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Cleavage of the Jaw1 C-terminal region enhances its augmentative effect on the Ca^{2+} release via IP_3 receptors

Takuma Kozono, Chifuyu Jogano, Wataru Okumura, Hiroyuki Sato, Hitomi Matsui, Tsubasa Takagi, Nobuaki Okumura, Toshifumi Takao, Takashi Tonozuka and Atsushi Nishikawa
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MS TITLE: Cleavage of the Jaw1 C-terminal region enhances its augmentative effect on the Ca2+ influx via inositol 1,4,5-trisphosphate receptors

AUTHORS: Takuma Kozono, Chifuyu Jogano, Wataru Okumura, Hiroyuki Sato, Hitomi Matsui, Tsubasa Takagi, Nobuaki Okumura, Toshifumi Takao, Takashi Tonozuka, and Atsushi Nishikawa
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We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers raise a number of criticisms that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

In this study, the authors investigate in great detail and in a very logical way the characteristics of the Jaw1 cleavage site, the cleavage mechanism involved and the functional role of the cleaved protein with respect to IP3R-dependent Ca2+ signaling.
Very nice work and potentially highly significant, as it concerns a novel stimulatory pathway acting on the IP3R.

Comments for the author

The manuscript is very well written and the research very well performed, using the appropriate techniques, including the development and use of mutant proteins and KO cells. I have only a few major concerns, and a larger number of minor comments, but which I expect to be also taken into account.

Major concerns.
1) Figure 6 could be improved. In panels B and C the upper band (with opsin) are not that easily visible. I do not know whether it is possible to obtain stronger signals and/or to present a figure with better resolution? If not, I would advocate to identify with tick marks on the blot the location of the various bands and to provide some more explanations in the relevant paragraph. Finally the number of independently performed experiments should be indicated.
Concerning panel 6E, it should be made clear somewhere why another Ab was used and why the MW of the cleaved Jaw1 is here so much smaller.

2) Lines 223-224 and figure S3: is the conclusion that the interaction with the IP3Rs is maintained for the AASS mutant fully justified? How many times this IP had been independently performed? Was an accurate quantification performed?
Visually it looks to me that in the absence of cleavage, much less type 2 and type 3 are immunoprecipitated. Incidentally, a better interaction of cleaved Jaw1 with IP3R2 and 3 might provide an explanation for the larger Ca2+ signal the authors observe with Jaw1 IE compared to AASS IE. To obtain a cleaner experimental system one can envisage (for the future, not for present paper) experiments in which the expression of the AASS mutant is compared in KO cells with that of Jaw1 1-509 as well for IP experiments as for Ca2+ signaling.

3) Concerning the Ca2+ traces: (a) the results obtained with 5 μM ATP (figure S4) are nearly identical to those obtained with 100 μM ATP (please note: on line 226, 100 mM ATP is indicated, which is impossible) and that is because 100 μM ATP is already a supramaximal dose. Authors should therefore best focus on the traces obtained with 5 μM ATP instead of putting those in the supplementary materials. (b) I appreciate that the authors are trying to distinguish various types of Ca2+ signals as no response, single peak, oscillating (not oscillated) and steady reduction type, but to convey the message correctly a quantification must be performed, i.e., in each condition the percentage of each type of Ca2+ signals occurring should be given. (c) Lines 53: I am not sure the definition of steady reduction holds, as it looks like the signal could go below 125% of the initial value though does this much more slowly. (d) Line 535: the definition of “no response” is made unnecessarily complicated. Only stating that no peak appeared in a time period of 6 min should be sufficient.

Minor concerns.
1) The authors consistently speak about Ca2+ influx, even in the title. This is a misnomer: in the Ca2+ field, Ca2+ influx means Ca2+ entering the cell from the extracellular space. What the authors investigate here is however Ca2+ release (from the ER). This is important, as in a few cells IP3Rs were described in the plasma membrane and are thus involved in Ca2+ entry.
2) IP3 is inositol 1,4,5 trisphosphate. Using the word triphosphate is erroneous as it would means that the 3 phosphate groups are linked to each other as in ATP (-P-P-P) which is not the case.
3) Line 101 and figure 1C: the abbreviations used (K1 to K5) should be explicitly explained.
4) Line 137: can the authors indicate the size of the NIDR (number of a.a. or MW)? And of the PA tag?
5) Line 139: please rephrase “in the point of the steric hindrance” as this makes not much sense to me
6) Line 148: I would tone down this sentence as the vast majority of the a.a. in that region are also conserved

7) Lines 165-167: the authors may here even wish to strengthen the sentence, as the Flag SEC11C is not rescuing even in spite of its much higher expression levels
8) Lines 181-185: it would be much more logical to present the protein expression levels just after the mRNA levels, but before the quantification of the Jaw1 cleavage.
9) Line 305-306: “prudent” is certainly not the word needed. On the contrary, it is in my opinion highly necessary to investigate the role of Jaw1 cleavage on Ca2+ signaling in various cell types!
10) Line 516: the use of probenecid should be explained, as I do not think this is routinely used

Reviewer 2

Advance summary and potential significance to field

The authors report a series of experiments examining aspects of the function of c/LRMP, a tail-anchored (TA) protein with 39 carboxyl (C)-terminal amino acids that is oriented to the lumen of the endoplasmic reticulum and outer nuclear membrane. outer nuclear membrane. They previously found that Jaw1, as a member of the KASH protein family, plays a role in maintaining nuclear shape via its C-terminal region and that Jaw1 functions as an augmentative effector of Ca2+ influx by interacting with the inositol 1,4,5-trisphosphate receptors. The C-terminal region is partially cleaved, so that in the cell in uncleaved and cleaved forms. The mechanism of cleavage has not been determined. They now demonstrate that the C-terminal region of Jaw1 is cleaved after its insertion by the signal peptidase complex (SPC) and that the SPC with the catalytic subunit SEC11A, but not SEC11C, specifically cleaves Jaw1. Finally they show that the cleavage event enhances the augmentative effect of Jaw1 on the Ca2+ release ability of the inositol 1,4,5-trisphosphate receptors.

Comments for the author

The manuscript provides convincing evidence for the conclusions but several issues require addressing.

Major comments.
1. The manuscript is spoiled by the large number if acronyms used, some only once or twice in the manuscript. The readability and broader interest would be much improved if fewer acronyms were used, and used only for the major proteins that are frequently mentioned in the manuscript. In this context much of the detail provided in the introduction could be summarised more briefly without specifically naming the many proteins involved in the pathways mentioned.

2. A schematic illustration of the pathways involved would be helpful.

3. No quantitative data is provided for the results shown in Figure 1, 2, 3 and 6, even though much of the direction of the study is dictated by the results presented in these figures. The relative amounts of material in each of the bands should be measured and data presented as mean and errors, with numbers of observations given, as it is for data in Figures 4, 5 and 7.

4. I wonder why the authors choose to use the ITPR for to describe the inositol 1,4,5-trisphosphate receptor. Unless there is a nomenclature change that I have missed, the common abbreviation is IP3 receptor or IP3R.

5. The conclusions from Figure 7 appear to assume that the only process governing the Ca2+ transient is release through the inositol 1,4,5-trisphosphate receptors? The possible contribution from other proteins that might alter the transient, such a pumps, transporters and Ca2+ buffers should also be considered along with the possible effects of Jaw1 on these processes. What is the functional significance of the oscillations described?

Minor comments
6. The statement “The human SPC consists of three accessory subunits” appears to be contradicted by the rest of the sentence “Signal peptidase complex subunits 1–3 (SPCS1–3) and SEC11A or SEC11C as a catalytic subunit” which implies that there are only 2 subunits in two different combinations, i.e. (SPCS1–3 plus SEC11A) or (SPCS1–3 plus SEC11C). Please clarify.

7. Lines 121-124 “However, both the uncleaved and cleaved forms of Jaw1 were detected in all the lanes of mutants, comparable with FLAG Ms Jaw1 although there were subtle differences in the proposition of the two forms among them (Fig. 2B). Here, we focused on two alanine residues (509/510) that were not substituted in the above mutants.” It is not clear why the authors decided to focus on the two alanine residues. Please clarify. Sould “here” be “therefore”???

8. Line 137 “The NIDR was fused to increase….”. Fused with what?

9. Line 138-140 “Furthermore, we expected that the addition of this intrinsically disordered region, which was described in our previous study 139 (Kozono et al., 2021), would be better than the addition of structural tags in the point of the steric hindrance in the protein insertion into the ER membranes due to its structural flexibility”. Better for what?

10. Line 164 “To validate this, the rescue experiment was performed”.
Please specify the general detail of the rescue experiment.

First revision

Author response to reviewers' comments

Response to Reviewer 1

We would like to express our deepest gratitude for carefully reading our manuscript and giving constructive comments. We describe the changes made in response to your comments point-by-point as stated below. We would appreciate it if you check them.

Reviewer 1 Advance Summary and Potential Significance to Field...

-In this study, the authors investigate in great detail and in a very logical way the characteristics of the Jaw1 cleavage site, the cleavage mechanism involved and the functional role of the cleaved protein with respect to IP3R-dependent Ca2+ signaling. Very nice work and potentially highly significant, as it concerns a novel stimulatory pathway acting on the IP3R.

Reviewer 1 Comments for the Author...

-The manuscript is very well written and the research very well performed, using the appropriate techniques, including the development and use of mutant proteins and KO cells. I have only a few major concerns, and a larger number of minor comments, but which I expect to be also taken into account.

Reply: Thank you very much for summarizing our manuscript. We agree with your assessment regarding our arguments. In order to solve your concern, we carried out an additional experiment and revised the manuscript as follows. We would appreciate it if you check them.

Major concerns.

-1) Figure 6 could be improved. In panels B and C the upper band (with opsin) are not that easily visible. I do not know whether it is possible to obtain stronger signals and/or to present a figure with better resolution? If not, I would advocate to identify with tick marks on the blot the location of the various bands and to provide some more explanations in the relevant paragraph. Finally, the number of independently performed experiments should be indicated. Concerning panel 6E, it should be made clear somewhere why another Ab was used, and why the MW of the cleaved Jaw1 is here so much smaller.

Reply: Thank you so much for your valuable advice. We put the tick marks on the blots in Figures 6B-6E, since it was difficult to make all the bands stronger and higher resolution without saturation. With this change, we added some explanations to the figure legend and the manuscript. Furthermore, we performed all blottings of Figure 6 three times with similar results. To describe these, we corrected the manuscript as follows.

>Line 216-217: “, thus, uncleaved Jaw1 with *N*-linked glycosylation and pre-inserted Jaw1, respectively” was added.

>Line 791-792: “B-E) Open triangles, the bands of ER-inserted uncleaved Jaw1 with *N*-linked glycosylation; closed triangles, the bands of the pre-inserted Jaw1(black) and cleaved Jaw1 (gray).” was added.

>Line 792-793: “The representative blot images from three independent experiments with similar results are shown.” was added.

Reply: In Figure 6E, FlpIn T-REx HEK293 expressing Hu Jaw1 opsin, without N-terminal HA and FLAG tandem tags unlike HA and FLAG tandem tagged Jaw1 opsin (FLAG Hu Jaw1 opsin) in other panels of Figure 6, was used. Therefore, an anti-Jaw1 Coil antibody but not an anti-FLAG antibody was used. On the other hand, concerning the band position of the cleaved Jaw1, it seems not to be much smaller, since the tick marks in Figure 6E indicate the almost same ratio of the interval among the three bands as that in other panels of Figure 6. Perhaps, it might be subtly affected by the gel concentration or loss of N-terminal tags, but it does not matter for our interpretation. To mention the reason why another Ab was used in Figure 6E, we corrected the manuscript as follows.

>Line 789-791: “Hu Jaw1 opsin expressed by the treatment with Dox in this cell does not bear the N- terminal tags unlike FLAG Hu Jaw1 opsin in (B) and (D), thereby, an anti-Jaw1 Coil antibody but not an anti-FLAG mouse antibody was used.” was added.

-2) Lines 223-224 and figure S3: is the conclusion that the interaction with the IP3Rs is maintained for the AASS mutant fully justified? How many times this IP had been independently performed? Was an accurate quantification performed? Visually it looks to me that in the absence of cleavage, much less type 2 and type 3 are immunoprecipitated. Incidentally, a better interaction of cleaved Jaw1 with IP3R2 and 3 might provide an explanation for the larger Ca2+ signal the authors observe with Jaw1 IE compared to AASS IE. To obtain a cleaner experimental system one can envisage (for the future, not for present paper) experiments in which the expression of the AASS mutant is compared in KO cells with that of Jaw1 1- 509 as well for IP experiments as for Ca2+ signaling.

Reply: Thank you for your important comment. We carried out this CoIP assay in two independent experiments with similar results, but it seems to us that no difference in the affinity of AASS mutant with any type of IP3Rs compared to that of wildtype Jaw1. Even if there is a subtle difference, CoIP assay is difficult to quantify the affinity precisely. To solve this issue, a more stoichiometric analysis to evaluate the protein-protein interaction would be required. We tried this in future including Jaw1 1-509. Again, thank you very much for your valuable suggestion. We corrected the manuscript as follows.

>Line 239: “markedly” was added.

>Figure legends in Fig. S4: “The representative blot images from two independent experiments with similar results were shown.” was added.

-3) Concerning the Ca2+ traces: (a) the results obtained with 5 µM ATP (figure S4) are nearly identical to those obtained with 100 µM ATP (please note: on line 226, 100 mM ATP is indicated, which is impossible) and that is because 100 µM ATP is already a supramaximal dose. Authors should therefore best focus on the traces obtained with 5 µM ATP instead of putting those in the supplementary materials. (b) I appreciate that the authors are trying to distinguish various types of Ca2+ signals as no response, single peak, oscillating (not oscillated) and steady reduction type, but to convey the message correctly a quantification must be performed, i.e., in each condition the percentage of each type of Ca2+ signals occurring should be given. (c) Lines 53: I am not sure the definition of steady reduction holds, as it looks like the signal could go below 125% of the initial value though does this much more slowly. (d) Line 535: the definition of“no response” is made unnecessarily complicated. Only stating that no peak appeared in a time period of 6 min should be sufficient.

(a)

Reply: Thank you for your comments. As you mentioned, the results obtained with 5 µM ATP and 100 µM ATP shown in the curves in Fig. 7G and Fig. S5A, respectively, are apparently similar. However, the averages of the maximum amplitude in Fig. 7H (Jaw1 KO, 6.88; Jaw1 IE, 8.158; AASS IE, 7.846) were higher than those in Fig. S5B (Jaw1 KO, 6.595; Jaw1 IE, 7.537; AASS IE, 7.07). Furthermore, the averages of AUCs in Fig. 7I (Jaw1 KO, 145.7; Jaw1 IE, 345.0; AASS IE, 296.2) were also higher than those in Fig. S5C (Jaw1 KO, 149.2; Jaw1 IE, 250.7; AASS IE, 230.0), especially in the groups of Jaw1 IE and AASS IE cells. This result means that the stimulation with 100 µM ATP causes longer signal retention in the cells expressing Jaw1 than that with 5 µM ATP. Thus, the stimulation with 100 µM ATP is not a supramaximal dose. Furthermore, the difference in the AUCs between Jaw1 IE and AASS IE was biggest under the condition of the stimulation with 100 µM ATP. Therefore, we decided to focus on the results

obtained with 100 μM ATP in the main figures since it is easier to understand the phenotypic difference between Jaw1 and ASSS IE cells. In addition, we appreciate your important notice regarding the incorrect typo of the concentration. We corrected the manuscript as follows.
>Line 244: “100 mM ATP” was changed into “100 μM ATP”.
(b) (c) (d)

Reply: Thank you for your valuable suggestion. According to your advice, we counted the Ca^{2+} signal occurrence in each group of each condition. Although all of the Ca^{2+} flux types (400 cells in total in each group) were counted, the scores in the types of “No response” and “Single” were all “0” and “1”, respectively. Therefore, we prepared the graphs showing only the scores in the types of “Oscillation” and “Steady Reduction”. Furthermore, under the condition of stimulation with 0.5 μM ATP (Fig. S4J) in the initial submission), the percentages of “Oscillation” and “Steady Reduction” were very low and the numbers were not sufficient to evaluate the differences. Therefore, we quantified only the results acquired from the condition of stimulation with 100 μM ATP (Fig. 7K in the initial submission) and 5 μM ATP (Fig. S4E in the initial submission). We would appreciate it if you check the graphs of “Supplementary Information for reviewers only”. As shown in the graphs, the Ca^{2+} signal occurrence in the type of “Steady Reduction” in Jaw1 IE and AASS IE cells tends to be more than that of Jaw1 KO cells. Thus, the expression of Jaw1 makes the Ca^{2+} signaling in the cells a more oscillating state, consistent with our conclusion in our previous report. On the other hand, no prominent differences in the Ca^{2+} signal occurrence in each type among Jaw IE and AASS IE cells were seen. The results do not partially fit with the results of the classification (Fig. 7K and S4E in the initial submission). These contradictions will sometimes confuse the readers. On the other hand, Figure 7G-I is sufficiently supporting our conclusion that the Ca^{2+} signaling in the cells expressing AASS mutant is slightly lower than those expressing wildtype Jaw1, without the graphs of the classification. Therefore, we decided to delete the results of the classification and corrected the manuscript as follows to convey our conclusions clearly.

Furthermore, the Ca^{2+} signal occurrence in the type of “Steady Reduction” is much higher than that of “Oscillation” in all the groups. It is consistent with our previous report that the high oscillating cells tend to be the type of “Steady Reduction”. Thus, we believe that our definition of “Steady Reduction” for the classification of Ca^{2+} flux types worked well. Again, thank you very much for your valuable and important suggestions.

>Line 248-257: “The kinetic curves of Ca^{2+} influx were different at the single cell level, as previously reported (Okumura et al., 2022).....Thus, the Ca^{2+} influx in AASS IE cells is not as highly oscillated as that of Jaw1 IE cells.” was deleted.
>Line 257: “Furthermore, these” was changed into “These”.

>Line 557-566: “For the classification of the Ca^{2+} influx types,..... To calculate the ratio, 100 cells in each of four measurements were taken and used as data.” Was deleted.
>Figure 7J, 7K, S4D, S4E, S4I, S4J was deleted and the corresponding figure legends were corrected.
>Line 801: “G-K)” was changed into “G-I)”.
>Line 894-806: “J) Five representative curves of relative Fluo-4 intensity in Jaw1 KO, Jaw1 IE, and AASS IE cells. K) Classification of the Ca^{2+} influx type in Jaw1 KO, Jaw1 IE, and AASS IE cells. Four independent experiments (n = 100).” was deleted.
>Line 806: “C-F, H, I, K)” was changed into “C-F, H, I)”.
>Line 806: “*P < 0.05; **P < 0.01;” was deleted.
>Line 808: “(C–E, H, I, K)” was changed into “(C–E, H, I)”.

-Minor concerns.

-1) The authors consistently speak about Ca^{2+} influx, even in the title. This is a misnomer: in the Ca^{2+} field, Ca^{2+} influx means Ca^{2+} entering the cell from the extracellular space. What the authors investigate here is however Ca^{2+} release (from the ER). This is important, as in a few cells IP3Rs were described in the plasma membrane and are thus involved in Ca^{2+} entry.

Reply: Thank you for your important advices. We corrected the manuscript as follows.

>Line 2, 71: "Ca^{2+} influx" was changed into "Ca^{2+} release".
>Line 30-31: "Ca^{2+} influx" was changed into "Ca^{2+} release from the endoplasmic reticulum".
>Line 96-97, 226, 236, 260, 262, 270, 273, 319, 323, 333: "Ca^{2+} influx" was changed into "Ca^{2+} release from the ER".
>Line 237, 242, 246, 258: Ca^{2+} influx was changed into "Ca^{2+} flux".

-2) IP3 is inositol 1,4,5 trisphosphate. Using the word triphosphate is erroneous as it would means that the 3 phosphate groups are linked to each other as in ATP (-P-P-P) which is not the case.

Reply: Thank you for your important notice. We noticed the mistake in Line 62. However, we deleted the synonym (IRAG2) of Jaw1 according to another reviewer's request to make "Introduction" brief without many detailed explanations and abbreviations.

-3) Line 101 and figure 1C: the abbreviations used (K1 to K5) should be explicitly explained.

Reply: Thank you for your advice to improve the manuscript. We revised Figure 1C to indicate that the luminal region of Jaw1 was replaced with that of other KASH proteins. Furthermore, we corrected the manuscript as follows.
>Figure 1C: "Luminal region" and "Luminal region of other KASH proteins (K1-K5)" were shown on the schematic representation of Ms Jaw1 and Ms Jaw1 KASH chimera (K1-K5), respectively.
>Line 107-108: "K1-K5" was changed into "KASH1–KASH5 (K1–K5)".

-4) Line 137: can the authors indicate the size of the NIDR (number of a.a. or MW)? And of the PA tag?

Reply: Thank you for your advice to improve the manuscript. The predicted molecular weights of NIDR and PA tag are 19.8 kDa (pI 4.18) and 1.2 kDa (pI 3.49), respectively. Therefore, the predicted molecular weight of Fragment 2 is 24.3 kDa (pI 4.16), in case Ms Jaw1 NIDR PA is cut at the site between two alanine residues (509/510). Although the band of Fragment 2 on SDS-PAGE appeared at a higher position than predicted (Fig. 3G,3H), it is probably due to its low pI derived from NIDR and PA tag. Actually, we have previously shown that GFP tagged Ms Jaw1 N-terminal region (corresponding to NIDR) migrates slowly and the band appears at the position 10-20 kDa higher than the predicted molecular weight (Kozono et al., *Sci. Rep.* 2021). To validate the band position of Fragment 2, we also used Ms Jaw1 PA without NIDR as a control, and defined the specific bands in the lane of Ms Jaw1 NIDR PA as its full length (closed gray triangle in Fig. 3G, 3H) and Fragment 2 (closed black triangle in Fig. 3G, 3H), respectively. To describe these, we corrected Figure 3F and the manuscript as follows.
>Figure 3F: We added the number of amino acids on the schematic representation of the Ms Jaw1 NIDR PA.
>Line 143: "(19.8 kDa)" and "(1.2 kDa)" were added.
>Line 151-153: "Although the band corresponding to Fragment 2 on SDS-PAGE appeared at a higher position than predicted, it is probably due to the low pI derived from NIDR (pI 4.18) and PA tag (pI 3.49)." was added.

-5) Line 139: please rephrase "in the point of the steric hindrance" as this makes not much sense to me

Reply: Thank you for your comment. We apologize for the insufficient explanation. We first prepared the plasmid encoding Ms Jaw1 GFP in which the GFP tag was fused with the C-terminal region of Jaw1 (Ms Jaw1 GFP) instead of NIDR to increase the molecular mass of Fragment 2 on the electrophoresis gel. However, the protein expression level of Ms Jaw1 GFP was too low to be collected by immunoprecipitated. The TA proteins are often inserted into the ER membrane after the translation unlike the membrane protein with the N-terminal orientation into the lumen. Therefore, we estimated that the existence of a C-terminal GFP tag, a structural tag, might be a barrier to the insertion into the ER membrane. Therefore, we expected that the addition of the intrinsically disordered region (NIDR), a structurally flexible region, would be better for the protein insertion into the ER membrane than GFP. However, we have no sufficient evidence of whether the NIDR would be better for that. Furthermore, this explanation will confuse readers and it would be sufficient only to explain the objective of the NIDR addition to increase the molecular mass of

Fragment2. Therefore, we decided to remove this explanation. On the other hand, we added a brief description of the NIDR with our reference.
>Line 144-145: “, a structural flexible region previously described in our study (Kozono *et al.*, 2021),” was added.
>Line 146-149: “Furthermore, we expected that the addition of this intrinsically disordered region, which was described in our previous study (Kozono *et al.*, 2021), would be better than the addition of structural tags in the point of the steric hindrance in the protein insertion into the ER membranes due to its structural flexibility.” was deleted.

-6) Line 148: I would tone down this sentence as the vast majority of the a.a. in that region are also conserved

Reply: Thank you for your comment. As you indicate, the conserved residues in this alignment (Figure 3H) are not only two alanine residues (509/510). The sentence would make the readers tone down. The alignment among Jaw1 in several species might not be very important data. Therefore, we removed Figure 3H. With this change, we corrected the manuscript as follows.
>Line 158: “Interestingly, these alanine residues are completely conserved among species (Fig. 3H).” was deleted.
>Line 746-749: “H) Alignment of amino acid sequences corresponding to the transmembrane domain (gray) and luminal region among Jaw1 in several species. The conserved amino acids are in yellow. The UniProt accession numbers for each gene are presented with the species name. The number of conserved amino acids in the luminal region is shown to the right. Asterisks show the two alanine residues.” was deleted.

-7) Lines 165-167: the authors may here even wish to strengthen the sentence, as the Flag SEC11C is not rescuing even in spite of its much higher expression levels

Reply: Thank you very much for your valuable suggestion. To strengthen that, we corrected the manuscript as follows.
>Line 177-178: “in spite of its much higher expression level compared to FLAG SEC11A” was added.

-8) Lines 181-185: it would be much more logical to present the protein expression levels just after the mRNA levels, but before the quantification of the Jaw1 cleavage.

Reply: Thank you for your valuable suggestion. We rearranged Figure5 to show the protein expression levels (Fig. 5D–G) just after mRNA levels (Fig. 5A–C), but before the quantification of the Jaw1 cleavage (Fig. 5D, 5H). The blot image of Jaw1 Coil in Figure 5D was moved between those of SEC11A and GAPDH. With this rearrangement of data, we corrected the manuscript as follows.
>Line 190-193: “Under this condition, the protein expression level of SPC components and the percentage of cleaved Jaw1 was evaluated. The protein expression levels of the SPC components: SPCS1, SPCS2, and SEC11A were also significantly reduced in SPCS1 KD#2 cells, SPCS2 KD #2 cells, and SPCS3 KD#1 and #2 cells (Fig. 5D–G).” was added.
>Line 193-194: “Consistent with the knockdown efficiency” was changed into “Furthermore”.
>Line 195: “Fig. 5D,E” was changed into “Fig. 5D,H”
>Line 195-197: “Importantly, the protein expression levels of the SPC components: SPCS1, SPCS2, and SEC11A were also significantly reduced in SPCS1 KD#2 cells, SPCS2 KD #2 cells, and SPCS3 KD#1 and #2 cells (Fig. 5D,F–H).” was deleted.
>Line 197-198: “the SPC accessory subunits are also involved in Jaw1 C-terminal cleavage, and that” was deleted.
>Line 199-200: “, and that the SPC accessory subunits are also involved in Jaw1 C-terminal cleavage” was added.
>Line 772-773: “E) Graph showing the percentage of Jaw1 C-terminal cleavage in (D).” was deleted.
>Line 773: “F-H” was changed into “E-G”.
>Line 773-774: “(F)”, “(G)”, and “(H)” was changed into “(E)”, “(F)”, and “(G)”, respectively.
>Line 774: “H) Graph showing the percentage of Jaw1 C-terminal cleavage in (D).” was added.

-9) Line 305-306: “prudent” is certainly not the word needed. On the contrary, it is in my opinion highly necessary to investigate the role of Jaw1 cleavage on Ca2+ signaling in various cell types!

Reply: Thank you for your advice to improve the manuscript. We corrected the manuscript as follows.
>Line 332: “prudent” was changed into “required”.

-10) Line 516: the use of probenecid should be explained, as I do not think this is routinely used.
Reply: Thank you for your advice to improve the manuscript. In calcium assay, probenecid, an organic anion transporter inhibitor, is often used to prevent the leakage of the loaded calcium dye into the extracellular environment, which allows a stable amount of loaded dye in the cells during the experiment. In our study, probenecid was used according to the manufacturer’s instructions. To describe it, we corrected the manuscript as follows.
>Line 546-548: “Probenecid, an organic anion transporter inhibitor, is often used for calcium assay to prevent the leakage of the loaded dye into the extracellular environment, which allows a stable amount of loaded dye in the cells during the experiments” was added.

Response to Reviewer 2

We would like to express our deepest gratitude for carefully reading our manuscript and giving constructive comments. We describe the changes made in response to your comments point-by-point as stated below. We would appreciate it if you check them.

Reviewer 2 Advance Summary and Potential Significance to Field...
-The authors report a series of experiments examining aspects of the function of c/LRMP, a tail-anchored (TA) protein with 39 carboxyl (C)-terminal amino acids, that is oriented to the lumen of the endoplasmic reticulum and outer nuclear membrane. They previously found that Jaw1, as a member of the KASH protein family, plays a role in maintaining nuclear shape via its C-terminal region and that Jaw1 functions as an augmentative effector of Ca2+ influx by interacting with the inositol 1,4,5-trisphosphate receptors. The C-terminal region is partially cleaved, so that in the cell in uncleaved and cleaved forms. The mechanism of cleavage has not been determined. They now demonstrate that the C-terminal region of Jaw1 is cleaved after its insertion by the signal peptidase complex (SPC) and that the SPC with the catalytic subunit SEC11A, but not SEC11C, specifically cleaves Jaw1. Finally, they show that the cleavage event enhances the augmentative effect of Jaw1 on the Ca2+ release ability of the inositol 1,4,5- trisphosphate receptors.

Reviewer 2 Comments for the Author...
-The manuscript provides convincing evidence for the conclusions but several issues require addressing.
Reply: Thank you very much for summarizing our manuscript. We agree with your assessment regarding our arguments. In order to solve your concern, we carried out an additional experiment and data analysis and revised the manuscript as follows. We would appreciate it if you check them.

Major comments.
-1. The manuscript is spoiled by the large number if acronyms used, some only once or twice in the manuscript. The readability and broader interest would be much improved if fewer acronyms were used, and used only for the major proteins that are frequently mentioned in the manuscript. In this context much of the detail provided in the introduction could be summarised more briefly without specifically naming the many proteins involved in the pathways mentioned.
Reply: Thank you for your important comments to improve our manuscript. To summarize the section “Introduction” more briefly, we first removed the explanations of Signal peptidase (SPase) and Signal peptides (SPs). Only information on the SPC would be sufficient to understand our results. Instead, the sentence to describe the characters of signal peptides was moved to the section of Discussion.
>Line 76-80: “Signal peptidase (SPase) is conserved in prokaryotes and eukaryotes. It removes signal peptides (SP), targeting sequences for protein destination (Paetzel et al., 2002). SPs typically contain
three distinctive regions: the n-region, which carries a positive charge; the h-region, which comprises abundant hydrophobic residues; and the c-region, which contains polar residues. SPase processes the c-region that contains small uncharged residues at the −1 and −3 positions to the cleavage site (Paetzel et al., 2002).” was deleted.
>Line 276-278: “Signal peptides typically contain three distinctive regions: the n-region,

which carries a positive charge; the h-region, which comprises abundant hydrophobic residues; and the c- region, which contains polar residues." was added.
>Line 278: "As mentioned above, the" was changed into "The".
Furthermore, we removed the sentence describing the PPPX motif of KASH proteins since it is too much detail, and is not necessary to understand our results. With this change, we also removed the reference.
>Line 56-58: "Particularly, the PPPX motif at the C-terminal of the KASH domain is highly conserved in all KASH proteins and is crucial for the interaction with SUN proteins (Cain et al., 2018; Morimoto et al., 2012; Sosa et al., 2012)." was deleted.
>Line 696-697: Sosa, B. A., Rothballer, A., Kutay, U. and Schwartz, T. U. (2012). LINC complexes form by binding of three KASH peptides to domain interfaces of trimeric SUN proteins. Cell 149, 1035-1047.
To use fewer acronyms, we corrected the manuscript as follows.
>Line 27: "/LRMP," was deleted.
>Line 62-63: ", also known as lymphoid-restricted membrane protein (Lrmp) or inositol 1,4,5- trisphosphate receptor-associated 2 (IRAG2)," was deleted.
>Line 52: "(ONM)" was deleted.
>Line 63: "ONM" was changed into "outer nuclear membrane".
>Line 55: "(PNS)" was deleted.
>Line 65, 67: "PNS" was changed into "perinuclear space".
>Line 81: "SPase" was changed into "signal peptidase".
>Line 81, 278, 285: "SPs" was changed into "signal peptides".
>Line 82-83: ", in the dependent manner of signal recognition particle (SRP) and SEC61, the protein conducting channel in the ER" was deleted.
>Line 286: "SRP" was changed into "signal recognition particle".

-2. A schematic illustration of the pathways involved would be helpful.

Reply: Thank you for your valuable suggestion. We added the schematic illustration of the Ca^{2+} signaling pathway upon GPCR stimulation in Supplementary Figure S3. With this change, we corrected the manuscript as follows.

>Line 235: "IP3Rs release Ca^{2+} from the ER into the cytoplasm when the IP3 is produced upon GPCR
stimulation (Fig. S3)." was added.
>Line 241, 259: "Fig. S3" and "Fig. S4" were changed into "Fig. S4" and "Fig. S5".
>Figure legends in Fig. S3 was added and figure numbers in Supplementary Figures were adjusted.

-3. No quantitative data is provided for the results shown in Figure 1, 2, 3 and 6, even though much of the direction of the study is dictated by the results presented in these figures. The relative amounts of material in each of the bands should be measured and data presented as mean and errors, with numbers of observations given, as it is for data in Figures 4, 5 and 7.

Reply: Thank you for your advice. We added the quantitative data of Figures 1D, 2B, 3B, and 3C in Figures 1E, 2C, 3D, and 3E, respectively. These data were acquired from the three independent experiments. On the other hand, we did not quantify the intensity of each band in Figure 6 since they were just the data to characterize the bands. Since the bands corresponding to uncleaved Jaw1 with *N*-linked glycosylation are detected in the lanes of opsin-tagged Jaw1 in the blots of Figure 6, it is sufficient evidence to argue that the cleavage event of the Jaw1 C-terminal region by SPC does not affect its insertion into the ER membrane. We also carried out all blottings in Figure 6 in three independent experiments with similar results. To describe these, we corrected the manuscript as follows.
>Line 111: "(Fig. 1D)" was changed into "(Fig. 1D,E)".
>Line 117: "(Fig. 1D)" was changed into "(Fig. 1F)".
>Line 129: "(Fig. 2B)" was changed into "(Fig. 2B,C)".
>Line 134: "Figure 3B" was changed into "Figure 3B and 3C".
>Line 139: "(Fig. 3C)" was changed into "(Fig. 3D,E)".
>Line 144: "(Fig. 3D)" was changed into "(Fig. 3F)".
>Line 149: "Figure 3E" was changed into "Figure 3G".
>Line 154: "(Fig. 3F)" was changed into "(Fig. 3H)".
>Line 156: "(Figs. 3G,S1,S2)" was changed into "(Figs. 3I,S1,S2)".

>Figure legend in Supplementary Figure S1: “(Fig. 3D)” and “(Fig. 3F)” was changed into “(Fig. 3F)” and “(Fig. 3H)”, respectively.
>Line 715: “D, E)” was changed into “D, F)”.
>Line 717: “(E)” was changed into “(F)”, and “(D)” was deleted.
>Line 717-720: “E) Graph showing the percentage of Jaw1 C-terminal cleavage in (D). The averages of three independent experiments per condition are shown in the graph. Error bar shows ±SD, ****P < 0.0001; statistical analysis, one-way ANOVA followed by Dunnett’s multiple comparison test.” was added.
>Line 725-728: “C) Graph showing the percentage of Jaw1 C-terminal cleavage in (B). The averages of three independent experiments per condition are shown in the graph. Error bar shows ±SD, “n.s.”, not significant; *P < 0.05; **P < 0.01; statistical analysis, one-way ANOVA followed by Dunnett’s multiple comparison test.” was added.
>Line 733: “B, C)” was changed into “B, D)”.
>Line 734: “(C)” was changed into “(D)”.
>Line 735-738: “C, E) Graphs showing the percentage of Jaw1 C-terminal cleavage in (B) and (D), respectively. The averages of three independent experiments per condition are shown in the graphs. Error bar shows ±SD, ****P < 0.0001; statistical analysis, two-tailed Student’s t-test.” was added.
>Line 738: “D)” was changed into “F)”.
>Line 739: “E, F)” was changed into “G, H)”.
>Line 742: “(E)” and “(F)” were changed into “(G)” and “(H)”, respectively.
>Line 744: “(F)” was changed into “(H)”.
>Line 744: “G)” was changed into “I)”.
>Line 792-793: “The representative blot images from three independent experiments with similar results are shown.” was added.

-4. I wonder why the authors choose to use the ITPR for to describe the inositol 1,4,5-trisphosphate receptor. Unless there is a nomenclature change that I have missed, the common abbreviation is IP3 receptor or IP3R.

Reply: Thank you for your important notice. Throughout the manuscript, we changed “ITPR” to “IP3R”. We would appreciate it if you check the points below.
>Line 17, 31, 38, 72, 74, 97, 236, 239, 241, 262, 274, 303, 321, 322, 324, 496, 497, 498, 512, 795,
800
>Figure 7B, 7C-E, S4A-C, S11B-D, and S12A-I
>Figure legends in Figure S4 and S5

-5. The conclusions from Figure 7 appear to assume that the only process governing the Ca2+ transient is release through the inositol 1,4,5-trisphosphate receptors? The possible contribution from other proteins that might alter the transient, such a pumps, transporters and Ca2+ buffers should also be considered along with the possible effects of Jaw1 on these processes. What is the functional significance of the oscillations described?

Reply: Thank you for your important comment. In our previous report (Okumura et al., 2022), we showed that Jaw1 interacts with IP3Rs via its coiled-coil domain, and Jaw1ΔCoil, a mutant lacking the coiled-coil domain, has no augmentative effect of Jaw1 on the Ca^{2+} signaling. Furthermore, we showed that the augmentative effect of Jaw1 on the Ca^{2+} release activity upon GPCR stimulation is maintained under the condition of the removal of extracellular Ca^{2+} or in the presence of the inhibitor of SERCA, a Ca^{2+} pump on the ER to retrieve the Ca^{2+} from the cytosol into ER. This result indicates
that the augmentative effect of Jaw1 on the Ca^{2+} signaling is independent of SOCE and SERCA activity. Therefore, in the previous report, we concluded that Jaw1 directly increases the Ca^{2+} release activity of IP3Rs. However, we have not yet completely excluded the possibility that Jaw1 affects the other additional proteins involved with the Ca^{2+} signaling, and the slightly lower effect of the AASS mutant on the Ca^{2+} signaling is brought by unknown factors except IP3Rs. Therefore, we added the limitations in this study in the

section of “Discussion”. We corrected the manuscript as follows.
>Line 316-318: “We previously reported that Jaw1 interacts with IP3Rs via its coiled-coil domain, and a mutant lacking the coiled-coil domain, has no augmentative effect of Jaw1 on the Ca^{2+} signaling, which indicates that Jaw1 directly increases the Ca^{2+} release activity of IP3Rs (Okumura *et al.*, 2022).” was added.
>Line 318: “In this study,” was added, and “The” was changed into “the”.
>Line 325-328: “On the other hand, we could not completely exclude the possibility that Jaw1 augments the Ca^{2+} signaling by affecting other proteins such as Ca^{2+} pumps, transporters, and Ca^{2+} buffering in addition to the activity of IP3Rs, and the slightly lower effect of the AASS mutant on the Ca^{2+} signaling is brought by unknown factors except IP3Rs” was added.
>Line 328-329: “Furthermore, the” was changed into “The”.
Regarding the significance of the oscillations, previous studies reported that the oscillation, as well as the strength of the Ca^{2+} signal, is important for Ca^{2+}-dependent protein activation (Kupzig et al., 2005; Oancea et al., 1998). The rise of cytoplasmic Ca^{2+} level activates the Ca^{2+} adaptor proteins and enzymes in the cytoplasm, which transmit various signals. We hypothesize that the oscillation- mediated duration of high cytoplasmic Ca^{2+} levels above a certain threshold could be a key factor to induce particular physiological cellular responses. Indeed, several cellular events, such as secretion and gene expression, reportedly take a few minutes to initiate a response after the stimulation. However, we decided to delete the graphs showing the classification of the Ca^{2+} flux type, taking into consideration of another reviewer’s comments. Therefore, we did not newly add this discussion regarding the Ca^{2+} oscillation.

Minor comments
-6. The statement “The human SPC consists of three accessory subunits” appears to be contradicted by the rest of the sentence “Signal peptidase complex subunits 1-3 (SPCS1-3) and SEC11A or SEC11C as a catalytic subunit” which implies that there are only 2 subunits in two different combinations, i.e. (SPCS1- 3 plus SEC11A) or (SPCS1-3 plus SEC11C). Please clarify.

Reply: Thank you for your advice to improve the manuscript. The human SPC exists as a heterotetramer consisting of three accessory subunits and a catalytic subunit. The three accessory subunits are Signal peptidase complex subunit 1 (SPCS1), SPCS2, and SPCS3, and the catalytic subunit is either SEC11A or SEC11C, thus, SPC exists in two different forms: SPCS1-3 plus SEC11A or SPCS1-3 plus SEC11C. To clarify this, we corrected the manuscript as follows.
>Line 85-86: “The human SPC consists of three accessory subunits: Signal peptidase complex subunits 1-3 (SPCS1-3) and SEC11A or SEC11C as a catalytic subunit; thus, it exists as a heterotetrameric assembly (Liaci et al., 2021).” was deleted.
>Line 87-90: “The human SPC exists as a heterotetramer consisting of three accessory subunits and a catalytic subunit (Liaci et al., 2021). The three accessory subunits are Signal peptidase complex subunit 1 (SPCS1), SPCS2, and SPCS3, and the catalytic subunit is either SEC11A or SEC11C, thus, SPC exists in two different forms: SPCS1-3 plus SEC11A or SPCS1-3 plus SEC11C.” was added.

-7. Lines 121-124 “However, both the uncleaved and cleaved forms of Jaw1 were detected in all the lanes of mutants, comparable with FLAG Ms Jaw1, although there were subtle differences in the proposition of the two forms among them (Fig. 2B). Here, we focused on two alanine residues (509/510) that were not substituted in the above mutants.” It is not clear why the authors decided to focus on the two alanine residues. Please clarify. Sould “here” be therefore”???

Reply: Thank you for your advice to improve the manuscript. The result in Figure 2 indicates that all the mutated sites in those mutants are not a candidate for the cleavage site of the Jaw1 C-terminal region. However, in those mutants, the two alanine residues (509/510) were not still substituted. Therefore, we focused on these alanine residues. To clarify this, we corrected the manuscript as follows.
>Line 129-130: “This result indicates that all the mutated sites in the above mutants are not a

candidate for the cleavage site." was added.
>Line 130: "Here" was changed into "Therefore".

-8. Line 137 "The NIDR was fused to increase….". Fused with what?

Reply: Thank you for your comment. The NIDR was fused between the C-terminal region of Jaw1 and the PA tag, as shown in Figure 3D. To clarify this, we corrected the manuscript as follows.
>Line 145: "between the C-terminal region of Jaw1 and the PA tag" was added.

-9. Line 138-140 "Furthermore, we expected that the addition of this intrinsically disordered region, which was described in our previous study 139 (Kozono et al., 2021), would be better than the addition of structural tags in the point of the steric hindrance in the protein insertion into the ER membranes due to its structural flexibility". Better for what?

Reply: Thank you for your comment. We apologize for the insufficient explanation. We first prepared the plasmid encoding Ms Jaw1 GFP in which the GFP tag was fused with the C-terminal region of Jaw1 (Ms Jaw1 GFP) instead of NIDR to increase the molecular mass of Fragment 2 on the electrophoresis gel. However, the protein expression level of Ms Jaw1 GFP was too low to be collected by immunoprecipitated. The TA proteins are often inserted into the membrane after the translation unlike the membrane protein with the N-terminal orientation into the lumen. Therefore, we estimated that the existence of a C-terminal GFP tag, a structural tag, might be a barrier to the insertion into the ER membrane. Therefore, we expected that the addition of the intrinsically disordered region (NIDR), a structurally flexible region, would be better for the protein insertion into the ER membrane than GFP. However, we have no sufficient evidence of whether the NIDR would be better for that. Furthermore, this explanation will confuse readers and it would be sufficient only to explain the objective of the NIDR addition to increase the molecular mass of Fragment2. Therefore, we decided to remove this explanation. On the other hand, we added a brief description of the NIDR with our reference.
>Line 144-145: ", a structural flexible region previously described in our study (Kozono et al., 2021)," was added.
>Line 146-149: "Furthermore, we expected that the addition of this intrinsically disordered region, which was described in our previous study (Kozono et al., 2021), would be better than the addition of structural tags in the point of the steric hindrance in the protein insertion into the ER membranes due to its structural flexibility." was deleted.

-10. Line 164 "To validate this, the rescue experiment was performed". Please specify the general detail of the rescue experiment.

Reply: Thank you for your advice to improve the manuscript. To describe the general detail of the rescue experiment, we corrected the manuscript as follows.
>Line 174-176: ", in which it is tested whether or not the phenotypic changes due to the loss of genes are restored by re- or complementary protein expression" was added.

Second decision letter

MS ID#: JOCES/2022/260439

MS TITLE: Cleavage of the Jaw1 C-terminal region enhances its augmentative effect on the Ca^{2+} release via inositol 1,4,5-trisphosphate receptors

AUTHORS: Takuma Kozono, Chifuyu Jogano, Wataru Okumura, Hiroyuki Sato, Hitomi Matsui, Tsubasa Takagi, Nobuaki Okumura, Toshifumi Takao, Takashi Tonozuka, and Atsushi Nishikawa
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

In this study, the authors investigate in great detail and in a very logical way the characteristics of the Jaw1 cleavage site, the cleavage mechanism involved and the functional role of the cleaved protein with respect to IP3R-dependent Ca2+ signaling.
Very nice work and potentially highly significant, as it concerns a novel stimulatory pathway acting on the IP3R.

Comments for the author

All my concerns have been appropriately addressed by the authors and I consequently support publication in JCS of the revised version of this Ms.

Reviewer 2

Advance summary and potential significance to field

The authors report a series of experiments examining aspects of the function of c/LRMP, a tail-anchored (TA) protein with 39 carboxyl (C)-terminal amino acids that is oriented to the lumen of the endoplasmic reticulum and outer nuclear membrane. They previously found that Jaw1, as a member of the KASH protein family, plays a role in maintaining nuclear shape via its C-terminal region and that Jaw1 functions as an augmentative effector of Ca2+ influx by interacting with the inositol 1,4,5-trisphosphate receptors. The C-terminal region is partially cleaved, so that in the cell in uncleaved and cleaved forms. The mechanism of cleavage has not been determined. They now demonstrate that the C-terminal region of Jaw1 is cleaved after its insertion by the signal peptidase complex (SPC) and that the SPC with the catalytic subunit SEC11A, but not SEC11C, specifically cleaves Jaw1. Finally, they show that the cleavage event enhances the augmentative effect of Jaw1 on the Ca2+ release ability of the inositol 1,4,5-trisphosphate receptors. The paper therefore makes a significant contribution to the field.

Comments for the author

The authors have made satisfactory corrections and additions to manuscript in response to my comments. I have no further comments