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Identification, functional expression and enzymic analysis of two distinct CaaX proteases from *Caenorhabditis elegans*

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Post-translational processing of proteins such as the Ras GTPases, which contain a C-terminal CaaX motif (where C stands for cysteine, a for aliphatic and X is one of several amino acids), includes prenylation, proteolytic removal of the C-terminal tripeptide and carboxy-methylation of the isoprenyl-cysteine residue. In the present study, we report the presence of two distinct CaaX-proteolytic activities in membrane extracts from *Caenorhabditis elegans*, which are sensitive to EDTA and Tos-Phe-CH_2Cl (tosylphenylalanylchloromethane; 'TPCK') respectively. A protein similar to the mammalian and yeast farnesylated-proteins converting enzyme-1 (FACE-1)/Ste24p CaaX metalloprotease, encoded by a hypothetical gene (CeFACE-1/C04F12.10) found in *C. elegans* chromosome I, probably accounts for the EDTA-sensitive activity. An orthologue of FACE-2/Rce1p, the enzyme responsible for the proteolytic maturation of Ras oncoproteins and other prenylated substrates, probably accounts for the Tos-Phe-CH_2Cl-sensitive activity, even though the gene for FACE-2/Rce1 has not been previously identified in this model organism. We have identified a previously overlooked gene in *C. elegans* chromosome V, which codes for a 266-amino-acid protein (CeFACE-2) with 30% sequence identity to human FACE-2/Rce1. We show that both CeFACE-1 and CeFACE-2 have the ability to promote production of the farnesylated yeast pheromone **a**-factor *in vivo* and to cleave a farnesylated peptide *in vitro*. These results indicate that CeFACE-1 and CeFACE-2 are *bona fide* CaaX proteases and support the evolutionary conservation of this proteolytic system in eukaryotes.

Key words: drug target, farnesylation, integral membrane protease, prenylation, proteolysis, Ras.

INTRODUCTION

A wide range of cellular and viral proteins, such as members of the Ras superfamily of oncogenes, certain nuclear lamins and the γ subunits of heterotrimeric G-proteins, contain a C-terminal CaaX motif (where C stands for cysteine, a for aliphatic and X is one of several amino acids) that directs an ordered series of three modifications. The first of these modifications is the addition of a C_{15} farnesyl or a C_{20} geranylgeranyl group to the cysteine residue, and this event is catalysed by the cytoplasmic farnesyl and geranylgeranyl transferases respectively. Prenylation is followed by a proteolytic cleavage that removes the tripeptide -a-a-X. The newly exposed C-terminal prenylated cysteine is finally carboxymethylated by a membrane-bound methyltransferase [1]. Since the anchorage of Ras proteins to the cytoplasmic leaflet of the membranes is required for their biological functions, including oncogenesis, the enzymic steps of the CaaX protein maturation cascade have attracted considerable interest as potential targets of anti-cancer therapy [2].

Two distinct enzymic activities have been identified that can mediate the proteolytic maturation of prenylated proteins in yeast and mammalian systems. The candidate genes encoding these enzymes were first identified from yeast [3]. One of these genes encodes a metalloprotease, named Afc1p or Ste24p, which cleaves the precursor of the yeast **a**-factor mating pheromone after the farnesylated cysteine residue and also at a second point close to the N-terminus of the precursor peptide [3–7]. The second gene encodes Rce1p, a protein that seems to be directly involved in the C-terminal processing of farnesylated **a**-factor, Ras proteins and other prenylated proteins. Using the yeast Ste24p and Rce1p sequences, we and others have reported the identification of the mammalian homologues of these two proteases, which have been named farnesylated-proteins converting enzyme-1 (FACE-1)/Zmpste24 and FACE-2/Rce1 respectively [5,8–10].

Gene targeting of the murine genes encoding the CaaX proteases provides valuable information on the *in vivo* substrates and biological relevance of these enzymes. It has been reported that disruption of FACE-2/Rce1 causes late embryonic to early postnatal lethality and that FACE-2-deficient cells show abnormal subcellular localization of Ras proteins and a reduced susceptibility to transformation by Ras oncogenes, suggesting that this protease may be a feasible anti-tumour target [11,12]. Disruption of FACE-1/Zmpste24 in mice causes a less severe and more specific phenotype [13,14]. Newborn FACE-1$^{-/-}$ mice are indistinguishable from their wild-type littermates, but after a few weeks of life their growth slows, and they eventually develop symptoms characteristic of diverse syndromes caused by

Abbreviations used: CVIS, Cys-Val-Ile-Ser; FACE, farnesylated-proteins converting enzyme; HA, haemagglutinin epitope, NEM, *N*-ethylmaleimide; PGK, phosphoglycerate kinase; RT, reverse transcriptase; Tos-Phe-CH_2Cl, tosylphenylalanylchloromethane ('TPCK').

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The nucleotide sequence data reported in this paper have been deposited in the EMBL, GenBank®, DDBJ and GSDB Nucleotide Sequence Databases under the accession number AJ487543.

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alterations in lamin A. Lamin A is a nuclear CaaX protein whose maturation resembles **a**-factor biogenesis since it includes an additional proteolytic event at a position outside the CaaX motif. The fact that this second proteolytic event does not take place in FACE-1-deficient cells demonstrates that this metalloproteinase has an important role in the maturation of this specific protein substrate [14]. Other than the yeast **a**-factor and lamin A precursors, additional substrates of FACE-1/Zmpste24 have not been identified.

All eukaryotes so far examined contain CaaX proteins and are thus predicted to express CaaX proteases. Several hundred CaaX proteins are encoded by the *Caenorhabditis elegans* genome and isoprenylation of *C. elegans* proteins has been demonstrated previously [15]. However, CaaX proteolytic activity has not been previously reported in invertebrates, and none of the approx. 20000 identified or predicted nematode genes shows a significant similarity to the mammalian or yeast protease FACE-2, which is probably responsible for the proteolytic maturation of prenylated Ras proteins. A gene coding for a putative metalloproteinase similar to FACE-1 (C04F12.10) has been predicted in *C. elegans* chromosome I, but no experimental evidence of its functionality has been reported. In the present study, we provide evidence for the presence of two separate CaaX proteolytic activities in *C. elegans* extracts. Furthermore, we have identified a previously overlooked gene encoding a nematode protease similar to FACE-2. We show that both CeFACE-1 and CeFACE-2 complement mutations of the respective orthologue genes in yeast, and we show that the yeast-expressed *C. elegans* CaaX proteases are able to process a farnesylated tetrapeptide *in vitro*. These results demonstrate the evolutionary conservation of the function of these enzymes and suggest that additional substrates of FACE-1 remain to be identified.

Table 1 Yeast strains used in the present study

Strain	Genotype	Reference
SM1058	*MAT**a** trp1 leu2 ura3 his4 can1*	[19]
SM1068	*MATα lys1*	[19]
SM1086	*MATα rme his6 met1 can1 cyh2 sst2-1*	[20]
SM3041	*pRS316* [*CEN URA3*] transformant of SM1058	[4]
SM3103	*MAT**a** trp1 leu2 ura3 his4 can1 ste24::LEU2*	[4]
SM3613	*MAT**a** trp1 leu2 ura3 his4 can1 rce1::TRP1*	[5]
SM3614	*MAT**a** trp1 leu2 ura3 his4 can1 rce1::TRP1 ste24::LEU2*	[5]
SM3637	*pRS316* [*CEN URA3*] transformant of SM3103	[4]
SM3643	*pRS316* [*CEN URA3*] transformant of SM3613	[5]
SM3650	*pRS316* [*CEN URA3*] transformant of SM3614	[5]
yWS106*	*pWS358* [*CEN URA3 CeFACE-2*]	This study
yWS107	*pWS359* [*CEN URA3 CeFACE-1*]	This study
yWS108	*pWS360* [*CEN URA3 CeFACE-2-HA*]	This study
yWS109	*pWS364* [*CEN URA3 HA-CeFACE-1*]	This study
yWS110	*pWS370* [2 μ *URA3* P_{PGK}-*CeFACE-1*]	This study
yWS111	*pWS369* [2 μ *URA3* P_{PGK}-*HA-CeFACE-2*]	This study

* All yWS strains are transformants of SM3614.

MATERIALS AND METHODS

Materials

Restriction endonucleases and other molecular cloning reagents were purchased from Roche Molecular Biochemicals (Indianapolis, IN, U.S.A.), [α-^{32}P]dCTP was from Amersham Biosciences (Little Chalfont, Bucks., U.K.), [^{3}H]farnesyl pyrophosphate was from New England Nuclear (Boston, MA, U.S.A.) and Bristol N2 strain of *C. elegans* was obtained from the Caenorhabditis Genetics Center (Minneapolis, MN, U.S.A.) and cultured at room temperature (20 °C) in nematode growth medium plates.

CaaX proteolytic assay

CaaX proteolytic assays were performed essentially as described previously [16]. Farnesyltransferase was partially purified from calf brains by ammonium sulphate (30–50 %) precipitation, hydrophobic-interaction and cation-exchange chromatography and used to farnesylate the tetrapeptide acetyl-Cys-Val-Ile-Ser (CVIS) using [^{3}H]farnesyl pyrophosphate as the donor of the prenyl group. The farnesylated substrate and product were analysed by TLC and exposed to Biomax X-ray film (Kodak, Rochester, NY, U.S.A.) at −80 °C for 1 week.

RNA isolation and reverse transcription

Total RNA was extracted from mixed-stage worms following standard procedures. Reverse transcriptase (RT)–PCR was performed with the SuperScript kit (Life Technologies, Rockville, MD, U.S.A.), using oligo(dT) as primer in the RT reaction. PCRs were performed in a GeneAmp 2400 PCR system from PerkinElmer/Cetus (Boston, MA, U.S.A.) for 40 cycles of denaturation (94 °C, 15 s), annealing (64 °C, 20 s) and extension (72 °C, 2 min). The coding sequence of *C. elegans* FACE-1 was amplified with primers F1CEfull-5 (5′-CTCATCTTCAGAATGGACGCAAG) and F1CEfull-3 (5′-GGGAATTTATTTATTCTTTGCCTGG). The central portion of the *C. elegans* FACE-2 cDNA was amplified with primers F2CE1 (5′-GGTCCAATAACCGAAGAAATCGT) and F2CE2 (5′-GTGGCGAATGCTCCAAACAGATA), the 5′-portion was amplified with primers F2CE2 and SL1 (5′-GGTTTAATTACCCAAGTTTGAG) and the 3′-portion was amplified with F2CE1 and F2CE4 (5′-AAAGAGGGCGAAAACGTAATAAA).

DNA sequencing and sequence analysis

PCR products were gel-purified, treated with Klenow fragment, 'kinased' and subcloned into pBlueScript or pcDNA3. DNA sequencing was performed using an ABI PRISM™ 310 Genetic Analyser (Applied Biosystems). Multiple alignments were performed using the ClustalX program [17] and edited with GeneDoc (www.psc.edu/biomed/genedoc/). Transmembrane regions were predicted using TMpred, at EMBnet [18].

Northern-blot analysis

Nylon filters containing 20 μg of total RNA from mixed-stage worms were prehybridized at 42 °C for 3 h in 50 % (v/v) formamide, 5 × SSPE (1 × SSPE is 150 mM NaCl/10 mM NaH_2PO_4/1 mM EDTA, pH 7.4), 10 × Denhardt's solution, 2 % (w/v) SDS and 100 μg/ml denatured herring sperm DNA, and then hybridized with radiolabelled CeFACE-1 or CeFACE-2 cDNAs for 20 h under the same conditions. Filters were washed with 0.1 × SSC, 0.1 % SDS for 2 h at 50 °C and processed for autoradiography.

Yeast strains and plasmids

The *Saccharomyces cerevisiae* strains used in the present study are listed in Table 1. Yeasts were grown at 30 °C as described previously [19,20]. Transformation of these strains with plasmids was performed according to the published methods [21]. The yeast expression plasmids used in the present study are listed in Table 2. Yeast expression vectors encoding epitope-tagged and

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Table 2 Yeast expression plasmids used in the present study

Plasmid	Genotype	Reference
pRS316	*CEN URA3*	[22]
pSM703	*2 μ URA3* P_{PGK}	[23]
pSM1093	*CEN URA3 STE24*	[4]
pSM1097	*CEN LEU2 HA-STE24*	[4]
pSM1275	*CEN URA3 RCE1*	[24]
pSM1314	*CEN URA3 RCE1-HA*	[24]
pWS358	*CEN URA3 CeFACE-2*	This study
pWS359	*CEN URA3 CeFACE-1*	This study
pWS360	*CEN URA3 CeFACE-2-HA*	This study
pWS361	*CEN LEU2 HA-CeFACE-1*	This study
pWS364	*CEN URA3 HA- CeFACE-1*	This study
pWS370	*2 μ URA3* P_{PGK}-*CeFACE-1*	This study
pWS369	*2 μ URA3* P_{PGK}-*HA-CeFACE-2*	This study

untagged CeFACE-1 and CeFACE-2 were generated by replacing the appropriate yeast open reading frame with that of CeFACE-1 or CeFACE2 by PCR-directed homologous recombination [25]. The parent plasmids used as the recipient vectors in this cloning strategy were pSM1093, pSM1097, pSM1275 and pSM1314 [4,24]. The inserts for this strategy were generated by PCR using the CeFACE-1 and CeFACE-2 cDNAs as templates. In pWS361, a second round of recombination was performed with pRS316 to change the marker from *LEU2* to *URA3* to generate pWS364. The plasmids pWS110 and pWS111 were generated by subcloning the CeFACE-1 and HA-CeFACE-2 cDNAs (where HA stand for haemagglutinin epitope) respectively into the polylinker of pSM703, a 2 μ vector containing the constitutive phosphoglycerate kinase (PGK) promoter [23].

a-Factor production assays

To test the ability of the *C. elegans* CaaX proteases to support **a**-factor production in yeast, halo and mating tests were performed using established biological assays [4,19]. For both tests, cells grown to saturation in selective media were harvested, washed twice with sterile water and resuspended to the original cell density (approx. 2×10^7 cells/ml). A portion of the resuspended cells (2 μl) was spotted on to a lawn of SM1086 (halo test) or SM1068 (mating test) that was previously spread on to a rich YPD [1 % (w/v) yeast extract, 2 % (w/v) peptone and 2 % (w/v) glucose] or minimal synthetic dropout agar plate respectively. Halo formation and diploid production were recorded after incubation at 30 °C for 24 and 48 h respectively.

RESULTS

Analysis of CaaX proteolytic activity in *C. elegans* extracts

CaaX proteases from both mammalian and yeast cells are localized to intracellular membranes [24]. To test for the existence of possible CaaX proteolytic activities in *C. elegans*, we prepared a membrane-enriched fraction from mixed-stage worms and assayed its ability to cleave a radiolabelled synthetic CaaX tetrapeptide. This direct biochemical assay revealed the presence of potent proteolytic activity against the farnesylated tetrapeptide in *C. elegans* membrane preparations (Figure 1). To obtain additional information on the characteristics of the proteolytic activities acting on this prenylated substrate, we incubated *C. elegans* membranes with several proteinase inhibitors before performing the enzymic assay. As shown in Figure 1, pre-incubation of the membrane samples with either EDTA or tosylphenylalanylchloromethane (Tos-Phe-CH_2Cl; 'TPCK') alone reduced but did not abolish CaaX proteolysis, whereas the combination of both substances completely abrogated this activity. These results are consistent with the existence of two independently acting *C. elegans* CaaX proteases, as has been described for mammals and yeasts.

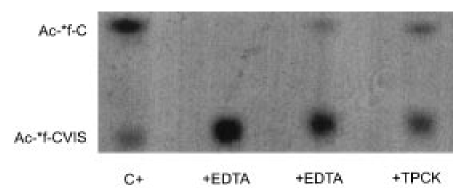

Figure 1 CaaX proteolytic activity in *C. elegans* membranes

Radioactive farnesylated peptide acetyl-[^{3}H]farnesyl-CVIS (Ac-*f-CVIS; 25 pmol) was treated with membranes (50 μg of protein) from *C. elegans* alone or in combination with the indicated protease inhibitors [50 mM EDTA or 300 μM Tos-Phe-CH_2Cl ('TPCK')]. The substrate (Ac-*f-CVIS) and the product (Ac-*f-C) of the reaction were separated by TLC.

Identification and characterization of the *C. elegans* gene encoding FACE-1

A BLAST search for orthologues of human FACE-1 encoded in the *C. elegans* genome produced a single significant match with the predicted gene C04F12.10. This gene, located in the nematode chromosome I, is predicted to encode a 442-amino-acid protein that is 41 % identical with human FACE-1. RT–PCR using mixed-stage worm total RNA as template allowed us to amplify a 1347 bp cDNA, containing the full coding sequence for this putative protease. The amino acid sequence deduced from the amplified cDNA contains the HEXXH Zn-binding motif characteristic of most families of Zn metalloproteases. When the CeFACE-1 amino acid sequence is compared with the corresponding sequences from mammals, insects, plants and yeast (Figure 2), a significant degree of conservation is observed for these distantly related evolutionary orthologues. Approximately 20 % of the residues are invariably conserved and the degree of identity between any two sequences is approx. 40 %.

Identification and characterization of the *C. elegans* gene encoding FACE-2

In contrast with the presence of a predicted FACE-1 orthologue, the *C. elegans* genome project did not uncover a gene similar to FACE-2. A TBLASTN search of the *C. elegans* genome revealed two separated, short stretches of genomic sequence with a limited degree of similarity to the human enzyme (the encoded polypeptide fragments would be 54 and 46 residues long and approx. 30 % similar to FACE-2 at the amino acid level). This region, located in the cosmid F48F5, was not predicted to contain transcribed genes by any of the available gene identification programs, and no expressed-sequence tags corresponding to this sequence were available in the public domain. To evaluate the possibility that this region encodes a FACE-2 type protein, two oligonucleotides corresponding to nucleotide sequences with the highest degree of similarity to the mammalian sequence were used for an RT–PCR experiment with RNA from mixed-stage worms. Although amplification of *C. elegans* genomic DNA with these primers should produce a 2308 bp fragment, a 221 bp cDNA fragment was obtained from the RT–PCR, demonstrating that this genomic sequence was effectively transcribed and spliced. To isolate the missing 5′-portion of the *C. elegans* FACE-2, we took

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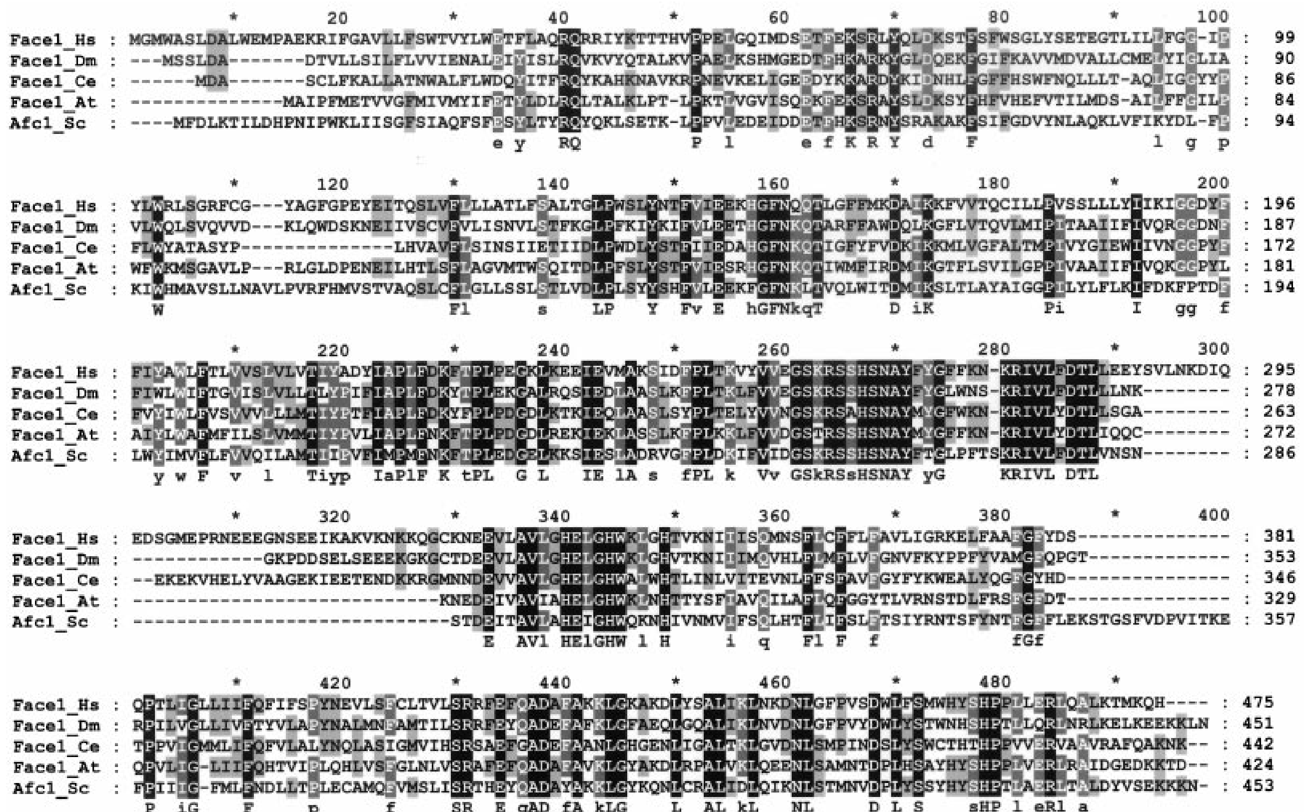

Figure 2 Sequence alignment of CeFACE-1 with related proteins from *Homo sapiens* (Hs), *D. melanogaster* (Dm), *A. thaliana* (At) and *S. cerevisiae* (Sc)

Residues identical in all the sequences analysed are indicated in black shading. Grey shading denotes residues conserved in some but not all sequences.

advantage of the trans-splicing phenomenon that adds a 22-nucleotide fragment named SL1 to the 5′-end of certain nematode transcripts [26]. Thus RT–PCR was performed with an SL1 sense primer and an antisense primer deduced from the partial CeFACE-2 cDNA. Using this approach, a 716 bp cDNA fragment was obtained, representing seven different exons contained in cosmid F48F5. Visual inspection of the 3′-flanking genomic sequence revealed a potential exon that was used for the design of an additional antisense oligonucleotide that allowed for amplification of the missing 3′-coding sequence. The complete cDNA fragment represents nine exons which are entirely within the above cosmid clone and contains an in-frame TAA stop codon.

The identified *C. elegans* cDNA encodes a 266-amino-acid protein, with a molecular mass of 30.917 kDa, and 22 % identical with human FACE-2 (Figure 3A). The closest homologue of this *C. elegans* polypeptide is *Drosophila melanogaster* Sras (46 % similarity and 25 % identity), whereas *S. cerevisiae* Rce1p shows the lowest similarity to its worm counterpart (34 % similarity, 16 % identity). Alignment of FACE-2 orthologues reveals that 27 residues are invariably conserved (Figure 3A). Remarkably, most of the conserved residues are clustered in the third-quarter of the polypeptide chains (residues 180–290 in the alignment). Several residues reported to be essential for the activity of this enzyme family [27] are present in the nematode sequence (Glu^{131}, His^{165}, His^{216} and Cys^{219}). Hydrophobic amino acid residues account for more than 40 % of the protein, and sequence analysis predicts several putative transmembrane helices, which is a characteristic feature of members of the FACE-2/Rce1 family (Figure 3B). As can be seen in Figure 3(B), despite the modest degree of sequence identity, the topologies predicted for these proteases are remarkably similar. In all likelihood, the identified cDNA is the nematode member of the FACE-2/Rce1 protease family.

Expression of CeFACE-1 and CeFACE-2

To investigate the expression of the genes encoding the putative nematode CaaX proteases CeFACE-1 and CeFACE-2, we performed a Northern-blot analysis of total RNA from mixed-stage *C. elegans* worms, using the full-length cDNAs as probes. As shown in Figure 4(A), the probes detected bands that were approx. 1.8 kb in size. These transcripts are of sufficient length to contain the complete coding information of each gene. The CeFACE-2 probe yielded an additional minor band, corresponding to a shorter mRNA species. Since a BLAST search of the *C. elegans* genome shows that it does not contain other sequences significantly similar to CeFACE-2, cross-reaction with a transcript from a different locus is unlikely, and this band probably reflects an alternative splicing or polyadenylation event.

In vivo activity of CeFACE-1 and CeFACE-2 in *S. cerevisiae*

To examine the ability of these cDNA fragments to encode proteins, high-expression *S. cerevisiae* vectors were constructed that contain the full-length coding sequence of these proteases in

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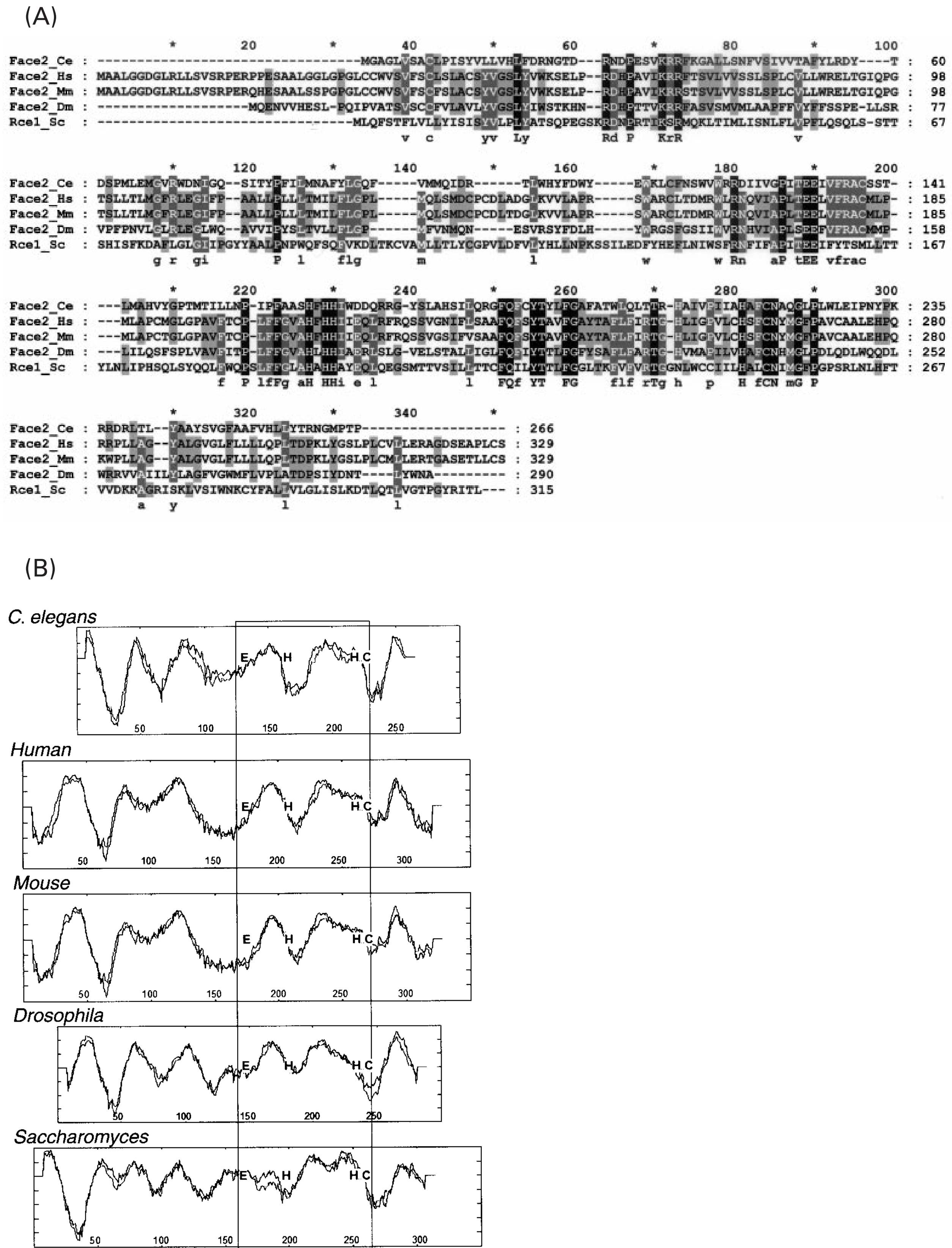

Figure 3 Structural comparison of CeFACE-2 to related proteins from *H. sapiens* (Hs), *M. musculus* (Mm), *D. melanogaster* (Dm) and *S. cerevisiae* (Sc)

(**A**) Sequence alignment. Black shading shows residues identical in all sequences analysed. Residues conserved in some but not all sequences are highlighted in grey shading. (**B**) Topology of the proteins aligned in (**A**) as predicted by TMpred [29]. The approximate positions of residues reported to be essential for the proteolytic activity of these proteases are indicated. The region with the highest level of sequence conservation is enclosed by a box.

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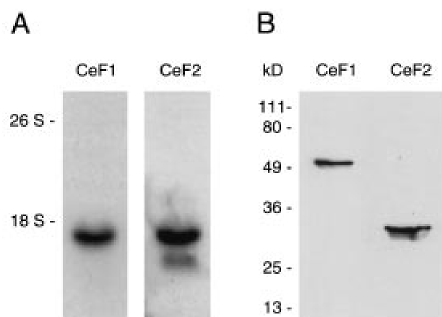

Figure 4 Expression analysis of *CeFACE-1* and *CeFACE-2*

(**A**) Northern-blot analysis of RNA (20 μg) from mixed-stage worms using probes against *CeFACE-1* and *CeFACE-2*. The positions of 26 S and 18 S *C. elegans* rRNAs are indicated. (**B**) Western-blot analysis, using an anti-HA monoclonal antibody, of membranes (15 μg of protein/lane) from yeast transformed with HA-tagged cDNAs encoding CeFACE-1 or CeFACE-2. The positions of protein standards are indicated.

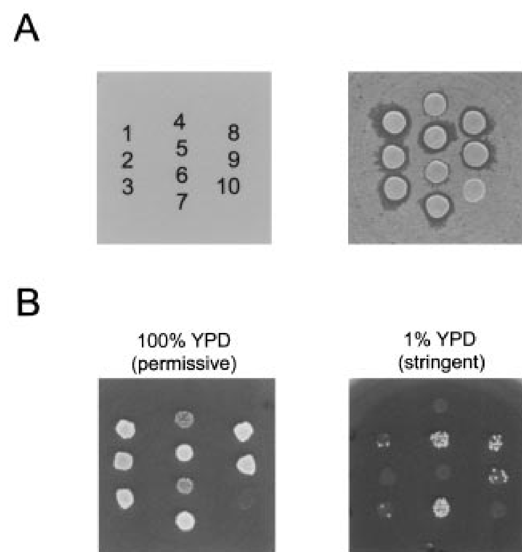

Figure 5 Complementation of the mating defect of *S. cerevisiae* lacking *RCE1* and *STE24* by CeFACE-1 and CeFACE-2

(**A**) Halo test. The indicated *MAT***a** yeast strains were spotted on a lawn of supersensitive *MAT*α *sst2* cells. Strains used are: 1, SM3041 (wild-type); 2, SM3637 (*ste24*Δ); 3, SM3643 (*rce1*Δ); 10, SM3650 (*ste24*Δ *rce1*Δ) and plasmid transformants of SM3614: yWS106 (4) and yWS108 (6) (transformed with native or HA-tagged CeFACE-2, respectively), yWS107 (5) and yWS109 (7) (transformed with native or HA-tagged CeFACE-1 respectively), and yWS110 (8) and yWS111 (9) (transformed with overexpressed CeFACE-1 and HA-tagged CeFACE-2 respectively). (**B**) Mating test. The same strains as in (**A**) were spotted on a lawn of *MAT*α *lys2* cells suspended in the indicated media before preparation of the lawn.

frame with an HA. Western-blot analysis of membrane-enriched fractions from yeast cells transformed with these plasmids demonstrated the production of proteins of approx. 50 and 30 kDa (Figure 4B), which is in excellent agreement with the molecular masses predicted for CeFACE-1 and CeFACE-2 (50.6 and 30.9 kDa respectively).

Since the cDNAs identified in the present study encode proteins with structural features characteristic of CaaX proteases of the FACE-1 and FACE-2 families, the functionality of these putative proteases was examined by testing their ability to process *in vivo* the *S. cerevisiae* **a**-factor mating pheromone. For this purpose, we transformed plasmids encoding CeFACE-1 or CeFACE-2 in a yeast strain that lacks both CaaX proteases [5], and we analysed the production of mature extracellular **a**-factor by the halo test. This assay is based on the growth arrest of a lawn of supersensitive *MAT*α *sst2* cells by extracellular **a**-factor, which results in a clear zone (halo) surrounding **a**-factor-producing cells [19,28]. When a wild-type *MAT***a** strain (SM1058) was spotted on the *MAT*α lawn, a strong halo was produced (Figure 5A). Mutant yeast strains lacking either *STE24* (SM3637) or *RCE1* (SM3643) still produced clear halos, although those produced by the former strain were smaller, which is in agreement with the greater involvement of Ste24p in **a**-factor biogenesis [5]. As expected, the double-mutant yeast (SM3650) failed to produce any detectable inhibition of the *MAT*α lawn.

Transformation of the double-mutant strain with a centromeric vector carrying the CeFACE-2 cDNA under the control of the natural yeast *RCE1* regulatory sequences (yWS106) resulted in the production of noticeable halos, which were not affected by the introduction of a HA tag in the expression construct (yWS108). Transformation of the double-mutant yeasts with an episomal plasmid containing the HA-tagged CeFACE-2 coding sequence under the strong PGK promoter (yWS111) resulted in the generation of larger halos, in accordance with the higher expression levels expected from this construct. Similarly, transformation of the mutant yeasts with a centromeric vector containing native (yWS107) or HA-tagged (yWS109) CeFACE-1 cDNAs under the natural *STE24* promoter resulted in the production of large halos, similar to those generated by the wild-type strain, which were not enhanced by the overexpression of this cDNA by the PGK promoter (Figure 5A).

We confirmed the ability of CeFACE-1 and CeFACE-2 to process yeast **a**-factor using a yeast spot mating test, in which the same *MAT***a** strains used for the halo test shown in Figure 5(A) were spotted on a lawn of *MAT*α *lys2* cells and mating was assessed by the generation of prototrophic diploids capable of growing on minimal media. As shown in Figure 5(B), both native and HA-tagged CeFACE-1 and CeFACE-2 cDNAs complemented the mating defect of the double-mutant yeast strain, paralleling the results of the halo assay. However, we did observe that the CeFACE-2 constructs only partially restored mating as compared with wild-type and single CaaX-protease-deficient strains. Under stringent mating conditions (1 % YPD), CeFACE-2 could restore mating only when overexpressed. The similarity of CeFACE-1 and CeFACE-2 to members of the FACE-1/Ste24 and FACE-2/Rce1 protease families, along with their ability to promote **a**-factor production, strongly suggests that these nematode proteins are *bona fide* CaaX proteases.

In vitro CaaX proteolytic activity of CeFACE-1 and CeFACE-2

To study the *in vitro* enzymic activity of each of the *C. elegans* proteases, membrane-enriched extracts containing either CeFACE-1 or CeFACE-2 were analysed for proteolytic activity. The extracts were prepared from yeast cells lacking the endogenous CaaX proteases (i.e. *ste24*Δ *rce1*Δ) that overexpressed CeFACE-1 or CeFACE-2. These extracts were examined for the ability to promote the cleavage of the farnesylated CVIS peptide used previously for the detection of CaaX proteolytic activity in *C. elegans* membranes. As shown in Figure 6, membranes from the double-mutant yeast cells (SM3650) did not process this prenylated tetrapeptide, whereas membranes from yeast overexpressing CeFACE-1 (yWS110) or CeFACE-2 (yWS111) cleaved

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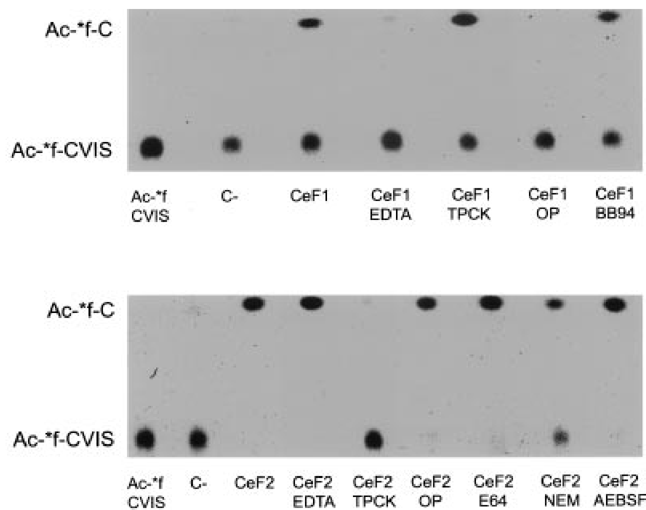

Figure 6 ***In vitro*** **CaaX proteolytic activity of CeFACE-1 and CeFACE-2**

The radioactive farnesylated peptide acetyl(Ac)-*f-CVIS (25 pmol) was treated with 5 μg of membranes derived from the yeast strains SM3650, which lacks both Ste24p and Rce1p (C-), yWS110 which expresses CeFACE-1 (CeF1), or yWS111, which expresses CeFACE2 (CeF2). Where indicated, membranes were preincubated with 50 mM EDTA, 300 μM Tos-Phe-CH_2Cl ('TPCK'), 4 mM *o*-phenantholine (OP), 0.1 μM BB94, 0.1 mM E64, 1 mM NEM or 1 mM 4-(2-aminoethyl)-benzenesulphonyl fluoride (AEBSF). The substrate (Ac-*f-CVIS) and the product (Ac-*f-C) of the reaction were separated by TLC.

the substrate efficiently. The CeFACE-1 proteolytic activity is dramatically reduced in the presence of EDTA or *o*-phenanthroline, indicating that this enzyme is a metalloprotease, as predicted from its primary structure. BB94, a specific inhibitor of matrix metalloproteases, did not exert any effect on CeFACE-1. CeFACE-2 was inhibited by Tos-Phe-CH_2Cl and, to a lesser extent, by the thiol-modifying compound *N*-ethylmaleimide (NEM), which would be compatible with this enzyme being a cysteine protease, as proposed previously for yeast Rce1p [27].

DISCUSSION

The *C. elegans* genome encodes more than 300 proteins containing the CaaX motif, including Ras and Rho proteins, γ subunits of heterotrimeric G-proteins, a B-type lamin, DnaJ homologues and a large number of hypothetical proteins. Since isoprenylation of nematode proteins has been shown previously [22], we hypothesized that a CaaX proteolytic activity existed in this organism. A direct radiometric assay using a farnesylated tetrapeptide allowed us to demonstrate the presence of at least two distinct CaaX proteolytic activities in *C. elegans* membranes. Two distinct CaaX proteolytic activities have been described in eukaryotic systems that have been attributed to the FACE-1/Ste24 and FACE-2/Rce1 protein families. A FACE-1/Ste24 homologue has been previously predicted in the worm genome, but it has not been functionally characterized. This gene, located in chromosome I, encodes a putative metalloprotease having multiple membrane spans that is highly similar in primary amino acid sequence and predicted topology to its mammalian, plant and yeast counterparts. Before the present study, *C. elegans* databases lacked an annotated FACE-2 protein. However, a careful search of the *C. elegans* genome allowed the identification of two short regions derived from chromosome V having limited similarity to the mammalian sequence. An RT–PCR approach demonstrated that this genomic sequence was transcribed and spliced and allowed us to identify the full-length cDNA, which encodes a 30.9 kDa protein whose amino acid sequence contains all the residues reported previously to be essential for the activity of FACE-2 proteases [27].

The FACE-2 sequences from different species display a modest degree of sequence similarity. Most of the invariably conserved residues and several residues demonstrated to be essential for the activity of these enzymes are located in a region corresponding approximately to the third quarter of the polypeptide chain [27]. Despite the low level of sequence conservation, all FACE-2 proteins present a very similar topology, with up to seven transmembrane helices predicted at equivalent positions (Figure 3B).

The cloned nematode cDNAs encode functional CaaX proteases, as determined by heterologous expression of the cloned cDNA in yeast (Figure 4). Using established biological assays for the production of the prenylated **a**-factor mating pheromone, we found that either CeFACE-1 or CeFACE-2 was sufficient to promote **a**-factor processing. In these studies, the only source of CaaX protease activity was provided by heterologous CeFACE-1 and CeFACE-2, since the yeast copies of these genes have been deleted. CeFACE-1 restored pheromone production very efficiently when expressed from the yeast *STE24* promoter on a low-copy plasmid. Similar findings have been reported for FACE-1/Ste24 from human and mouse [7,13,24]. The ability of FACE-1 homologues to perform these activities faithfully has been suggested to indicate that **a**-factor-like molecules may exist in systems outside yeast, but such molecules are yet to be described. In contrast, CeFACE-2 restored **a**-factor production less efficiently. The weaker complementation by CeFACE-2 is most likely the result of poor CeFACE-2 expression in yeast (A. Lapham and N. K. Schmidt, unpublished work). Consistent with this hypothesis, we find that the expression of CeFACE-2 from a strong constitutive promoter yields increased **a**-factor production.

Both nematode CaaX proteases are able to cleave a farnesylated peptide with the sequence Cys-Val-Ile-Ser, which is designed on the basis of the C-terminus of mammalian transducin [16] and is present in at least three *C. elegans* proteins (T05H10.8, C16C4.12 and F46A9.1). CeFACE-1-mediated cleavage of this substrate is inhibited by metal-chelating agents (EDTA and *o*-phenanthroline), indicating that this enzyme is a metalloprotease, like other members of the FACE-1/Ste24 family. CeFACE-2 activity is resistant to metal chelators and sensitive to Tos-Phe-CH_2Cl and NEM, which would be compatible with this enzyme being a cysteine protease, as previously proposed for yeast Rce1p [27]. Additional studies will be required to obtain a definitive conclusion on this issue, since it has been suggested that the proteins of this family display structural features characteristic of metalloproteases [8,29].

Whether FACE-1 or FACE-2 are redundant or have partially overlapping functions in *C. elegans* still needs to be addressed. This question is particularly intriguing for FACE-1, since this enzyme is present in all eukaryotes but the only documented substrates are **a**-factor in *S. cerevisiae* [3–5] and prelamin A in mammalian cells [14]. Since **a**-factor homologues have not been identified in higher eukaryotes and *C. elegans* lamin is more closely related to B-type lamins than to lamin A, it seems probably that metalloproteases of the FACE-1 family have unreported roles. The availability of the reagents generated in the present study will be useful for further studies directed to clarify the role of CaaX proteases in this and other model organisms.

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REFERENCES

1 Zhang, F. L. and Casey, P. J. (1996) Protein prenylation: molecular mechanisms and functional consequences. Annu. Rev. Biochem. **65**, 241–269
2 Cox, A. D. and Der, C. J. (2002) Farnesyltransferase inhibitors: promises and realities. Curr. Opin. Pharmacol. **2**, 388–393
3 Boyartchuk, V. L., Ashby, M. N. and Rine, J. (1997) Modulation of Ras and a-factor function by carboxyl-terminal proteolysis. Science **275**, 1796–1800
4 Fujimura-Kamada, K., Nouvet, F. J. and Michaelis, S. (1997) A novel membrane-associated metalloprotease, Ste24p, is required for the first step of NH_2-terminal processing of the yeast a-factor precursor. J. Cell Biol. **136**, 271–285
5 Tam, A., Nouvet, F. J., Fujimura-Kamada, K., Slunt, H., Sisodia, S. S. and Michaelis, S. (1998) Dual roles for Ste24p in yeast a-factor maturation: NH_2-terminal proteolysis and COOH-terminal CAAX processing. J. Cell Biol. **142**, 635–649
6 Schmidt, W. K., Tam, A. and Michaelis, S. (2000) Reconstitution of the Ste24p-dependent N-terminal proteolytic step in yeast a-factor biogenesis. J. Biol. Chem. **275**, 6227–6233
7 Tam, A., Schmidt, W. K. and Michaelis, S. (2001) The multispanning membrane protein Ste24p catalyzes CAAX proteolysis and NH_2-terminal processing of the yeast a-factor precursor. J. Biol. Chem. **276**, 46798–46806
8 Freije, J. P., Blay, P., Pendás, A. M., Cadiñanos, J., Crespo, P. and López-Otín, C. (1999) Identification and chromosomal location of two human genes encoding enzymes potentially involved in proteolytic maturation of farnesylated proteins. Genomics **58**, 270–280
9 Kumagai, H., Kawamura, Y., Yanagisawa, K. and Komano, H. (1999) Identification of a human cDNA encoding a novel protein structurally related to the yeast membrane-associated metalloprotease, Ste24p. Biochim. Biophys. Acta **1426**, 468–474
10 Otto, J. C., Kim, E., Young, S. G. and Casey, P. J. (1999) Cloning and characterization of a mammalian prenyl protein-specific protease. J. Biol. Chem. **274**, 8379–8382
11 Kim, E., Ambroziak, P., Otto, J. C., Taylor, B., Ashby, M., Shannon, K., Casey, P. J. and Young, S. G. (1999) Disruption of the mouse Rce1 gene results in defective Ras processing and mislocalization of Ras within cells. J. Biol. Chem. **274**, 8383–8390
12 Bergo, M. O., Ambroziak, P., Gregory, C., George, A., Otto, J. C., Kim, E., Nagase, H., Casey, P. J., Balmain, A. and Young, S. G. (2002) Absence of the CAAX endoprotease Rce1: effects on cell growth and transformation. Mol. Cell. Biol. **22**, 171–181
13 Leung, G. K., Schmidt, W. K., Bergo, M. O., Gavino, B., Wong, D. H., Tam, A., Ashby, M. N., Michaelis, S. and Young, S. G. (2001) Biochemical studies of Zmpste24-deficient mice. J. Biol. Chem. **276**, 29051–29058
14 Pendás, A. M., Zhou, Z., Cadiñanos, J., Freije, J. P., Wang, J., Hultenby, K., Astudillo, A., Wernerson, A., Rodríguez, F., Tryggvason, K. et al. (2002) Defective prelamin A processing and muscular and adipocyte alterations in Zmpste24 metalloproteinase-deficient mice. Nat. Genet. **31**, 94–99
15 Aspbury, R. A., Prescott, M. C., Fisher, M. J. and Rees, H. H. (1998) Isoprenylation of polypeptides in the nematode *Caenorhabditis elegans*. Biochim. Biophys. Acta **1392**, 265–275
16 Georgopapadakou, N. H., Hall, C. C., Lambros, T., Liu, W. and Watkins, J. D. (1994) A radiometric assay for Ras-processing peptidase using an enzymatically radiolabeled peptide. Anal. Biochem. **218**, 273–277
17 Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. and Higgins, D. G. (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic. Acids Res. **25**, 4876–4882
18 Hofmann, K. and Stoffel, W. (1993) TMbase – a database of membrane spanning proteins segments. Biol. Chem. Hoppe-Seyler **374**, 166–171
19 Michaelis, S. and Herskowitz, I. (1988) The a-factor pheromone of *Saccharomyces cerevisiae* is essential for mating. Mol. Cell. Biol. **8**, 1309–1318
20 Powers, S., Michaelis, S., Broek, D., Santa Anna, S., Field, J., Herskowitz, I. and Wigler, M. (1986) RAM, a gene of yeast required for a functional modification of RAS proteins and for production of mating pheromone a-factor. Cell (Cambridge, Mass.) **47**, 413–422
21 Elble, R. (1992) A simple and efficient procedure for transformation of yeasts. Biotechniques **13**, 18–20
22 Sikorski, R. S. and Hieter, P. (1989) A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics **122**, 19–27
23 Zhang, Y., Nijbroek, G., Sullivan, M. L., McCracken, A. A., Watkins, S. C., Michaelis, S. and Brodsky, J. L. (2001) Hsp70 molecular chaperone facilitates endoplasmic reticulum-associated protein degradation of cystic fibrosis transmembrane conductance regulator in yeast. Mol. Biol. Cell **12**, 1303–1314
24 Schmidt, W. K., Tam, A., Fujimura-Kamada, K. and Michaelis, S. (1998) Endoplasmic reticulum membrane localization of Rce1p and Ste24p, yeast proteases involved in carboxyl-terminal CAAX protein processing and amino-terminal a-factor cleavage. Proc. Natl. Acad. Sci. U.S.A. **95**, 11175–11180
25 Oldenburg, K. R., Vo, K. T., Michaelis, S. and Paddon, C. (1997) Recombination-mediated PCR-directed plasmid construction *in vivo* in yeast. Nucleic Acids Res. **25**, 451–452
26 Blumenthal, T. (1995) Trans-splicing and polycistronic transcription in *Caenorhabditis elegans*. Trends Genet. **11**, 132–136
27 Dolence, J. M., Steward, L. E., Dolence, E. K., Wong, D. H. and Poulter, C. D. (2000) Studies with recombinant *Saccharomyces cerevisiae* CaaX prenyl protease Rce1p. Biochemistry **39**, 4096–4104
28 Nijbroek, G. L. and Michaelis, S. (1998) Functional assays for analysis of yeast ste6 mutants. Methods Enzymol. **292**, 193–212
29 Pei, J. and Grishin, N. V. (2001) Type II CAAX prenyl endopeptidases belong to a novel superfamily of putative membrane-bound metalloproteases. Trends Biochem. Sci. **26**, 275–277

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