A Quantitative FRET Assay for the Upstream Cleavage Activity of the Integral Membrane Proteases Human ZMPSTE24 and Yeast Ste24

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Abstract

The integral membrane protease ZMPSTE24 plays an important role in the lamin A maturation pathway. ZMPSTE24 is the only known enzyme to cleave the last 15 residues from the C-terminus of prelamin A, including a farnesylated and carboxyl methylated cysteine. Mutations in ZMPSTE24 lead to progeroid diseases with abnormal prelamin A accumulation in the nucleus. Ste24 is the yeast functional homolog of ZMPSTE24 and similarly cleaves the a-factor pheromone precursor during its posttranslational maturation. To complement established qualitative techniques used to detect the upstream enzymatic cleavage by ZMPSTE24 and Ste24, including gel-shift assays and mass spectrometry analyses, we developed an enzymatic in vitro FRET-based assay to quantitatively measure the upstream cleavage activities of these two enzymes. This assay uses either purified enzyme or enzyme in crude membrane preparations and a 33-amino acid a-factor analog peptide that is a substrate for both Ste24 and ZMPSTE24. This peptide contains a fluorophore (2-aminobenzoic acid—Abz) at its N-terminus and a quencher moiety (dinitrophenol—DNP) positioned four residues downstream from the cleavage site. Upon cleavage, a fluorescent signal is generated in real time at 420 nm that is proportional to cleavage of the peptide and these kinetic data are used to quantify activity. This assay should provide a useful tool for kinetic analysis and for studying the catalytic mechanism of both ZMPSTE24 and Ste24.

Key words ZMPSTE24/Ste24, Metalloproteases, Membrane proteins, Fluorescence quenching, Fluorescence resonance energy transfer (FRET), Assay

1 Introduction

Human ZMPSTE24 is a unique intramembrane zinc metalloprotease that is localized to both the endoplasmic reticulum and inner nuclear membranes. This enzyme plays important dual roles in the maturation of the nuclear scaffold protein lamin A. Like all CaaX proteins, the precursor of lamin A, prelamin A, undergoes a series of posttranslational modifications including farnesylation by farnesyltransferase, endoproteolysis of the AAX residues by ZMPSTE24

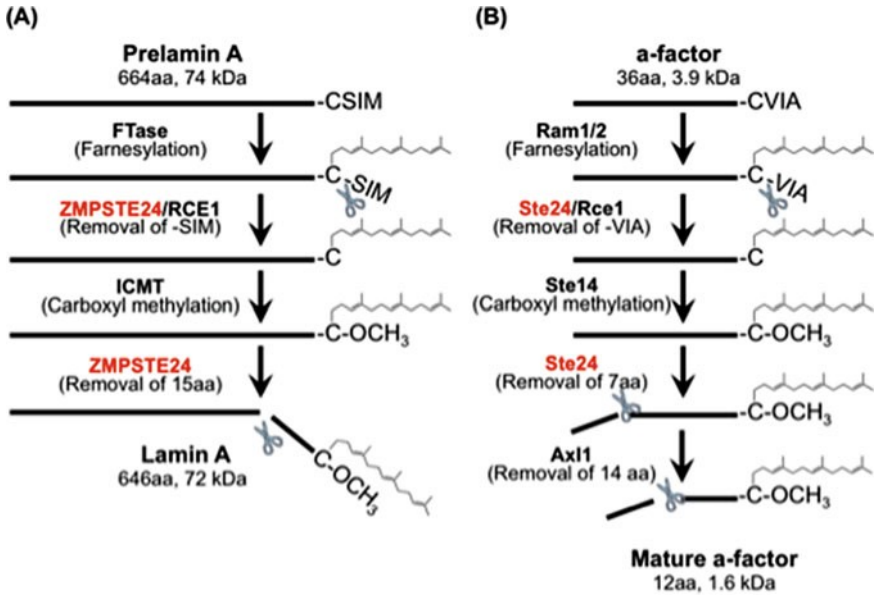

Fig. 1 Comparison of the maturation pathways for lamin A and a-factor. (a) Prelamin A processing pathway to form mature lamin A. Prelamin A is a 74 kDa protein with a CSIM motif at the C-terminus. The pathway includes a series of posttranslational modifications, called CAAX processing, which includes farnesylation, endoproteolysis, and carboxyl methylation. Furthermore, an additional cleavage event is required to remove the last 15 residues, which is performed by ZMPSTE24 only. (b) Yeast a-factor biogenesis pathway. The a-factor precursor is a 36-mer peptide. Like prelamin A and other CAAX proteins, the CVIA motif at the C-terminus triggers the CAAX processing, followed by two additional cleavages by Ste24 and Axl1 to form mature a-factor (Adapted from [1])

or RCE1, and carboxyl methylation by isoprenylcysteine carboxyl methyltransferase (ICMT). Prelamin A then undergoes a discrete site-specific upstream cleavage mediated only by ZMPSTE24. This step removes 15 residues from the C-terminus, including the farnesylated and carboxyl methylated cysteine, releasing mature lamin A into the nucleoplasm (Fig. 1a) [1, 2]. Ste24, which is the functional homolog of ZMPSTE24 from *S. cerevisiae*, cleaves the mating pheromone a-factor during its posttranslational maturation process. Like ZMPSTE24, Ste24 catalyzes two distinct cleavages of the a-factor peptide precursor. The first cleavage is the endoproteolysis of the AAX tail. The second cleavage is to remove the first 7 residues from the N-terminus (Fig. 1b) [1, 3].

Improper prelamin A processing caused by mutations in the gene encoding ZMPSTE24 results in progeroid diseases [4, 5]. Since ZMPSTE24 and another novel CAAX protease, RCE1, could both efficiently cleave the CAAX motif from prelamin A, the ability of ZMPSTE24 to perform the upstream cleavage determines the level of accumulated uncleaved prelamin A in progeroid diseases. Therefore, it is critical to develop effective tools to detect the upstream cleavage of ZMPSTE24. Currently,

immunoblot analysis is commonly used to detect the upstream cleavage by ZMPSTE24 in mammalian cells, since the mature prelamin A is 2 kDa smaller than its precursor [6, 7]. A mass spectrometry binding assay has been reported to monitor the proteolysis by ZMPSTE24 [8]. However, both methods are laborious, technically demanding and difficult for quantification. Similar assays have been utilized for a-factor cleavage in yeast [9–11].

Herein, we introduce a FRET-based assay to quantify the upstream cleavage activity of ZMPSTE24 and Ste24. The method is adapted from a previously reported FRET assay for CAAX proteases that measures the fluorescence from a dequenched peptide [12, 13]. We utilize an a-factor sequence-based peptide as the probe because a-factor can be processed by both ZMPSTE24 and Ste24 in yeast [4, 10, 11].

The 33-mer a-factor analog (Peptide 1) has a 2-aminobenzoic (Abz) fluorophore at the N-terminus and a dinitrophenol (DNP) quencher located on the opposite side of the cleavage site. This peptide was synthesized using methods previously described by Distefano and coworkers [14, 15]. The peptide does not contain the AAX tail, but instead contains a C-terminal *S*-farnesyl-cysteine methyl ester, which mimics the processed substrate for the upstream cleavage. Because of internal quenching from the DNP group, the fluorescence of the Abz group in the intact peptide is significantly diminished. Following site-specific proteolysis by ZMPSTE24 or Ste24, increased fluorescence is observed over time due to the separation of the Abz/DNP fluorophore–quencher pair and the signal can be detected continuously in real time using a fluorimeter. These data are used to quantitatively determine the enzymatic activities of each enzyme.

This chapter first describes the purification process of the integral membrane proteases ZMPSTE24 and Ste24 expressed in the yeast *S. cerevisiae*. Each enzyme is tagged at the N-terminus with ten histidine residues and three tandem repeats of the hemagglutinin epitope (HA). Crude membranes derived from these cells containing over-expressed enzymes are prepared and subsequently solubilized in buffer containing detergent. TALON® cobalt resin is used for enzyme purification, exploiting the presence of the His-tags [16]. These enzymes can also be expressed in insect cells and purified by other chromatographic methods [17–19].

The second part of the chapter discusses the preparation of the FRET peptide, how to calibrate the assay and how to use the assay to determine enzyme activity and kinetic parameters. For peptide preparation, lyophilized peptides might contain from 10% to as much as 70% water and salts by weight. Therefore, it is difficult to determine the actual peptide concentration based on its mass. Using the Beer–Lambert law and the known extinction coefficient for DNP-lysine, the actual peptide concentration can be calculated [12]. Next, before performing the assay for further studies, a

standard curve (Peptide 2) for conversion of RFU into specific activity is constructed. It is also important to generate another calibration curve (Peptide 2 + 3) for fluorescence correction since this Abz/DNP pair exhibits a strong inner filter effect, which leads to decreases in fluorescence when using a high concentration of fluorogenic substrate [12, 20].

The reaction setup in this FRET assay enables real-time monitoring of the peptide cleavage and direct determination of kinetic parameters. Together, this assay provides a convenient way for studying the upstream cleavage of both ZMPSTE24 and Ste24, which will aid in the elucidation of the precise catalytic mechanism of these novel enzymes.

2 Materials

2.1 Purification of ZMPSTE24 and Ste24

1. *Saccharomyces cerevisiae* strain (Δ*ste24*Δ*rce1*) overexpressing His-tagged ZMPSTE24 or Ste24 (*2* μ *URA3* P_{PGK}-His_{10}- HA_3-*ZMPSTE24* or *STE24*) [4, 16].
2. Synthetic complete medium without uracil (SC-URA): For 1 L SC-URA, mix 20 g of glucose, 5 g of ammonium sulfate, 1.7 g of yeast nitrogen base, and 0.7 g of SC-URA powder in a 2-L flask. Add deionized water to 1 L then autoclave the medium.
3. Lysis buffer: 300 mM sorbitol, 100 mM NaCl, 6 mM $MgCl_2$, 10 mM Tris–HCl pH 7.5, 10 μg/mL aprotinin, 2 mM AEBSF, and 1 mM DTT. Add protease inhibitors and DTT freshly. Keep on ice.
4. Liquid nitrogen.
5. French press (SLM Aminco).
6. Disposable 1-mL syringe and 18, 20, 22, and 25 gauge needles.
7. Buffer S: 300 mM sorbitol, 100 mM NaCl, 6 mM $MgCl_2$, 10 mM Tris–HCl, pH 7.5, 10% glycerol, 10 μg/mL aprotinin, and 2 mM AEBSF. Add protease inhibitors and glycerol fresh. Keep on ice.
8. 10% dodecyl maltoside (DDM): Dissolve 250 μg DDM in 2.5 mL of autoclaved water (*see* Note 1). Always prepare fresh just before use and keep on ice.
9. 5 M Imidazole: Dissolve 17.2 g of imidazole in about 25 mL of autoclaved water. Adjust pH to 7.5 by adding HCl. Add autoclaved water to bring the final volume to 50 mL. Store at 4 °C and avoid exposure to light.
10. TALON® cobalt resin (Clontech). Store at 4 °C.
11. Buffer A: 40 mM imidazole and 1% (w/v) DDM in buffer S. Always prepare fresh buffer and keep on ice.

12. Buffer B: 500 mM KCl, 40 mM imidazole, and 1% (w/v) DDM in buffer S. Always prepare fresh buffer and keep on ice.
13. Buffer C: 500 mM KCl, 40 mM imidazole, and 0.1% (w/v) DDM in buffer S. Always prepare fresh buffer and keep on ice.
14. Buffer E: 250 mM imidazole and 0.1% (w/v) DDM in buffer S. Always prepare fresh buffer and keep on ice.
15. Disposable 10-mL chromatography column.
16. Amicon Ultra-15 centrifugal filter (30-kDa): Purchased from EMD Millipore.
17. Lab armor beads: Store at −80 °C.
18. 5× SDS-PAGE buffer: 0.5 M Tris–HCl, pH 6.8, 25% β-mercaptoethanol, 0.1% bromophenol blue, 30% sucrose, and 10% SDS. Store at −20 °C.

2.2 Preparation of FRET Peptides Stock Solution

1. FRET peptides: Abz-MQPSTATAAPK(DNP)EKTSSEKKD-NYIIKGVFWDPAC(Fr)-OMe (Peptide 1), Abz-MQPSTAT (Peptide 2) and AAPK(DNP)EKTSSEKKDN-YIIKGVFWD-PAC(Fr)-OMe (Peptide 3) (Fig. 2). Synthesized in the Distefano lab (unpublished data) using previously described methods [14, 15].
2. Dimethylformamide (DMF): Store at room temperature in supplied container.

2.3 FRET Assay

1. FRET peptides: Stock solution of Peptides 1, 2 and 3 prepared as in Subheading 3.2. Dilute to 1 mM in 100% DMF. Store at −80 °C. Incubate at room temperature for 10 min before usage and avoid exposure to light.

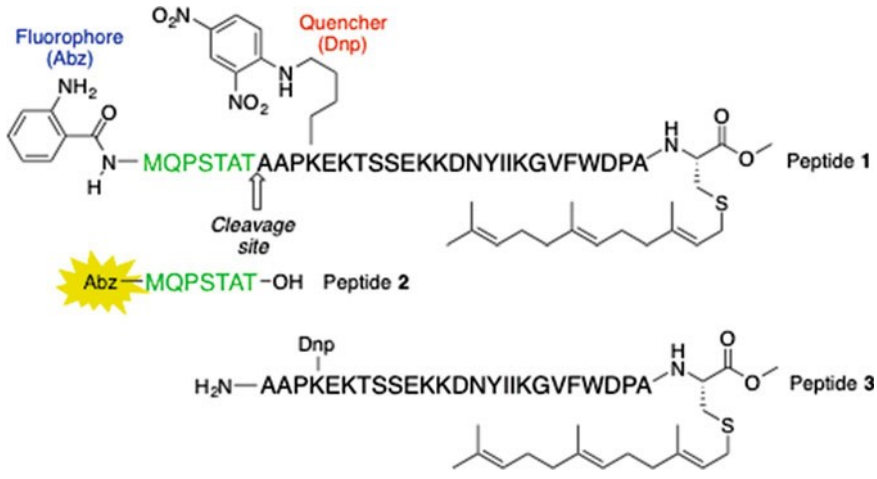

Fig. 2 Schematic representation of the FRET assay for ZMPSTE24 and Ste24 cleavage. The internally quenched FRET substrate, Peptide 1, contains an Abz fluorophore and a DNP quencher positioned at the N-terminus, and 4 residues downstream from the cleavage site, respectively. Cleavage by ZMPSTE24 or Ste24 between the T7 and A8 residues results in two cleavage products, Peptides 2 and 3. Fluorescence released from the dequenched Abz fluorophore can be measured at 420 nm

2. Assay buffer: 150 mM Tris–HCl, pH 7.5. Dissolve 18.171 g of Tris base in about 800 mL of deionized water. Adjust the pH to 7.5 with HCl. Add deionized water to 1 L. Store at 4 °C.
3. 625 μg/μL *E. coli* polar lipid extract (Avanti Polar Lipids). For 50 mg/mL lipid stock, add 2 mL of autoclaved water and 4 μL of 1 M β-mercaptoethanol (final: 2 μM) to 100 mg of lipid extract (one ampule). Gently resuspend by repeated pipetting on ice. Aliquot 500 μL to new eppendorf tubes. To prepare 625 μg/μL lipid stock, mix 50 μL of 50 mg/mL stock with 3950 μL of autoclaved water. Aliquot 500 μL to new eppendorf tubes. Purge the tubes with nitrogen and store at −80 °C. Thaw and keep on ice before use (*see* Note 2).
4. 0.15 μg/μL ZMPSTE24 and Ste24 protein: Prepared as in Subheading 3.1. Freshly dilute concentrated proteins with 10 mM Tris–HCl, pH 7.5 to 0.15 μg/μL stock. Keep on ice.
5. Black polystyrene 96-well half-area plate with flat bottom: purchased from Corning.
6. Synergy™ H4 hybrid microplate reader (BioTek). All experiments described in Subheadings 3.3 to 3.5 are designed using this microplate reader. Other multiwell fluorimeters with an excitation wavelength around 320 nm and capable of measuring emission around 420 nm may also be suitable.

3 Methods

3.1 Purification of Ste24 and ZMPSTE24

1. Grow Δ*ste24*Δ*rce1* strain of *Saccharomyces cerevisiae* overexpressing His-tagged ZMPSTE24 or Ste24 from fresh (less than 2-months old) working stock plate in 15 mL of SC-URA medium. Incubate with shaking at 220 rpm for 20 h at 30 °C.
2. Transfer the overnight culture into fresh 1 L of SC-URA medium for inoculation. Incubate with shaking at 220 rpm for 20 h at 30 °C. Measure the optical density (OD) at 600 nm and multiply by the culture volume to obtain the total number of OD_{600} units (*see* Note 3).
3. Harvest the yeast cells in late-log phase (3 to 5 OD_{600}/mL) by centrifugation at 3750 *g* for 5 min at 4 °C using 500-mL centrifuge bottles. Decant the supernatant. Combine the pellets into a 50-mL plastic conical tube (*see* Note 4).
4. Resuspend the pelleted cells by adding 1 mL of cold lysis buffer for per 800 OD_{600}'s of culture. Place the sample on ice for 15 min to swell. Then freeze and thaw the sample twice using liquid nitrogen.
5. Lyse the cells using a French Press. Prechill the French press cell on ice. Apply the sample to the French Press cell and bring

the cell to 12,000 psi. While maintaining the pressure, adjust the outlet flow rate to about 1 drop/s. Collect cell lysate in a 50-mL plastic conical tube that is kept on ice (*see* Note 5). Pass the lysate through the chilled French Press as described one additional time.

6. Centrifuge the lysate at 500 × *g* for 10 min at 4 °C. Transfer the supernatants to new conical tubes and centrifuge again to completely remove cell debris and unbroken cells. Transfer the supernatants to ultracentrifugation tubes. Ultracentrifuge at 100,000 × *g* for 1 h at 4 °C to obtain a pellet that includes the crude membranes containing the overexpressed ZMPSTE24 or Ste24. Remove the supernatant by decanting.
7. Add 300 μL of prechilled 10 mM Tris–HCl, pH 7.5 to the pellet. Resuspend the pellet with 1-mL syringe by passing through bent needles (18-, 20-, 22-, and 25-gauge) on ice. Then transfer the resuspended crude membranes to a new eppendorf tube using the 25-gauge needle (*see* Note 6). A 1-L yeast culture could yield about 10 mg crude membranes.
8. Solubilize 10 mg of crude membrane in 200 μL of 10% DDM (final: 1%), 8 μL of 5 M imidazole (final: 20 mM) and add buffer S to final volume of 2 mL. Incubate the sample on a rotary mixer for 1 h at 4 °C. Remove the insoluble fraction by ultracentrifugation at 100,000 × *g* for 45 min at 4 °C.
9. Prepare the cobalt resin by adding 2 mL of a 50% suspension of the resin into a 15-mL plastic conical tube. Spin down at 350 × *g* for 2 min at 4 °C then remove the supernatant. Wash the resin twice by adding 2 mL of buffer S to the resin. Gently mix, then spin down at 350 × *g* for 2 min at 4 °C. Decant the supernatant (*see* Note 7).
10. Transfer the supernatant fraction containing the solubilized ZMPSTE24 or Ste24 from step 8 to the preequilibrated cobalt resin. Incubate the sample on a rotary mixer for 1 h at 4 °C. His-tagged ZMPSTE24 or Ste24 should bind and be retained on the resin.
11. Centrifuge at 350 × *g* for 2 min at 4 °C then remove the unbound fraction. Then wash the resin by adding 5 mL of buffer A. Incubate the sample on a rotary mixer for 10 min at 4 °C. Spin down at 350 × *g* for 2 min at 4 °C then remove the supernatant. Repeat washing with another 5 mL of buffer A, B, and C.
12. Transfer the resin to a 10-mL chromatography column. Elute the protein with 5 mL of buffer E and collect the elution at 4 °C.
13. Transfer the elution sample to a prerinsed 30-kDa concentrator and perform centrifugation at 5000 × *g* for 20 min at 4 °C.

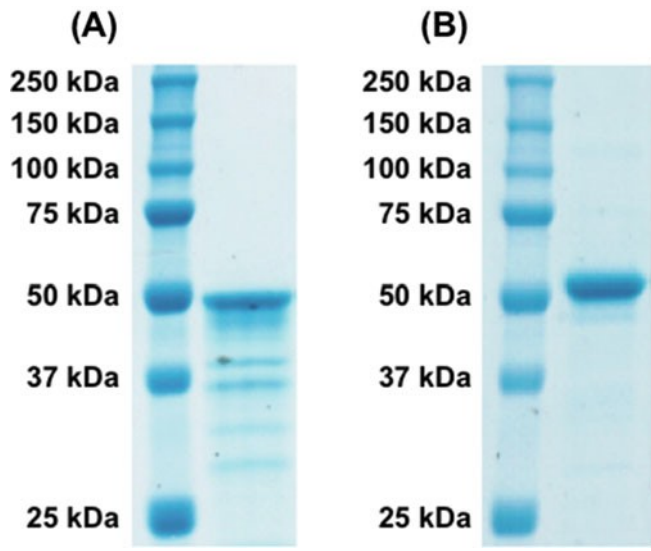

Fig. 3 Purification of ZMPSTE24 and Ste24 proteins. Coomassie blue stained SDS-PAGE shows all proteins were >90% pure. (a) Purified His-HA-ZMPSTE24 runs at approximate 50 kDa. (b) Purified His-HA-Ste24 runs at the expected size of 58 kDa

Aliquot 10 μL of concentrated protein to new eppendorf tubes, then flash-freeze on −80 °C lab armor beads (*see* Note 8). 10 mg of crude membrane should yield about 50 μg pure ZMPSTE24 or Ste24 protein.

14. Determine the protein concentration using Amido Black protein assay (*see* Note 9) [21]. To prepare SDS-PAGE samples, dilute purified protein to 0.5 μg/μL. Take 10 μL of diluted protein and mix with 2.5 μL of 5× SDS-PAGE buffer. Incubate at room temperature for 30 min and vortex every 5 min. Evaluate the purity of purified ZMPSTE24 or Ste24 by SDS-PAGE, followed by Coomassie blue staining (Fig. 3).

3.2 Preparation of FRET Peptide Stock Solutions

1. Dissolve the Peptides (1, 2 and 3) in 100% DMF to prepare about 20 μg/μL stock solutions.
2. Measure the absorbance of Peptides 1 and 3 at 360 nm to determine the molar concentration using the molar extinction coefficient of $\varepsilon = 17{,}530\ M^{-1}\ cm^{-1}$ for the DNP group. To determine the molar concentration of Peptide 2, measure the absorbance at 320 nm and use the molar extinction coefficient of $\varepsilon = 21{,}870\ M^{-1}\ cm^{-1}$ for the Abz group for conversion (*see* Note 10) [22].
3. Avoid exposure to light. Store the stock solutions at −80 °C for up to 1 year.

3.3 Conversion and Calibration of Relative Fluorescence Units (RFUs) into Specific Activity

1. For the standard curve, prepare a serial dilution of Peptide 2. Dilute Peptide 2 in assay buffer to 250, 150, 75, 37.5, 18.75, and 9.375 μM with a final volume of 30 μL. Use 30 μL of assay buffer for the 0 μM stock. Keep at room temperature and avoid exposure to light.
2. For the calibration curve, prepare a serial dilution of Peptides 2 and 3 mixture. Dilute both peptides in assay buffer to 500, 300, 150, 75, 37.5, and 18.75 μM with a final volume of 15 μL. Then combine the solutions with the same concentrations in new eppendorf tubes. Those tubes should contain 30 μL of 250 to 9.375 μM of both Peptides 2 and 3. Use 30 μL of assay buffer for the 0 μM stock. Keep at room temperature and avoid exposure to light.
3. Transfer the serial dilutions of Peptide 2 (from step 1) and the serial dilutions of the mixed Peptides 2 and 3 (step 2) to the 96-well plate. Cover with lid to prevent light exposure. Keep at room temperature.
4. Prepare master mix. Rapidly reconstitute 80 μL of 0.15 μg/μL purified ZMPSTE24 or Ste24 protein into 160 μL of 625 μg/μL *E. coli* polar lipid extract in a 15-mL plastic conical tube. Add 1040 μL of assay buffer into the tube. Gently vortex and incubate on ice for 10 min.
5. Warm up the master mix in a 30 °C water bath for 5 min. Transfer 80 μL of master mix to 12 wells in the 96-well plate.
6. Place the 96-well plate into the microplate reader and wait about 3 min for the temperature to reach 30 °C.
7. Use an 8-channel pipette to transfer 20 μL of FRET peptide prepared from step 3 to each well containing 80 μL of master mix (*see* Note 11). Thus, the total volume of each sample is 100 μL; final peptide concentrations range from 50 to 0 μM.
8. Mix by shaking at medium speed for 30 s. Measure the fluorescence readings at excitation and emission wavelengths of 320 ± 9 nm and 420 ± 9 nm at 30 °C (*see* Note 12).
9. Plot the fluorescence reading against final peptide concentration (Fig. 4). The extinction coefficient (ε) obtained from the standard curve (Peptide 2) can be used for converting from RFU to concentration of dequenched peptide product.
10. A correction factor (*C*) for the inner filter effect is calculated at each concentration of peptide used in the assay, as given by the following relationship:

$$C = \frac{F(\text{Peptide 2})}{F(\text{Peptide 2 + 3})}$$

where *F*(Peptide 2) is the fluorescence reading of Peptide 2 and *F*(Peptide 2 + 3) is the fluorescence reading of Peptide 2 in the

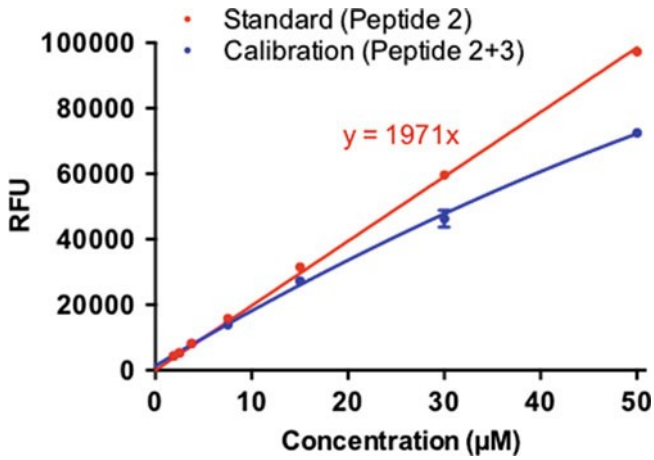

Fig. 4 Assay calibration. The standard curve (*red*) correlates the relative fluorescence unit (RFU) to a range of concentrations of the dequenched product, Peptide 2. The extinction coefficient (ε) was determined as 1971 RFU/μM from the slope of the standard curve. The calibration curve (*blue*) was measured using an equimolar mixture of Peptides 2 and 3 over the same range of concentrations. All fluorescence readings were taken under standard assay conditions with purified Ste24

presence of equimolar amount of Peptide 3. *C* will be applied to the same concentrations of FRET peptides to yield the corrected fluorescence value.

3.4 Determination of Upstream Cleavage Activity

1. Freshly dilute Peptide 1 in assay buffer to 150 μM with a final volume of 30 μL. Keep at room temperature and avoid exposure to light.
2. Rapid reconstitute 7.5 μL of 0.15 μg/μL purified ZMPSTE24 or Ste24 protein into 15 μL of 625 μg/μL *E. coli* polar lipid extract in an eppendorf tube. Add 97.5 μL of assay buffer into the tube. Gently vortex and incubate on ice for 10 min.
3. Warm up the protein/liposome sample in a 30 °C water bath for 5 min. Transfer 80 μL to a well in the 96-well plate.
4. Place the 96-well plate into the microplate reader and wait about 3 min for the temperature to reach 30 °C.
5. Initiate the assay by adding 20 μL of 150 μM Peptide 1 prepared from step 1. Final volume in each well is 100 μL. Each reaction contains 0.75 μg of purified enzyme reconstituted in 6.25 mg of *E. coli* lipid and 30 μM substrate in 100 mM Tris–HCl, pH 7.5 (*see* Note 13). Mix by shaking at medium for 30 s. Measure the fluorescence readings at excitation and emission wavelengths of 320 ± 9 nm and 420 ± 9 nm at 30 °C every 30 s (*see* Note 14). Constantly record the increase in fluorescence with time (Fig. 5).

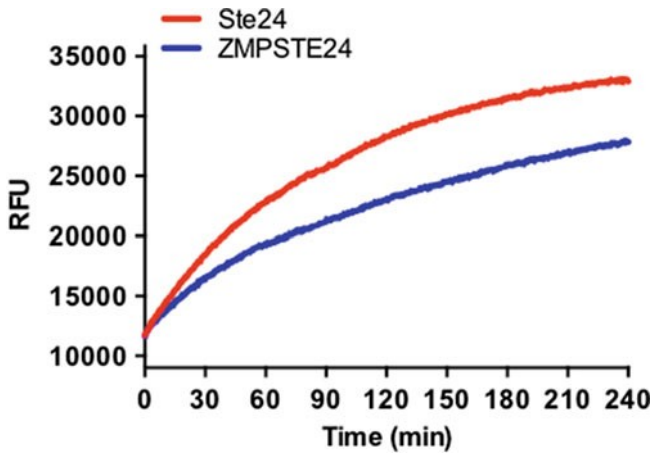

Fig. 5 Real-time monitoring of the upstream cleavage of the FRET substrate by ZMPSTE24 and Ste24. Fluorescence readings over a time course of 4 h were detected using an excitation wavelength of 320 nm and an emission wavelength of 420 nm. The fluorescence increased remarkably during the cleavage by purified ZMPSTE24 (*blue*) and Ste24 (*red*) proteins. Initial rates were found to be within the first 10 min of reaction

6. Determine the cleavage rate of ZMPSTE24/Ste24 using the slope from the first linear region (usually the first 10 min) (*see* Note 15). Convert the cleavage rate to the specific activity using the following equation:

$$\text{Specific activity}\left(\frac{\text{pmol}}{\text{mg}\times\text{min}}\right) = \text{Rate}\left(\frac{\text{RFU}}{\text{min}}\right) \times C \times \frac{1}{\varepsilon\left(\frac{\text{RFU}}{\mu\text{M}}\right)} \times \frac{10^6\left(\frac{\text{pmol}}{\mu\text{mol}}\right)}{10^4\,(\text{L})} \times \frac{1}{0.0075\,(\text{mg})}$$

where the correction factor (C) and the extinction coefficient (ε) are defined in Subheading 3.3. The specific activity is expressed as pmol of substrate cleaved per min by per mg of enzyme.

3.5 *Kinetic Analysis*

1. Freshly dilute Peptide 1 in assay buffer to 150, 75, 37.5, 18.75, and 9.375 μM with a final volume of 30 μL. Use 30 μL of assay buffer for the 0 μM stock. Keep at room temperature and avoid exposure to light.
2. Transfer solutions made in step 1 to the 96-well plate. Cover with lid to prevent light exposure. Keep at room temperature.
3. Prepare master mix. Rapid reconstitute 40 μL of 0.15 μg/μL purified ZMPSTE24 or Ste24 protein into 80 μL of 625 μg/μL *E. coli* polar lipid extract in a 15-mL plastic conical tube. Add 520 μL of assay buffer into the tube. Gently vortex and incubate on ice for 10 min.

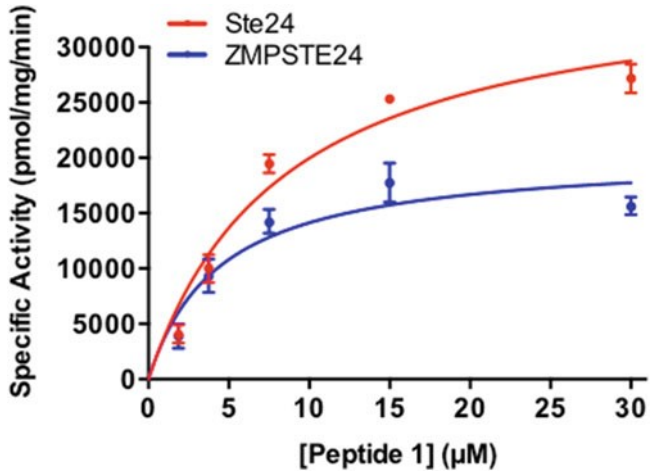

Fig. 6 Kinetic analysis of the upstream cleavage of the FRET substrate by ZMPSTE24 and Ste24. Specific activities and substrate concentrations were plotted and fitted to the Michaelis-Menten equation using the Prism 6 software. From these data, the values of kinetic parameters were determined. For ZMPSTE24 (*blue*), V_{max} is 27,000 pmol/mg/min and K_m is 6.7 μM. For Ste24 (*red*), V_{max} is 38,000 pmol/mg/min and K_m is 9.1 μM

4. Warm up the master mix in a 30 °C water bath for 5 min. Transfer 80 μL of master mix to 6 wells in the 96-well plate.
5. Place the 96-well plate into the microplate reader and wait about 3 min for the temperature to reach 30 °C.
6. Use 8-channel pipette to transfer 20 μL of FRET peptide solution (Peptide 1) prepared from step 2 to each well containing 80 μL of master mix. Thus, total volume of each sample is 100 μL. Final peptide concentrations are from 30 to 0 μM.
7. Mix by shaking at medium speed for 30 s. Constantly measure the fluorescence readings at excitation and emission wavelengths of 320 ± 9 nm and 420 ± 9 nm at 30 °C for at least 10 min until seeing the first linear region.
8. Calculate specific activity at each peptide concentration as Subheading 3.4 description. Plot the specific activities against the peptide concentration. Fit the curve to the Michaelis–Menten equation using GraphPrism software to determine kinetic parameters (Fig. 6).

4 Notes

1. Always prepare DDM solution freshly to avoid hydrolysis and oxidation. Gently invert the solution or use rotator until DDM goes into solution. This will prevent bubble formation.

2. After each use, repurge the tube with nitrogen and store at −80 °C immediately to protect lipid from oxidation.
3. Generally speaking, the density of yeast overexpressing wild-type Ste24 or ZMPSTE24 will reach about 4 OD_{600} after 20 h incubation. Some mutant strains grow slower and might to longer incubation time.
4. The pellet can be stored at −80 °C for long-term storage.
5. If the volume of cell lysate is more than 40 mL, separate the sample into multiple tubes. Confirm the suggested minimum and maximum volume of the French Press cell before use.
6. The crude membranes can be stored at −80 °C for up to 2 years. Protein concentration of the crude membrane can be determined by the Bradford assay and should be between 25 and 35 mg/mL.
7. Do not allow the resin to dry after adding the lysate. It is also critical to avoid using strong reducing agents, such as DTT, or chelators, such as EDTA, which will disrupt the function of the cobalt resin.
8. Proteins may also be flash-frozen in liquid nitrogen with additional glycerol to a final concentration of 10–20%.
9. The Amido Black protein assay can be used to accurately determine low amounts of protein in the presence of high levels of both ionic and nonionic detergents, including DDM used for purification. Measuring the absorbance at 280 nm by NanoDrop spectrophotometer can also be used to determine the protein concentration. However, it tends to have a higher error at very low protein concentrations.
10. Depending on the spectrophotometer used, peptides might need to be diluted or prepared in larger volumes to construct a standard curve to determine the concentrations. The final concentration of FRET peptides should be around 5 mM for Peptides 1 and 3 and 20 mM for Peptide 2.
11. Since the samples contain detergent, it is easy to add bubbles to the wells. To avoid air bubbles, dispense the liquid while touching the bottom corner of the well and only pressing the pipette to its first stop. If there are still bubbles, use a pipette top to remove them, or perform a quick spin using a suitable centrifuge.
12. Changing assay conditions might result in emission intensity and spectral shifts. Recording an emission spectrum to confirm the maximum peak will help to select the best wavelength for the assay.
13. We do not suggest using more than 50 μM substrate in each reaction. This leads to a strong decrease in the fluorescence

intensity due to the increase in the inner filter effect. Moreover, the FRET peptide might not be able to dissolve in the assay buffer because of its limited solubility.

14. The time interval should be selected according to the reaction rate, the total sample number and the microplate reader model. We generally use 10 s to 1 min for measurement.
15. It is important to initially run the reactions to completion to determine the linear region. Since the 96-well plates are uncovered during analysis, evaporation can cause sample concentrations to increase, which affects data collection. Use plate sealing film if needed. If seeing an "S" shape curve, incubate the sample at 30 °C or shake in the microplate reader for a longer time.

References

1. Barrowman J, Michaelis S (2009) ZMPSTE24, an integral membrane zinc metalloprotease with a connection to progeroid disorders. Biol Chem 390:761–773
2. Michaelis S, Hrycyna CA (2013) A protease for the ages. Science 339:1529–1530
3. Michaelis S, Barrowman J (2012) Biogenesis of the *Saccharomyces cerevisiae* pheromone a-factor, from yeast mating to human disease. Microbiol and Mol Biol Rev 76:626–651
4. Barrowman J, Wiley PA, Hudon-Miller SE, Hrycyna CA, Michaelis S (2012) Human ZMPSTE24 disease mutations: residual proteolytic activity correlates with disease severity. Human Mol Genet 21:4084–4093
5. Davies BS, Fong LG, Yang SH, Coffinier C, Young SG (2009) The posttranslational processing of prelamin a and disease. Annu Rev Genomics Hum Genet 10:153–174
6. Barrowman J, Hamblet C, Kane MS, Michaelis S (2012) Requirements for efficient proteolytic cleavage of prelamin a by ZMPSTE24. PLoS One 7:e32120
7. Bergo MO, Gavino B, Ross J, Schmidt WK, Hong C, Kendall LV, Mohr A, Meta M, Genant H, Jiang Y, Wisner ER, Van Bruggen N, Carano RA, Michaelis S, Griffey SM, Young SG (2002) Zmpste24 deficiency in mice causes spontaneous bone fractures, muscle weakness, and a prelamin a processing defect. Proc Natl Acad Sci U S A 99:13049–13054
8. Mehmood S, Marcoux J, Gault J, Quigley A, Michaelis S, Young SG, Carpenter EP, Robinson CV (2016) Mass spectrometry captures off-target drug binding and provides mechanistic insights into the human metalloprotease ZMPSTE24. Nat Chem 8:1152–1158
9. Chen P, Sapperstein SK, Choi JD, Michaelis S (1997) Biogenesis of the *Saccharomyces cerevisiae* mating pheromone a-factor. J Cell Biol 136:251–269
10. Schmidt WK, Tam A, Michaelis S (2000) Reconstitution of the Ste24p-dependent N-terminal proteolytic step in yeast a-factor biogenesis. J Biol Chem 275:6227–6233
11. Tam A, Schmidt WK, Michaelis S (2001) The multispanning membrane protein Ste24p catalyzes CAAX proteolysis and NH2-terminal processing of the yeast a-factor precursor. J Biol Chem 276:46798–46806
12. Arachea BT, Wiener MC (2017) Acquisition of accurate data from intramolecular quenched fluorescence protease assays. Anal Biochem 522:30–36
13. Hollander I, Frommer E, Mallon R (2000) Human ras-converting enzyme (hRCE1) endoproteolytic activity on K-ras-derived peptides. Anal Biochem 286:129–137
14. Diaz-Rodriguez V, Ganusova E, Rappe TM, Becker JM, Distefano MD (2015) Synthesis of peptides containing C-terminal esters using trityl side-chain anchoring: applications to the synthesis of C-terminal ester analogs of the *Saccharomyces cerevisiae* mating pheromone a-factor. J Org Chem 80:11266–11274
15. Diaz-Rodriguez V, Mullen DG, Ganusova E, Becker JM, Distefano MD (2012) Synthesis of peptides containing C-terminal methyl esters using trityl side-chain anchoring: application to the synthesis of a-factor and a-factor analogs. Org Lett 14:5648–5651

16. Hudon SE, Coffinier C, Michaelis S, Fong LG, Young SG, Hrycyna CA (2008) HIV-protease inhibitors block the enzymatic activity of purified Ste24p. Biochem Biophys Res Commun 374:365–368
17. Clark KM, Jenkins JL, Fedoriw N, Dumont ME (2017) Human CaaX protease ZMPSTE24 expressed in yeast: structure and inhibition by HIV protease inhibitors. Protein Sci 26:242–257
18. Pryor EE Jr, Horanyi PS, Clark KM, Fedoriw N, Connelly SM, Koszelak-Rosenblum M, Zhu G, Malkowski MG, Wiener MC, Dumont ME (2013) Structure of the integral membrane protein CAAX protease Ste24p. Science 339:1600–1604
19. Quigley A, Dong YY, Pike AC, Dong L, Shrestha L, Berridge G, Stansfeld PJ, Sansom MS, Edwards AM, Bountra C, von Delft F, Bullock AN, Burgess-Brown NA, Carpenter EP (2013) The structural basis of ZMPSTE24-dependent laminopathies. Sci- ence 339:1604–1607
20. Hildebrandt ER, Arachea BT, Wiener MC, Schmidt WK (2016) Ste24p mediates proteolysis of both isoprenylated and non-prenylated oligopeptides. J Biol Chem 291:14185–14198
21. Schaffner W, Weissmann C (1973) A rapid, sensitive, and specific method for the determination of protein in dilute solution. Anal Biochem 56:502–514
22. Arellano M, Coll PM, Yang W, Duran A, Tamanoi F, Perez P (1998) Characterization of the geranylgeranyl transferase type I from *Schizosaccharomyces pombe*. Mol Microbiol 29:1357–1367