THE EMBO JOURNAL

A salt stress-activated GSO1-SOS2-SOS1 module protects the Arabidopsis root stem cell niche by enhancing sodium ion extrusion

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DOI: 10.15252/embj.2022113004

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Review Timeline:

Submission Date:	7th Nov 22
Editorial Decision:	14th Dec 22
Revision Received:	6th Mar 23
Editorial Decision:	27th Mar 23
Revision Received:	31st Mar 23
Accepted:	19th Apr 23

Editor: William Teale

Transaction Report:

(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. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

1st Editorial Decision **14th Dec 2022**

Dear Prof. Kudla,

Thank you again for the submission of your manuscript entitled "A salt stress-activated GSO1-SOS2-SOS1 module confers Na+ extrusion for root meristem protection" (EMBOJ-2022-113004). We have now received reports from three referees, which I copy below.

As you can see from their comments, while referee # 1 raises important questions about the integration of GSO1 into the SOS1/SOS2 signalling module, all referees express interest in the experiments you report. Based on this overall interest, I would like to invite you to address the comments of all referees in a revised version of the manuscript. I should add that it is The EMBO Journal policy to allow only a single major round of revision and that it is therefore important to resolve the main concerns at this stage. I believe the concerns of the referees are reasonable and addressable, but if you would like to discuss any of their points, please do not hesitate to contact me and we can arrange a Zoom meeting. Please follow the instructions below when preparing your manuscript for resubmission.

I would also like to point out that as a matter of policy, competing manuscripts published during this period will not be taken into consideration in our assessment of the novelty presented by your study ("scooping" protection). We have extended this 'scooping protection policy' beyond the usual 3 month revision timeline to cover the period required for a full revision to address the essential experimental issues. Please contact me if you see a paper with related content published elsewhere to discuss the appropriate course of action.

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: https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess

Again, please contact me at any time during revision if you need any help or have further questions. Thank you very much again for the opportunity to consider your work for publication. I look forward to your revision.

Best regards,

William Teale

William Teale, Ph.D.
Editor
The EMBO Journal

When submitting your revised manuscript, please carefully review the instructions below and include the following items:

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point response to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

4) a complete author checklist, which you can download from our author guidelines (https://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/Author Checklist%20-%20EMBO%20J-1561436015657.xlsx). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

5) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript.

6) We require a 'Data Availability' section after the Materials and Methods. Before submitting your revision, primary datasets produced in this study need to be deposited in an appropriate public database, and the accession numbers and database listed under 'Data Availability'. Please remember to provide a reviewer password if the datasets are not yet public (see https://www.embopress.org/page/journal/14602075/authorguide#datadeposition). If no data deposition in external databases is needed for this paper, please then state in this section: This study includes no data deposited in external repositories. Note that the Data Availability Section is restricted to new primary data that are part of this study.

Note - All links should resolve to a page where the data can be accessed.

7) When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
http://bit.ly/EMBOPressFigurePreparationGuideline

Please remember: Digital image enhancement is acceptable practice, as long as it accurately represents the original data and conforms to community standards. If a figure has been subjected to significant electronic manipulation, this must be noted in the figure legend or in the 'Materials and Methods' section. The editors reserve the right to request original versions of figures and the original images that were used to assemble the figure.

8) For data quantification: please specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments (specify technical or biological replicates) underlying each data point and the test used to calculate p-values in each figure legend. The figure legends should contain a basic description of n, P and the test applied. Graphs must include a description of the bars and the error bars (s.d., s.e.m.).

9) We would also encourage you to include the source data for figure panels that show essential data. Numerical data can be provided as individual .xls or .csv files (including a tab describing the data). For 'blots' or microscopy, uncropped images should be submitted (using a zip archive or a single pdf per main figure if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available at .

10) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online (see examples in https://www.embopress.org/doi/10.15252/embj.201695874). A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc. in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here: .

- Additional Tables/Datasets should be labelled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

11) At EMBO Press we ask authors to provide source data for the main manuscript figures. Our source data coordinator will contact you to discuss which figure panels we would need source data for and will also provide you with helpful tips on how to upload and organize the files.

12) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at .

Additional instructions for preparing your revised manuscript:

Please make sure you upload a letter of response to the referees' comments together with the revised manuscript.

Please also check that the title and abstract of the manuscript are brief, yet explicit, even to non-specialists.

When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
https://bit.ly/EMBOPressFigurePreparationGuideline
See also guidelines for figure legends: https://www.embopress.org/page/journal/14602075/authorguide#figureformat

At EMBO Press we ask authors to provide source data for the main manuscript figures. Our source data coordinator will contact you to discuss which figure panels we would need source data for and will also provide you with helpful tips on how to upload and organize the files.

IMPORTANT: When you send the revision we will require
- a point-by-point response to the referees' comments, with a detailed description of the changes made (as a word file).
- a word file of the manuscript text.
- individual production quality figure files (one file per figure)
- a complete author checklist, which you can download from our author guidelines
(https://www.embopress.org/page/journal/14602075/authorguide).
- Expanded View files (replacing Supplementary Information)
Please see out instructions to authors
https://www.embopress.org/page/journal/14602075/authorguide#expandedview

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Further information is available in our Guide For Authors: https://www.embopress.org/page/journal/14602075/authorguide

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (14th Mar 2023). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions. Use the link below to submit your revision:

https://emboj.msubmit.net/cgi-bin/main.plex

Referee #1:

The manuscript by Chen et al. presented a study of GSO1-SOS2-SOS1 module in salt stress response and tolerance in Arabidopsis. Through screening of a T-DNA insertional mutant pool of receptor-like kinases, the authors identified a T-DNA insertion mutant of GSO1 gene showing hypersensitive to salt stress. The authors further identified GSO1 interacting proteins using IP-MS and found that SOS2, a critical salt tolerance determinant, is among the GSO1-interacting proteins. By using an array of genetic, physiological and biochemical assays, the authors found that GSO1 is a dual functional protein important for the CS formation and salt stress response, and the role of GSO1-SOS2 module in salt tolerance is independent of it function in CS formation. GSO1 phosphorylates SOS2 at Thr16 and activates SOS2, and phosphorylation of SOS1 by activated SOS2 increases salt tolerance. The authors also concluded that GSO1-SOS2-SOS1 pathway is independent of SOS3.
Overall, this is a thorough investigation of GSO1-SOS2 in salt tolerance, and the findings are novel and important addition to our current knowledge about salt tolerance in plants. The manuscript was nicely written, although some sections could be shortened (see comments below). All figures and supplemental figures were well organized. The manuscript could be further improved by addressing the following comments.
1. The major role of GSO1-SOS2-SOS1 in salt tolerance is attributed to roots, especially root meristematic zone, which was emphasized in the results and discussion (Fig. 7). However, phenotypically, gso1 mutant showed at least comparable, if not more severe, inhibition of shoot growth by salt stress (Fig. 1). This was not well explained in the manuscript and needs to be addressed in the revision.
2. The authors underlined the independence of GSO1-SOS2-SOS1 on SOS3, but yeast assays showed that SOS3 enhances the role of GSO1 in salt tolerance. It is possible that binding of SOS3 with SOS2 releases SOS2 self-inhibition, which provides the SOS2 as a substrate for GSO1 phosphorylation. This could be included as a possible mechanism.
3. FigEV5-A, could GSO1KD phosphorylate SOS1C300?
4. Results line 359-371, Fig. 4E, why SOS2Δ308T16D could not mimick phosphorylated T? this is a commonly used mimic in many studied. The authors need better explain in the results.
5. Line 394, "phenotype of in incomplete", "in" should be deleted.
6. Fig. 5F, gso1 mutant had similar level of Na+ in xylem with casp1,3 mutant, while in Fig.5D, gso1 showed higher Na+ in shoot than casp1,3. Assuming most of Na+ in leaves come from the transpiration stream (Xylem), could the authors explain how gso1 accumulates more Na+ in shoot?
7. The role of GSO1 in CS formation and suberization (Line 381-502) could be shortened to highlight the role of GSO1 in salt SOS2-SOS1 mediated salt response.
8. In the Discussion section, line 704, 706, 709, cited figure should be figure 7 not figure 8.

Referee #2:

General Summary and Opinion

This comprehensive study describes a new function for the plasma membrane receptor-like kinase GSO1/SGN3 that is best known for its role in the SCHENGEN response pathway regulating CS formation and integrity. Here they demonstrate a meristem-specific role for GSO1 in the Salt Overly Sensitive (SOS) signaling pathway via direct activation of the SOS2-SOS3 module. The authors have at each step in the manuscript robustly interrogated the interaction between GSO1 and the SOS proteins in both heterologous systems and in-planta. Importantly, they have carefully presented evidence of an interaction between sodium detoxification and developmental differentiation that is necessary to interpret how plants co-ordinate developmental responses to salinity. This manuscript would have a wide-readership interest from researchers in the salinity tolerance and endodermal barriers communities. I ask that the authors address the minor comments below.

Minor comments (general)

Considering the number of protein variants used in this study, an index table of the mutations and their function would be extremely useful. Like-wise, an index of the plant mutants could serve as an excellent reference to help the reader navigate between a difference in naming of plant mutants and transgenic lines (e.g. Fig2C is "pSGN3:SGN3-mVenus" instead of pGSO1:GSO1-mVenus" understood to be due to the prior naming of the protein/construct/line, the complementation line renaming from Fig 1A "com-1" and "com-2" to Fig3G "gso1-GSO1" and the changing of the allelic naming of the mutant from Fig1A "gso1-1" to Fig3C "gso1").

Minor comments (specific)

Introduction

Line45-47- Please clarify what is meant by "developmental adaptation reactions" and replace with a more informative description.

Line47-49- Please clarify what is meant by "higher order organ-scale detoxification processes" and replace with a more informative description.

Line 87- "transpiration dependent salt tolerance" is a confusing term for what many in the salinity community as "ion-exclusion" and the genotypes capable of active exclusion to reduce shoot Na+ but maintain transpiration have enhanced "shoot ion independent tolerance" (Roy, Negrao and Tester 2014 or Munns and Tester 2008 Annu Rev. Plant Biology).

Line 363- change "wild-type SOS2" to "SOS2Δ308" since "wild-type SOS2" could be interpreted as a non-modified version of SOS2.

Discussion

Line598-601- The authors have concluded that Thr16 is the phosphorylation site by GSO1 despite that the SOS2T16D version could not mimic the phosphorylation status of SOS2. Can the authors de-emphasise that the T16 site is the only/major phosphorylation site by GSO1?

Line 704-709- change all mentions of figure 8 to figure 7

Figures

Figure 6 E-H- change the Y axis "exrepssion" to "expression"

Fig5J- The display of fluorescent units from whole roots stained with fluorol yellow is not a robust method due to the effect of field of view and cell layers/thickness (that is likely to be affected by salinity treatment) on the fluorescence. How did the authors normalize for this variation? I have more confidence in EV8 and EV9 than Fig5J.

Fig4A- Please explain the slight phosphorylation of GSO1KD by SOS2 when the kinase domain is inactive.

Fig4E- Please include the M-GSO1KD expressed alone in yeast in this figure as shown in Fig3A.

Fig3A - Have the authors confirmed that the myristolyation of GSO1 does not alter its function or ability to interact with SOS2 as compared to the native/unmodified GSO1? Can the authors please provide the yeast assays for unmodified GSO1 in Fig 3A or in a supplementary file?

Fig 3B - Why does SOS2 appear to inhibit SOS3 in the +SOS2Δ308,SOS3 yeast assay at 75mM NaCl if this SOS2 variant can not bind SOS3. Also, why was the concentration of NaCl lowered to 75 mM NaCl when Fig3A was 100 mM NaCl?

Fig1A. The variation in primary tip length of replicates in wildtype, com-1 and com-2 is not reflected in the data points or error bars in Fig1B. If you have selected one representative plate then I expect to see this variance reflected in the data graph.

Fig2B. Please include the PI staining for confirmation of PM localization.

Fig2D. Please include gso-1 mutant in the plate for ease of comparison with the double mutant.

FigEV1 and FigEV2. Please explain the inclusion of scabp8 line as control.

FigEV3. Is Actin2 stably expressed between control and saline conditions?

Methods

Please report the Na+ concentration in the background MS agar. This is particularly relevant when using +0.5mM and +1mM assays (EV7).

Non-essential recommendations

Line 146- recommend that the authors please provide a list of receptor-like kinases screened for salt tolerance in the supplementary file as a reference to those interested in RLKs or those who come across candidates in GWAS studies for salinity tolerance for example.

Fig5D- Ideally, the authors should conduct a hydroponic experiment with salinity to assess biomass and shoot sodium content. Plants are more actively transpiring in hydroponic systems than on agar plates and you will see a more severe phenotype. Secondly, in hydroponics, you can maintain the specific activity of calcium (supplementing with calcium chloride for example) to ensure that the induced responses are specifically due to changes in rhizosphere Na+ and not Ca2+ deficiency.

Fig 7- This model is excellent but it does take some time to interpret. I suggest improvements to define the columns or rows. For example, could the salt tolerance contributor columns be coloured to separate them? Could the rows defining the root

positioning of the responses to NaCl be defined with a dotted line? Any variations on these suggestions to improve the clarity is welcomed.

Referee #3:

Title: A salt stress-activated GSO1-SOS2-SOS1 module confers Na+ extrusion for root meristem protection

• Overall opinion

The current research is investigating the interplay between salt adaptation mechanisms in Arabidopsis. The research idea is novel and of significant importance. Basic research of salt adaptation mechanisms will serve as a great source for desalination ideas in industry. Moreover, it would provide insights for creation of a salt tolerant construct that could be transferred to many crops and contribute to solving freshwater scarcity problem. Therefore, research of salt tolerant and adaptation mechanism is crucial nowadays.

• Minor concerns that should be addressed

Introduction

Line 41

It would be better if the authors listed a few of the essential activities of the plant that would be impaired by sodium accumulation; this would feature the significance of studying the salt-stress mechanism in the conducted research.

Lines 47-52

These lines should be removed from the introduction as there is no importance in identifying a literature gap while not addressing this gap in the current research

Lines 56-61

The rice comparison and other transport systems are not meaningful to the purpose of the current research; therefore, it is better to be omitted.

Lines 62-128

While discussing various mechanisms of salt extrusion, the authors moved back and forth between plants generally and Arabidopsis specifically in an unorganized manner. It would be better if the authors focus on discussing mechanisms of salt extrusion in Arabidopsis only throughout the introduction. An extrapolatory conclusion could be done about other plants in the discussion part. Therefore, these paragraphs need reorganization in a way that addresses salt extrusion mechanisms in Arabidopsis specifically.

Lines 129-142

This part is summarizing the results of the study; it is better to replace this part with the objectives of the study and the significant impact of studying the salt stress mechanism in Arabidopsis.

Results

Lines 165-168 should be in the beginning of the section (before line 146) to introduce the function of GSO1 and GSO2 before illustrating the results.

Lines 177-183

The authors should elaborate more how the SOS2/CIPK24 was identified as potential GSO1 interacting protein. They can do so via illustrating the mass spectrometry results in more details; they can add the illustration as caption to table S1 or add it directly in the results.

Lines 242-247 (Figure 2D)

It would be better to confirm the results by comparing the growth between WT, gso1, sos2 gso1 and sos2; not only WT, sos2 gso1 and sos2. If these data are available, it should be added.

Line 299

It is better if Kinase dead mutation was emphasized i.e. lack the phosphorylation ability but do not interfere with substrate recognition.

Lines 390-402 (Figure 5B-F)

Although SOS pathway does not contribute to TSDT; it would have been better if sos2 were added to all experiments from 5B to 5E. Useful insights might have come out. Also, why esb1 was not included into 5F experiment.

Discussion

The discussion was written greatly and in deep details; some minor re-organization could be considered. Start with lines 549-553, then lines 577-579, lines 554-558, then lines 580- end of discussion. Lines 559-576 could be omitted.

Material and Methods

It was illustrated in a clear and brief way.

• Additional suggestions

Language:

Overall, the language is good and no need for revision by a native speaker. However, there are lots of running sentences (ex. Lines 100-105); sometimes the conjunction and connectors are not properly employed, and sentence structure is not correct on a few occasions. The authors can use Grammarly to enhance the clarity, connection, and structure of the sentence; and it would also help with sentence fragmentation.

Abbreviations:
It is better to list all abbreviations as footnotes according to order of appearance in the article. This will allow readers to easily return to any abbreviation when needed.
Figures nomenclature:
For figures starting with EV, it is better to name it S (for supplementary); if it is not supplementary data then better to use one numbering system (Figure1, Figure2,.....).
[editor's note: Regarding Referee 3's last few comments, please see the 'Guide for Authors" section of our website for instructions on formatting. If you would like a native speaker to look over your text, we would recommend using a professional proof-reading service (although I am not convinced it is necessary in this case.)]

Referee #1:

The manuscript by Chen et al. presented a study of GSO1-SOS2-SOS1 module in salt stress response and tolerance in Arabidopsis. Through screening of a T-DNA insertional mutant pool of receptor-like kinases, the authors identified a T-DNA insertion mutant of GSO1 gene showing hypersensitive to salt stress. The authors further identified GSO1 interacting proteins using IP-MS and found that SOS2, a critical salt tolerance determinant, is among the GSO1-interacting proteins. By using an array of genetic, physiological and biochemical assays, the authors found that GSO1 is a dual functional protein important for the CS formation and salt stress response, and the role of GSO1-SOS2 module in salt tolerance is independent of it function in CS formation. GSO1 phosphorylates SOS2 at Thr16 and activates SOS2, and phosphorylation of SOS1 by activated SOS2 increases salt tolerance. The authors also concluded that GSO1-SOS2-SOS1 pathway is independent of SOS3.
Overall, this is a thorough investigation of GSO1-SOS2 in salt tolerance, and the findings are novel and important addition to our current knowledge about salt tolerance in plants. The manuscript was nicely written, although some sections could be shortened (see comments below). All figures and supplemental figures were well organized. The manuscript could be further improved by addressing the following comments.

We very much appreciate the positive and encouraging feedback of this reviewer and have edited (shortened) some parts of the manuscript.

1. The major role of GSO1-SOS2-SOS1 in salt tolerance is attributed to roots, especially root meristematic zone, which was emphasized in the results and discussion (Fig. 7). However, phenotypically, gso1 mutant showed at least comparable, if not more severe, inhibition of shoot growth by salt stress (Fig. 1). This was not well explained in the manuscript and needs to be addressed in the revision.

We agree that in the originally submitted version, as consequence of our intention to clarify and emphasize the newly discovered function and mechanism of stem cell protection, we may have not sufficiently enough discussed the impact of GSO-mediated SOS2 regulation on the (known) contribution of SOS1 activity to preventing leaf Na^+ accumulation. It has been reported that reduced SOS1 activity enhances leaf Na^+ accumulation (Shi et al., 2002, El Mahi et al., 2019). Accordingly, reduced GSO1/SOS2-mediated SOS1 activation in our study results in higher leaf Na^+ accumulation causing the phenotypes of leaves. It has been discussed that SOS1 contributes to protecting the vasculature from Na^+ accumulation and in this way reduces root to leaf Na^+ translocation. We have revised the text to refer to this known contribution of SOS1 to "leaf protection" although – to the best of our knowledge – the underlying mechanistic details remain to be clarified. This interesting aspect of SOS1 function is however not the focus of our study and not related to the meristem-protective mechanism uncovered in our work.

2. The authors underlined the independence of GSO1-SOS2-SOS1 on SOS3, but yeast assays showed that SOS3 enhances the role of GSO1 in salt tolerance. It is possible that binding of SOS3 with SOS2 releases SOS2 self-inhibition, which provides the SOS2 as a substrate for GSO1 phosphorylation. This could be included as a possible mechanism.

In the originally submitted version, we had already attempted to provide a possible explanation for this observation in lines 292-295 ("This conclusion does of course not exclude that GSO1 enhances the activity of already assembled SOS3-SOS2 complexes as it is suggested by superior salt tolerance of yeasts co-expressing GSO1, SOS2, and SOS3."). This SOS3-SOS2 complex assembly would obviously

also involve release of self-inhibition and in addition brings SOS2 to the plasma membrane where it could be more efficiently undergo phosphorylation by GSO1. We have now added one sentence that more clearly outlines both possible mechanisms (lines 299-300).

3. FigEV5-A, could GSO1KD phosphorylate SOS1C300?

GSO1KD does not phosphorylate SOS1C300. This information has been added in the results part (line 331-332) and as new supplementary figure (Appendix Figure S3B).

4. Results line 359-371, Fig. 4E, why SOS2Δ308T16D could not mimic phosphorylated T? this is a commonly used mimic in many studied. The authors need better explain in the results.

Indeed, the intention of introducing S to D or T to D substitutions into proteins is to mimic the phosphorylated status of this residue. However, the negative charge in the aspartate side chain is not in every case sufficient to efficiently mimic the consequences of a phosphate group attachment to S/T. (In our experience the "success rate" is around 50 %.) In these cases, the S/T to D exchange functionally behaves like the S/T to A exchange, meaning that it represents a non-phosphorylatable status. Examples from own work are published, for example, in Li et al., 2020 and Ju et al., 2022 and examples from the work of other labs would be available in Jiang et al., Plant Physiol. 2017; Cai et al., Proc Natl Acad Sci U S A. 2014; and Bustos et al, Proc Natl Acad Sci U S A. 2017.

5. Line 394, "phenotype of in incomplete", "in" should be deleted.

Thank you. We have corrected this mistake.

6. Fig. 5F, gso1 mutant had similar level of Na+ in xylem with casp1,3 mutant, while in Fig.5D, gso1 showed higher Na+ in shoot than casp1,3. Assuming most of Na+ in leaves come from the transpiration stream (Xylem), could the authors explain how gso1 accumulates more Na+ in shoot?

The xylem sap in Figure 5F was obtained from the wild type and mutants grown in soil after 1-day NaCl treatment. This result reflects the level of sodium ions entering the stele with the transpiration flow through the apoplast pathway and demonstrates that Casparian strip contributes to and improves ion transport during active plant evapotranspiration. The *gso1* mutant, like other Casparian strip mutants, does not allow more sodium ions to enter the stele. Figure 5D showed Na^+ accumulation of plants grown on MS medium measured after 8-day NaCl treatment under a low transpiration condition. We reason that GSO1 affects Na^+ accumulation or transport in multiple tissues in the root, which eventually leads to the accumulation of Na^+ in the shoot. These two experiments, we believe, indicate that the salt-sensitive phenotype of *gso1* is not only due to a defect in Casparian strip formation.

7. The role of GSO1 in CS formation and suberization (Line 381-502) could be shortened to highlight the role of GSO1 in salt SOS2-SOS1 mediated salt response.

Emotionally, we agree with this comment. However, the assumption that GSO1 (by influencing CS formation and suberization) may affect ion fluxes as consequence of modulated barrier function is a widespread misconception in the plant biology community. Although it is clearly not the focus of our research to elucidate the role of CS and suberization, we had to respond to such concerns in several

of our previous publications on ion signaling. Therefore, we have streamlined this paragraph a bit, but still feel that this is valuable information for the reader.

8. In the Discussion section, line 704, 706, 709, cited figure should be figure 7 not figure 8.

Thank you, this mistake has been corrected.

Referee #2:

General Summary and Opinion
This comprehensive study describes a new function for the plasma membrane receptor-like kinase GSO1/SGN3 that is best known for its role in the SCHENGEN response pathway regulating CS formation and integrity. Here they demonstrate a meristem-specific role for GSO1 in the Salt Overly Sensitive (SOS) signaling pathway via direct activation of the SOS2-SOS3 module. The authors have at each step in the manuscript robustly interrogated the interaction between GSO1 and the SOS proteins in both heterologous systems and in-planta. Importantly, they have carefully presented evidence of an interaction between sodium detoxification and developmental differentiation that is necessary to interpret how plants co-ordinate developmental responses to salinity. This manuscript would have a wide-readership interest from researchers in the salinity tolerance and endodermal barriers communities. I ask that the authors address the minor comments below.

Obtaining such a reassuring feedback from a reviewer is a very positive experience for which are very thankful.

Minor comments (general)
9. Considering the number of protein variants used in this study, an index table of the mutations and their function would be extremely useful. Like-wise, an index of the plant mutants could serve as an excellent reference to help the reader navigate between a difference in naming of plant mutants and transgenic lines (e.g. Fig2C is "pSGN3:SGN3-mVenus" instead of pGSO1:GSO1-mVenus" understood to be due to the prior naming of the protein/construct/line, the complementation line renaming from Fig 1A "com-1" and "com-2" to Fig3G "gso1-GSO1" and the changing of the allelic naming of the mutant from Fig1A "gso1-1" to Fig3C "gso1").

This is a most constructive suggestion that we have happily considered. We have now provided a table of resources as Table EV3 and referred to this table in the Material and Methods section. Moreover, the suggested edits have been implemented.

Minor comments (specific)
Introduction
10. Line45-47- Please clarify what is meant by "developmental adaptation reactions" and replace with a more informative description.

We have now more explicitly specified (by providing examples) to which developmental adaptation we refer (please see lines 50-51).

11. Line47-49- Please clarify what is meant by "higher order organ-scale detoxification processes" and replace with a more informative description.

We intended to emphasize that detoxification does not only occur at the cellular level (the single cell), but that each single cell is part of a tissue and of an organ and that on this level, also mechanisms for salt tolerance have to exist and function. We have revised this sentence (line 53).

12. Line 87- "transpiration dependent salt tolerance" is a confusing term for what many in the salinity community as "ion-exclusion" and the genotypes capable of active exclusion to reduce shoot Na+ but maintain transpiration have enhanced "shoot ion independent tolerance" (Roy, Negrao and Tester 2014 or Munns and Tester 2008 Annu Rev. Plant Biology).

We kindly but clearly disagree. The term “transpiration-dependent salt tolerance” has been introduced in an authoritative review article by José Dinneny (Dinneny, 2015) and this mechanism has been further discussed in more recent reviews (Manishankar et al., 2018; Köster et al., 2019). It was initially introduced to intelligibly describe the function and impact of the NADPH oxidase RBOHF in plant salt tolerance and emphasized that the role of RBOHF for conferring salt tolerance was positively dependent on the transpiration rate of the plants (Jiang et al., 2012). Moreover, a most recent publication from the same group further detailed this mechanism by describing the function of ESBL in this process and clarifying that the degree of root cell suberization does not impact on TDST (Wang et al., 2022).

13. Line 363- change "wild-type SOS2" to "SOS2Δ308" since "wild-type SOS2" could be interpreted as a non-modified version of SOS2.

This is very valuable suggestion that we have accordingly implemented.

Discussion

14. Line598-601- The authors have concluded that Thr16 is the phosphorylation site by GSO1 despite that the SOS2T16D version could not mimic the phosphorylation status of SOS2. Can the authors de-emphasise that the T16 site is the only/major phosphorylation site by GSO1?

For a detailed explanation of the inability of SOST16D to mimic the phosphorylation status we kindly refer to our answer in response to point 4 of referee #1. Our conclusion that Thr16 is the major GSO1 phosphorylation site in SOS2 was also based on the abundance of phosphorylated peptides in our mass spec analysis. Here, we noticed that the peptide containing pThr16 was much more abundant than any other phosphorylated peptide in a MASCOT search. This result is now included in the manuscript (line 358-359) and was added to Table EV2.

15. Line 704-709- change all mentions of figure 8 to figure 7

We are thankful for bringing this mistake to our attention and have corrected it.

Figures

16. Figure 6 E-H- change the Y axis "exrepssion" to "expression"

Thank you.

17. Fig5J- The display of fluorescent units from whole roots stained with fluorol yellow is not a robust method due to the effect of field of view and cell layers/thickness (that is likely to be affected by

salinity treatment) on the fluorescence. How did the authors normalize for this variation? I have more confidence in EV8 and EV9 than Fig5J.

We very much appreciate this deep-thinking comment and apologize that we did not explain our data clear enough. To circumvent the limitations in quantifying the fluorol yellow intensity and obtain reliable data we used the accumulated fluorescence intensity from a maximum projection of z-stacks through the quantified regions of the root and thereby avoided artefacts due to cell layers/thickness. We edited the respective figure legends and methods descriptions to clearly describe this method.

18. Fig4A- Please explain the slight phosphorylation of GSO1KD by SOS2 when the kinase domain is inactive.

We can exclude autophosphorylation of GSO1KD as a reason and can only speculate that this faint accumulation of radioactivity is a consequence of very minor non-specific phosphorylation under *in vitro* conditions.

19. Fig4E- Please include the M-GSO1KD expressed alone in yeast in this figure as shown in Fig3A.

We have now included the M-GSO1KD sample in the new Fig 4E and Appendix Figure S3F.

20. Fig3A - Have the authors confirmed that the myristolyation of GSO1 does not alter its function or ability to interact with SOS2 as compared to the native/unmodified GSO1? Can the authors please provide the yeast assays for unmodified GSO1 in Fig 3A or in a supplementary file?

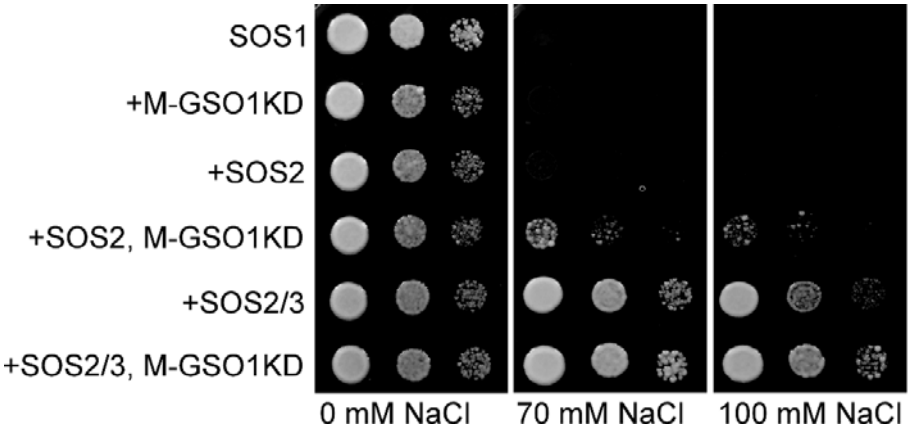
The requested controls are now presented in a new Appendix Figure S2A and B and are mentioned in the text (lines 264-265). Importantly, as expected, these experiments confirm that myristoylation does not affect the ability of GSO1 to interact with SOS2. Moreover, we have added the yeast assays for unmodified GSO1 to our experimental data and present them now as Appendix Figure S2A.

21. Fig 3B - Why does SOS2 appear to inhibit SOS3 in the +SOS2Δ308,SOS3 yeast assay at 75mM NaCl if this SOS2 variant can not bind SOS3. Also, why was the concentration of NaCl lowered to 75 mM NaCl when Fig3A was 100 mM NaCl?

With regard to question 1: It is a well-known inherent feature of such assays that the expression of an additional third protein in yeast globally reduces the expression level of these proteins compared to a situation where only two out of the three proteins are expressed. This is a consequence of the limited translational capacity of the cells in assays that use multicopy expression plasmids. Observations similar to the results that we have obtained here, have been published in Guo et al., 2004, Figure 2 (Transgenic Evaluation of Activated Mutant Alleles of SOS2 Reveals a Critical Requirement for Its Kinase Activity and C-Terminal Regulatory Domain for Salt Tolerance in Arabidopsis thaliana). In our specific case, this means that the additional expression of SOS3 results in a slight reduction of SOS2 expression, which than results in the “impression” of a negative effect of SOS3 expression. However, this technical “side effect” does not influence the major conclusions of this experiment.

The yeast strain expressing SOS2 and SOS3 can grow on a higher salt concentration medium than the yeast strain expressing SOS2T/DΔ308, which was reported previously (Quintero et al, 2002). When we treated the yeast lines (+SOS2, SOS3) co-expressing with or without M-GSO1KD under 70 mM

NaCl, we found that the difference was not very obvious compared with that under 100 mM NaCl treatment. We here provide additional picture under 70 mM NaCl treatment. Therefore, we presented the results in which the yeast strains were treated with 100 mM NaCl in Fig.3A. The yeast line (+SOST/DΔ308) grew poorly on 100 mM NaCl, so we used 70 mM NaCl treatment for comparison.



22. Fig1A. The variation in primary tip length of replicates in wildtype, com-1 and com-2 is not reflected in the data points or error bars in Fig1B. If you have selected one representative plate then I expect to see this variance reflected in the data graph.

This is because for the bar graph in Figure 1B more plants were analyzed than represented in the representative photograph illustrating the phenotype (Figure 1A). For this reviewer's information only, we here provide additional pictures of representative plates.

This result was tested at least for five independent biological repeats. The *gso1* mutants were more sensitive to 100 mM NaCl treatment, with shorter root length and less fresh weight compared to that of wild type.

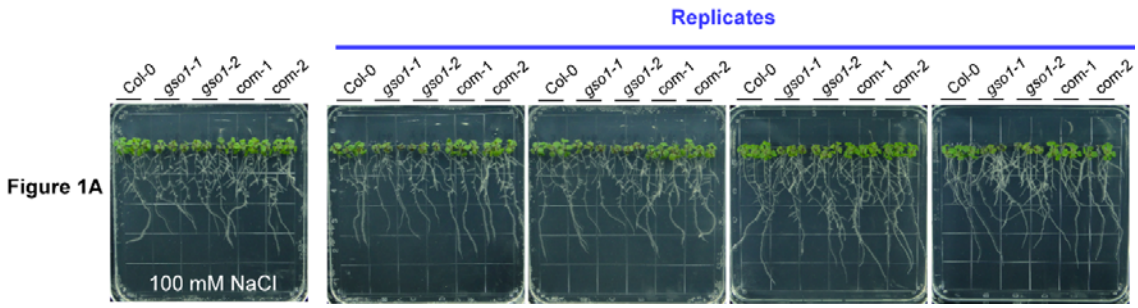


Figure 1A

23. Fig2B. Please include the PI staining for confirmation of PM localization.

We included the plasma membrane marker CBL1n-OFP (Batistic et al., 2010). This dataset is now added to Figure 2B and is described in the figure legend.

24. Fig2D. Please include gso-1 mutant in the plate for ease of comparison with the double mutant.

Thank you very much for bringing this to our attention. We added these data.

25. FigEV1 and FigEV2. Please explain the inclusion of scabp8 line as control.

The *cbl10/scabp8* mutant serves as a control for shoot salt sensitivity (Kim et al., 2007; Quan et al., 2007).

26. FigEV3. Is Actin2 stably expressed between control and saline conditions?

This appears to be a misunderstanding, since Actin2 was only used as reference expression in non-saline conditions.

Methods

27. Please report the Na+ concentration in the background MS agar. This is particularly relevant when using +0.5mM and +1mM assays (EV7).

In all figures, we added a "+" before the supplied NaCl concentrations to clarify that this represents the enhancement of NaCl content compared to control conditions. Moreover, we determined the background concentration of Na in 0.3 % phytagel to be 0.345 mM. We have added this information to the Material and Methods section.

Non-essential recommendations

28. Line 146- recommend that the authors please provide a list of receptor-like kinases screened for salt tolerance in the supplementary file as a reference to those interested in RLKs or those who come across candidates in GWAS studies for salinity tolerance for example.

The list of screened mutant lines is now provided as Table EV4.

29. Fig5D- Ideally, the authors should conduct a hydroponic experiment with salinity to assess biomass and shoot sodium content. Plants are more actively transpiring in hydroponic systems than on agar plates and you will see a more severe phenotype. Secondly, in hydroponics, you can maintain the specific activity of calcium (supplementing with calcium chloride for example) to ensure that the induced responses are specifically due to changes in rhizosphere Na+ and not Ca2+ deficiency.

We appreciate this suggestion. However, as this reviewer already stated, in hydroponic cultivation, much lower concentrations of NaCl would cause severe phenotypes compared to cultivation on plate (See for example Steinhorst et al., 2022). This would significantly limit the comparability of results that have been obtained at different NaCl concentrations. As described in the material and methods section, the assays under conditions of "enhanced evaporation" were conducted according to the established protocols of Jiang et al., 2012. (This work also established that *sos2* phenotypes occur independent of the transpirational status of plants.) We are therefore confident, that this reflects appropriate conditions.

30. Fig 7- This model is excellent but it does take some time to interpret. I suggest improvements to define the columns or rows. For example, could the salt tolerance contributor columns be coloured to separate them? Could the rows defining the root positioning of the responses to NaCl be defined with a dotted line? Any variations on these suggestions to improve the clarity is welcomed.

We are very thankful for this most thoughtful comment and are of cause delighted by this kind feedback. We have intensively discussed further improvements for enhancing clarity and comprehensibility. We hope that the advanced version of the model provided in this revision finds the consent of this reviewer.

Referee #3:

Title: A salt stress-activated GSO1-SOS2-SOS1 module confers Na+ extrusion for root meristem protection

• Overall opinion

The current research is investigating the interplay between salt adaptation mechanisms in Arabidopsis. The research idea is novel and of significant importance. Basic research of salt adaptation mechanisms will serve as a great source for desalination ideas in industry. Moreover, it would provide insights for creation of a salt tolerant construct that could be transferred to many crops and contribute to solving freshwater scarcity problem. Therefore, research of salt tolerant and adaptation mechanism is crucial nowadays.

Thank you.

• Minor concerns that should be addressed

Introduction

31. Line 41

It would be better if the authors listed a few of the essential activities of the plant that would be impaired by sodium accumulation; this would feature the significance of studying the salt-stress mechanism in the conducted research.

We are thankful for this constructive suggestion by this reviewer and expanded this paragraph accordingly (lines 44-45).

32. Lines 47-52

These lines should be removed from the introduction as there is no importance in identifying a literature gap while not addressing this gap in the current research

By also considering the comment of reviewer #1 (10.) we decided not to remove but to reformulate this paragraph.

33. Lines 56-61

The rice comparison and other transport systems are not meaningful to the purpose of the current research; therefore, it is better to be omitted.

We agree with this feedback and have revised these lines accordingly.

34. Lines 62-128

While discussing various mechanisms of salt extrusion, the authors moved back and forth between plants generally and Arabidopsis specifically in an unorganized manner. It would be better if the authors focus on discussing mechanisms of salt extrusion in Arabidopsis only throughout the introduction. An extrapolatory conclusion could be done about other plants in the discussion part.

Therefore, these paragraphs need reorganization in a way that addresses salt extrusion mechanisms in Arabidopsis specifically.

We reorganized some citations in these paragraphs as suggested and mainly focused on Arabidopsis. However, we feel that our understanding of plant tolerance mechanisms should not solely rely on findings from Arabidopsis.

35. Lines 129-142
This part is summarizing the results of the study; it is better to replace this part with the objectives of the study and the significant impact of studying the salt stress mechanism in Arabidopsis.

The problem that is addressed in this study is described in the question formulated before the summarizing paragraph. It would add redundancy to the text if we after this question would formulate that we aimed to answer this question. We feel that it is of value to provide the reader with a short outlook what to expect in the results part.

Results
36. Lines 165-168 should be in the beginning of the section (before line 146) to introduce the function of GSO1 and GSO2 before illustrating the results.

We kindly disagree because it is logically required to first identify the gene before we can refer to what is already known about this gene.

37. Lines 177-183
The authors should elaborate more how the SOS2/CIPK24 was identified as potential GSO1 interacting protein. They can do so via illustrating the mass spectrometry results in more details; they can add the illustration as caption to table S1 or add it directly in the results.

In response to this suggestion, we have in the revised version expanded the caption of table EV1.

38. Lines 242-247 (Figure 2D)
It would be better to confirm the results by comparing the growth between WT, gso1, sos2 gso1 and sos2; not only WT, sos2 gso1 and sos2. If these data are available, it should be added.

Thank you very much for bringing this to our attention. We here refer to our reply to point 24 by reviewer #2.

39. Line 299
It is better if Kinase dead mutation was emphasized i.e. lack the phosphorylation ability but do not interfere with substrate recognition.

In response to this sensible comment, we have addressed if the GSO1K979N kinase dead mutant still recognizes and interacts with its substrate SOS2 and added this data as Appendix Figure 2B (please see line 305).

40. Lines 390-402 (Figure 5B-F)
Although SOS pathway does not contribute to TSDT; it would have been better if sos2 were added to

all experiments from 5B to 5E. Useful insights might have come out. Also, why esb1 was not included into 5F experiment.

For the data presented in Fig.5B-5E, we measured the Na^+ accumulation of mutants under 100 mM NaCl for 8 days. This NaCl concentration would be lethal for the *sos2* mutant.

We added the Na^+ concentration of *esb1* in Fig 5F.

Discussion

41. The discussion was written greatly and in deep details; some minor re-organization could be considered. Start with lines 549-553, then lines 577-579, lines 554-558, then lines 580- end of discussion. Lines 559-576 could be omitted.

We thank this reviewer very much for this constructive suggestion. We have reorganized the discussion accordingly.

Material and Methods

It was illustrated in a clear and brief way.

• Additional suggestions

Language:

42. Overall, the language is good and no need for revision by a native speaker. However, there are lots of running sentences (ex. Lines 100-105); sometimes the conjunction and connectors are not properly employed, and sentence structure is not correct on a few occasions. The authors can use Grammarly to enhance the clarity, connection, and structure of the sentence; and it would also help with sentence fragmentation.

We appreciate this positive feedback. Indeed, in some cases the number of required citations appears to affect the readability especially at this stage of manuscript formatting. However, we find it appropriate to refer to important contributions from colleagues in the field.

Abbreviations:

43. It is better to list all abbreviations as footnotes according to order of appearance in the article. This will allow readers to easily return to any abbreviation when needed.

The formatting guidelines for The EMBO Journal ask to provide the meaning of all abbreviations when first mentioned in the text.

Figures nomenclature:

44. For figures starting with EV, it is better to name it S (for supplementary); if it is not supplementary data then better to use one numbering system (Figure1, Figure2,.....).

The formatting guidelines for The EMBO Journal require the nomenclature as it is used in the manuscript. Nevertheless, we are thankful for this suggestion to improve the logical order of figures and data.

Additional information for all referees:

During the preparation of the source data for our revision we noticed that the results depicted in Figure 4B involved a different substrate protein and thus accord to an experiment not discussed in our manuscript. Specifically, in the experiments for the previous Figure 4B, SOS1C300 and CBL10/SCaBP8 protein (which is also phosphorylated by SOS2) was loaded in the experiment. During figure assembly, CBL10/SCaBP8 was included in the data part of the figure but labeled as SOS1C300 in the figure legend. We have now included the correct original data, in which SOS1C300 was used as substrate. These data were already existing at the timepoint of initial submission. The newly included data do not change in any way the statement of this figure panel.

Dear Prof. Kudla,

We have now received re-review reports from two referees. As you will see from their comments (which I have pasted below), you have addressed their concerns satisfactorily.

Before we can progress towards publication, there are some minor editorial points which need to be addressed:
In this regard would you please:

- remove all red highlights from the manuscript text,

- rename the Conflict of Interest Section the "Disclosure and competing interests statement" according to the instructions on our website,

- remove the Author Credit /CrediT section from the manuscript,

- make sure callouts for Fig. 6 panels are in alphabetical order,

- add page numbers to the Table of Contents in Appendix 1,

- split files for SD figure 3 into 2 zip files as they are large, and

- supply source data for Figure 2B. There is a blank image without the expected noise for YFP / SOS3-YNBGSO1-YCB; this should be confirmed.

EMBO Press is an editorially independent publishing platform for the development of EMBO scientific publications.

Best wishes,

William Teale

William Teale, PhD
Editor
The EMBO Journal
w.teale@embojournal.org

Use the link below to submit your revision:

https://emboj.msubmit.net/cgi-bin/main.plex

Referee #1:

This revision addressed my concerns mostly related to explanation/clarification. I am satisfied with the changes made in the manuscript and the response to my comments.

Referee #2:

The reviewer has appropriately responded to all of my suggestions and modified the manuscript figures and text.
There only appears to be some confusion with the numbering of the EV files in the manuscript as compared to those submitted individually. Please check this.
I thank the reviewers for taking the time to carefully consider my suggested changes and I look forward to seeing this manuscript published.

All editorial and formatting issues were resolved by the authors.

EMBO Press Author Checklist

Corresponding Author Name: Jörg Kudla, Yan Guo
Journal Submitted to: The EMBO Journal
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The data shown in figures should satisfy the following conditions:
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- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
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Each figure caption should contain the following information, for each panel where they are relevant:
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- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
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  - definition of error bars as s.d. or s.e.m.

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New materials and reagents need to be available; do any restrictions apply?	Not Applicable	

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Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Not Applicable	

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For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Material and Methods and Figure legends

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