EMBO reports

A GID E3 ligase assembly ubiquitinates an Rsp5 E3 adaptor and regulates plasma membrane transporters

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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.)

Dear Dr. Langlois

Thank you for the submission of your research manuscript to our journal. We have now received the full set of referee reports that is copied below.

As you will see, the referees acknowledge that the findings are potentially interesting and consider the structural and biochemical analysis of Gid10 overall well documented. However, the referees also point out that the functional part linking Gid10 to Art2, Rsp5 and plasma membrane nutrient transporters has not been sufficiently developed and stronger evidence to support the proposed physiological role of GID-SR10, as outlined by the referees, will be needed for publication here. The referee reports are very constructive, providing various strategies how to strengthen the proposed physiological role of GID-SR10, which should be addressed.

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as detailed above and in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

We invite you to submit your manuscript within three months of a request for revision. This would be December 13th in your case. However, we are aware of the fact that many laboratories are not fully functional due to COVID-19 related shutdowns and we have therefore extended the revision time for all research manuscripts under our scooping protection to allow for the extra time required to address essential experimental issues. Please contact us to discuss the time needed and the revisions further.

***IMPORTANT NOTE: we perform an initial quality control of all revised manuscripts before re-review. Your manuscript will FAIL this control and the handling will be DELAYED if the following APPLIES:

1) A data availability section is missing.
2) Your manuscript contains error bars based on n=2. Please use scatter blots showing the individual datapoints in these cases. The use of statistical tests needs to be justified.

When submitting your revised manuscript, please carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision.***

When submitting your revised manuscript, we will require:

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).
Please download our Figure Preparation Guidelines (figure preparation pdf) from our Author Guidelines pages https://www.embopress.org/page/journal/14693178/authorguide for more info on how to prepare your figures.

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses 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 (). 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 (). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines
()

6) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. 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 labeled 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.

7) Before submitting your revision, primary datasets (and computer code, where appropriate) produced in this study need to be deposited in an appropriate public database (see < https://www.embopress.org/page/journal/14693178/authorguide#dataavailability>).

Specifically, we would kindly ask you to provide public access to the following datasets:
- Structural data
- Proteomics

Please remember to provide a reviewer password if the datasets are not yet public.

The accession numbers and database should be listed in a formal "Data Availability " section (placed after Materials & Method) that follows the model below (see also < https://www.embopress.org/page/journal/14693178/authorguide#dataavailability>). Please note that the Data Availability Section is restricted to new primary data that are part of this study.

Data availability

The datasets (and computer code) produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

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

8) We would also encourage you to include the source data for figure panels that show essential data. Numerical data should 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 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 .

9) Regarding data quantification
The following points must be specified in each figure legend:
- the name of the statistical test used to generate error bars and P values,
- the number (n) of independent experiments (please specify technical or biological replicates) underlying each data point,
- the nature of the bars and error bars (s.d., s.e.m.)
Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.
- Please also include scale bars in all microscopy images.

10) 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 .

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We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

Please use this link to submit your revision: https://embor.msubmit.net/cgi-bin/main.plex

Yours sincerely,

Martina Rembold, PhD
Senior Editor
EMBO reports

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Referee #1:

In this study by Langlois et al., Gid10 is identified as a new substrate receptor (SR) for the GID E3 Ub ligase complex. They determine that Gid10 binds to the same surface of Gid5 as Gid4, a previously established SR for the GID complex. The authors determine that Gid10 does not appear to target the same substrates that are targeted by Gid4.

Gid10 expression is induced in response to heat shock, osmotic shock, and amino acid or nitrogen starvation.

The authors then used proteomics to identify Nhp10 and Art2 as potential Gid10 substrates.

By Y2H, they find that Gid10, but not Gid4, interacts with the N-terminus of Art2.

They then determine a 1.3 A structure of this N-terminal "degron" peptide portion of Art2 bound to Gid10. The structure resembles that of Gid4 bound to the Fbp1 N-terminal degron, but the Gid10 interaction with its substrate is more extensive, which explains the relatively high affinity the authors find between Gid10 and the Art2 degron.

They then use in vitro ubiquitination assays to determine that the GID-SR10 complex (with Gid10) can ubiquitinate Art2 but GID-SR4 (with Gid4) cannot. They find that mutation of Pro-2 of Art2 prevents ubiquitination.

They further find that Art2 and the GID complex have a role in down regulation of lysine importers.

Ultimately, the authors have identified a new GID substrate receptor and demonstrated a role for GID function in regulation of amino acid uptake independent of its role in regulating the switch to glycolysis.

I found this to be a comprehensive, rigorous study that presents important new data and interprets it appropriately. My only suggestion for improvement regards the fact that in Figure 5A, the growth of the Art2-P2S mutant on the thialysine plate does not appear to be much different from the wild-type. Perhaps a mutant of Art2 lacking its N-terminal ~7 amino acids would have a stronger phenotype?

Referee #2:

The manuscript from Langlois and coworkers reports that the GID E3 ligase targets the Art2 protein for ubiquitylationand degradation, mediated by the GID10 subsrate receptor. It is shown that GID10, an N-degron substrate receptor, recognizes the N-terminus of Art2, with the position 2 proline being particularly critical, and a structure is determined of the N-terminal Art2 peptide with GID10. The biochemical and structural analyses are generally well designed and executed and there are no substantive critcisms of this aspect of the work. However, the paper concludes with some physiologic experiments that are confounding and not fully explored, as detailed below. There are also several problems with presentation and clarity that are detailed further below.

Major comments.

1. A significant issue is that the authors are reporting that the GID E3 ligase targets an Rsp5 adaptor (Art2) yet there is not a single experiment that centers on Rsp5 (other than a minor side experiment that shows that Rsp5 is not, itself, a target of GID). That is, all links of this work to Rsp5 is presumed and never tested or established. No genetic interactions between the GID ligase or GID substrate receptors and Rsp5 are probed or tested. This is a particularly important point as the genetic and physiology experiments that are performed (the last section of the Results section) are unexpected and a bit inexplicable (see next point).

2. This problem centers on the experiments with thialysine. First, the basis of the thialysine experiments should be clearly laid out for the reader. The rationale is that cells will be hypersensitive to thialysine, a toxic lysine analog, when Lyp1 - the lysine permease - is stabilized at the plasma membrane. Thus, mutations in Art2, the Rsp5 adaptor for targeting Lyp1 for ubiquitin-mediated downregulation, render cells sensitive to thialysine (as shown in Figure 5A), as well as mutations in Rsp5, itself (work from others and not confirmed here). Therefore, according to a model where GID-SR10 is targeting Art2 for ubiquitin-mediated degradation, mutations in GID subunits or GID10 should stabilize Art2 and should therefore not render cells hypersensitive to thialysine - they would be expecgted to have the opposite effect (or at least not a hypersensitivity compared to wt). So, these results are not consistent with a simple model where the GID ligase is targeting Art2 for ubiquitin-mediate degradation. The authors of course recognize this, but the problem is that there is no resolution and no attempt is made to step back and ask if any of this really has anything to do with the Rsp5 axis. There are quite a few simple experiments that could be done here... is there a synthetic lethality from GID mutations and rsp5 hypomorphs, or do GID mutations rescue rps5 mutants? Is Art2 ubiquitylated by K48 chains by GID in cells, and is there K63 ubiquitylation of Art2 by Rsp5? Is there a role for the Rsp5-associated DUB (Ubp2) in this system? Ubp2 also has a proline at position 2. A PPxY motif within Art2 allows Art2 to bind to Rsp5; what is the effect of a PPxY deletion on turnover of Art2 by GID? Some of these things and no doubt many others could be quickly done to hopefully clarify what is going on in this system. Again, the authors are clearly aware of all the complexities here and spend much of the discussion trying to account for them, but, again, some simple things can be tried. At a minimum, a direct genetic link to Rsp5 must somehow be confirmed.

Writing/clarity: there are many places where the authors have not been careful to spell out their logic or the relevant background information.

1.There is little discussion of the overall similarity of GID10 to other GID proteins. This is related to the first sentence of the Results section, where it is stated that previous studies suggested that GID10 might be an SR for the GID E3; what suggested this? And again in the second paragraph of the Results it is stated that GID4 and GID10 share many sequence and structural similarities, but I have not idea what features these proteins have in common.

2. Many readers will not know what a DIA proteomics experiment is. It only takes a sentence or two to spell out what DIA means and what info such an experiment gives you.
3. This sentence in the introduction states "Furthermore, Rsp5 contains an intrinsic ubiquitin binding site and in many cases ubiquitination of ART proteins promotes their activity [10,24,27,28], suggesting that adaptor ubiquitination may serve as an additional layer of regulation." This deserves some expansion and clarification. Other than just a statement that things could be complicated, I really don't know what is being suggested here.
4. The N-end recognition by GID ligases is poorly introduced. It is not until the Results sectiuon that it is stated that you "selected for proteins with contain a proline in position 2 or 3" before telling us in the next paragraph that GID-SR4 is in fact an N-degron E3. This aspect of GID function should probably be brought up in the introduction.

5. There is no reference for the "GFP protection assay" and it is not explained very clearly.

Referee #3:

In this manuscript, the authors characterize a specific manifestation of the multi-subunit GID E3 ligase and identify a role in the ART-Rsp5 pathway in budding yeast. The GID complex is a highly conserved E3 consisting of at least seven subunits. It functions in glucose metabolism by targeting key enzymes of gluconeogenesis for proteasomal degradation, using the substrate receptor (SR) subunit Gid4. Rsp5 is the only NEDD4-family ubiquitin ligase in budding yeast and involved in many cellular processes like endocytosis, intracellular trafficking and the heat shock response. ART proteins are adaptor proteins targeting Rsp5 to its substrates.

By interaction assays (yeast two hybrid and pull-downs), the authors confirm the previous assumption that Gid10, a putative SR, physically interacts with the GID complex via the Gid5 subunit, using the same interface as the SR subunit Gid4. Next, the authors looked into potential functions of Gid10 in budding yeast. By using quantitative MS under heat stress, the authors identified the ART-protein Art2 as a regulatory target of Gid10.
The authors nicely demonstrate by Y2H, ITC and structural work that Gid10 can directly bind to the N-terminal proline of Art2, as expected for an SR of the GID complex. THey show that the interaction with Art2 is specific for Gid10. Furthermore, GID-SR10 specifically ubiquitinates Art2 and its N-terminal peptide (aa2-30) in vitro. Last, the authors focused on the functional interplay between the GID complex and ART-Rsp5 signaling. They show that the GID complex shares the delayed growth phenotype of Art2 on thialysine, suggesting a potential role in lysine metabolism. Additional functional assays show that the GID complex is involved in the flux of plasma membrane nutrient transporters.

Overall, the presented in vitro data nicely demonstrate that the GID complex with the alternative SR, Gid10, selectively targets the Art2 N-terminus for ubiquitination. However, the functional part of the study has not been taken far enough. Specifically, the assays addressing the contribution of the GID complex to the ART-Rsp5 signaling pathway are not fully supporting the suggested model that the GID complex directly acts via ubiquitination of Art2. To confirm such a model additional functional

studies are required.

Major comments:

1. The in vivo data on the interplay of Gid4 and Gid10 (Figure 1) support the suggested model that the two SRs compete for interaction with GIDAnt. This model is further strengthen by the interaction studies (Figure 1B). However, to prove the suggested model, in vitro competition assays with Gid4 and Gid10 for binding to GID-Ant would be required.

2. The conclusions drawn from the functional assays on membrane transporter trafficking (Figure 5) about the interplay between the GID complex and the ART-Rsp5 pathway are not fully supported by the presented data. A similar phenotype of single gene deletions does necessarily imply a functional relation (e.g. epistasis) between the two genes. In order to draw such conclusion a direct comparison to a double deletion background (ΔArt2ΔGidX) would be required. The same holds true for the experiments conducted in the art1 deletion background. In this case, a side-by-side comparison, including quantification of the Western Blot data, of the respective double mutant strains (ΔArt1ΔArt2 and ΔArt1ΔGidX) to the triple deletion (ΔArt1ΔArt2ΔGidX) would be required.

3. The Western blot data from the membrane internalization assays need to be quantified to draw a conclusion on the effect of the different strain backgrounds. Especially the data from the Can1-GFP assay (Figure EV5D) are difficult to interpret because a gid2 or gid5 deletion in an art1 deletion background shows a reduction of Can1-GFP already at time point 0. This would affect the amount of GFP accumulating during the heat shock.

4. The authors discuss two potential models of how GID-SR10 acts on Art2. Art2 ubiquitination by GIDSR10 may lead to its deactivation or promote its degradation. The authors have everything at hand to the test if Art2 undergoes proteasomal degradation after ubiquitination by GID-SR10. They could utilize their established quantitative MS pipeline to quantify the protein levels of Art2 under conditions of proteasomal inhibition in the presence and absence of Gid10. Next, they could use the established in vitro ubiquitination assay of the Art2 N-terminal peptide (Figure 4D) to identify the targeted lysines on Art2 and the type of ubiquitination. The N-terminal peptide harbors only two lysines. The band pattern in figure 4D suggests that Art2 undergoes poly-ubiquitination by GID-SR10. By using ubiquitin K-to-R mutations the authors can easily identify the ubiquitin linkage(s) formed by GID-SR10.

Minor comments:

1. In the first paragraph of the introduction, the authors need to add some references.

2. In Figure 1B, the authors show that C-terminal truncations of Gid10 and Gid4 abolish the respective interaction with GID-Ant. They conclude in the text that Gid10 and Gid4 bind to "the same surface on Gid5 through homologous residues on each SR". The authors have to provide the amino acid composition of the deleted residues in the text and the respective figure caption (Gid4: Δ359-362 "FEFA"; Gid10: Δ359-362 "FEIA"). Otherwise, the reader has to look for this information in a database.

3. "Gid4 and Gid10 share many sequence and structural elements and might carry out redundant functions in the cell." A schematic representation of the important sequence and structural elements of Gid4 and Gid10 would be helpful for the reader.

4. "Gid10 was efficiently bound the Art2, but not the Nhp10, N-terminus (Fig 3A, EV3A)." Better: Gid10 efficiently bound...

5. Figure 4D: Which N-terminal peptide of Art2 was used in this assay? Art22-30 (figure label) or Art22-28 (figure caption)

6. "Thus, we tested if the GID complex plays a role in Lyp1 import and degradation by examining phenotypes on the toxic lysine analog, thialysine (S-Aminoethyl-l-cysteine)." - please refer to Figure 5A here.

7. Figure 5B: It looks as if the growth defect of ΔGid4ΔGid10 is less pronounced than the growth defect of the other GID subunit deletions. Also, it seems that the images for the other GID subunits are derived from a spot assay on a different plate. If that is the case, the assay should be repeated on a single plate. If not, the authors need to include a statement about splicing of the image. Moreover, it is critical that whatever image manipulation was done on the different parts was identical - here, background and contrast look different.

8. Figure 5D: the dash of the 40 kDa marker label is located in the Pgk1 blot image.

9. "In the absence of Art1, deletion of a core subunit resulted in increased toxicity during growth on thialysine (Fig 5C), similar to that observed in an ART1ART2 double deletion (Fig EV5C)." - please refer to Figure 5C for the GID subunit phenotype.

10. "Furthermore, deletion of Art1 also resulted in increased toxicity in the Gid10 deletion strain, but not in the Gid4 deletion (Fig EV5D)." >> this refers to Fig 5D, not Fig EV5D.

General response to reviewers:

We thank the Reviewers for their encouraging comments and for many suggestions for improving our manuscript. To address your questions, we performed many new experiments and revised the text and figures. Responses are indicated in blue.

Referee #1:

In this study by Langlois et al., Gid10 is identified as a new substrate receptor (SR) for the GID E3 Ub ligase complex. They determine that Gid10 binds to the same surface of Gid5 as Gid4, a previously established SR for the GID complex. The authors determine that Gid10 does not appear to target the same substrates that are targeted by Gid4.

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They further find that Art2 and the GID complex have a role in down regulation of lysine importers.

Ultimately, the authors have identified a new GID substrate receptor and demonstrated a role for GID function in regulation of amino acid uptake independent of its role in regulating the switch to glycolysis.

I found this to be a comprehensive, rigorous study that presents important new data and interprets it appropriately.

We are very pleased with the reviewer's enthusiasm for our work. Thank you!

My only suggestion for improvement regards the fact that in Figure 5A, the growth of the Art2-P2S mutant on the thialysine plate does not appear to be much different from the wild-type. Perhaps a mutant of Art2 lacking its N-terminal ~7 amino acids would have a stronger phenotype?

We performed the experiment requested by the reviewer. Surprisingly, a mutant lacking the entire degron restored growth on thialysine to wildtype levels, despite the subtle growth defect of the Art^{P2S} mutant. Interestingly, the deleted amino acids (PFITSRP) contain two potential phosphorylation sites. Indeed, residues 9-20 of Art2 contain five annotated phosphorylation sites (MPFITSRPVA KNSSHSLSET), indicating that the N-terminus of Art2 is heavily phosphorylated. We hypothesize that the first 8 residues also contain phosphorylation sites (although these have not been detected as such in previous studies, it seems their position before the first arginine would limit identification of the peptide by proteomics) conferring additional regulation of Art2.

Referee #2:

The manuscript from Langlois and coworkers reports that the GID E3 ligase targets the Art2 protein for ubiquitylationand degradation, mediated by the GID10 subsrate receptor. It is shown that GID10, an N-degron substrate receptor, recognizes the N-terminus of Art2, with the position 2 proline being particularly critical, and a structure is determined of the N-terminal Art2 peptide with GID10. The biochemical and structural analyses are generally well designed and executed and there are no substantive critcisms of this aspect of the work. However, the paper concludes with some physiologic experiments that are confounding and not fully explored, as detailed below. There are also several problems with presentation and clarity that are detailed further below.

Major comments.

1. A significant issue is that the authors are reporting that the GID E3 ligase targets an Rsp5 adaptor (Art2) yet there is not a single experiment that centers on Rsp5 (other than a minor side experiment that shows that Rsp5 is not, itself, a target of GID). That is, all links of this work to Rsp5 is presumed and never tested or established. No genetic interactions between the GID ligase or GID substrate receptors and Rsp5 are probed or tested. This is a particularly important point as the genetic and physiology experiments that are performed (the last section of the Results section) are unexpected and a bit inexplicable (see next point).

(response below next point)

2. This problem centers on the experiments with thialysine. First, the basis of the thialysine experiments should be clearly laid out for the reader. The rationale is that cells will be hypersensitive to thialysine, a toxic lysine analog, when Lyp1 - the lysine permease - is stabilized at the plasma membrane. Thus, mutations in Art2, the Rsp5 adaptor for targeting Lyp1 for ubiquitin-mediated downregulation, render cells sensitive to thialysine (as shown in Figure 5A), as well as mutations in Rsp5, itself (work from others and not confirmed here). Therefore, according to a model where GID-SR10 is targeting Art2 for ubiquitin-mediated degradation, mutations in GID subunits or GID10 should stabilize Art2 and should therefore not render cells hypersensitive to thialysine - they would be expecgted to have the opposite effect (or at least not a hypersensitivity compared to wt). So, these results are not consistent with a simple model where the GID ligase is targeting Art2 for ubiquitin-mediate degradation. The authors of course recognize this, but the problem is that there is no resolution and no attempt is made to step back and ask if any of this really has anything to do with the Rsp5 axis. There are quite a few simple experiments that could be done here... is there a synthetic lethality from GID mutations and rsp5 hypomorphs, or do GID mutations rescue rps5 mutants? Is Art2 ubiquitylated by K48 chains by GID in cells, and is there K63 ubiquitylation of Art2 by Rsp5? Is there a role for the Rsp5-associated DUB (Ubp2) in this system? Ubp2 also has a proline at position 2. A PPxY motif within Art2 allows Art2 to bind to Rsp5; what is the effect of a PPxY deletion on turnover of Art2 by GID?

Some of these things and no doubt many others could be quickly done to hopefully clarify what is going on in this system. Again, the authors are clearly aware of all the complexities here and spend much of the discussion trying to account for them, but, again, some simple things can be tried. At a minimum, a direct genetic link to Rsp5 must somehow be confirmed.

We thank the reviewer for these comments. We have clarified the rationale for the thialysine experiments in the text. To investigate the impact of the GID E3 ligase on the Rsp5 axis, we performed several experiments:

1) We tested if GID mutants interacted genetically with an Rsp5 mutant that is defective in ubiquitin binding. We found that the effects of deleting GID subunits is likely independent of Rsp5's ubiquitin binding ability. These data are summarized in Figure 6F of the revised manuscript.
2) We tested if the mutations in GID subunits affect the Art2-Rsp5 interaction between the PPx(Y/F) domain of Art2 and the WW domain of Rsp5. While deletion of a GID core subunit, and mutations in either the Rsp5 WW domain, or the Art2 PPx(Y/F) domain cause phenotypes on thialysine alone, we found that combining these mutations did not aggravate the defect, suggesting that the GID E3 ligase acts by influencing the Art2-Rsp5 interaction, confirming a genetic link between the GID E3 ligase and Rsp5. These data are summarized in Figure 6G and 6H of the revised manuscript.
3) We tested if GID^{SR10} forms K48 ubiquitin chains on Art2 using ubiquitin mutants. The data suggest that Gid^{SR10} forms a mixture of K48 and non-K48 chains. These data are shown in Figure EV4A of the revised manuscript.
4) We tested if the Rsp5-associated DUB Ubp2 interacts with the GID substrate receptors by yeast two hybrid. We found no interaction. These data are shown in Figure EV4C of the revised manuscript.

Writing/clarity: there are many places where the authors have not been careful to spell out their logic or the relevant background information.

1.There is little discussion of the overall similarity of GID10 to other GID proteins. This is related to the first sentence of the Results section, where it is stated that previous studies suggested that GID10 might be an SR for the GID E3; what suggested this? And again in the second paragraph of the Results it is stated that GID4 and GID10 share many sequence and structural similarities, but I have not idea what features these proteins have in common.

We have added a figure showing the alignment between Gid4 and Gid10, highlighting the similar sequence and structural elements (Appendix figure S1).

2. Many readers will not know what a DIA proteomics experiment is. It only takes a sentence or two to spell out what DIA means and what info such an experiment gives you.

This has been clarified in the text.

3. This sentence in the introduction states "Furthermore, Rsp5 contains an intrinsic ubiquitin binding site and in many cases ubiquitination of ART proteins promotes their activity [10,24,27,28], suggesting that adaptor ubiquitination may serve as an additional layer of regulation." This deserves some expansion and clarification. Other than just a statement that things could be complicated, I really don't know what is being suggested here.

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We thank the reviewer for this suggestion. This has been clarified in the text.

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Referee #3:

In this manuscript, the authors characterize a specific manifestation of the multi-subunit GID E3 ligase and identify a role in the ART-Rsp5 pathway in budding yeast. The GID complex is a highly conserved E3 consisting of at least seven subunits. It functions in glucose metabolism by targeting key enzymes of gluconeogenesis for proteasomal degradation, using the substrate receptor (SR) subunit Gid4. Rsp5 is the only NEDD4-family ubiquitin ligase in budding yeast and involved in many cellular processes like endocytosis, intracellular trafficking and the heat shock response. ART proteins are adaptor proteins targeting Rsp5 to its substrates.

By interaction assays (yeast two hybrid and pull-downs), the authors confirm the previous assumption that Gid10, a putative SR, physically interacts with the GID complex via the Gid5 subunit, using the same interface as the SR subunit Gid4. Next, the authors looked into potential functions of Gid10 in budding yeast. By using quantitative MS under heat stress, the authors identified the ART-protein Art2 as a regulatory target of Gid10.
The authors nicely demonstrate by Y2H, ITC and structural work that Gid10 can directly bind to the N-terminal proline of Art2, as expected for an SR of the GID complex. THey show that the interaction with Art2 is specific for Gid10. Furthermore, GID-SR10 specifically ubiquitinates Art2 and its N-terminal peptide (aa2-30) in vitro. Last, the authors focused on the functional interplay between the GID complex and ART-Rsp5 signaling. They show that the GID complex shares the delayed growth phenotype of Art2 on thialysine, suggesting a potential role in lysine metabolism. Additional functional assays show that the GID complex is involved in the flux of plasma membrane nutrient transporters.

Overall, the presented in vitro data nicely demonstrate that the GID complex with the alternative SR, Gid10, selectively targets the Art2 N-terminus for ubiquitination. However, the functional part of the study has not been taken far enough. Specifically, the assays addressing the contribution of the GID complex to the ART-Rsp5 signaling pathway are not fully supporting the suggested model that the GID complex directly acts via ubiquitination of Art2. To confirm such a model additional functional studies are required.

Major comments:

1. The in vivo data on the interplay of Gid4 and Gid10 (Figure 1) support the suggested model that the two SRs compete for interaction with GIDAnt. This model is further strengthen by the interaction studies (Figure 1B). However, to prove the suggested model, in vitro competition assays with Gid4 and Gid10 for binding to GID-Ant would be required.

We performed the experiment requested by the reviewer. Using recombinantly purified proteins, we found that Gid4 was able to compete with Gid10 for binding to Gid^{Ant}. In addition, we now show that overexpression of Gid10 not only stabilizes Gid4 levels, but also concomitantly leads to a reduction in the levels of the Gid4 substrate Fbp1. These data are shown in Figure 2C-D of the revised manuscript.

2. The conclusions drawn from the functional assays on membrane transporter trafficking (Figure 5) about the interplay between the GID complex and the ART-Rsp5 pathway are not fully supported by the presented data. A similar phenotype of single gene deletions does necessarily imply a functional relation (e.g. epistasis) between the two genes. In order to draw such conclusion a direct comparison to a double deletion background (ΔArt2ΔGidX) would be required. The same holds true for the experiments conducted in the art1 deletion background. In this case, a side-by-side comparison, including quantification of the Western Blot data, of the respective double mutant strains (ΔArt1ΔArt2 and ΔArt1ΔGidX) to the triple deletion (ΔArt1ΔArt2ΔGidX) would be required.

We appreciate the reviewer's suggestions and we performed the experiments requested by the reviewer. Although there is a clear effect of GID deletion on Lyp1 import and degradation, there is also an additive effect of GID and Art2 (Figures EV5E, EV5F, and EV5G of the revised manuscript). Given the results, we refocused the manuscript on the direct functions of the GID E3 ligase.

3. The Western blot data from the membrane internalization assays need to be quantified to draw a conclusion on the effect of the different strain backgrounds. Especially the data from the Can1-GFP assay (Figure EV5D) are difficult to interpret because a gid2 or gid5 deletion in an art1 deletion background shows a reduction of Can1-GFP already at time point 0. This would affect the amount of GFP accumulating during the heat shock.

We thank the reviewer for this suggestion. Unfortunately, despite multiple attempts, we were not able to obtain robust quantification. A major challenge was the vastly different exposure times required to image Lyp1-GFP, free GFP, and Pgk1. Based on the reviewer's comment, we removed the Can1-GFP assay from the manuscript.

4. The authors discuss two potential models of how GID-SR10 acts on Art2. Art2 ubiquitination by GIDSR10 may lead to its deactivation or promote its degradation. The authors have everything at hand to the test if Art2 undergoes proteasomal degradation after ubiquitination by GID-SR10. They could utilize their established quantitative MS pipeline to quantify the protein levels of Art2 under conditions of proteasomal inhibition in the presence and absence of Gid10.

To test if Art2 undergoes proteasomal degradation, we used both the established promoter reference technique (PRT) and our MS pipeline in the presence of the proteasomal inhibitor MG-132. While average values suggested Art2 likely undergoes proteasomal degradation, the error bars in both assays were unfortunately too high for us to feel comfortable including these results in the manuscript.

Next, they could use the established in vitro ubiquitination assay of the Art2 N-terminal peptide (Figure 4D) to identify the targeted lysines on Art2 and the type of ubiquitination. The N-terminal peptide harbors only two lysines. The band pattern in figure 4D suggests that Art2 undergoes poly-ubiquitination by GID-SR10. By using ubiquitin K-to-R mutations the authors can easily identify the ubiquitin linkage(s) formed by GID-SR10.

To address this comment (and a related comment from reviewer #2), we tested if GID^{SR10} forms K48 ubiquitin chains on Art2 using ubiquitin mutants. The data suggest that Gid^{SR10} forms a mixture of K48 and non-K48 chains. These data are shown in Figure EV4A.

Minor comments:

1. In the first paragraph of the introduction, the authors need to add some references.

The references have been added.

2. In Figure 1B, the authors show that C-terminal truncations of Gid10 and Gid4 abolish the respective interaction with GID-Ant. They conclude in the text that Gid10 and Gid4 bind to "the same surface on Gid5 through homologous residues on each SR". The authors have to provide the amino acid composition of the deleted residues in the text and the respective figure caption (Gid4: Δ359-362 "FEFA"; Gid10: Δ359-362 "FEIA"). Otherwise, the reader has to look for this information in a database.

The requested information has been added to the figure legend.

3. "Gid4 and Gid10 share many sequence and structural elements and might carry out redundant functions in the cell." A schematic representation of the important sequence and structural elements of Gid4 and Gid10 would be helpful for the reader.

We have added an alignment of Gid4 and Gid10, highlighting the sequence and structural elements. This is show in Appendix Figure S1.

4. "Gid10 was efficiently bound the Art2, but not the Nhp10, N-terminus (Fig 3A, EV3A)." Better: Gid10 efficiently bound...

The sentence has been changed in the text.

5. Figure 4D: Which N-terminal peptide of Art2 was used in this assay? Art22-30 (figure label) or Art22-28 (figure caption)

We apologize for the confusion. Art2$^{2-29}$ was used in the assay. This has been corrected in the figure and text.

6. "Thus, we tested if the GID complex plays a role in Lyp1 import and degradation by examining phenotypes on the toxic lysine analog, thialysine (S-Aminoethyl-l-cysteine)." - please refer to Figure 5A here.

We have corrected this in the text.

7. Figure 5B: It looks as if the growth defect of ΔGid4ΔGid10 is less pronounced than the growth defect of the other GID subunit deletions. Also, it seems that the images for the other GID subunits are derived from a spot assay on a different plate. If that is the case, the assay should be repeated on a single plate. If not, the authors need to include a statement about splicing of the image. Moreover, it is critical that whatever image manipulation was done on the different parts was identical - here, background and contrast look different.

The figure has been corrected to show the wildtype control in comparison with the ΔGid4ΔGid10 double deletion. These data are shown as Figure 6B-C of the revised manuscript.

8. Figure 5D: the dash of the 40 kDa marker label is located in the Pgk1 blot image.

This has been corrected on the figure.

9. "In the absence of Art1, deletion of a core subunit resulted in increased toxicity during growth on thialysine (Fig 5C), similar to that observed in an ART1ART2 double deletion (Fig EV5C)." - please refer to Figure 5C for the GID subunit phenotype.

The figure call has been corrected in the text.

10. "Furthermore, deletion of Art1 also resulted in increased toxicity in the Gid10 deletion strain, but not in the Gid4 deletion (Fig EV5D)." >> this refers to Fig 5D, not Fig EV5D.

The figure call has been corrected in the text.

Dear Dr. Langlois

Thank you for the submission of your revised manuscript to EMBO reports. We have now received the reports from the referees that were asked to assess the revised version.

As you will see, both referees acknowledge that the revised manuscript has been strengthened. Referee 3 notes that the functional data provided are limited and I agree with this point, note however that referee 2 and former referee 1 supported publication in EMBO reports. Given the support from at least two referees and that no major concerns regarding the data present in the manuscript as such remain, we have decided to proceed with the publication of your study in EMBO reports, pending that the remaining concerns of referee 2 are addressed.

From the editorial side, there are also a number of things that we need before we can proceed with the official acceptance of your study:

- Data availability section: Please provide links that resolve to the datasets in RCSB and PRIDE.

- References: please only list the first 10 authors followed by et al.

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We look forward to seeing a final version of your manuscript as soon as possible.

Kind regards,

Martina Rembold, PhD
Senior Editor
EMBO reports

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Referee #2:

The authors have thoughtfully addressed the previous reviewer comments and have conducted additional experiments. My main criticism of the revised manuscript is that the authors utilize a mutation in Rsp5 that affects ubiquitin binding (F618A) to propose a possible model in which interaction of Art2 with Rsp5 is mediated by the ubiquitin on Art2. They do not point out that F618 mutations have a influences the ability of Rsp5 to catalyze polyubiquitination and is likely temperature sensitive for growth (like an F618D mutant; DOI: 10.1038/embor.2011.23). As described, a reader would be led to conclude (from Figure 6F) that the extreme sensitivity of the Rsp5-F618 mutant to thialysine is due to the inability of Art2-Ub to bind to Rsp5. This does not seem like a very likely possibility (nor was it explored with in vitro experiments).
Minor comments:
Grammatically, the sentence in the text referring to Figure 1B say that the mutations discussed disrupt ubiquitination by GID-SR4, but no ubiquitination experiments are shown in Fig. 1B.

It is stated that Rsp5, Ubp2, and Art3 all contain N-terminal proline residues. I think what you mean to say is that they all contain a proline at position 2, and therefore may potentially exist in cells with an N-terminal proline. Is there evidence that the proline is ever exposed as an N-terminal proline in any of these proteins?

Referee #3:

The authors have performed a good number of additional experiments in response to the reviewers' comments. Unfortunately,

their attempts to clarify the genetic relationship between the GID complex and Rsp5-Art2 have not worked out as intended because of problems with reproducibility, background or significance of the results. The authors have therefore taken out some of these data. This makes the manuscript overall more solid, but unfortunately reduces its impact as the biochemical part is mainly a nice and accurate confirmation of a previously postulated model. There is nothing wrong with this per se, and the manuscript could be published in this form; however, it seems a pity to not put some more effort and time into clarifying the functional aspects as requested by two of the referees in their main points.

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Abstract suggestion

Cells rapidly remodel their proteomes to align their cellular metabolism to environmental conditions. Ubiquitin E3 ligases enable this response, by facilitating rapid and reversible changes to protein stability, localization, or interaction partners. In S. cerevisiae, the GID E3 ligase regulates the switch from gluconeogenic to glycolytic conditions through induction and incorporation of the substrate receptor subunit Gid4, which promotes the degradation of gluconeogenic enzymes. Here, we show that an alternative substrate receptor, Gid10, which is induced in response to changes in temperature, osmolarity and nutrient availability, regulates the ART-Rsp5 ubiquitin ligase pathway, a component of plasma membrane protein quality control. The levels of the adaptor Art2 are elevated upon GID10 deletion. A crystal structure shows the basis for Gid10-Art2 interactions, and we demonstrate that Gid10 directs a GID E3 ligase complex to ubiquitinate Art2. We also find that the GID E3 ligase affects Art2-dependent amino acid transport. The data reveal GID as a system of E3 ligases with metabolic regulatory functions outside of glycolysis and gluconeogenesis, controlled by distinct stress-specific substrate receptors.

We thank the reviewers for their enthusiasm about our work and their positive feedback. Author responses are shown in blue.

Referee #2:

The authors have thoughtfully addressed the previous reviewer comments and have conducted additional experiments. My main criticism of the revised manuscript is that the authors utilize a mutation in Rsp5 that affects ubiquitin binding (F618A) to propose a possible model in which interaction of Art2 with Rsp5 is mediated by the ubiquitin on Art2. They do not point out that F618 mutations have a influences the ability of Rsp5 to catalyze polyubiquitination and is likely temperature sensitive for growth (like an F618D mutant; DOI: 10.1038/embor.2011.23). As described, a reader would be led to conclude (from Figure 6F) that the extreme sensitivity of the Rsp5-F618 mutant to thialysine is due to the inability of Art2-Ub to bind to Rsp5. This does not seem like a very likely possibility (nor was it explored with in vitro experiments).

We have clarified the functions and phenotype of the Rsp5^{F618A} mutant in the text (page 10):

"Notably, ubiquitination of other Rsp5 adaptor proteins promotes their interaction with Rsp5 in a manner depending on Rsp5's ubiquitin-binding exosite (MacDonald *et al*, 2020). Thus, one intriguing possibility is that ubiquitinated Art2 may also employ this exosite in binding to Rsp5. Ubiquitin binding to such HECT E3 exosites has pleiotropic mechanistic roles, including allosterically activating ubiquitin transferase activity, contributing to processivity of the polyubiquitination reaction, and recruiting ubiquitinated partner proteins including adaptor proteins. Nonetheless, an Rsp5 point mutant (F618A) that impairs ubiquitin binding is useful for probing genetic interactions (Kim *et al*, 2011; Maspero *et al*, 2011; Kathman *et al*, 2015; Zhang *et al*, 2016; French *et al*, 2009). While the Rsp5^{F618A} mutation alone showed a strong growth defect on thialysine, the combination of a deletion of a GID core subunit and Rsp5^{F618A} resulted in stronger toxicity on thialysine than either mutation alone (Fig. 6F), suggesting that GID-dependent ubiquitination of Art2 is acting independently from the ubiquitin binding function of Rsp5."

Minor comments:
Grammatically, the sentence in the text referring to Figure 1B say that the mutations discussed disrupt ubiquitination by GID-SR4, but no ubiquitination experiments are shown in Fig. 1B.

The ubiquitination experiments were shown in the reference cited at the end of the sentence. The figure call has been moved to clarify this (page 4):

"While Gid4 and Gid10 were able to bind WT GID^{Ant} to a similar extent, binding was significantly abrogated to GID^{Ant} containing Gid5 point mutations (Gid5$^{W606A, Y613A, Q649A}$) on the concave binding surface (Fig 1B), which also disrupts ubiquitination by GID^{SR4} (Qiao *et al*, 2020)."

It is stated that Rsp5, Ubp2, and Art3 all contain N-terminal proline residues. I think what you mean to say is that they all contain a proline at position 2, and therefore may potentially exist in cells with an N-terminal proline. Is there evidence that the proline is ever exposed as an N-terminal proline in any of these proteins?

This has been clarified in the text (page 8):

"Intriguingly, Rsp5, the Rsp5-associated deubiquitinase Ubp2, and the α-arrestin Art3 also contain a proline at position 2, and therefore may parallel other GID E3 substrates and be processed by methionine aminopeptidase to exist in cells with an N-terminal proline. Thus, we tested if Gid10 and/or Gid4 bind the N-terminal sequences of Rsp5, Ubp2 or members of

the α-arrestin family."

Referee #3:

The authors have performed a good number of additional experiments in response to the reviewers' comments. Unfortunately, their attempts to clarify the genetic relationship between the GID complex and Rsp5-Art2 have not worked out as intended because of problems with reproducibility, background or significance of the results. The authors have therefore taken out some of these data. This makes the manuscript overall more solid, but unfortunately reduces its impact as the biochemical part is mainly a nice and accurate confirmation of a previously postulated model. There is nothing wrong with this per se, and the manuscript could be published in this form; however, it seems a pity to not put some more effort and time into clarifying the functional aspects as requested by two of the referees in their main points.

Thank you for your comments. In our revised manuscript, we demonstrated epistasis between the GID E3 ligase and Rsp5, suggesting a direct link between the two pathways. Unfortunately, the complexity and redundancy of the ART-Rsp5 system, as well as the biochemical nature of the Art2 protein, preclude further *in vivo* studies.

Dr. Christine R Langlois
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Germany

Dear Dr. Langlois,

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The data shown in figures should satisfy the following conditions:

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- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
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- a specification of the experimental system investigated (eg cell line, species name).
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- an explicit mention of the biological and chemical entity(ies) that are being measured.
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- 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.).
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  - are tests one-sided or two-sided?
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In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

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Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

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7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Yeast strains are based on the parent strain BY4741 (Euroscarf Cat#00000)

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