Intramembrane proteases catalyse the signal-generating step of various cell signalling pathways, and continue to be implicated in diseases ranging from malaria infection to Parkinsonian neurodegeneration1–3. Despite playing such decisive roles, it remains unclear whether or how these membrane-immersed enzymes might be regulated directly. To address this limitation, here we focus on intramembrane proteases containing domains known to exert regulatory functions in other contexts, and characterize a rhomboid protease that harbours calcium-binding EF-hands. We find calcium potently stimulates proteolysis by endogenous rhomboid-4 in Drosophila cells, and, remarkably, when rhomboid-4 is purified and reconstituted in liposomes. Interestingly, deleting the amino-terminal EF-hands activates proteolysis prematurely, while residues in cytoplasmic loops connecting distal transmembrane segments mediate calcium stimulation. Rhomboid regulation is not orchestrated by either dimerization or substrate interactions. Instead, calcium increases catalytic rate by promoting substrate gating. Substrates with cleavage sites outside the membrane can be cleaved but lose the capacity to be regulated. These observations indicate substrate gating is not an essential step in catalysis, but instead evolved as a mechanism for regulating proteolysis inside the membrane. Moreover, these insights provide new approaches for studying rhomboid functions by investigating upstream inputs that trigger proteolysis.

Cell membranes are both controlled borders with the outside world as well as dynamic platforms for organizing cell signalling, metabolic pathways, and ultrastructure assembly. All of these key events rely on enzymes that reside directly within the cell membrane, yet achieving a mechanistic understanding of how these specialized enzymes function within this environment has proved challenging.

Intramembrane proteases catalyse the committed, signal-generating step of several key signalling pathways by cleaving transmembrane proteins within the membrane11,12. Their importance is emphasized by repeated implication in disease. γ-Secretase generates the amyloid-β peptide in Alzheimer’s disease13,14, but more recently has been successfully targeted in a spectrum of cancers15, because its activating cleavage of the Notch receptor triggers signalling2; Site-2 protease family metalloenzymes liberate transcription factors from the membrane to control cholesterol and fatty-acid composition of membranes1, and signalling circuits that control virulence in pathogenic bacteria16. Rhomboid serine proteases are a family of master regulators that initiate epidermal growth factor signalling during Drosophila development17,18, but more recently have been implicated in cleaving adhesins during malaria invasion19, and regulating mitochondrial quality control to guard against Parkinson’s disease3.

Since peptide bond cleavage is irreversible in the cell, precise regulation of protease activity is paramount. Yet it is generally thought that intramembrane proteases are constitutively active enzymes over which the cell cannot exert direct regulation2. Instead, two mechanisms control activity. The first is transcriptional, as exemplified by Drosophila rhomboid-1: the constitutively active protease is made only when and where needed3. This mechanism has historically served as a beautiful atlas of epidermal growth factor signal initiation during development. The second mechanism is centred on controlling access to substrate by segregating it from protease20. Malaria, for example, sequesters adhesins in secretory organelles before invasion, while their secretion onto the surface leads to the first encounter with an active rhomboid protease2.

The key property missing from these two mechanisms is the ability to respond rapidly to changing conditions: transcriptional and cell localization changes are ill adapted to provide immediate responses that are hallmarks of cell signalling. Moreover, it is essentially unprecedented for proteases to be devoid of direct enzymatic regulation in the cell, raising the possibility that this apparent discrepancy reflects our lack of understanding rather than absence of a regulatory mechanism.

Although Escherichia coli rhomboid protease GlpG has served as a tractable model for studying the structure--function of intramembrane proteolysis21, no information is available on its cellular role. This knowledge gap prohibits deciphering regulatory mechanisms. Instead, as a new approach to this question, we searched for rhomboid

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**Figure 1** Calcium rapidly stimulates intramembrane proteolysis in Drosophila cells by endogenous DmRho4. a, Diagram comparing the predicted calcium-binding loop residues of DmRho4 to an EF-hand consensus (in red). b, Calcium ionophore treatment of Drosophila S2R+ cells induced cleavage of GFP–Spitz, but not its cleavage-site mutant, by endogenous DmRho4. Graph shows expression levels of Drosophila rhomboid genes in S2R+ cells (RNA-seq data from modENCODE, http://modencode.org). AU, arbitrary units. c, Ionophore-induced Spitz cleavage was detectable within 5 min (red triangle) and linear for 3 h. d, RNAi knockdown of DmRho4 but not DmRho1 abolished calcium-induced cleavage of GFP–Spitz. e, Plasmid expression of DmRho4 rescued calcium-induced cleavage of GFP–Spitz in S2R+ cells undergoing RNAi. f, Calcium-stimulated Spitz cleavage abolished by DmRho4 RNAi could not be rescued by DmRho1 overexpression. All images are anti-GFP western analyses, with substrate and cleavage bands denoted by ‘x’ (see Fig. 3d for untransfected cells).
Proteins that contain additional domains with precedent for regulating protein activity and focused on a conserved subset of over two dozen animal rhomboid enzymes with EF-hand domains appended to their cytosolic amino (N) termini (Fig. 1a and Extended Data Fig. 1). EF-hands are helix–loop–helix motifs in which calcium binding at the loop serves either a structural or a regulatory role. In the latter, calcium binding separates the helices and exposes a new surface for binding a regulatory partner. EF-hands typically occur in pairs to form a stable bundle, perhaps the best characterized of which are the EF-hands of calmodulin.

Since rhomboid function is best understood in *Drosophila*, we sought to study the EF-hand containing *Drosophila* rhomboid-4 (DmRho4) under physiological conditions by searching for cell lines that endogenously express DmRho4. We focused on the well-characterized S2R+ cell line, which also expresses the housekeeping mitochondrial rhomboid and low amounts of DmRho1, but no other rhomboid (Fig. 1b). Treating S2R+ cells with a calcium ionophore potently stimulated processing of the epidermal growth factor ligand Spitz by more than 50-fold, but not its transmembrane mutant (Fig. 1b), and processing was rapid, becoming detectable within 5 min (Fig. 1c). Targeting DmRho4 with RNA interference (RNAi) removed this stimulation completely, while parallel RNAi against Spitz by more than 50-fold, but not a panel of other rhomboid proteases, without any other protein factors present (Fig. 2a). This stimulation was selective since the N-terminal is cytosolic while the C terminus of DmRho4 is extracellular (blue marks nuclei).

The resulting thermograms revealed two sites for calcium binding with a thermodynamic basis of calcium binding to the EF-hand domain (Fig. 2b). This effect was direct because pure DmRho4 stimulated proteolytic activity of DmRho4 in proteoliposomes (error bars, s.d. for experimental replicates). EF-hand mutants to cleave GFP–Spitz in response to calcium ionophore stimulation in S2R+ cells was quantified by anti-GFP western analysis (see also Extended Data Fig. 2b for DmRho4 levels). Graphs show activity of selectively compromised loop 4 and 6 mutants under calcium-stimulated conditions in cells (upper graph) versus unstimulated conditions (lower graph, measured as cleavage product accumulation in culture media after 24 h; see also Extended Data Fig. 2c). Error bars, s.d. for experimental replicates. Filled and open triangles denote substrate and cleavage bands, respectively, throughout.

Our goal was to study direct regulation of rhomboid enzymes. We therefore next tested the unlikely possibility that calcium directly regulates pure DmRho4 expressed and purified from bacteria and reconstituted into liposomes. Remarkably, addition of calcium directly stimulates pure DmRho4 expressed and purified from bacteria and reconstituted into liposomes. The resulting thermograms revealed two sites for calcium binding with a thermodynamic basis of calcium binding to the EF-hand domain (Fig. 2b). This effect was direct because pure DmRho4 stimulated proteolytic activity of DmRho4 in proteoliposomes (error bars, s.d. for experimental replicates). EF-hand mutants to cleave GFP–Spitz in response to calcium ionophore stimulation in S2R+ cells was quantified by anti-GFP western analysis (see also Extended Data Fig. 2b for DmRho4 levels). Graphs show activity of selectively compromised loop 4 and 6 mutants under calcium-stimulated conditions in cells (upper graph) versus unstimulated conditions (lower graph, measured as cleavage product accumulation in culture media after 24 h; see also Extended Data Fig. 2c). Error bars, s.d. for experimental replicates. Filled and open triangles denote substrate and cleavage bands, respectively, throughout.
recently rhomboid proteases have been postulated to exist as dimers \(^ {17} \). in contrast to this model, co-expressing two DmRho4 molecules in S2R cells did not result in dimerization. functionally, we also did not observe calcium stimulation of protease activity in trans, which is a classical test of allostery resulting from oligomerization: a catalytically inactive rhomboid enzyme that can still bind calcium could not stimulate the activity of a DmRho4 enzyme carrying a mutation that compromised calcium binding (Extended Data Fig. 3c).

these observations collectively suggest that DmRho4 is not regulated by interaction with any other proteins. to test this further in Drosophila cells, we overexpressed inactive DmRho4 to outcompete the endogenous enzyme for any binding partners (Fig. 3d). remarkably, about 200-fold more inactive DmRho4 had no effect whatsoever on the ability of the endogenous DmRho4 to process Spitz. this observation, in particular, indicates that rhomboid regulation under physiological conditions is not mediated by dimerization, substrate affinity, or additional factors (although factors that fine tune responses in different contexts remain possible).

in contrast to intermolecular target binding, calcium could directly stimulate the activity of a single DmRho4 enzyme through intramolecular allostery. Calpains are the precedent for this type of activation, with calcium binding resulting in a conformation change that aligns the catalytic residues \(^ {18} \). Since the structure of a eukaryotic rhomboid enzyme has never been solved, we used a biochemical cross-linking approach to test whether calcium aligns the catalytic residues of DmRho4. Cysteines installed at the catalytic serine and histidine positions had no effect on the structural stability of DmRho4 (Extended Data Fig. 4a) and could readily and reversibly be oxidized to form a disulphide bridge (Fig. 4a). importantly, calcium did not affect the amount of cross-linking, revealing that the DmRho4 catalytic residues are pre-aligned with no influence from calcium binding.

Stimulation of the catalytic rate constant \( k_{cat} \) by calcium was strikingly reminiscent of the increase in \( k_{cat} \) we measured for gate-open mutants of GlpG \(^ {16} \). this is an attractive parallel, because gate-opening is the rate-limiting step for rhomboid intramembrane proteolysis, and the loops that we predict bind calcium also connect the presumed transmembrane gating helix to the rest of the enzyme. one consequence of gate-opening is that substrates can enter deeper into the transmembrane site, which is reflected in a shift of cleavage site deeper into the substrate transmembrane segment \(^ {19} \). accordingly, calcium-stimulated proteolysis shifted the cleavage site 3 residues deeper into the transmembrane segment for DmRho4 but not other rhomboid proteases, consistent with calcium specifically stimulating DmRho4 proteolysis by facilitating gate-opening (Fig. 4b).

to explore the functional consequence of this shift, we examined proteolysis of a series of transmembrane substrates that we engineered to have cleavage sites inside the membrane, outside the membrane, or both in the same molecule. remarkably, the external site was used very well in the absence of calcium, while the intramembrane site was barely cleaved (Fig. 4c and Extended Data Fig. 4b). moreover, loop 4 and 6 mutants that compromised calcium regulation in cells also readily cleaved the external site (Extended Data Fig. 4c), which independently confirms that the catalytic residues are competent for catalysis in the absence of calcium. however, addition of calcium shifted the cleavage site from the external site almost exclusively to the intramembrane site for DmRho4 while having no effect on the cleavage site selection of other rhomboid enzymes (Fig. 4c and Extended Data Fig. 4d).

Although no information is available on how E. coli GlpG is regulated, we also extended these analyses to this widely studied enzyme by comparing wild-type with gate-open mutants \(^ {20} \). Both wild-type and gate-open GlpG cleaved the external site with similar efficiency (Fig. 4c), while proteolysis at the intramembrane site was specifically stimulated by gate-open mutants (Fig. 4d). Moreover, although gate-open mutants of E. coli GlpG stimulated proteolysis of transmembrane substrates by approximately tenfold (Fig. 4d), a soluble casein substrate can still bind calcium could not stimulate the activity of a DmRho4 enzyme carrying a mutation that compromised calcium binding (Extended Data Fig. 3c).

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Gating has been controversial, because rhomboid proteases can be responsible for calcium release of Notch intracellular domain. Interestingly, the ‘non-canonical’ mode of calcium binding to DmRho4 loops is reminiscent of synaptotagmin activation, which also involves loops that are placed into close contact with lipid molecules. In fact, rhomboid activation may also involve lipids, which may explain the apparent high calcium levels needed for full enzyme activation in vitro: synaptotagmin exhibits an intrinsic $K_d$ of 530 μM for calcium at the C2 site that decreases to 3–4 μM when appropriate lipids are present. Although ultimately structural analysis is required to reveal the precise architecture of calcium binding, likely involvement of lipid, and impact on gating in DmRho4, so far no rhomboid enzyme with an intact extramembranous domain has produced well-diffracting crystals. Our studies provide incentive to move beyond GlpG and focus structural biology efforts on these more complex rhomboid proteases.

A particularly exciting implication of these enzymatic properties is that rhomboid proteases can directly integrate upstream signals from other signalling pathways. In fact, this may have medical implications, since Ventrient/RHBDL3, a human rhomboid that contains potential calcium-binding residues in its EF-hands and cytoplasmic loops, is expressed in the nervous system and may be linked to a mental retardation syndrome. In this light, studying upstream regulation provides a powerful new approach towards revealing biological functions of rhomboid proteases that have evaded discovery. In fact, previous efforts could have missed important roles because they were studying rhomboid functions under unstimulated conditions. Activation is not limited to calcium signalling, since a diversity of recognizable domains have been appended to different rhomboid proteins including zinc fingers, β-propellers, and tetracopeptide repeats. It should be noted that not all extramembranous domains necessarily serve direct regulatory functions. For example, trafficking signals have been found in the cytosolic domains of parasitic rhomboid enzymes.

Finally, while we focused our studies on rhomboid proteases, substrate gating has been proposed for other intramembrane proteases, raising the possibility that gating may be a general mechanism for directly regulating intramembrane proteolysis.

Online Content Methods, along with any additional 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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Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to S.U. (surban@jhmi.edu).
METHODS
Rhomboid expression constructs. The Drosophila rhomboid-4 open reading frame (ORF) was subcloned from SD06923 (Drosophila Genomics Research Center) into pGEX-6P-1 or PET21 for bacterial expression, or into pRMH43 for expression in Drosophila S2R⁺ cells. In the N-terminal 3×HA-tag was introduced to allow detection of DmRh4 by anti-HA western analysis. Residue substitutions were introduced by site-directed mutagenesis using Pfu Ultra in a Stratagene 96 Gradient Robocycler (Agilent Technologies) and were confirmed by sequencing the entire ORF. The EF-hand domain of DmRh40 (residues 68–154) or its entire N-terminal domain (residues 1–176) were deleted by site-directed mutagenesis to generate the ΔEF and ΔN mutants, respectively.

Drosophila cell culture and manipulation. Drosophila S2R⁺ cells (purchased from the Drosophila Genomics Research Center) were cultured at 25°C in Schneider’s insect medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS, Sigma). For transfection experiments, S2R⁺ cells were seeded into six-well plates and transfected with a total of ~2 μg of plasmid DNA (0.5 μg each of pRMH43–GFP–Spitz and pRMH43–Star plus 1 μg pBluescript, and ~5–25 ng of pRMH43–3×HA–DmRh4 if applicable) and 5 μl ofXE GENE HP (Roche). Transfection complexes were formed at room temperature in 100 μl of DMEM for 15 min before being added dropwise to cells. Expression from the metallothionine serum (FBS, Sigma). For transfection experiments, S2R⁺ cells were transfected with 3 μl of XtremeGENE HP (Roche). GFP western analysis of media fractions. and the GFP–Spitz that had been released by proteolysis was quantified by anti-

DmRho4 activity, Schneider’s serum-free media was conditioned for 24 h, which resulted in a doubling of GFP–Spitz processing (Fig. 3d). To detect basal (unstimulated) cells was estimated by titrating wild-type 3×HA–DmRh4 by transfection: matching the level of transfected 3×HA–DmRh4 to the endogenous DmRh4 expression level resulted in a doubling of GFP–Spitz processing (Fig. 3d). To detect basal (unstimulated) DmRh4 activity, Schneider’s serum-free media was conditioned for 24 h, and the GFP–Spitz that had been released by proteolysis was quantified by anti-GFP western analysis of media fractions.

Drosophila cell RNAi. Templates for in vitro transcription were amplified by PCR with primers corresponding to the divergent N-terminal regions of DmRh1 (ORF nucleotides 1–451) and DmRh40 (ORF nucleotides 1–501) and incorporated T7 promoter sequences. RNA was generated using the RibomAX T7 kit (Promega) according to the manufacturer’s instructions, and purified using the RNasy protocol (Qiagen). Double-stranded RNA (dsRNA) was formed by mixing equal amounts of each strand in annealing buffer (1 mM HEPES pH 7.3, 0.5 mM EDTA) and boiling for 5 min, followed by slow cooling. dsRNA was analyzed by agarose gel electrophoresis. S2R⁺ cells seeded in six-well plates were washed with serum-free Schneider’s media, and ~25 μg of dsRNA was added to each well containing 1 ml of serum-free Schneider’s insect media. After a 1–2 h incubation, 3 ml of Schneider’s media + 10% FBS was added, and the cells were incubated at 25°C for 3 days. On the third day, cells were assayed by transfection as described above.

Drosophila cell microscopy. S2R⁺ cells were seeded onto glass coverslips, transfected with 3×HA–DmRh4–1×Flag (which was verified to be proteolytically active and calcium regulated), induced with 0.5 mM CuSO4 for ~24 h, and fixed in 4% formaldehyde in PBS for 20 min. Cells were blocked with 1% bovine serum albumin in the presence or absence of 0.1% TritonX-100, and stained with 1/200 anti-HA and anti-Flag antibodies overnight. The resulting immune complexes washed with serum-free Schneider’s media, and ~0.5 μl of dsRNA was added to each well containing 1 ml of serum-free Schneider’s insect media. After a 1–2 h incubation, 3 ml of Schneider’s media + 10% FBS was added, and the cells were incubated at 25°C for 3 days. On the third day, cells were assayed by transfection as described above.

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Rhomboid proteases were reconstituted in liposomes and used to perform successive 2 μl injections into the reaction cell at 25°C. Control experiments titrating calcium against liposomes were performed to determine the heat of titrant dilution, which, subtracted from the heat of reaction, yielded the effective heat of calcium binding. Data were fitted using Origin analysis software.

Thermostability analysis. Wild-type and engineered variants of DmRh4 were subjected to quantitative thermostability analysis as described previously. Briefly, 600 pmol FITC-TatA substrate was incubated for 1 h at room temperature in 25°C Tris pH 7.4, 150 mM MgCl₂, 0.1% dodecylβ-D-maltoside (DDM) for 1 h at 37°C overnight. Bacterial rhomboid proteases were expressed in E. coli with 1 mM IPTG for 16–18 h except DmRho4, which was assayed at 25°C for 2–4 h. APP–Sp7–Flag reaction products were resolved by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and quantified by anti-Flag western analysis using an Odyssey infrared laser scanner (LiCor Biosciences), while products for the real-time assay using fluorescein isothiocyanate (FITC)-TatA were quantified using an Odyssey infrared laser scanner (LiCor Biosciences) and imaged using a Typhoon Imager (GE Healthcare) with settings of 488 nm for excitation and 526 nm for emission.

Isothermal titration calorimetry. Isothermal titration calorimetry used a Microcal ITC200 instrument (GE Healthcare). The reaction cell contained 20–30 μM DmRh4 in proteoliposomes and the reference cell contained water. A motorized syringe loaded with 1 mM calcium was used to perform successive 2 μl injections into the reaction cell at 25°C. Control experiments titrating calcium against liposomes were performed to determine the heat of titrant dilution, which, subtracted from the heat of reaction, yielded the effective heat of calcium binding. Data were fitted using Origin analysis software.

Rhomboid-substrate co-immunoprecipitation. S2R⁺ cells were co-transfected with catalytically inactive pRMH3–Flag–DmRh4–H358A and pRMH3–GFP–Spitz or, as a negative control, with pRMH3–GFP–Spitz alone, and were either untreated or treated with 6 μM 1,10-phenanthroline in insect saline or Ca-free insect saline, both with 0.5% DDM. Cell lysates were solubilized for 1 h at room temperature in 25 mM Tris pH 7.4, 150 mM MgCl₂. Complete EDTA-free protease inhibitor cocktail (Roche), and 0.25% DDM in either the presence of 1 mM CaCl₂ or 1 mM MgCl₂. Cell debris was removed by centrifugation at 16,000g for 20 min at 4°C. Immunoprecipitations were done using anti-Flag M2 agarose for 1 h at room temperature. Beads washed in the presence of 1 mM CaCl₂ or 1 mM MgCl₂ were resuspended in SDS sample buffer, then load and bound fractions were resolved by SDS–PAGE followed by anti-Flag/anti-GFP western analysis. For co-immunoprecipitation analysis in proteoliposomes, catalytically inactive HA-tagged DmRh4 (S299A) was co-reconstituted with APP–Sp7–Flag as described above in buffer at 37°C overnight. Buffer changes outside the transmembrane domain was constructed by inverse PCR to substitute residues 20–25 (FFAEDV) of APP–Sp7–Flag with the sequence IATAFP from P. stuartii TatA. APP–TatA–Flag had only the external TatA site.

Intramembrane proteolysis assays. Rhomboid proteases were co-reconstituted with substrates into liposomes using the inducible reconstitution system that we described recently. Bacterial rhomboid proteases were reconstituted in liposomes formed from an E. coli polar lipid extract, while DmRh4 was reconstituted in liposomes formed from a yeast polar lipid extract or 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine lipids (Avanti Polar Lipids). All enzymes were assayed for 1 h at 37°C, except DmRh4, which was assayed at 25°C for 2–4 h. APP–Sp7–Flag reaction products were resolved by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and quantified by anti-Flag western analysis using an Odyssey infrared laser scanner (LiCor Biosciences), while products for the real-time assay using fluorescein isothiocyanate (FITC)-TatA were quantified using an Odyssey infrared laser scanner (LiCor Biosciences) and imaged using a Typhoon Imager (GE Healthcare) with settings of 488 nm for excitation and 526 nm for emission.

Light scattering data were fitted to a two-state Boltzmann curve using Stargazer (Harbinger Biotech). Light scattering data were fitted to a two-state Boltzmann curve using Stargazer BioActive software to derive transition temperature midpoints (Tm).
resuspended in SDS sample buffer and the input and bound fractions were compared by anti-HA and anti-Flag western analysis.

**Rhomboid co-immunoprecipitation.** S2R⁺ cells were co-transfected with expression constructs encoding triple HA-tagged and Flag-tagged DmRho4, and as a control, with HA-tagged DmRho4 alone as described above, and were then either untreated or treated with 6 μM ionomycin in insect saline or Ca-free insect saline. Cells were solubilized with 0.25% DDM (as described above) and immunoprecipitations were performed with anti-Flag M2 agarose for 1 h at room temperature. Beads washed in the presence of 1 mM CaCl₂ or 1 mM MgCl₂ were resuspended in SDS sample buffer. Load and bound fractions were detected by anti-HA and anti-Flag western analysis. Pure samples of HA-tagged and Flag-tagged DmRho4 were also co-reconstituted into proteoliposomes in the absence or presence of 0.5 mM CaCl₂, then solubilized with 1% DDM for 30 min at room temperature, immunoprecipitated with anti-Flag M2 agarose (as described above) and then subjected to anti-HA/anti-Flag western analysis.

**Disulphide cross-linking.** A cysteine-less mutant of DmRho4 was generated by substituting the three amino-terminal native cysteine residues with serine residues (C104S, C150S, C176S) and the native transmembrane cysteine residue with a valine residue (C334V). Using this construct as a template, the active site residues S299 and H358 were replaced with cysteine residues, either singly, to generate S299C and H358C, or in combination, to generate the double mutant S299C/H358C. Pure cysteine-substituted proteins were reduced by treatment with 5 mM TCEP for 30 min at room temperature and then passed through a Zeba spin column (Pierce) equilibrated in 50 mM Tris, 150 mM NaCl, 0.1% DDM. Proteins were then oxidized, either in detergent micelles or after reconstitution into proteoliposomes, by the addition of 50 or 100 μM copper phenanthroline, respectively, for 15 min at room temperature in the absence or presence of 0.5 mM calcium. Note that owing to the random orientation of rhomboid in reconstituted proteoliposomes, only half of the DmRho4 was accessible to the oxidizing reagent. Control reactions were done in parallel with no copper but with 50 mM DTT to prevent spontaneous oxidation. Reactions were stopped by the addition of SDS–sample buffer. As an additional control, the oxidation observed for the double mutants was reversed by the addition of 50 mM DTT for 10 min to the oxidized proteins in SDS sample buffer. Samples were analysed by SDS–PAGE and stained using either IRDye Blue Protein Stain (LiCor) or Krypton Infrared Protein Stain (Pierce).

**Mass spectrometry.** Full-length substrate and C-terminal cleavage products were purified from *in vitro* proteolysis assays by anti-Flag immunoprecipitation and analysed by matrix-assisted laser desorption/ionization–time of flight (MALDI–TOF) mass spectrometry using sinapinic acid matrix as described previously19,32. No statistical methods were used to predetermine sample size.

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Extended Data Figure 1 | The rhomboid-4 subfamily of rhomboid proteases. 
a. ClustalW multiple sequence alignment of the conserved N-terminal EF-hand domains of 24 members of the rhomboid-4 subfamily (generated in Biology Workbench, http://workbench.sdsc.edu). Identical residues are shaded in green, highly conserved residues in yellow, and similar residues in cyan. The EF-hand calcium-binding loop consensus sequence is given below the alignment.

b. Rooted tree of the 24 rhomboid-4 homologues. Genus and species names are colour-coded as follows: primates (blue), other mammals (green), birds (purple), fish (pink), non-vertebrate chordate (cyan), nematode (orange), and insects (red), with vernacular names given in parentheses, followed by National Center for Biotechnology Information (NCBI) accession numbers.
Extended Data Figure 2 | Activity and thermostability analysis of DmRho4 mutants. 
a. Comparison of calcium stimulation of DmRho4 versus its EF-hand domain deletion mutant (ΔEF), and a mutant lacking the entire cytosolic domain (AN). Upper diagram shows position of domains (demarked by residue numbers) and the corresponding deletion constructs. Transmembrane segments are shown as grey rectangles. GFP–Spitz substrate and cleavage products (green bands in the anti-GFP western) are denoted by black and white triangles, respectively. DmRho4 protein levels are shown as red bands (anti-HA western).
b. Analysis of DmRho4 loop 2, 4, and 6 mutant protein levels from Fig. 2f (calcium stimulation conditions). 
c. DmRho4 loop 2, 4, and 6 mutants were assayed for cleavage of GFP–Spitz under basal (unstimulated) conditions for ~24 h in the absence of calcium. Cleavage product (green bands, white arrowhead) was detected in media fractions for most of the mutants at levels comparable to the wild-type enzyme. Corresponding DmRho4 protein levels are shown as red bands (anti-HA western analyses). 
d. Wild-type DmRho4 and engineered variants were expressed and purified from bacteria, subjected to quantitative thermal stability analysis, and transition temperature midpoints ($T_m$) were derived (error bars, s.d. of four experimental replicates). The thermal stability of mutant DmRho4 proteases was indistinguishable from that of wild-type DmRho4.
Extended Data Figure 3 | Calcium does not regulate DmRho4 through intermolecular interactions. a, Anti-Flag co-immunoprecipitation analysis of HA–DmRho4 and APP–Spi7–Flag substrate from proteoliposomes in the presence or absence of 0.5 mM calcium. An inactive mutant of DmRho4 (S299A) was used to facilitate substrate complex isolation. The amount of HA-tagged DmRho4 co-immunoprecipitated with the Flag-tagged substrate was not affected by the presence of 0.5 mM calcium. L, load; B, bound. b, Anti-Flag co-immunoprecipitation of Flag–DmRho4 and HA–DmRho4 from proteoliposomes. HA-tagged DmRho4 did not co-immunoprecipitate with Flag-tagged DmRho4 in both the absence and presence of 0.5 mM calcium. c, Mixing a catalytic mutant (H358A) and a calcium-binding mutant (E382A) cannot rescue calcium stimulation in trans (star indicates lane where a product would be expected with the mixed single mutants).
Extended Data Figure 4 | Lateral substrate gating underlies direct regulation of intramembrane proteolysis. 
a. Thermostability analysis of single and double cysteine mutants of DmRho4 (error bars, s.d. of four experimental replicates). 
b. Average relative proportions of cleavage at the external cleavage site (orange) compared with the internal cleavage site (blue) are shown for DmRho4 in the absence (no Ca) and presence (+ Ca) of 1 mM calcium (error bars, standard error of replicate experiments). The external site was favoured in the absence of calcium (approximately 80%) while internal cleavage was preferred in the presence of calcium (approximately 70%).
c. DmRho4 loop 4 and loop 6 calcium-binding site mutants retained calcium-independent cleavage of a substrate harbouring only an external cleavage site. Full-length substrate (filled triangle) and cleavage product (open triangle) are indicated.
d. Cleavage of a substrate with external and internal cleavage sites was compared for E. coli GlpG, P. stuartii AarA, and V. cholerae Rho1 in the absence (no Ca) or presence (+ Ca) of 0.5 mM calcium. The relative proportions of cleavage at the two sites varied between the bacterial rhomboid proteases, but in no case did calcium alter the cleavage site preference.