cell that can perform the second endoproteolytic conversion of prelamin A is highly
unlikely due to the unique specificity and localization requirements for prelamin A processing, yet this possibility can not be ruled out with the current set of data. In summary, the possibility that more than one enzyme can perform AAXing on prelamin A is ruled out by our set of in vitro data along with the MAD phenotype, and the possibility that a redundant enzyme is present that is capable of the second endoproteolytic reaction can not be ruled out. Nonetheless, it is entirely possible that Zmpste24 is the sole enzyme within the cell that performs both steps in prelamin A maturation.

The immunofluorescence data presented in this paper show that Zmpste24 overexpressed in CHO cells has not only an ER localization but a nuclear envelope localization as well. Previous reports have demonstrated that human Zmpste24 is localized to the ER and the golgi (Kumagai and others 1999). However, unlike yeast Ste24, Zmpste24 does not contain a canonical C-terminal ER retention motif (Tam and others 1998) leading to the possibility that Zmpste24 may be found at other locations within the cell other than the ER. It is to be noted that because Zmpste24 contains 7 transmembrane domains, it is topologically constrained to membranous structures. The nuclear localization of Zmpste24 as well as its membrane topology are in agreement with data from previous work done in our lab that demonstrates purified nuclear envelope fractions contain endoproteolytic activity (Kilic and others 1997; Kilic and others 1999), and that the endoproteolytic conversion of prelamin A to lamin A can be accomplished solely in the nucleus (Lutz and others 1992). One possible processing pathway for prelamin A that would encompass all of the known data would be a model in which prelamin A is first synthesized and farnesylated in the cytosol, travels to the ER membrane to undergo AAXing by Zmpste24 or some other AAXing enzyme, undergoes
carboxymethylation on the ER by ICMT, and then travels to the nucleus to undergo endoproteolysis by Zmpste24. For this model to be valid, an explanation describing why prelamin A does not become converted to lamin A before entering the nucleus would need to be established because Zmpste24 is clearly present in the ER. One possible explanation is that another factor or chaperone is interacting with prelamin A and/or Zmpste24 in such a way as to render endoproteolysis as a nuclear specific event. Another model with regards to the subcellular localization of prelamin A maturation is one in which all prelamin A processing occurs solely in the nucleus. Such a model is consistent with findings that incubating cells in the presence of the prenylation inhibitor lovastatin causes non prenylated prelamin A to accumulate in the nucleoplasm, after which the commencement of prenylation causes all prelamin A to lamin A conversion to take place in the nucleus within 3 hours (Lutz 1992). Full processing of nonprenylated prelamin A to lamin A solely in the nucleus is made possible by the observation that farnesyltransferase can be localized to the nucleoplasm (Sinensky and others 1994b). The actual pathway may be a hybrid between these two models and would open up the possibility that the extent to which either pathway is being used is a highly regulated process based on tissue type, differentiation, and state of proliferation.

Previous data from our lab conducted with nuclear envelope extracts demonstrate that the prelamin A substrate must be prenylated for endoproteolysis to occur, and that farnesyl cysteine methyl ester acts as a non-competitive inhibitor of endoproteolytic activity, suggesting that there may be a prenyl-specific binding domain that is separate and distinct from the active site (Kilic and others 1997). Certainly, the data presented here for Zmpste24 is in agreement with this prenylation requirement in that: incubating cells...
with lovastatin abolishes the Zmpste24 mediated conversion of prelaminAct to laminAct (Fig 16); and a prelaminAct substrate bearing a nonprenylated carboxyl-terminal cysteine is unable to be processed to laminAct by Zmpste24 in vitro (Fig 22). These findings agree with the prenylation requirement for endoproteolytic conversion but do not address the specificity or constraints of the prenyl moiety. The prelaminAct substrate constructed in the insect system possessed a farnesyl cysteine methyl ester (Fig’s 9 and 10) and, therefore, the results reported here shed light on the specificity of Zmpste24 towards a farnesyl-moiety as the prenyl substituent. However, in previous studies with crude nuclear envelope preparations, an artificial substrate mimicking the last 18 residues prelamin A bearing a farnesyl isoprenoid is 19 times more reactive ($V_{max}/K_m$) than the same substrate containing a geranylgeranyl moiety (Kilic and others 1997). Two hypothesis could explain this substrate recognition specificity: 1) The “two site hypothesis” in which a prenyl binding site directly resides on Zmpste24 along with the active site, and 2) The “other factor hypothesis” in which some other prenyl-specific recognition cofactor or membrane anchor is involved in properly presenting the substrate to the active site of Zmpste24. The most likely candidate for a prenylation dependent anchoring protein is NARF, a nuclear membrane associated protein that binds the C-terminal tail of prelamin A with prenylation dependent specificity (Barton and Worman 1999). It would be interesting to explore the phenotype of NARF−/− mice to determine if they exhibit similar phenotypes to known laminopathies. Additionally, it would be informative to analyze the fate of prelamin A in cells derived from these mice by examining the extent of processing. Finding both a laminopathy like phenotype and defective processing or prelamin A would demonstrate whether or not the interaction between farnesylated prelamin A and NARF is necessary
for downstream prelamin A processing events (i.e., AAXing, carboxymethylation, and endoproteolysis).

These similarities between the yeast and human forms of the enzyme are limited in extent as the results of this present study reveal that the mechanistic features of these two enzymes diverge significantly. Performing an inhibition profile on Zmpste24 revealed that the endoproteolytic activity is solely chymotrypsin like (Fig 23), while the AAXing reaction is distinctly Zn\(^{+2}\) dependent (Fig 15). This latter result was as expected because the AAXing activity of human Zmpste24 against the yeast substrate a-factor has been firmly established as a Zn\(^{+2}\) dependent process (Tam and others 1998; Schmidt and others 2000). As a confirmation to the data observed in the inhibition response profiles, a mutant of Zmpste24 containing a deletion of the HEXXH motif was incompetent for AAXing, but faithfully maintained endoproteolytic activity (Fig 25). The unexpected observation that endoproteolysis is mediated by a serine like functionality naturally led to the hypothesis that a second, yet distinct, active site existed within the same enzyme. Since we were not able to reconstitute the second endoproteolytic activity from detergent extracted and purified Zmpste24 with any success, we could not rule out the possibility that Zmpste24 was functioning as a cofactor, membrane anchor, or shuttling effector to present the substrate to the bona fide prelamin A endoprotease. With this possibility in mind, we attempted to affinity label Zmpste24 with FFCK, a fluorescently derived analogue to the chymotrypsin specific suicide inhibitor TPCK. Inhibition by this family of chloromethyl ketones function by specifically and covalently alkylating the active site histidine within the catalytic triad, thereby rendering it non-functional (Grabarek and others 2002b). Our results indicated that Zmpste24 can be efficiently labeled with FFCK
and that such labeling is abolished if the enzyme is first incubated in the presence of TPCK. Mass spectral analysis of the purified FFCK labeled band mass fingerprinted this as Zmpste24, although a definitively FFCK labeled peptide was not able to be identified in the spectra. However, the observation that the chymotrypsin affinity label FFCK was able to directly tag the Zmpste24 enzyme supported the hypothesis that the serine like active site resides directly within Zmpste24. This is in agreement with previous experiments on purified nuclear envelope fractions that demonstrated the endoproteolytic activity is mediated by a chymotrypsin like enzyme (Kilic and others 1999). These data taken together with the fact that the endoproteolytic reaction is inhibited by TPCK and FFCK, provides strong evidence that there is a chymotrypsin like active site within Zmpste24.

Further evaluation of this “two active site” hypothesis should be conducted in the future using site directed mutagenesis of the putative serine and/or histidine residues, and then assaying for endoproteolytic activity. Other labeling compounds to aid in the identification of the putative serine residue include diisopropylfluorophosphonate (DFP), and aminoethylbenzenesulfonylfluoride (AEBSF), specific compounds that covalently attach to the serine catalytic residue of serine-like enzymes. Radiolabeled forms of these compounds can be used to follow HPLC separated peptides from a tryptic digest of labeled Zmpste24. Positive fractions can be pooled and subjected to MS/MS or Edman degradation to identify the corresponding residues. Future studies encompassing the new questions raised by this current research will provide a more in depth knowledge of the nature and function of these two active sites.
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