CAAX proteins have essential roles in multiple signalling pathways, controlling processes such as proliferation, differentiation and carcinogenesis. The ~120 mammalian CAAX proteins function at cellular membranes and include the Ras superfamily of small GTPases, nuclear lamins, the γ-subunit of heterotrimeric GTPases, and several protein kinases and phosphatases. The proper localization of CAAX proteins to cell membranes is orchestrated by a series of post-translational modifications of the carboxy-terminal CAAX motifs (where C is cysteine, A is an aliphatic amino acid and X is any amino acid). These reactions involve prenylation of the cysteine residue, cleavage at the AAX tripeptide and methylation of the carboxyl-prenylated cysteine residue. The major CAAX protease activity is mediated by Rce1 (Ras and a-factor converting enzyme 1), an intramembrane protease (IMP) of the endoplasmic reticulum.

Information on the architectural and proteolytic mechanism of Rce1 has been lacking. Here we report the crystal structure of a Methanococcus maripaludis homologue of Rce1, whose endopeptidase specificity for farnesylated peptides mimics that of eukaryotic Rce1. Its structure, comprising eight transmembrane α-helices, and catalytic site are distinct from those of other IMPS. The catalytic residues are positioned on TM4, TM5 and TM7 (Fig. 3a, c). Seven transmembrane helices form an antiparallel helix bundle that has approximately two-fold symmetry and is approximately 35 Å in length, 26 Å in width and 46 Å in height, allowing it to be embedded in the phospholipid membrane (Fig. 2c). Except for two peripheral membrane α-helices (αA and αB) (Fig. 2a, b), Rce1 is topologically distinct from other IMPS and, to our knowledge, represents a novel protein fold. The molecule is approximately 35 Å in length, 26 Å in width and 46 Å in height, allowing it to be embedded in the phospholipid membrane (Fig. 2c). Except for αA and αB, which interconnect TM2 with TM3 and TM7 with TM8, respectively, short loops link the transmembrane helices.

The ~100-residue ABI domain corresponds to TM4, TM5, TM6 and TM7 (Fig. 3a, c). These four transmembrane helices form an antiparallel helix bundle that has approximately two-fold symmetry and is surrounded by the less conserved helices TM1, TM2, TM3 and TM8 (Extended Data Fig. 3). The three conserved motifs of the ABI domain are positioned on TM4, TM5 and TM7 (Fig. 3a, c). Seven transmembrane helices delineate a large conical catalytic cavity (volume 1,400 Å³) (Fig. 2a, c and Fig. 3a). Rce1 inactivation has been also been shown in mice to result in the mislocalization of Ras proteins from the plasma membrane. The consequent disruption of Ras signalling inhibited the Ras-induced formation of fibroblasts but accelerated progression of K-Ras-induced myeloproliferative disease.

In addition, Rce1-deficient mice have been found to develop lethal dilated cardiomyopathy, and Rce1 is also essential for the survival of photoreceptor cells in mice.

To understand the structure and catalytic mechanism of Rce1, we examined the expression and solubility properties of ~30 Rce1 homologues (including human, yeast and prokaryotic homologues) using fluorescence-detection size-exclusion chromatography (FSEC) and differential scanning fluorimetry (data not shown). Rce1 from the archaea Methanococcus maripaludis (MmRce1) was identified as a suitable candidate for structural studies, and the full-length protein (276 residues, 15% sequence identity with human RCE1) was crystallized in complex with a conformation-sensitive monoclonal antibody Fab fragment (Extended Data Fig. 1). The structure of MmRce1–Fab was determined by molecular replacement using the Fab fragment as a search model, and the complex was refined to 2.5 Å resolution (Extended Data Table 1).

A fluorescence-based protease assay showed that MmRce1 hydrolyses a farnesylated peptide modelled on the C terminus of human RhoA (Fig. 1a, b and Extended Data Fig. 2). Similar to its eukaryotic orthologues, peptide hydrolysis depends on a farnesylated cysteine residue; however, in contrast to human RCE1 (ref. 21), MmRce1 did not proteolytically cleave geranylgeranylated peptides (data not shown).

Eukaryotic Rce1 is an endopeptidase that specifically cleaves immediately C-terminally to the farnesyl cysteine (at position 1 (P1)) mass spectrometry analysis indicated that MmRce1 is also an endopeptidase, although with a slightly relaxed specificity, cleaving the CAAX motif C-terminal to both P1 and P1′ (Fig. 1b, c). MmRce1 is inhibited by N-acetyl-S-farnesyl-L-cysteine (AFC), the minimal analogue of farnesylated peptides (Fig. 1a). The ability of MmRce1 to cleave specifically farnesylated peptides validates it as a model for understanding the mechanism of CAAX processing by eukaryotic Rce1.

The structure of MmRce1 comprises eight conserved transmembrane α-helices (TM1–8), with two peripheral membrane α-helices (αA and αB) (Fig. 2a, b). Rce1 is a type II CAAX prenyl endopeptidase that was first identified in Saccharomyces cerevisiae together with the type I CAAX-processing enzyme, Ste24p (also known as ZMPSTE24 or Afc1p). This proteolyzing enzyme is a zinc metalloproteinase with a specific role in the processing of prelamin A in all eukaryotes and a-factor in yeast. Rce1, by contrast, has a much wider specificity, processing all farnesylated and geranylgeranylated CAAX proteins. However, extensive sequence and biochemical analyses have been unable to classify Rce1 within the three conventional IMP families. These are the rhomboids, the intramembrane metalloproteinases (which include S2P) and the aspartyl proteases (which include presenilin and SPP). The membrane proteases ZMPSTE24 (refs 10, 11) and FlaK12 have their catalytic sites at the membrane interface. Rce1 belongs to the ABI (abortive infection) family of putative IMPS and has homologues in all three domains of life. The ABI family is defined by three conserved motifs (Extended Data Fig. 2) that constitute the catalytic site of the ABI proteases and whose importance has been demonstrated by mutational analysis of yeast Rce1p (15, 16). Rce1 inactivation has also been shown in mice to result in the mislocalization of Ras proteins from the plasma membrane. The consequent disruption of Ras signalling inhibited the Ras-induced formation of fibroblasts but accelerated progression of K-Ras-induced myeloproliferative disease.

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access to the catalytic site from the cytoplasm at the base of the cavity. One side of the conical cavity is open to the membrane through a gap between TM2 and TM4. Access to the periplasm (the endoplasmic reticulum lumen) is blocked by the conserved residue R145, which interacts with T210 and the invariant E141 (Fig. 2a, b).

To understand the relevance of the MmRce1 structure in the context of the MmRce1–Fab complex, we assayed MmRce1 in the presence of the Fab. The formation of the MmRce1–Fab complex had no effect on the catalytic activity of MmRce1 (Fig. 1a and Extended Data Fig. 1), even though MmRce1 and the Fab form extensive contacts (1,100 Å²) that would prevent conformational changes in the seven transmembrane helices that delineate the catalytic cavity. Thus, in the crystal structure of MmRce1, the catalytic residues are correctly aligned for cleaving a farnesylated peptide, although it is possible that conformational changes are required to accommodate larger, prenylated protein substrates. Both

Figure 1 | MmRce1 is an endoprotease specific for farnesylated peptides. (a) The proteolytic activity of wild-type MmRce1 (Rce1; set to 100%) compared with the MmRce1–Fab complex (+ Fab), MmRce1 incubated with a molar excess of AFC (+ AFC) and MmRce1 incubated with a non-farnesylated peptide (+ Non-F peptide), as determined by fluorescence resonance energy transfer (FRET) assay. The data are presented as mean ± s.d. for n = 3 experiments. (b) A schematic representation of the RhoA-derived farnesylated peptide. The two cleavage sites identified by mass spectrometry are marked with red arrows. (c) Semi-quantitative mass spectrometry data for the uncleaved and truncated farnesylated peptides (where, for example, P10/P9 denotes cleavage between P9 and P10 after incubation with MmRce1). Many of the truncated forms were also present in the no enzyme control sample (− Enzyme). These might be by-products of peptide synthesis that are isobaric with truncations corresponding to positions P2/P3 and P6/P5 to P10/P9. Only the P1/P2 and P1/P1 truncations (ARSGAKASGCLVS) and ARSGAKASGCLVS are found in the + Enzyme sample. The data are presented as the mean ± 2 s.d. from n = 4 experiments.

Figure 2 | MmRce1 is an IMP with eight transmembrane α-helices. (a, b) Two views showing ribbon representations of MmRce1 (molecule C from the asymmetric unit). The side chains of the five invariant ABI domain residues (and the conserved R145 and T210) are shown as ball-and-stick representations (with oxygen in red and nitrogen in blue). The catalytic water (W) molecule is shown as a red sphere. Hydrogen bonds are shown as red dashed lines. (c) View of the molecule parallel to the membrane. The molecular surface of MmRce1, colour-coded by electrostatic potential ranging from blue (most positive) to red (most negative) to white (uncharged). The lipid membrane (−30 Å) is indicated by the grey background, based on the distribution of nonpolar residues and transmembrane helices. The E140 side chain and the catalytic water molecule are shown. The catalytic water molecule is located −10 Å into the membrane.

Rce1 has no sequence similarity to other proteases. It therefore represents a novel protease, and interestingly it has no paralogues in eukaryotes. Both cysteine-enzyme-based and metalloenzyme-based catalytic mechanisms have been proposed for Rce1 (refs 13, 15). However, the bacterial rhomboid GlpG24–27 and S2P8 have been proposed to undergo conformational shifts to mediate substrate gating, and structural changes in presenilin8 and Flak7 are necessary to align catalytic site residues.
absence of an evolutionarily conserved cysteine residue (Fig. 3c and Extended Data Fig. 3) precludes a thiol-based mechanism. Strong evidence also indicates that Rce1 is not a metalloenzyme. MmRce1 activity is unaffected by EDTA or Zn\textsuperscript{2+} (Extended Data Fig. 4a). Its concentration-dependent inactivation by L,10-phenanthroline, a hydrophobic metal chelator, results from nonspecific protein unfolding (Extended Data Fig. 4). Furthermore, we did not detect Zn\textsuperscript{2+} ions bound to MmRce1 by using proton-induced X-ray emission (PIXE) or total reflection X-ray fluorescence (TXRF) (data not shown), and no Zn\textsuperscript{2+} ions were identified in the MmRce1 crystal structure.

All five conserved residues of the ABI domain (Fig. 3c) have been implicated in catalysis.\textsuperscript{13,16} Three residues of S. cerevisiae Rce1p—E156, H194 and H248 (equivalent to E140, H173 and H227 of MmRce1)—are critical for catalysis, whereas mutation of either E157 or N252 (E141 and N231 of MmRce1) impairs catalytic activity\textsuperscript{15,16} (see Fig. 3c). We confirmed that MmRce1 catalytic activity is strictly dependent on the absence of an evolutionarily conserved cysteine residue (Fig. 3c and Extended Data Fig. 3). Furthermore, we did not detect Zn\textsuperscript{2+} ions bound to MmRce1 by using proton-induced X-ray emission (PIXE) or total reflection X-ray fluorescence (TXRF) (data not shown), and no Zn\textsuperscript{2+} ions were identified in the MmRce1 crystal structure.

Although Rce1 belongs to a novel protease family, the proposed catalytic mechanism has similarities to that of other proteases.
in its invariant HX 3N motif, which is located on successive turns GlpG, in which the proposed oxyanion hole is formed by H150 and and Extended Data Fig. 7). A striking similarity exists with the protease suggests a similar role for asparagine and/or histidine residues (Fig. 4a Our inspection of the ZMPSTE24 and Ste24p catalytic sites (refs 10, 11) molecule for cleavage of the scissile bond (Fig. 4a and Extended Data Data Fig. 7). Thus MmRce1, analogous to metalloproteinases, aspartyl in the serine–histidine catalytic dyad in GlpG (Fig. 4b and Extended conformations of MmRce1 (cyan) and GlpG (magenta; PDB ID 2O7L).27. The catalytic water molecule from MmRce1 is located in a similar position to the catalytic serine (S201) hydroxyl in GlpG. Hydrogen bonds are shown as dashed lines. c. Cartoon representation of MmRce1 with a farnesylated peptide (GAKASGC(farnesyl)LVS) modelled at its catalytic site. The ABI domain helices are shown in blue, and the conserved TM4 fragment (L132 to T138) is shown in dark pink. The nonpolar residues in TM2 and TM4 that flank the farnesyl lipid are labelled. d. Detailed view of the catalytic site of MmRce1 shown in c. The catalytic residues (E140 and H173), the catalytic water molecule and the oxyanion residues (H227 and N231) are shown. Hydrogen bonds are shown as dashed yellow lines.

Data Fig. 7). The membrane-associated zinc metalloproteinases S2P, ZMPSTE24 and Ste24p (refs 10, 11) use a glutamate-activated water molecule for cleavage of the scissile bond (Fig. 4a and Extended Data Fig. 7), as does the recently described fungal glutamic peptide SGP. In S2P, an asparagine residue is proposed to stabilize the oxyanion. Our inspection of the ZMPSTE24 and Ste24p catalytic sites (refs 10, 11) suggests a similar role for asparagine and/or histidine residues (Fig. 4a and Extended Data Fig. 7). A striking similarity exists with the protease GlpG, in which the proposed oxyanion hole is formed by H150 and N154 in its invariant HX 3N motif, which is located on successive turns of a transmembrane helix, analogous to the proposed oxyanion hole of H227 and N231 in MmRce1. Superimposing H227 and N231 onto their equivalent residues in the HX 3N motif of GlpG reveals that the position of the proposed glutamate-activated nucleophilic water of MmRce1 exactly matches that of the nucleophilic hydroxyl group of the S201 side chain of GlpG (Fig. 4b). In support of the idea that H173 of MmRce1 activates the nucleophilic water for attack on the peptide substrate, the imidazole side chain of H173 superimposes with H254, in the serine–histidine catalytic dyad in GlpG (Fig. 4b and Extended Data Fig. 7). Thus MmRce1, analogous to metalloproteinases, aspartyl proteases and SGP, uses a carboxylate to activate a nucleophilic water molecule; however, both MmRce1 and SGP are unlike other proteases in that they do not polarize the carbonyl group of the scissile peptide.

To understand how MmRce1 recognizes its substrates, we modelled a farnesylated peptide, based on the CAAX motif of RhoA, at the catalytic site of Rce1. The peptide adopts a β-hairpin conformation with the scissile bond positioned adjacent to the putative nucleophilic water molecule (Fig. 4c, d). The model is consistent with the proposed catalytic mechanism and suggests that the farnesyl lipid enters MmRce1’s catalytic site from the membrane, sealing the opening between the nonpolar faces of TM2 and TM4. The interaction of the farnesyl lipid at this site would position the isoprenyl cysteine relative to the catalytic water molecule, contributing to defining the CAAX motif cleavage site. There is sufficient space to accommodate the aliphatic residues of the CAAX motif adjacent to TM5, TM6 and TM7, whereas the six residues aminoterminal to the CAAX motif and the C-terminal X residue would occupy the solvent-filled conical cavity, which leads into the cytoplasm. The large volume of the cavity, which is capable of accommodating diverse residues, is consistent with the variation in the sequence of Rce1 substrates N-terminal to the CAAX motif. Mutations of TM5, which
might alter the size of the AAX-motif binding pocket, modify the specificity of yeast Rce1p for CAAX\textsuperscript{TM}. In support of the model that the opening between TM2 and TM4 creates the farnesyl lipid-binding site, substitution of either L132 of TM4 with a bulky geranylgeranylated peptide, we generated a model of human RCE1 (Extended Data Fig. 8). Compared with MmRce1, the model predicts a longer TM4 helix that might provide a more extensive hydrophobic surface to promote favourable interactions with the C20 prenyl chain of a geranylgeranyl lipid.

This study establishes Rce1 as a founding member of a novel family of glutamate-dependent IMPs. Insights into the structure and mechanism of Rce1 have implications for the development of antagonists of CAAX motif processing, which have the potential to disrupt Ras signalling pathways.

**METHODS SUMMARY**

MmRce1 was expressed in *Escherichia coli* and purified to homogeneity by affinity chromatography and gel filtration. BALB/c mice were immunized with reconstituted MmRce1 proteoliposomes, and antibodies were raised to recognize conformational epitopes of the protein. MmRce1 was mixed with the Fab fragment Fab645-2, and the complex was crystallized as described in Methods. Diffraction data were collected on beamline I04-1 at the Diamond Light Source (UK). The structure was solved by molecular replacement, using an antibody Fab fragment structure (Protein Data Bank (PDB) ID 3V9g) as the search model. The data collection and refinement statistics are summarized in Extended Data Table 1. A fluorescence resonance energy transfer (FRET) assay was used to demonstrate the proteolytic activity of MmRce1 towards the C-terminal CAAX cleavage site of a farnesylated peptide modelled on human RhoA. MmRce1 was shown to be an endoprotease by mass spectrometry, and a farnesylated peptide was modelled into the catalytic site of the protein. The human RCE1 homologue was modelled in its apo state and as a complex with a geranylgeranylated peptide.

**Online Content** Any additional Methods, Extended Data display items and Source Data are available in the online version of the paper; references unique to these sections appear only in the online paper.

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METHODS

Cloning. Homologues of human RCE1 were identified based on their membership of the InterPro database CAAX N-terminal protease family (InterPro ID IPR003675), members of which contain the conserved ABI domain. Thirty open reading frames were PCR-amplified from bacterial and archaeal strains obtained from DSMZ and cloned using the In-Fusion method (Clontech) into a pTriEx-derived vector encoding a tobacco etch virus (TEV)-protease-cleavable C-terminal green fluorescent protein (GFP)-His: tag (pOPIP-GFP).

Expression and homologue screening. Constructs were screened for expression levels while varying four parameters: (1) isopropyl-β-D-thiogalactopyranoside (IPTG) concentration, 100, 400 or 1,000 μM; (2) induction temperature, 20, 25 or 30 °C; (3) induction duration, 4 or 16 h; and (4) E. coli expression strain, BL21(DE3), C41(DE3) or C43(DE3). Expression levels were quantified by measuring in-cell fluorescence in a Caryen fluorimeter fitted with a plate reader. The expression of full-length constructs was assayed by separating samples on SDS–PAGE gels and performing in-gel fluorescence imaging.

The best-expressed Rce1 homologues were further tested with a detergent screen for optimization of both solubilization efficiency and sample monodispersity. A range of detergents with differing properties was assayed (OM, DM, UDM, DDM, TMAO, TMAO-9, TMAO-10, DDM, and ONG-3; all from Anatrace), and the solubilization efficiency was measured by quantifying the GFP signal of samples following solubilization and pelleting of insoluble material. Sample monodispersity was tested using fluorescence-detection size-exclusion chromatography (FSEC) on a Superdex 200 10/300 GL column coupled with an off-line fluorometer to follow the elution profiles of the GFP-fused membrane proteins. Taken together, these methods identified that M. maripaludis S2 Rce1 (MmRce1) was the best expressed protein and that it could be efficiently solubilized in 1% (w/v) n-undecyl-β-D-maltopyranoside (UDM).

Expression and purification of MmRce1 and mutants. MmRce1–GFP–His: protein was expressed in E. coli strain C41(DE3). Cultures were grown in L-Broth medium at 30 °C until the optical density (A600) reached 0.5 and were then induced with 1 mM IPTG and left shaking overnight at 20 °C. Cells were collected by centrifugation at 4,000g (30 min at 4 °C) and stored at −80 °C. The harvested cell pellet was resuspended in lysis buffer (15 ml buffer per 1 l of cell culture) (50 mM Tris–HCl, pH 8.0, 200 mM NaCl, 50 μg ml−1 lysozyme, 20 μg ml−1 DNase I and two protease inhibitor cocktail tablets (Roche) per 60 ml lysate). The mixture was passed three times through an EmulsiFlex homogenizer (Avestin) at 15,000 p.s.i. and centrifuged at 12,000g (15 min at 4 °C) to remove cell debris. The resultant supernatant was centrifuged at 100,000g (50 min at 4 °C) to remove the non-solubilized membrane fraction.

The protein was bound to Ni-nitrilotriacetic acid (NTA) Superflow resin (Qiagen) in Buffer A (50 mM Tris–HCl, pH 8.0, 150 mM NaCl and 0.025% (v/v) UDM), washed in Buffer A plus 50 mM imidazole and finally eluted from the column in Buffer A plus 200 mM imidazole. The C-terminal GFP:His tag was cleaved by TEV protease during overnight dialysis at 4 °C, and GFP–His and His–tagged TEV protease were removed by passing through a second Ni-NTA Superflow column. MmRce1 was further purified by size-exclusion chromatography using a Superdex 200 10/300 GL size-exclusion column (GE Healthcare) in Buffer B (20 mM MES, pH 6.5, 200 mM NaCl, 0.025% (v/v) UDM and 0.86% (v/v) n-octyl-β-D-glucopyranoside (OG)). OG was added to Buffer B to reduce the total micelle size and improve protein monodispersity, as assayed using multiangle light scattering (data not shown). The MmRce1 fractions collected were analysed by SDS–PAGE. The protein was stored at 4 °C for up to 2 days or flash-frozen and kept at −80 °C for longer periods.

Antibody generation. All animal experiments described here were approved by the Institutional Animal Care and Use Committee of Kyoto University Graduate School of Medicine.

To raise antibodies against conformational epitopes of MmRce1, 4-week-old female BALB/c mice were immunized with 0.1 mg recombinant MmRce1 proteoliposomes four times, at 10-day intervals. Two days after the last injection, the mouse spleens were removed, and the splenocytes were fused with mouse myeloma P3U1 cells using polyethylene glycol (PEG) 11.

To screen for antibodies that specifically recognized native receptors (and to determine those that recognized flexible loops, N and C termini and unstructured regions of MmRce1), a denatured liposome enzyme-linked immunosorbent assay (ELISA) method was used, as described previously 12. Candidate clones producing conformational antibodies against MmRce1 were screened by small-scale SEC, and the clones that became established were isolated by limiting dilution to produce monoclonal hybridoma cell lines. The binding affinities of the established clones for MmRce1 were measured using a BiaCore T100 system (GE Healthcare) 12.

For large-scale antibody production, the hybridomas were transplanted into BALB/c mice. IgG was collected from mouse ascites by 40% (w/v) ammonium sulfate precipitation and purified by Protein G Sepharose Fast Flow chromatography (GE Healthcare). The Fab645-2 fragment was obtained from IgG using a Fab Preparation Kit (Pierce).

Purification of the MmRce1–Fab complex. Purified MmRce1 and Fab645-2 were mixed at a molar ratio of 1:1.5 and were incubated on ice for 30 min before size-exclusion chromatography. The complex eluted on a Superdex 200 10/300 GL size-exclusion column (GE Healthcare) equilibrated with Buffer B, and the collected fractions were analysed by SDS–PAGE.

Crystallization of the MmRce1–Fab complex. The MmRce1–Fab645-2 complex was concentrated up to 8 mg ml−1 using Vivaspin 50 kDa cut-off centrifugal concentrators (Sartorius Stedim Biotech). The first crystallization trials were carried out by using the sitting-drop vapour–diffusion method with 96-well plates (Greiner) at 20 °C, screening against the commercial screens MemGold, MemStart, MemSys and MemPlus (Molecular Dimensions). Crystals were obtained under various conditions, but further crystallization screening and optimization identified one set of crystallization conditions as the most promising in terms of crystal quality and productivity (MemPlus Fl: 12.5 mM 3-N-(methylphosphino) propanesulfonic acid (MOPS), pH 7.0, 350 mM NaCl and 28% (v/v) PEG 1000). The final crystallization conditions were 12.5 mM MOPS, pH 7.0, 350 mM NaCl and 30% (v/v) PEG 400. Well-diffacting crystals were obtained in 24-well plate hanging drops by vapour diffusion at 20 °C; they grew to maximum dimensions in 3 weeks and were directly flash-frozen and stored in liquid nitrogen.

Data collection and processing. Diffraction data were collected from a single cryo-cooled crystal on beamline I04-1 at the Diamond Light Source (UK). Two identical data sets were collected on the same crystal with a single 3° oscillation difference of 45° between them. Two thousand images were collected for each data set, with an oscillation range of 0.1° per image, to a maximum resolution of 2.5 Å. Both data sets were indexed with XDS 11. The data sets could be indexed equally well in P1 with two different unit cell parameters (a = 72.9 Å, b = 72.9 Å, c = 101.7 Å, β = 79.4°, γ = 76.2° and a = 113.0 Å, b = 90.0 Å, c = 99.3 Å, β = 89.0°, γ = 102.3°, γ = 90.0°). Further data analysis with XTRIAGE 13 confirmed pseudotranslational with NCS vector 0.5, 0.5, 0.0. Therefore, the data were re-indexed with the larger unit cell and merged and scaled with SCALA 15 from the CCP4 program suite 16.

Structure determination and refinement. The structure was determined by molecular replacement using an antibody Fab fragment structure (PDB ID 3V9G) as a search model using Phaser 17. The Matthews coefficient calculated for the larger unit cell indicated the presence of four MmRce1–Fab645-2 complexes in the asymmetric unit (MmRce1 chains labelled C, F, I and L). Phaser successfully placed all four Fab645-2 molecules with a Z-score of 6.8. The electron density map obtained from Phaser showed θ-helix-like features for MmRce1. However, this map was not interpretable and had poorly defined molecular boundaries for MmRce1. To improve the phases, solvent flattening and NCS averaging were carried out with DM 18. For this purpose, a solvent mask for MmRce1 was calculated by placing dummy atoms at the putative MmRce1 positions. Density-modified maps clearly showed all of the θ-helices and some of the loops of MmRce1. Using this map, iterative manual model building was performed with COOT 19 and refined with PHENIX 20. Systematically, at every stage of model building, simulated annealed omit maps were calculated to check for model bias and also for phase improvement. Water molecules and detergent molecules were added towards the end of the refinement (Extended Data Fig. 9). The data collection and refinement statistics are shown in Extended Data Table 1. Ramachandran map definitions were defined using MOLPROBITY 21.

Site-directed mutagenesis. All MmRce1 point mutants were produced using the QuickChange Site-Directed Mutagenesis Kit (Agilent Technologies).

All point mutants were able to interact with the conformation-sensitive antibody Fab645-2 on a gel filtration column (Superdex 200 10/300 GL), suggesting that their overall fold did not differ from that of native MmRce1.

In vitro assay of MmRce1 enzymatic activity. A fluorescence resonance energy transfer (FRET) assay was used to demonstrate that purified MmRce1 could cleave the CAAX cleavage site of a peptide substrate designed based on the C terminus of human RHoA. The sequence of the peptide was DABCYL-ARSGAKSGC(farnesyl) LVS-EDANS (where DABCYL is 4-[4-(dimethylamino)phenyl]azo)benzoic acid and EDANS is 5-[(2-aminooethylamino)naphthalene-1-sulfonic acid. Cambridge Peptides). The farnesylated cysteine was dimethyl sulphoxide (DMSO) and stored at a final concentration of 10 mM at −80 °C. In the intact substrate, DABCYL quenches the fluorescence of EDANS. Proteolytic cleavage at the C-terminal side of the farnesylated cysteine separates the fluorophore and quencher, thus resulting in an increase in fluorescence.
Wild-type MmRce1 and mutants were purified in Buffer B, as described above, and made up to a stock concentration of 1 μM. The peptide stock was diluted in Buffer B to a final concentration of 10–90 μM. Assays were performed in 96-well opaque microplates (Nunc) using a POLARstar Omega plate reader (BMG Labtech). The reactions were performed at 25 °C, and 200 measurements were obtained for each run with a 5-s delay between each measurement. The rate of substrate hydrolysis was determined by monitoring the fluorescence as a function of time (excitation wavelength, 330 nm; emission wavelength, 490 nm). Neither peptide nor MmRce1 showed any significant changes in fluorescence over the period of the assay when incubated alone.

The relative fluorescence units (RFU) obtained from these assays were converted to concentration units (μM) by measuring the total fluorescence change of the substrate during long reaction times with enzyme, which allowed nearly complete conversion of the known concentrations of substrate to product. The data were analysed using PRISM software (GraphPad). Graphs were plotted after subtraction of the uncatalysed peptide control data and were fitted to a nonlinear regression one-phase decay equation, because hydrolysis of the peptide by MmRce1 obeyed Michaelis–Menten kinetics. The apparent binding constant (K\text{cat}) was 19.7 ± 1.0 μM and the apparent turnover constant (kcat) was 0.175 ± 0.0027 s⁻¹.

FRET assays (Fig. 1a, Fig. 3d, Extended Data Fig. 4 and Extended Data Fig. 8) were performed at the following concentrations: MmRce1, MmRce1–Fab and all mutants, 1 μM; farnesylated and non-farnesylated peptides, 50 μM; ACF, ZnSO₄, EDTA, 1,7-phenanthroline and 1,10-phenanthroline, 300 μM and 5 mM.

**Liquid chromatography–mass spectrometry analysis.** The RhoA peptide (50 μM) and MmRce1 (1 μM) were mixed in Buffer B at 25 °C for 2 h before mass spectrometry analysis.

Reversed phase chromatography was performed using an HPL200 platform (Agilent). Peptide reaction solutions, incubated with and without enzyme, were diluted in 1 to 50, and 5 μl was injected for analysis (an estimated 5 pmol of the initial unreacted peptide was loaded on the column). Peptides were resolved on a 75 mm internal diameter, 15 cm C18 packed emitter column (3 mm particle size; Nikkyo Technos) over 30 min using a linear gradient of 96:4 to 30:70 buffer LC-A:LC-B (buffer LC-A, 2% acetonitrile and 0.1% formic acid; and buffer LC-B, 80% acetonitrile and 0.1% formic acid) at 250 nl min⁻¹. Peptides were ionized by electrospray ionization using 2.3 kV applied immediately pre-column via a microTEE into the nanospray source. The sample was infused into an LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific) directly from the end of the tapered tip silica column (6–8 μm) by using GROMOS96 43a1 force field. Before the MD simulations, the ligand–protein complex was solvated in POPC lipid bilayer using InfaTGE (Biocomputing Group, University of Calgary) and GROMACS 4.6. During minimization and MD, positional restraints were applied to the protein atoms. Selection of the final peptides was based on the interaction energy and the conformational stability of the ligand during the MD simulations.

**Modelling human RCE1.** A model of human RCE1 was determined using MODELLER⁴⁴ based on the MmRce1 coordinates. The model was further refined using GROMACS 4.6 (ref. 42). A model for a geranylgeranylated peptide GAKASGC (geranylgeranyl)LVLS was based on the farnesylated peptide described above and docked into MmRce1 and the human RCE1 model at the site of the farnesylated peptide GAKASGC(farnesyl)LVLS in MmRce1. The complex was subject to energy minimization as described above for the MmRce1–GAKASGC(farnesyl)LVLS peptide complex. Circular Dichroism (CD). CD experiments on wild-type MmRce1 and six mutants (E140A, H173A, H227A, N231A, L45W and L132W) were performed using a J-715 spectrophotometer at 25 °C in 20 mM MES, pH 6.5, 100 mM KF, 0.02% (w/v) UDM and 0.86% (w/v) OG. The CD spectra for secondary structure determination were recorded between 190 nm and 300 nm, using a 0.01 nm path length cell at a protein concentration of 1 mg ml⁻¹. Three spectra were recorded for each protein, in 0.5 nm increments, and averaged.

The analysis of CD spectra was performed by the programs CONTIN/L²⁵ and SELCON3 (refs 46, 47) incorporated in the software DICHROWEB⁴⁸ using SP175 as the reference protein set⁴⁹. The CD spectra were zeroed between 260 nm and 300 nm and are presented as Δε (mol−1 cm−1) against wavelength (nm).

**CPM-based thermostability assays.** CPM-based thermostability assays on wild-type MmRce1 and all mutants were carried out as previously described⁵⁰. Purified MmRce1 (10 μl at 1 mg ml⁻¹) was added to 140 μl buffer (20 mM MES, pH 6.5, 200 mM NaCl, 0.02% (w/v) UDM and 0.86% (w/v) OG) in a 96-well black plate (Nunc). N-[4-(7-Diethylamino-4-methyl-3-coumarinyl)phenyl]malonamide (CPM) dye (4 mg ml⁻¹ in DMSO) was diluted 100-fold in the same buffer and warmed to room temperature. Dye (3 μl at 40 μg ml⁻¹) was added to the protein, and fluorescence emission was measured almost immediately at 463 nm (excitation 387 nm) on the SpectraMax 2e plate reader (Molecular Devices) at 40 °C. Recordings were taken every 5 min for 2 h, with a 15-s shaking interval between each measurement. A single exponential decay curve was plotted for each protein run and fitted to the Boltzmann sigmoidal equation using PRISM software. For the wild type and the N231A and N231D mutants, the screen was also performed at pH 8 in 20 mM HEPES, pH 8.0, 200 mM NaCl, 0.02% (w/v) UDM and 0.86% (w/v) OG. The mean and s.d. of three experiments were considered for data analysis.

**Thermal shift screen.** Thermal shift assays for wild-type MmRce1 and all mutants were carried out using a 7500 Fast Real-Time PCR System (Applied Biosystems). All available excitation and emission wavelengths of the instrument were used during each run. Purified MmRce1 or mutant MmRce1 (10 μl at 1 mg ml⁻¹) was added to 50 μl buffer (20 mM MES, pH 6.5, 200 mM NaCl, 0.02% (w/v) UDM and 0.86% (w/v) OG) on an Applied Biosystems 7500 Fast-96-well PCR plate (Thermo Scientific). CPM dye (4 mg ml⁻¹ in DMSO) was diluted tenfold in the same buffer, and 7 μl dye at 100 μg ml⁻¹ was added to the protein. The plate was sealed with a Microseal ‘B’ clear adhesive seal (Bio-Rad), and fluorescence emission was measured almost immediately. The samples were heated from 10 °C to 95 °C at a rate of 1 °C min⁻¹. Purified MmRce1 (10 μl at 30 μl) was added to 50 μl buffer (20 mM MES, pH 6.5, 200 mM NaCl, 0.02% (w/v) UDM and 0.86% (w/v) OG) on an Applied Biosystems 96-well PCR plate. After heating at 95 °C for 1 min, the plate was cooled to 4 °C and then heated again at 0.5 °C per minute until the samples reached a release temperature (T_melt) value as taken as the minima in the derivative plots (derivative melt profiles). For the wild type and the N231A and N231D mutants, the screen was also performed at pH 8 in 20 mM HEPES, pH 8.0, 200 mM NaCl, 0.02% (w/v) UDM and 0.86% (w/v) OG. The mean and s.d. of three experiments were considered for data analysis.

**Geranylgeranylated peptide synthesis and assays.** Solid phase peptide synthesis of three geranylgeranylated peptides was performed as described previously⁵¹. The amino acid sequence of two peptides was based on the C terminus of RhoA (ARGSAGSC[geranylgeranyl]LVLS and the sequence of the third peptide was based on the A sequence of the yeast a-factor (VITKGPVPDPA[D]geranyl-geranyl]VIA). One RhoA-derived peptide was further labelled with DABCYL and EDANS to resemble the farnesylated RhoA peptide used in the FRET experiments described above. All three lyophilized peptides were dissolved in DMSO and stored at a final concentration of 10 μM at −80 °C.
The two non-fluorescent peptides (RhoA and yeast α-factor) were used for mass spectrometry analysis (as described above); the FRET RhoA peptide was diluted to 10–60 μM in Buffer B, and its proteolytic cleavage by MmRce1 (1–5 μM) was assayed by FRET (assay and conditions described above).

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Extended Data Figure 1 | Structure of the MmRce1–Fab645-2 complex. Overall ribbon diagram of the MmRce1–Fab645-2 complex, with MmRce1 viewed parallel to the membrane. MmRce1 is depicted in blue, the antibody Fab fragment heavy chain (Fab(H)) in yellow, and the antibody Fab fragment light chain (Fab(L)) in green. MmRce1 interacts with Fab645-2 through its cytoplasmic side.
Extended Data Figure 2 | Fluorescence resonance energy transfer (FRET) proteolytic assay of purified MmRce1. The FRET assay was performed using a CAAX synthetic peptide modelled on the C terminus of RhoA (Fig. 1b and Methods). The plotted graph was fitted to a nonlinear regression one-phase decay equation, because hydrolysis of the peptide by MmRce1 obeyed Michaelis–Menten kinetics. The apparent binding constant ($K_m$) was $19.7 \pm 1.0 \, \mu M$, and the apparent turnover constant ($k_{cat}$) was $0.175 \pm 0.0027 \, s^{-1}$. The data are presented as the mean $\pm$ s.d. of $n = 3$ experiments. The fluorescence conversion factor (relative fluorescence units versus peptide concentration; inset curve) was determined by measuring the FRET between DABCYL and EDANS at different peptide concentrations (10–90 $\mu M$) and plotting the standard curve (fluorescence versus concentration). The data are presented as the mean $\pm$ s.d. of $n = 3$ experiments, and $R^2$ indicates the quality of the fit.
Extended Data Figure 3 | Structure-based multiple sequence alignment.
Structure-based multiple sequence alignment of Rce1 homologues representing all three domains of life: Homo sapiens Rce1 (HsRce1; UniProt ID Q9Y256), Saccharomyces cerevisiae Rce1p (ScRce1; UniProt ID Q03530), Streptomyces coelicolor Rce1 (ScoRce1; UniProt ID Q9XAK4), Methanothermus maripaludis Rce1 (MmRce1; UniProt ID Q6LZY8) and Lactobacillus plantarum Rce1 (LpRce1; UniProt ID C6VK86). The transmembrane helices are depicted as cyan barrels. The residues that were mutated and disrupt activity are indicated by green and orange arrows: the green arrows indicate catalytic residues, and the orange arrow indicates a putative farnesyl lipid-binding residue in TM4. HsRce1 and MmRce1 have 15% sequence identity.
Extended Data Figure 4 | Effects of zinc and chelating factors on the activity and stability of MmRce1. Comparison of the proteolytic activities of wild-type MmRce1 and MmRce1 incubated with a molar excess of ZnSO$_4$, EDTA, 1,10-phenanthroline or 1,7-phenanthroline. 1,7-Phenanthroline, a stereoisomer of 1,10-phenanthroline that does not chelate Zn$^{2+}$, inactivated MmRce1 to the same extent as 1,10-phenanthroline, which does chelate Zn$^{2+}$. A molar excess of Zn$^{2+}$ (5 mM) did not reverse the inactivating effect of 0.3 mM 1,10-phenanthroline on MmRce1. The data are presented as the mean ± s.d. of $n = 3$ experiments.

Representative derivative melt profiles of MmRce1 incubated without (black) and with 5 mM 1,7-phenanthroline (red) or 1,10-phenanthroline (blue) (b). Experiments were performed over a temperature range starting from 10 °C and ending at 95 °C. The melting temperatures ($T_m$) were taken from the minimum derivative plot (c), plotted as the negative of the first derivative of the normal fluorescent curve, converted by Protein Thermal Shift software. The $T_m$ values were determined as the mean ± s.d. of $n = 3$ replicates. d, e, Two views showing ribbon and surface representations of MmRce1 (molecule C from the asymmetric unit). The side chain of C213 is shown as an orange stick model. C213 is buried within the structure and inaccessible unless the protein unfolds and/or undergoes transient ‘breathing’ motions.
Extended Data Figure 5 | Thermal stability comparisons.

**a, b,** Representative melting curves of wild-type MmRce1 and mutants obtained from the CPM-based thermostability assay (Methods) fitted to a Boltzmann sigmoidal equation. The raw data for each protein are shown as dots, and the fitted curves are shown as black lines. The measurements were obtained over a period of 120 min at 40 °C.

**c–f,** Representative derivative melt profiles of wild-type MmRce1 and mutants (**c**) and wild-type MmRce1 and MmRce1 incubated with increasing amounts of urea (1–5 M) (**e**). Experiments were performed over a temperature range starting from 10 °C and ending at 95 °C. The melting temperatures (Tm) were taken from the minimum derivative plot (**d, f**), plotted as the negative of the first derivative of the normal fluorescent curve, converted by Protein Thermal Shift software. The Tm values were determined as the mean ± s.d. of n = 3 replicates.
Extended Data Figure 6 | Circular dichroism. a, Far ultraviolet circular dichroism spectra of wild-type MmRce1 and mutants (E140A, H173A, H227A, N231A, L45W and L132W). The measurements were made at 25 °C in 20 mM MES (pH 6.5), 100 mM KF, 0.029% (w/v) UDM and 0.86% (w/v) OG. Contributions to the spectra by the buffer were subtracted using control scans. All curves represent $\Delta \varepsilon$ measured in units of $\text{litre mol}^{-1} \text{cm}^{-1}$. b, Estimate of the proportion of $\alpha$-helical structure in wild-type MmRce1 and mutants. Analysis of the circular dichroism spectra was performed using CONTIN/LL. The error bars represent a presumed inaccuracy in the protein concentration determination of 10%.
The proposed catalytic mechanism of MmRce1.

The catalytic mechanism and overall structure of MmRce1 compared with those of GlpG (PDB ID 2O7L)\textsuperscript{27}, PSH (presenilin/SPP homologue) (PDB ID 4HYC)\textsuperscript{9} and ZMPSTE24 (PDB ID 4AW6)\textsuperscript{11}. The MmRce1 water molecule, which is hydrogen-bonded to both E140 and H173, is the nucleophile. The general base is the carboxylate of E140. H227 and N231 (both marked “oxy”) are proposed to stabilize the oxyanion transition state. Dashed lines represent hydrogen bonds or other electrostatic interactions. The arrows indicate the movement of electron pairs. The ribbon diagrams of MmRce1, GlpG, PSH and ZMPSTE24 are viewed parallel to the membrane, oriented from the top (the endoplasmic reticulum (ER) lumen) to the bottom (cytoplasm).
Extended Data Figure 8 | Disruption of the farnesyl lipid-binding site.

a, b, Both L-to-W mutants are modelled, and the mutations are depicted in orange as side-chain stick models and surface representations. c, The proteolytic activity of wild-type MmRce1 and mutants towards a farnesylated peptide. The data are presented as the mean ± s.d. of n = 3 experiments.

d, Superposition of the MmRce1 crystal structure (orange) and a human RCE1 (HsRce1) homology model (blue). e, Cartoon representation of the HsRce1 homology model with a geranylgeranylated peptide modelled at its catalytic site. The geranylgeranylated peptide (GAKASGC(geranylgeranyl)LVS) is depicted as a green ribbon with the N and C termini labelled, and the geranylgeranyl lipid is depicted as a yellow ball-and-stick model.
Extended Data Figure 9 | Final 2Fo-Fc electron density maps. **a**, Stereoview showing 2Fo-Fc electron density calculated after the final refinement run (contoured at the 1σ level) (grey), with the final MmRce1 model overlaid (blue). **b**, Stereoview of the 2Fo-Fc electron density (contoured at the 1σ level) at the MmRce1 catalytic site. The catalytic residues (E140 and H173), the catalytic water molecule (W) and the oxyanion residues (H227 and N231) are labelled, as well as a second water molecule (W2) that interacts directly with W. **c**, Stereoview of 2Fo-Fc electron density in the vicinity of the MmRce1 transmembrane helices.
**Extended Data Table 1 | Data collection and refinement statistics**

| Description                        | Value                  |
|------------------------------------|------------------------|
| Beam line                          | DLS 104-1              |
| Wavelength (Å)                     | 0.9199                 |
| Space Group                        | P1                     |
| **a** (Å)                          | 113.0                  |
| **b** (Å)                          | 90.0                   |
| **c** (Å)                          | 99.0                   |
| α (°)                              | 89.0                   |
| β (°)                              | 102.3                  |
| γ (°)                              | 90.0                   |
| **Z**                              | 4 MmRce1-Fab complexes|
| Resolution limits (Å)              | 44.1-2.50              |
|                                    | (2.64-2.50)            |
| Rmerge                             | 0.103 (0.77)           |
| I/I₀ (I)                           | 11.1 (2.5)             |
| Total number unique                | 130,242 (18,589)       |
| Completeness (%)                   | 98.3 (95.9)            |
| Multiplicity                       | 9.2 (7.9)              |
| Anomalous completeness (%)         | 97.0 (93.2)            |
| Anomalous multiplicity             | 4.6 (3.9)              |

**Refinement Statistics**

| Description                        | Value                  |
|------------------------------------|------------------------|
| Resolution limits (Å)              | 41.1-2.5               |
| **Number of reflections**          |                        |
| Working set                        | 128,355                |
| Test set                           | 6,440                  |
| Rwork/Rfree                        | 0.226/0.267            |
| **Number of atoms**                |                        |
| Protein                            | 21,358                 |
| Ligand                             | 397                    |
| **Mean B-factors**                 |                        |
| Protein atoms (Å²)                 | 70.4                   |
| Ligand atoms (Å²)                  | 71.5                   |
| **RMSD from ideal values**         |                        |
| Bond length (Å)                    | 0.004                  |
| Bond angles (°)                    | 0.886                  |
| **Ramachandran plot Statistics**   |                        |
| Preferred (%)                      | 98.5                   |
| Allowed (%)                        | 1.5                    |

The values in parentheses are for the highest resolution shell.