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Allosteric regulation of rhomboid intramembrane proteolysis

Elena Arutyunova, Pankaj Panwar, Pauline M. Skiba, Nicola Gale, Michelle W. Mak and M. Joanne Lemieux

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 13 February 2014

Thank you for submitting your manuscript to The EMBO Journal. We have now considered it within our editorial team, including our Chief Editor Dr. Pulverer, and unfortunately come to the conclusion that we cannot offer publication in The EMBO Journal. We certainly appreciate the importance and interest of understanding rhomboid intermembrane proteolysis on the mechanistic level, but we are not convinced that your current detailed enzymatic analysis provides a sufficiently major advance on this topic to constitute a strong candidate for publication in The EMBO Journal. In particular, we cannot consider the main finding of preferential and cooperative cleavage of a native substrate (TatA by AarA) conceptually surprising in light of the earlier work by Strisovsky et al that already established a certain degree of substrate specificity and implicated dual recognition determinants as well as the involvement of a protease exosite. We realize that you now extend this to show that cooperative cleavage of natural substrates requires rhomboid dimers and that it may be governed by allosteric regulation based on competition experiments. However, we feel that these further indications will currently be primarily interesting to the more immediate field but less so to the wider readership of our broad journal, given that the molecular basis of this allosteric regulation and proposed exosite binding remains to be understood. In light of these reservations, I am afraid we have to consider this study presently better suited for a somewhat more biochemical publication. I am sorry to have to disappoint you on this occasion and wish you every success in publishing this work.

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Appeal 18 February 2014

Sorry for the delay in acknowledging your email. I was away from the lab and could not respond until now.

I would like to thank you for taking the time to provide a rationale behind the decision to decline having our paper out for review. While we agree with your assessment that some work has been conducted in this field, we do note that the recent paper from Dr. Urban's group in Cell clearly shows there is strong controversy in this field.

First, we address the controversial issue of specificity. We have written a very respectful paper acknowledging our colleagues, and maybe missed strong justification rationalizing our experiments. The Strisovsky manuscript (Mol Cell 2009) focused on the cleavage site required for TatA cleavage, while we address whether rhomboids have broad specificity, i.e. is there discrimination between different enzymes for a single substrate. This issue was addressed in the Dickey paper (Cell 2013). While our steady state FRET based kinetic analysis shows similar turnover rates compared to that found in proteoliposomes, they conclude rhomboids are not specific, and our work directly contradicts this finding. The rationale behind this lies in the details of the proteoliposome work. We have identified several errors, including a very basic one of using pH to halt the reaction, where it is clear from their gels (Figure 1) that cleavage is still occurring at the pH 4.0, thus leading to erroneous kinetic assessment. For the Urban paper the fact that the E. coli rhomboid was used to study TatA cleavage is odd, since AarA enzyme is including in the full assessment of specificity. Again we have tried to be very political in reflecting our colleague's work, and respect the great deal of work that went into this manuscript.

We feel the Strisovsky manuscript does not fully address the issue of an exosite since they indirectly assume an exosite exists as a result of cleavage studies using substrate TatA harbouring deletions in the transmembrane region. This deletion could have caused a variety of changes altering the structure of the protein, and by no means proved the existence of an exosite. Kinetics is truly needed to determine if allostery exists.
Our competition binding studies provide strong evidence towards this conclusion.

Kinetic analysis of intramembrane proteases is indeed very rare, as seen by the DIckey paper in high impact journal Cell. The Urban paper is strong, but falls short on identifying the main issue, how does cooperativity contribute to substrate cleavage. Our experiments disrupting the dimer directly address this issue.

Lastly, aside from presenilin, allosteric activation has not been detected for any intramembrane protease. We feel this will have a big impact for the scientific community interested in peptidases and not only those interested in membrane imbedded proteases. Rhomboids are linked to many diseases, which also expands the interest to our findings.

We hope you would reconsider sending this out for review.

Additional correspondence 24 March 2014

Thank you for patience while we have been looking into your points arguing for a reconsideration of your manuscript EMBOJ-2014-88149, "Allosteric regulation of rhomboid intramembrane proteolysis". After discussing it once more, we have no objections to seeking in-depth input from external referees, and will thus proceed with sending the study out for peer review right away. I will be in touch as soon as we are in a position to make an informed editorial decision, whose outcome - as you hopefully appreciate - I am not able to predict at this point.

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2nd Editorial Decision 16 March 2014

Your manuscript on rhomboid allosteric regulation has now been assessed by three expert referees, whose comments are copied below. All three referees achnowledge the potential importance and interest of your results and conclusions, however only one of them is currently fully convinced by all the experimental evidence in their support. On the other hand, referees 1 and 3 raise a number of overlapping major issues, in particular with the kinetic analyses and their interpretation, that would need to be decisively clarified/improved before publication may be warranted.

Should you be able to satisfactorily address all these concerns (and should the key conclusions still hold up following the requested revision work), we would be interested in considering a revised version of this manuscript further.

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Since we allow only a single round of revision, please let us know in advance should you foresee a problem in meeting this three-month deadline, and we may be able to grant an extension. Please also do not hesitate to contact me with any other questions you may have regarding this decision or the referees' comments.

Finally, when preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

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REFEREE REPORTS:

Referee #1:

Arutyunova et al present an interesting and provocative manuscript on the regulation of activity of bacterial rhomboid proteases GlpG and AarA. Rhomboids are at present the main mechanistic and structural models for investigation of intramembrane proteolysis. The mechanism of substrate recognition, catalysis and regulation of rhomboids is currently a very hot and contentious topic (Dickey et al, Cell 2013), and the contribution of Arutyunova et al. thus has the potential to be at the forefront of current research in this area. The main conclusions of this manuscript are that homotropic allosteric activation occurs in rhomboid dimers, that it involves an intramembrane exosite that forms/is functional only in the dimers, and that rhomboids exist as dimers in biological membranes, hence this allosteric behavior is biologically relevant. This is novel and intriguing, and in principle would be appropriate for EMBO J, but not all the conclusions are fully supported by the data: some experimental approaches rest on assumptions that may not be valid or are technically not well executed and require more control experiments. I would like to illustrate this by commenting on three key experimental areas of the paper and other, minor points.

1. Rhomboids AarA, ecGlpG and hiGlpG show positive cooperativity against the full-length TatA substrate.

This conclusion is a major aspect of the paper and has not previously been observed, but the data on which it rests are not fully compelling. The initial observation of positive cooperativity in substrate cleavage by the rhomboids AarA, ecGlpG and hiGlpG presented in Fig. 1 uses endpoint assay with chemiluminescent western blot readout. The experiments use conditions of high substrate conversion at low substrate concentrations, which could lead to an underestimation of initial reaction rates, potentially resulting in artefactual appearance of sigmoidal Michaelis-Menten curves

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(Purich, D. L. (2010). Enzyme Kinetics: Catalysis & Control. Amsterdam, Elsevier Science Bv., p. 218). For this kind of detailed and quantitative analysis, the Michaelis curves presented in Fig. 1 should be re-measured at lower substrate conversions. Furthermore, for it to be legitimate to use western blot readout for such detailed kinetics measurements, the chemiluminescence readout needs to be linearly proportional to the amount of antigen. Thus, a calibration curve with known amounts of purified TatA in the range of intensities shown in the figure should be shown in the supplemental material.

2. Fluorescently labeled casein is used as a model non-transmembrane substrate and the comparison of kinetic parameters of cleavage of casein and the transmembrane substrate TatA is used for qualitative interpretations of the mechanism.

As in previous studies, BODIPY modified casein is used as a model for a soluble, non-transmembrane substrate. The authors argue that the cleavage of casein does not show cooperativity because casein does not bind to the allosteric exosite of rhomboid, but this is not the only possible interpretation of the data. Alternatively, if there are more cleavage sites in casein, and those sites differ in kinetic parameters, the read-out might be a superposition of two or more Michaelis curves, which could mask the sigmoidal nature of individual curves. How many cleavage sites are there in the BODIPY-casein? How was reaction velocity in casein cleavage and kcat calculated? I.e. how was the amount of cleaved casein quantitated?
The mechanism of inhibition of casein cleavage by AarA and GlpG by the native substrate of AarA, TatA (Fig. 5), is used as a strong argument for the role of intramembrane exosite in the cooperative behavior of rhomboids. However, the curves at 3 and 10 uM psTatA in Fig. 5a do not seem to fit the data very well. Can the quality of fits with different kinetic models be shown to illustrate that the chosen one is clearly the best?

3. DM induces monomer formation, while DDM stabilizes rhomboid dimers.

The fact that DM-induced dimer dissociation is accompanied by a decrease in activity does not necessarily mean that dimer dissociation is the specific cause of the loss of activity. In other words, DM could have two independent effects, on dimer stability and on the stability of the exosite. After all, detergents and lipids have been shown to bind between TMDs 2 and 5 in GlpG structures and, as the authors suggest, this has been proposed to be the site of substrate interaction.

p. 7 - Rhomboids purified in DM have regained activity when assayed in DDM suggesting re-association of the dimers. Can the re-association of the dimers be shown by gel filtration of native PAGE? This would help to strengthen the case for a direct relationship between dimerization and activity.

p. 7 - Fig. S3 shows that DM inhibits rhomboid activity at concentrations near its CMC, which is interpreted as being due to the dissociation into monomers. But it could be just due to the increase in the concentration of the detergent phase (i.e. the phase in which the cleavage takes place), which dilutes the enzyme and substrate to lower effective concentrations. What is the effect of increasing the DDM concentration in the assay on substrate conversion/activity (similar experiment to the one shown for DM in fig. S3)?

Minor points:

p.5 - It is very surprising that the FRET-TatA substrate is not cleaved by GlpGs while the full-length TatA is. What is the authors' explanation for this?

p. 5 - Dickey et al. already showed that several rhomboids display specificity by showing that their Kms for TatA are quite similar, but kcats differ by 2 orders of magnitude. This implies that kcat/Km differ. i.e. rhomboids display specificity.

The pH dependence of rhomboid activity shown in Fig. 2C is very intriguing. The recent Cell paper by Dickey et al showed that GlpG is completely inactive against Spitz and TatA at pH 4, while it can be reactivated at pH 7.5, and this was used as a basis for a very detailed analysis of rhomboid proteolysis in lipid membranes. Arutyunova et al surprisingly show that the activity of GlpG and AarA is about the same at pH 4 and 7.5 (Fig. 2C). How do the authors explain this large

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discrepancy?

What is shown in Fig. S5a? It is not described in the supplemental figure legend.

p.3 and 10 - RHBDD2 is not thought to be a protease so it us unclear whether its oligomerisation behavior is relevant to this work.

In summary, this paper presents an intriguing and potentially important model, but in its current form the conclusions are not fully supported by the data. The authors make a convincing case that rhomboids dimerize, and that, as suggested before (Strisovsky et al 2009, Lemberg et al 2012, Dickey et al 2013), there is a binding site outside of the active site that can be called an exosite. What is less convincing is that the exosite is formed by the dimers only, or that dimerization is essential for activity. If these issues could be made more solid, this paper would be appropriate for EMBO Journal.

One approach that could provide supportive evidence would be to generate substrate variants (with mutations in their TMD) with different degree of cooperativity against different rhomboids. Since the authors say that different rhomboids vary in specificity and also in cooperativity against TatA, it implies that their exosites have different binding properties. Showing this, and how it correlates with cleavage activity, would be powerful.

Referee #2:

This is an excellent study revealing the nature of substrate recognition by rhomboids, a topic of keen interest, particularly in the wake of a new study in Cell from the lab of Sinisa Urban suggesting that substrate affinity plays no role. Here the authors have taken advantage of the ability of 3 different microbial rhomboids to dimerize in the detergent DDM but not DM along with the use of a soluble and a transmembrane substrate to address this important issue, carrying out rigorous and careful kinetic analysis along with competition experiments. The authors find that the natural substrate (psTatA) for the AarA rhomboid is not processed by AarA rhomboid unless the protease is a dimer. The monomer does not cleave psTatA but does cleave the soluble unnatural substrate casein, demonstrating dimerization is needed for substrate selectivity. Consistent with this idea, kinetic data for cleavage of psTatA fits the Hill equation better than the Michaelis-Menten equation. Competition experiments show noncompetitive inhibition by psTatA of casein cleavage by AarA rhomboid, further arguing for the existence of an exosite that regulates substrate specificity for this rhomboid. Interestingly, the E. coli rhomboid ecGlpG, which displayed a minimal Hill coefficient with the unnatural psTatA as substrate, was competitively inhibited by psTatA in this experimental paradigm. Together these results strongly argue for a dimer-dependent exosite where natural transmembrane substrate binds to regulate rhomboid activity. The results in Table 1 further refute the findings of the Urban lab, showing reasonable affinity (Km) of psTatA substrate for AarA rhomboid and ecGlpG. The publication of this study is very important to clarify the issue of the nature of rhomboid-substrate interactions.

Referee #3:

In this manuscript Arutyunova and colleagues provide enzyme kinetics data to suggest an allosteric mechanism for rhomboid intramembrane serine protease. This is an important topic because such information is currently lacking in the intramembrane protease field. Unfortunately, the authors did not provide sufficient detail about how the kinetics data were collected and treated mathematically. For example, based on what it was presented in the method and legend to Figure 1, it appears that the authors used the intensities of the lower bands ("C" cleavage product) to generate the Hill plots in panel b. This is incorrect because the INITIAL reaction rate should be used for the Hill equation to be valid. Similar problems seem to exist in other experiments. These issues need to be addressed in a revised manuscript.

1st Revision - authors' response 29 April 2014

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We thank you for considering publication of our manuscript in EMBOJ. We would like to thank all the reviewers for their insightful comments. In this manuscript we make three main points regarding rhomboid cleavage of substrates: 1) they cleave with specificity. This has not been described with kinetic studies elsewhere in the literature. This point has been recognized by reviewer #2. 2) We show positive cooperativity in psTatA binding by evolutionary diverse rhomboids, and 3) we show an exocite is involved in the cleavage. While we also show dimerization may be needed to accommodate this cleavage, after the reviewer's comments we decided to provide alternatives to our working model (Page 10). Nevertheless, we observe striking features in rhomboid-mediated substrate cleavage that suggest rhomboid protease is quite dynamic during this process. This has been emphasized in this re-submission as well. We are happy that all three reviewers find this manuscript to be of great interest: "interesting and provocative", "excellent" and "important". The comments have been beneficial in making a stronger paper for publication. We address all issues as discussed in detail below. Our responses are in italics.

Referee #1:

Arutyunova et al present an interesting and provocative manuscript on the regulation of activity of bacterial rhomboid proteases GlpG and AarA. Rhomboids are at present the main mechanistic and structural models for investigation of intramembrane proteolysis. The mechanism of substrate recognition, catalysis and regulation of rhomboids is currently a very hot and contentious topic (Dickey et al, Cell 2013), and the contribution of Arutyunova et al. thus has the potential to be at the forefront of current research in this area. The main conclusions of this manuscript are that homotropic allosteric activation occurs in rhomboid dimers, that it involves an intramembrane exosite that forms/is functional only in the dimers, and that rhomboids exist as dimers in biological membranes, hence this allosteric behavior is biologically relevant. This is novel and intriguing, and in principle would be appropriate for EMBO J, but not all the conclusions are fully supported by the data: some experimental approaches rest on assumptions that may not be valid or are technically not well executed and require more control experiments. I would like to illustrate this by commenting on three key experimental areas of the paper and other, minor points.

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1. Rhomboids AarA, ecGlpG and hiGlpG show positive cooperativity against the full-length TatA substrate.

This conclusion is a major aspect of the paper and has not previously been observed, but the data on which it rests are not fully compelling. The initial observation of positive cooperativity in substrate cleavage by the rhomboids AarA, ecGlpG and hiGlpG presented in Fig. 1 uses endpoint assay with chemiluminescent western blot readout. The experiments use conditions of high substrate conversion at low substrate concentrations, which could lead to an underestimation of initial reaction rates, potentially resulting in artefactual appearance of sigmoidal Michaelis-Menten curves (Purich, D. L. (2010). Enzyme Kinetics: Catalysis & Control. Amsterdam, Elsevier Science Bv., p. 218). For this kind of detailed and quantitative analysis, the Michaelis curves presented in Fig. 1 should be re-measured at lower substrate conversions. Furthermore, for it to be legitimate to use western blot readout for such detailed kinetics measurements, the chemiluminescence readout needs to be linearly proportional to the amount of antigen. Thus, a calibration curve with known amounts of purified TatA in the range of intensities shown in the figure should be shown in the supplemental material.

We understand the limitations of the end-point assay and to make sure that the initial rates at low substrate concentrations were not underestimated we performed the assay for AarA at a lower time point, 1hr. ***Supplemental Fig 1c*** *demonstrates that for AarA, kinetic parameters measured at 1hr were very similar to those measured at 2hr time point. Only for hiGlpG, where TatA is a poor substrate and the reaction is so slow, this could not be accommodated. We do note that cooperativity is still observed at the 1hr time saturation curve. Therefore, including the FRET-based assay results which also show cooperativity, we feel the Hill plot is not artifactual.*

We agree that product formation needs to be assessed at the linear range of the reaction. To this end, during optimization of the assay the concentration of enzymes were chosen at levels that gave linearity between the amount of generated product and time. ***Supplemental Fig 1a and b*** *demonstrate that the time used in kinetic assays for AarA and ecGlpG was linearly proportional to the observed velocity.*

*During the optimization for all kinetic assays, we checked the proportionality between the measured signal and the amount of used substrate. For the western blot analysis, we have confirmed that there is proportionality between the measured chemiluminescence signal and the amount of TatA substrate used in the reaction (****Supplemental Figure 1d****). For kinetic assay with FL-casein as substrate such optimization measurements were taken for our previous publication (*Lazareno-Saez C, Arutyunova E, Coquelle N, Lemieux MJ (2013) Domain swapping in the cytoplasmic domain of the Escherichia coli rhomboid protease. J Mol Biol 425: 1127-1142*). For FRET-based assay, the proportional relationships between the fluorescent signals and the concentration of substrate are represented on* ***Supplemental Fig. 3d.***

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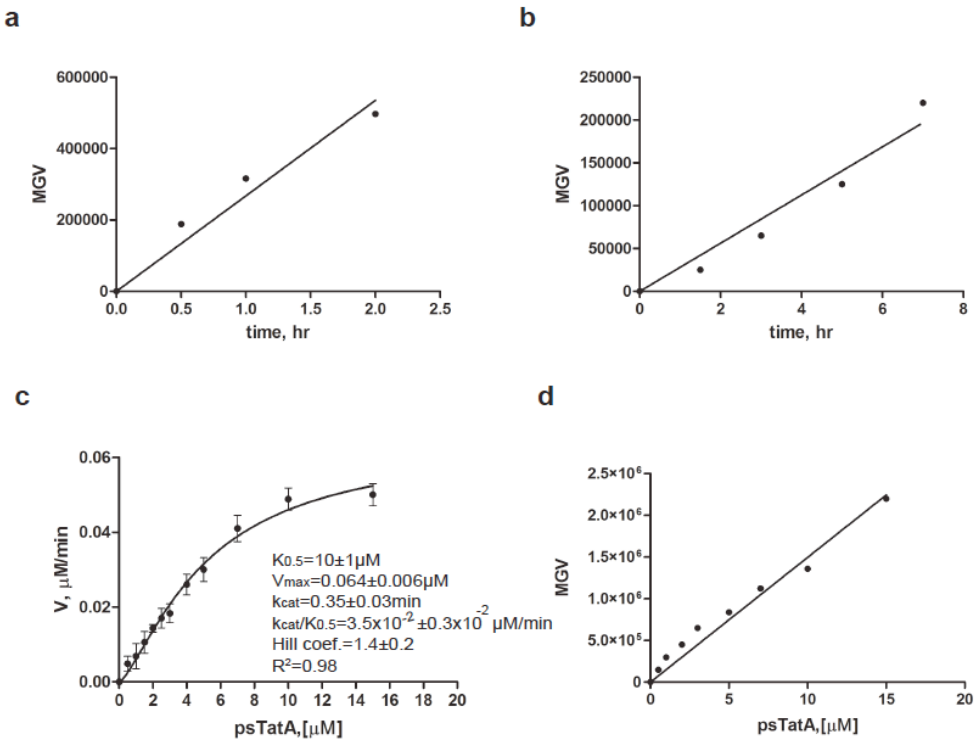

Supplemental Figure 1.

2. Fluorescently labeled casein is used as a model non-transmembrane substrate and the comparison of kinetic parameters of cleavage of casein and the transmembrane substrate TatA is used for qualitative interpretations of the mechanism.

As in previous studies, BODIPY modified casein is used as a model for a soluble, non-transmembrane substrate. The authors argue that the cleavage of casein does not show cooperativity because casein does not bind to the allosteric exosite of rhomboid, but this is not the only possible interpretation of the data. Alternatively, if there are more cleavage sites in casein, and those sites differ in kinetic parameters, the read-out might be a superposition of two or more Michaelis curves, which could mask the sigmoidal nature of individual curves. How many cleavage sites are there in the BODIPY-casein? How was reaction velocity in casein cleavage and kcat calculated? I.e. how was the amount of cleaved casein quantitated?

At this concentration, casein is cut once. All of these questions have been addressed in our previous publication: Lazareno-Saez C, Arutyunova E, Coquelle N, Lemieux MJ (2013) Domain swapping in the cytoplasmic domain of the Escherichia coli rhomboid protease. J Mol Biol 425: 1127-1142.

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From the methods of our JMB paper:
ecGlpG proteolytic activity assay using BODIPY FL casein as a substrate: The reaction mixture contained 0.179 to 8.95 μM of BODIPY FL casein (Invitrogen, Inc.), reaction buffer (50mM Tris-HCl, pH 8.0, 150mM NaCl, 10% Glycerol, 0.1% DDM) and 0.179 μM of ecGlpG. The total volume of reaction, the concentration of detergent, enzyme concentration and the time of reaction were optimized. The substrate was mixed with the reaction buffer and incubated at 37°C for 1 hour in the dark. The reaction was started with the protease. Fluorescence emission at 513nm was measured at 37°C every 5 min during 2 hours in FluoStar fluorescence microplate reader with an excitation wavelength of 503 nm. Fluorescence detection of each substrate concentration without enzyme was used as a negative control. The linear correlation between the emitted fluorescence and the amount of product was verified. SigmaPlot was used for data analysis as well as statistical analysis (One-way ANOVA). To convert the fluorescence units of generated product into μM, the cleavage reaction was performed under the same conditions, using the highest and the lowest substrate concentrations; the cleaved products were resolved with SDS-PAGE, visualized with Luminescent Image Analyzer, the amount of appeared product in μM was calculated and compared to fluorescence produced for the same substrate concentration.

The mechanism of inhibition of casein cleavage by AarA and GlpG by the native substrate of AarA, TatA (Fig. 5), is used as a strong argument for the role of intramembrane exosite in the cooperative behavior of rhomboids. However, the curves at 3 and 10 uM psTatA in Fig. 5a do not seem to fit the data very well. Can the quality of fits with different kinetic models be shown to illustrate that the chosen one is clearly the best?

Individual plots for competitive inhibition fit with 0.99-0.98 regression. To unequivocally demonstrate this is indeed non-competitive inhibition, one must conduct a global fit; in this scenario the fit is still strong but not perfect as would be expected in individual fits. See Fersht A (1998). Practical methods for kinetics and equilibria. In Structure and mechanism on protein structure, Julet MR (ed), 6, pp 191-215.: W. H. Freeman and company. We have attempted a plot with global competitive inhibition, however this cannot be fit at all due to such a poor agreement. The graph clearly shows non-competptive inhibition.

3. DM induces monomer formation, while DDM stabilizes rhomboid dimers.

The fact that DM-induced dimer dissociation is accompanied by a decrease in activity does not necessarily mean that dimer dissociation is the specific cause of the loss of activity. In other words, DM could have two independent effects, on dimer stability and on the stability of the exosite. After all, detergents and lipids have been shown to bind between TMDs 2 and 5 in GlpG structures and, as the authors suggest, this has been proposed to be the site of substrate interaction.

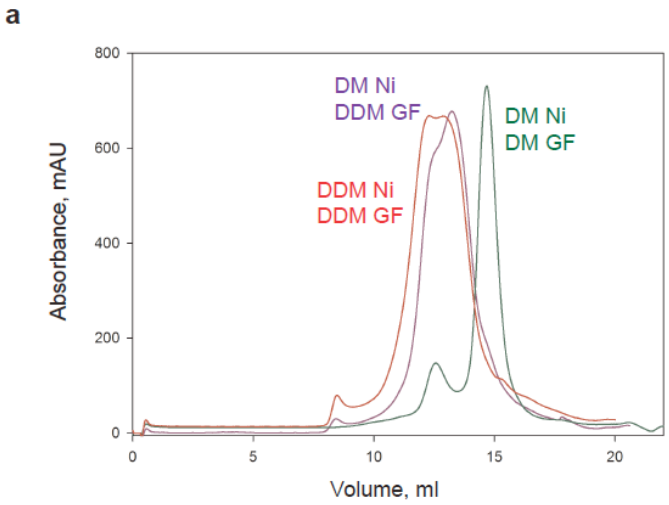
We agree with this hypothesis and although there is a loss of activity, it could be attributed to other properties of DM, even though we did not see inhibition with DM at low concentrations. In one such scenario DM in the monomeric form of the protein could bind to the exosite.

We have added information to the discussion stating these alternate possibilities for the model in the discussion section, page 11:

It is possible that dimerization is not essential for TM substrate cleavage but could enhance cleavage. An alternative interpretation to our observations is that DM not only disrupts dimerization but could also bind to the exosite at high concentrations above CMC.

p. 7 - Rhomboids purified in DM have regained activity when assayed in DDM suggesting re-association of the dimers. Can the re-association of the dimers be shown by gel filtration of native PAGE? This would help to strengthen the case for a direct relationship between dimerization and activity.

*We have included in the supplemental information gel filtration of hiGlpG rhomboid showing re-association of dimers when exchanged from DM into DDM (**Supplemental Fig. 5a**). Furthermore, we also provide dynamic light scattering analysis of the hiGlpG in both DDM and diluted in DM, since the change in the activity assay occurred after sample was added to activity buffer in DM. This experiment shows under the activity assay conditions, where a dilution was used, DM can convert dimers into monomers, and thus rules out the possibility that the appearance of the monomeric form was due to the running through the gel filtration column.*



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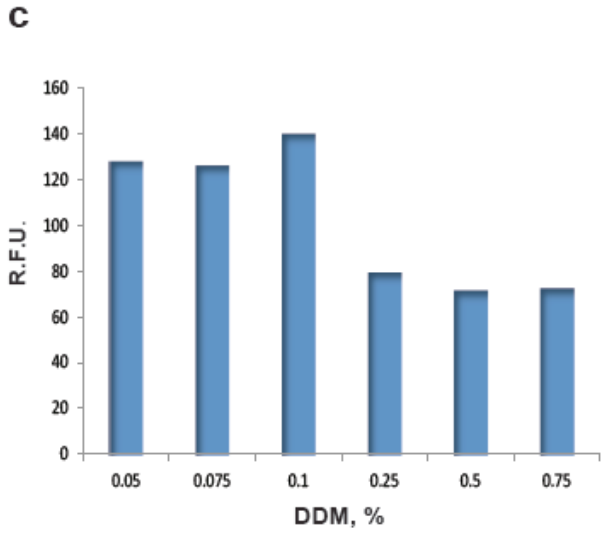
p. 7 - Fig. S3 shows that DM inhibits rhomboid activity at concentrations near its CMC, which is interpreted as being due to the dissociation into monomers. But it could be just due to the increase in the concentration of the detergent phase (i.e. the phase in which the cleavage takes place), which dilutes the enzyme and substrate to lower effective concentrations. What is the effect of increasing the DDM concentration in the assay on substrate conversion/activity (similar experiment to the one shown for DM in fig. S3)?

*We have optimized detergent concentration used in the FRET-based assay (**Supplemental Fig 3c**). Overall, reaching higher levels of DDM, 0.25% and above, does not abolish activity as was seen with the DM detergent. In Fig. S3, our aim was to show that low concentrations of DM do not inhibit rhomboid activity, thus confirming DM is not acting like a small molecule inhibitor. In this experiment (**Supplemental Fig 3c**,) DM at different concentrations, was added to activity buffer directly. The protein concentration was not diluted and samples were treated similarly to that in **Figure 1**. Therefore the loss of activity was not due to dilution of enzyme. We would expect if DM itself was inhibiting the proteolysis, this would occur at low detergent concentrations. However we only see a decrease in activity near concentrations near the CMC of DM. We do note that we observe a partial loss of activity as we reach higher DDM concentrations and this is most likely a result a stripping of lipids which are known to stabilize the membrane proteins (Lemieux et al., Protein Science 2003 (12):2748-56.).*



Minor points:

p.5 - It is very surprising that the FRET-TatA substrate is not cleaved by GlpGs while the full-length TatA is. What is the authors' explanation for this?

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There is most likely some steric hindrance with the FRET pair, and given the low $K_{0.5}$ for psTatA with ecGlpG and hiGlpG values there is insufficient binding to allow for the psTatA-FRET to be cleaved.

p. 5 - Dickey et al. already showed that several rhomboids display specificity by showing that their Kms for TatA are quite similar, but kcats differ by 2 orders of magnitude. This implies that kcat/Km differ. i.e. rhomboids display specificity.

We agree with the reviews comments completely, yet in the Dicky manuscript this was interpreted to indicate no specificity. In our manuscript we clearly define that rhomboids are specific by virtue of the k_{cat}/K_M values.

The pH dependence of rhomboid activity shown in **Fig. 2C** is very intriguing. The recent Cell paper by Dickey et al showed that GlpG is completely inactive against Spitz and TatA at pH 4, while it can be reactivated at pH 7.5, and this was used as a basis for a very detailed analysis of rhomboid proteolysis in lipid membranes. Arutyunova et al surprisingly show that the activity of GlpG and AarA is about the same at pH 4 and 7.5 (**Fig. 2C**). How do the authors explain this large discrepancy?

*At present we have no direct experimental explanation for this discrepancy. In Cell paper it is intriguing that the Spitz protein was used as a representative substrate to show inactivity at pH 4.0. If Spitz is a poor substrate one would expect a low amount of cleavage at pH 4.0. Phase diagrams with lipids at different pHs reveal in changes in their physical property; changes to curvature result in non-bilayer arrangements. Therefore, the change in cleavage is most likely a result of the physical effects on the lipids rather than on the protonation of the histidine in the enzyme itself. (*See

1. Phase diagrams and lipid domains in multicomponent lipid bilayer mixtures. Feigenson GW. Biochim Biophys Acta. 2009 Jan;1788(1):47-52. doi: 10.1016/j.bbamem.2008.08.014. Epub 2008 Sep 5.
2. pH-induced destabilization of phosphatidylethanolamine-containing liposomes: role of bilayer contact. Ellens H, Bentz J, Szoka FC. Biochemistry. 1984 Mar 27;23(7):1532-8.

What is shown in Fig. S5a? It is not described in the supplemental figure legend.

This is the activity measurement for FL-casein cleavage for the monomeric forms for each rhomboid assessed. This information has been added to the Supplemental figure legend.

p.3 and 10 - RHBDD2 is not thought to be a protease so it us unclear whether its oligomerisation behavior is relevant to this work.

Indeed RHBDD2 is an iRhom. The membrane domain segments are predicted to have similar topologies compared to active rhomboids suggesting structural similarities amongst the different rhomboid family members.

In summary, this paper presents an intriguing and potentially important model, but in its current form the conclusions are not fully supported by the data. The authors make a convincing case that rhomboids dimerize, and that, as suggested before (Strisovsky et al 2009, Lemberg et al 2012, Dickey et al 2013), there is a binding site outside of the active site that can be called an exosite. What is less convincing is that the exosite is formed by the dimers only, or that dimerization is essential for activity. If these issues could be made more solid, this paper would be appropriate for EMBO Journal.

*Our data present strong evidence of the exosite or different modes of substrate binding and recognition. The strength of the competitive binding assay is to show this kinetically which has not been done before. It is uncertain if this exosite requires the dimeric form but the current evidence supports this hypothesis. The above data, in particular where we show that rhomboids re-associate to form dimers in DDM (**Supplemental Fig. 5**), a scenario where activity is regained, strengthens this hypothesis. A second main conclusion from this study is that among three different bacterial rhomboids, there is specificity for the TatA substrate, which is in contrast to that stated in the Dickey publication (Cell 2014).*

We acknowledge that the dimerization may not be essential but it is a tantalizing result that will be the focus of future experimentation in the lab.

One approach that could provide supportive evidence would be to generate substrate variants (with mutations in their TMD) with different degree of cooperativity against different rhomboids. Since the authors say that different rhomboids vary in specificity and also in cooperativity against TatA, it implies that their exosites have different binding properties. Showing this, and how it correlates with cleavage activity, would be powerful.

We agree and this is the focus of future studies. Given the recent publication in Cell and the discrepancies with our study we found it was imperative to present our current findings.

Referee #2:

This is an excellent study revealing the nature of substrate recognition by rhomboids, a topic of keen interest, particularly in the wake of a new study in Cell from the lab of Sinisa Urban suggesting that substrate affinity plays no role. Here the authors have taken advantage of the ability of 3 different microbial rhomboids to dimerize in the detergent DDM but not DM along with the use of a soluble and a transmembrane substrate to address this important issue, carrying out rigorous and careful kinetic analysis

along with competition experiments. The authors find that the natural substrate (psTatA) for the AarA rhomboid is not processed by AarA rhomboid unless the protease is a dimer. The monomer does not cleave psTatA but does cleave the soluble unnatural substrate casein, demonstrating dimerization is needed for substrate selectivity. Consistent with this idea, kinetic data for cleavage of psTatA fits the Hill equation better than the Michaelis-Menten equation. Competition experiments show noncompetitive inhibition by psTatA of casein cleavage by AarA rhomboid, further arguing for the existence of an exosite that regulates substrate specificity for this rhomboid. Interestingly, the E. coli rhomboid ecGlpG, which displayed a minimal Hill coefficient with the unnatural psTatA as substrate, was competitively inhibited by psTatA in this experimental paradigm. Together these results strongly argue for a dimer-dependent exosite where natural transmembrane substrate binds to regulate rhomboid activity. The results in Table 1 further refute the findings of the Urban lab, showing reasonable affinity (Km) of psTatA substrate for AarA rhomboid and ecGlpG. The publication of this study is very important to clarify the issue of the nature of rhomboid-substrate interactions.

We thank the reviewer for noting our detailed and rigorous kinetic analysis. This reviewer also recognizes that we have shown specificity for rhomboid substrate recognition with reasonable Kms. We agree our works helps to clarify the current literature.

Referee #3:

In this manuscript Arutyunova and colleagues provide enzyme kinetics data to suggest an allosteric mechanism for rhomboid intramembrane serine protease. This is an important topic because such information is currently lacking in the intramembrane protease field. Unfortunately, the authors did not provide sufficient detail about how the kinetics data were collected and treated mathematically. For example, based on what it was presented in the method and legend to Figure 1, it appears that the authors used the intensities of the lower bands ("C" cleavage product) to generate the Hill plots in panel b. This is incorrect because the INITIAL reaction rate should be used for the Hill equation to be valid. Similar problems seem to exist in other experiments. These issues need to be addressed in a revised manuscript.

As this is an end point assay we optimized the time to ensure the product (the lower cleaved band) was being measured in the linear part of the reaction curve. As described above, this information has been added to the ***Supplemental figure 1****. In addition we have added information to the methods to clarify that the lower product band was measured. We feel the revised manuscript makes this point clear in both thr\e results and methods sections.*

Authors revisions:
Lastly, small errors were discovered in the legends of tables 2 and 3. These have been corrected. We have also corrected minor typographical errors in Figures 1, 2 and 4.

3rd Editorial Decision 12 May 2014

Thank you for submitting your revised manuscript for our consideration. It has now been reviewed once more by original referee 1, who considers the work significantly improved and most major concerns satisfactorily addressed. The referee does however retain one specific concern, which in my view warrants additional minor revision work, as detailed in the report below.

I hope you will be able to make the necessary further minor revisions (which I consider justified given the importance of the topic and the potential significance of the conclusions) and resubmit a final version of the manuscript as early as possible - we should then hopefully be in a position to accept the manuscript and swiftly proceed with its production for publication in The EMBO Journal.

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REFEREE REPORTS:

Referee #1:

The authors have dealt with nearly all my comments to my satisfaction, and I now consider the article suitable for publication in EMBO Journal.

I would like to mention one point though where the manuscript may benefit from a minor revison. As I have stressed before, in initial rate measurements using an end-point assay one must minimise substrate CONVERSION in the reaction (that is the percentage of substrate converted to products) to approximate initial reaction rate conditions. In practice, using a gel-based assay (with its limitations), one would probably aim for conditions such as those shown in Fig. 1c for hiGlpG, but absolutely not those used for AarA, where ~90% of the substrate has been converted to the product. To alleviate my concern (and same concern of reviewer #3), the authors now provide the analysis for AarA at a 2-fold shorter reaction time (new FigS1c). But this does not really address my concern, because substrate conversion is probably still very high in those conditions, and the corresponding gel where one could judge this is not provided. Since ecGlpG and psAarA are quite active against TatA, it seems to be relatively easy and feasible to repeat the analyses reported in Fig.1 in kinetic conditions (substrate conversion similarly as used for hiGlpG) that would even remotely approximate initial rate conditions.

However, even with this criticism, the set of data that the paper provides now, accompanied with several new control experiments in the supplemental material that are consistent with the rest of the paper, the results are now compelling enough to warrant publication in EMBO J. It will be an interesting and discussion-stirring paper.

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2nd Revision - authors' response 16 May 2014

We thank you for considering publication of our manuscript in EMBOJ. We would like to thank the first reviewer for further comments. We have conducted the kinetic analysis of AarA cleavage of psTatA with a 15 min reaction time. Indeed we do observe a slight increase in kinetic turnover and efficiency. This has been reflected in Table 1 and on page 4 in the manuscript. Overall this does not change the overall outcome of the results but, as we agree with the reviewer 1, ensures we are measuring rates at the initial linear phase of the curve for this end-point assay.

We feel that the manuscript is now ready for publication in EMBOJ and thank you for your assistance.

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