nature portfolio

Peer Review File

Lipid Metabolism and Contact Site Homologs Are Present at the Chloroplast Envelope-Thylakoid Interface

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This manuscript seeks to identify proteins involved in lipid metabolism at membrane contact sites (MCS) located at the interface between the chloroplast envelope and the thylakoid membranes in A. thaliana. After introducing the potential role of MCS in thylakoid biogenesis, the authors employ two complementary strategies to identify candidate MCS proteins within chloroplasts.
The first approach involves examining the subcellular localization of 19 proteins predicted to be chloroplast-resident and associated with lipids or membranes, using fluorescent protein fusions in transient expression system in N. benthamiana. Among these, five proteins exhibited strong chloroplast localization. However, the study does not assess their potential localization at MCS specifically, nor does it pursue further functional characterization beyond confirming chloroplast targeting.
The second approach relies on a proteomic analysis following the purification of four chloroplasts sub-fractions by ultracentrifugation. Proteins identified in the intermediate (light thylakoid) fraction were proposed to be involved in lipid metabolism and potentially associated with MCS. The authors validated their fractionation approach by localizing a subset of proteins identified in this fraction.
Ultimately, two candidate proteins, TVPFP and PMFP, were selected for further study. Electron microscopy of the corresponding mutants revealed alterations in the number and length of putative MCS. However, no additional physiological or biochemical analyses were performed. Further investigations—particularly during greening or under variable growth conditions, as discussed in the introduction—would be essential to substantiate the hypothesized role of these proteins in MCS and in thylakoid biogenesis. At a minimum, assessments of photosynthetic parameters, lipid composition, and pigment content should be included. In addition, correlative light electron microscopy, or higher resolution fluorescent microscopy, should be performed to confirm that the punctate signals obtained in fluorescence correspond to an enrichment of the candidates at MCSs.

Major Comments
1. Proteomic and Enrichment Claims
The principal result of the study is the demonstration that the purified light thylakoid fraction contains a protein composition distinct from that of the thylakoid and inner envelope fractions. However, the title of the manuscript is misleading in stating that the fraction is “enriched in lipid metabolism and contact site homologs.” According to the enrichment analysis (Figure 4G), Calvin cycle–related proteins represent the most enriched functional category, whereas fatty acid metabolism ranks only fourth. This discrepancy calls into question the claim of specific enrichment in lipid metabolism. In addition, key enzymes of chloroplasts lipid metabolism, including MGD1 and SQD1 are apparently not find in the proteomic data (Table 1), which is highly surprising.
2. Data Analysis and Objectivity
The proteomic dataset requires a comprehensive reanalysis with an unbiased approach. The current interpretation appears selective and insufficiently supported by quantitative comparisons. In its present form, the study does not reach the analytical rigor expected for publication in Nature Communications.
3. Purification Strategy
The strategy employed for MCS purification is not clearly described and may not be optimal. The fractionation scheme omits several potentially informative intermediate fractions, which could result in the loss of key candidate proteins. The rationale for the chosen fractionation workflow should be explicitly justified, and methodological details (e.g., sample volumes, gradients, and fraction boundaries) should be clarified.

4. Data Presentation and Transparency
The presentation of proteomic data is insufficiently transparent. Table S4 should be reformatted for clarity, with proteins sorted according to their most enriched compartment and their relative abundance compared with total chloroplast proteins. This would facilitate discrimination between genuine enrichment and potential contamination. Moreover, the criteria used to derive Table S5 from Table S4 are not described beyond homology to known MCS components, making the selection process opaque.
5. Candidate Selection and Validation
The rationale for selecting proteins for localization studies and knockout mutant generation is unclear. The localization in the different sub-fractions of the candidate identified by microscopy is not discussed. The exclusion of other potentially interesting candidates should be explained. The analysis of the two chosen mutants is limited to ultrastructural observations, which alone do not provide evidence for a role in MCS or lipid metabolism. Additional phenotypic, physiological, and metabolic analyses are required to substantiate these claims.
6. Interpretation of Localization Data
Localization analyses were performed in transient overexpressing system that is known to produce artifacts. Thus, this should be done in stable lines where the proteins are expressed under their own promotor. In addition, the imaging was not performed at a sufficient resolution to assess clearly sub-chloroplastic localization. Therefore, the statement that “the lightThy” fraction is enriched with chloroplast proteins distributed at discrete locations within the chloroplast and distinct from typical envelope localization patterns or chlorophyll fluorescence of thylakoid membranes” is not fully supported by the data. The observed discrete localization patterns could equally correspond to other chloroplast subdomains (e.g., nucleoids, sites of chlorophyll synthesis, or sugar transport regions). Without direct colocalization with envelope, thylakoids… markers, and higher resolution microscopy, no definitive conclusions can be drawn regarding MCS localization Correlative-light electron microscopy is stable lines expressing the candidates under their own promotor will be the best evidence of localization of these candidates at MCSs.

Minor Comments
• Table 1: The protein LACS9 appears twice. Since the proteomic analysis was performed in pea, the corresponding pea protein identifiers should be indicated.
• Figure 4: The fraction used for the final ultracentrifugation step is not clearly specified. It remains unclear why proteomic analysis was not conducted on the intermediate fraction between the inner envelope (IE) and thylakoid fractions from the initial centrifugation. Alternatively, a continuous gradient from intact chloroplasts might have provided a more comprehensive view of subchloroplast compartments.
• Figure 4 Overlap: Only one protein appears to be shared between the heavy IE and lightThy fractions. The identity of this protein should be specified.
• Data Completeness: The relationship between Figures 4, Table S4, and Table S5 should be explicitly described to allow reproducibility of the selection pipeline.

Overall Assessment
The study addresses an important question regarding the molecular composition of chloroplast membrane contact sites and their potential role in lipid metabolism and thylakoid development. However, the current dataset and analyses do not yet support the major conclusions. The manuscript would benefit from (i) a clearer methodological description, (ii) a more objective and transparent proteomic analysis, and (iii) additional experimental validation of candidate proteins.
With substantial revision and additional data, the study could make a meaningful contribution to the understanding of chloroplast MCS. In its current form, however, it falls short of the analytical depth and experimental rigor required for publication in Nature Communications.

Reviewer #2

(Remarks to the Author)
LaBrant et al. describes the identification of putative protein players involved in inner envelope-thylakoid contact sites, a little studied type of membrane contact site (MCS) found within plant chloroplasts and hypothesized to mediate lipid and fatty acid trafficking and thylakoid membrane biogenesis. How lipids move within the chloroplast and facilitate thylakoid biogenesis has been a long-standing and important question, but for which little headway has been made thus far.

The authors describe a two-pronged effort to identify putative mediators of IE-thy MCSs to address this long-standing question: i) a bioinformatics search for MCS /transporter homologs (followed by confocal validation of chloroplast localization) and ii) biochemical fractionation and proteomics analysis of light fractions among the thylakoid and heavier fractions among the IE. The resulting lists of candidates should provide a rich set of proteins for functional characterization in IE-thy MCSs. Importantly, the authors selected two candidate proteins and provide experimental support (cellular ultrastructure anomalies from electron micrographs) for roles in IE-thy MCSs, most notably the TVPFP protein which shows a greatly expanded length of MCSs in young chloroplasts of the cotyledon, providing experimental support for their lists. It is notable that, despite the clear impact on ultrastructure caused by disruption of tvpmp (Figure 6), the plant growth and development does not appear to be disturbed. Some discussion of why this may be true should be included in the Discussion.

The science is sound, though there are several concerns to raise and suggestions to strengthen the impact and enhance the clarity:

Concerns -

1. An extensive proteomics dataset was developed as part of this work though I don't see any reference to its availability or way for reviewers to review the raw/metadata. In my experience, it is standard practice to make proteomics data available for review of a manuscript.
2. I am confused with the statement that banding patterns of certain lanes are "characteristic" of IE or thy. Characteristic based on what? At the least, some references to previous papers with a descriptive comparison seems warranted for a reader to judge if this is indeed the case. And, the asterisks that are supposed to highlight such characteristic banding patterns mark only a few selected bands within a lane. Such a pattern doesn't sound very "characteristic" of any sub-compartment fraction. Furthermore, the asterisks are marking a few proteins in lane 11 (IE) and lane 19 (thy), even though these are not annotated as the genuine IE and thy fractions marked in lane 12 (IE) and lane 16 (thy). I question how appropriate is the execution of the biochemical fractionation without understanding correctly what was done or being able to judge if the thy and IE fractions are indeed what they are claimed to be.

Suggestions -
3. Why do the authors not address possible overlap of protein identifications between the bioinformatics search and biochemical fractionation? It seems like an obvious question for a reader whether there are proteins that come from the first-prong and the second, and if there such overlapping proteins it seems that these would be especially high-priority candidates to act in MCSs.
4. In the introduction, I believe another relevant citation for MCSs in ER-chloroplast lipid transfer would be: DOI: 10.1038/s41467-024-50425-7 and for cryo-ET tomography would be: DOI: 10.7554/eLife.105496
5. There seems to be some disconnect between the description of biochemical fractionation in the main text on one-hand and the M&M and Figure 3 legend on the other hand. Specifically, how was the homogenization for the second homogenization performed? hypertonic lysis? or freeze/thaw dounce homogenization? Or perhaps both? At any rate, it should be made clear and consistent between the different locations.
6. There is a reference to enrichment of proteins in various biochemical fractions but the percentage of what? Percentage of number of proteins? Percentage of protein abundance ie NSAFs? Or something else?
7.The authors describe an enrichment of lipid-related enzymes in lightThy of 3-fold compared to IE fraction. However, there are also about 3x as many identified proteins in the lightThy fraction as IE fraction, so I question if there really is this described enrichment of lipid related proteins in lightThy.
8. In the M&M, IE and thylakoid fractions are said to be "diluted" and pelleted (130,000 xg, 30 mins). What were they diluted in?
9. In Figure 1 it seems the partial and none categories are statistically significantly different from the full category but no asterisks are present. Is this correct?
10. In Figure 3 - panel A, the arrows seem to suggest (incorrectly) that the IE and thy were subsequently pooled before the second separation step. The authors could also mark the lower density broken chloroplast band in the initial centrifugation, along with the intact chloroplast band. Also, is 'M' indicating the protein marker lane? And finally, the legend stats, "These fractions were homogenized again", however from what I can tell only IE and thy were homogenized again, not OE as indicated in the legend. Finally, on this point, it would be very helpful for the reader if the approximate level/position of those fractions selected for proteomics were marked alongside the centrifuged samples. It seems that there is space within the figure to a blow-up of the tubes perhaps, which may be an effective and simple way to achieve this?
11. Figure 4F and G - I believe there are supposed to be colors associated with the FDR that are missing, correct?
12. Figure 4C legend - what are the fold-change and p-value respective to? is this relative to all other sub-compartments, or ...?
13. Figure 6 legend - some detail is needed where it is said that plants were grown under "standard conditions". For example, was this continuous light as for the TEM in panel C?

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This study addresses an important question about the molecular composition of chloroplast membrane contact sites and their potential role in lipid metabolism and thylakoid development. However, this article attempts to combine two independent stories that are only thematically linked. The first story attempts to identify chloroplast-localized lipid transporters using a bioinformatic approach. The researchers selected 19 candidates to verify their localizations and identified five with strong chloroplast localizations, including TVPFP and PMFP. The researchers analyzed the ultrastructure of the chloroplasts by electron microscopy (EM) and the photosynthetic activity of the KO mutant of these two proteins, observing minor defects. However, there is no evidence that these two proteins are involved in lipid transport.
The second approach is subfractionation, in which the authors isolated membrane contact sites from the inner envelope of the chloroplast or the thylakoid. This approach, combined with proteomics, is now well described and explained, yielding 16 interesting candidates. However, nothing has been done with these candidates except localizing three other proteins that aren't even among the 16 candidates.
For publication, at least the interconnection of the two stories and the function of one candidate are needed.
Minor comment:
- In Table S5, there are some empty lines with logP. What is the meaning of logP, and how is it calculated?
- I do not understand how the pairwise analyses in Table S4 are done if the number of analyses is not the same. For instance, there are four thy fractions versus six lightthy fractions. The fold change should be determined experimentally because each fractionation has its own biological variability. In Table S4, what is the difference between "is enriched" and

"is more"?
- I still don't understand the meaning of the enrichment in Figure 4C. I understand that it represents the redistribution of the localization of the identified proteins in the fraction. However, I don't understand what they are enriched against. The answer to reviewer 2.6 is unclear to me.
- On line 297, it says that TGD3 is enriched in heavyIE and lightThy. In this case, it should be present in Table 2, yet Table S4 states that IE is more.

Reviewer #2

(Remarks to the Author)
My concerns have been adequately addressed.

Response to reviewers

Dear Editor and Reviewers,

We thank you for your thorough and constructive evaluation of our manuscript. We have revised the manuscript extensively to improve clarity, transparency, and rigor, and to better align our conclusions with the strength of the data. We also added new physiological analyses for the *tvpfp* and *pmfp* mutants, expanded the description of the fractionation workflow, clarified the proteomics analyses and selection pipeline, corrected figure and table issues, and ensured public deposition (with reviewer access) of the proteomics dataset.

Below, we provide a point-by-point response. Reviewer comments are reproduced (in bold), followed by our responses.

Reviewer 1: This manuscript seeks to identify proteins involved in lipid metabolism at membrane contact sites (MCS) located at the interface between the chloroplast envelope and the thylakoid membranes in A. thaliana. After introducing the potential role of MCS in thylakoid biogenesis, the authors employ two complementary strategies to identify candidate MCS proteins within chloroplasts.
The first approach involves examining the subcellular localization of 19 proteins predicted to be chloroplast-resident and associated with lipids or membranes, using fluorescent protein fusions in transient expression system in *N. benthamiana*. Among these, five proteins exhibited strong chloroplast localization. However, the study does not assess their potential localization at MCS specifically, nor does it pursue further functional characterization beyond confirming chloroplast targeting.

Response: We appreciate the reviewer's interest in our work. There seems to be some confusion regarding the identification of TVPFP and PMFP, both of which came from the *N. benthamiana* subcellular localization study, and are visible in Figure 1, Table S1, and Table S2. We apologize for the confusion, and have clarified their origin in the text, stating, "Two candidates identified in our initial confocal microscopy screen, TVPFP and PMFP, … … were selected for additional characterization."

Reviewer 1: The second approach relies on a proteomic analysis following the purification of four chloroplasts sub-fractions by ultracentrifugation. Proteins

identified in the intermediate (light thylakoid) fraction were proposed to be involved in lipid metabolism and potentially associated with MCS. The authors validated their fractionation approach by localizing a subset of proteins identified in this fraction. Ultimately, two candidate proteins, TVPFP and PMFP, were selected for further study. Electron microscopy of the corresponding mutants revealed alterations in the number and length of putative MCS. However, no additional physiological or biochemical analyses were performed. Further investigations—particularly during greening or under variable growth conditions, as discussed in the introduction—would be essential to substantiate the hypothesized role of these proteins in MCS and in thylakoid biogenesis. At a minimum, assessments of photosynthetic parameters, lipid composition, and pigment content should be included. In addition, correlative light electron microscopy, or higher resolution fluorescent microscopy, should be performed to confirm that the punctate signals obtained in fluorescence correspond to an enrichment of the candidates at MCSs.

Response: We agreed that additional functional data would add value, we have therefore added chlorophyll fluorescence and pigment analyses for *tvpfp* and *pmfp* loss-of-function mutants. We have described these results in text "Maximum quantum yield (F_v/F_m) was equivalent across genotypes, but dynamic measurements of photosystem II performance indicated subtle alterations. *tvpfp* plants displayed a reduced maximum ΦPSII under actinic light with more rapid ΦPSII recovery after the light-to-dark transition, whereas *pmfp* plants showed slower ΦPSII induction but reached a higher maximum ΦPSII than wild type (Figure S6, Table S9). In the dark, both mutants exhibited faster NPQ relaxation relative to wild type, and pigment measurements showed *pmfp* plants had increased chlorophyll and carotenoid content in both lines (Figure S7, Table S10)." As the text indicates, we have added two supplemental figures and associated statistical tables.

The reviewer's comment that further physiological assessments are "essential", may indicate some confusion over the typical overlapping functions of lipid transporters at membrane contact sites. To avoid similar confusion in readers, we have improved the discussion to directly address the moderate physiological phenotypes observed, "The significant expansion of MCS length in *tvpfp* mutants (Figure 6C-D, despite the moderate developmental and photosynthetic phenotypes (Figures 6B, S6 and S7), aligns with established paradigms in MCS biology. Lipid transfer at membrane interfaces is often governed by functionally redundant protein families. A classic example is the yeast OSH family; while the septuple mutant is inviable, single or even sextuple mutants exhibit few

growth defects, suggesting overlapping roles in lipid homeostasis (Beh et al., 2001). Furthermore, the loss of primary tethers often triggers compensatory morphological changes. In mammalian cells, the loss of E-Syt tethers does not disrupt basic cellular viability, likely due to the presence of secondary tethers that maintain membrane proximity (Sclip et al., 2016). The expanded MCSs observed in *tvpfp* plastids (Figure 6C-D) may therefore reflect a compensatory response, where physical contact area is increased to facilitate lipid flux via parallel, perhaps less efficient, transport pathways. It is also possible that the physiological impact of *tvpfp* disruption is conditional, causing significant phenotypes under specific conditions that demand high-flux lipid transport or in combination with other mutations, a common feature of MCS proteins in other organisms (Manford et al., 2012)." Given this reality and the fact that i) our proteomic analysis showed multiple lipid transporters, and ii) there is a known vesicle system within chloroplasts that likely contributes to lipid transfer, we did not do lipid analysis in the single mutant backgrounds as it was unlikely to be informative.

Reviewer 1. In addition, correlative light electron microscopy, or higher resolution fluorescent microscopy, should be performed to confirm that the punctate signals obtained in fluorescence correspond to an enrichment of the candidates at MCSs.

Response: We also agree that definitively assigning the punctate fluorescence to MCS/TIES is desirable. However, it requires higher-resolution imaging and stable, native-expression lines (e.g., colocalization with envelope/thylakoid markers and/or CLEM or immunogold). Because developing and validating these approaches is beyond the scope of this revision, we have avoided definitive claims of MCS localization and instead describe the puncta as discrete chloroplast subdomains that are consistent with, but do not prove, contact-site association.

With respect to the reviewer's doubts, we note this study identifies useful sub-enrichments of chloroplast proteins, describing subcellular locations of MCS lipid transport homologs and identifying unique intermediate-density chloroplast membranes enriched in additional MCS homologs. We believe this, in addition to our enhanced validation strategy of TVPFP and PMFP make an exciting community resource into a mechanism of lipid transport to thylakoids often hypothesized (Sakamoto, 2025, GARAB et al., 2025, LaBrant et al., 2018), and with no previous candidate proteins.

Sakamoto, W., 2025. Thylakostasis: key factors in thylakoid membrane organization with emphasis on biogenesis and remodeling proteins in vascular plants. Plant Cell Physiol 66, 1602–1618. https://doi.org/10.1093/pcp/pcaf098

GARAB, G., BÖDE, K., DLOUHÝ, O., NÁSZTOR, Z., KARLICKÝ, V., DÉR, A., ŠPUNDA, V., 2025. Lipid polymorphism of plant thylakoid membranes. The dynamic exchange model – facts and hypotheses. Physiol Plant 177, e70230. https://doi.org/10.1111/ppl.70230

LaBrant, E., Barnes, A.C., Roston, R.L., 2018. Lipid transport required to make lipids of photosynthetic membranes. Photosynth Res 138, 345–360. https://doi.org/10.1007/s11120-018-0545-5

Reviewer 1. 1. Proteomic and Enrichment Claims

The principal result of the study is the demonstration that the purified light thylakoid fraction contains a protein composition distinct from that of the thylakoid and inner envelope fractions. However, the title of the manuscript is misleading in stating that the fraction is "enriched in lipid metabolism and contact site homologs." According to the enrichment analysis (Figure 4G), Calvin cycle–related proteins represent the most enriched functional category, whereas fatty acid metabolism ranks only fourth. This discrepancy calls into question the claim of specific enrichment in lipid metabolism.

Response. Thank you for this important point. We agree that our original title/text could be read as implying that lipid metabolism dominates the lightThy fraction. Our intent was to report statistical over-representation from standard enrichment analyses, where "enriched" is commonly used to describe over-representation results (Combes et al., 2021). We also note that our traditional enrichment analysis was performed without a custom background (e.g., versus all Arabidopsis proteins), meaning that comparing enrichment magnitude across pathways was complicated by the lack of organelle-specific metabolic terms (e.g., the Calvin cycle is chloroplast-specific, while "lipid metabolism" annotations span multiple compartments), making them necessarily less statistically enriched. To address this problem, we removed the traditional analysis and did a more relevant enrichment analysis using Metascape (Zhou et al., 2019), which allows correction for the inherent abundance bias of biological fractionation data by use of a custom background. The new enrichment results report fraction-specific enrichment, rather than whole-organism enrichment (Figure 4D). The updated results paragraph begins, "To characterize functional specialization, we performed Gene Ontology (GO) enrichment analysis using the total identified proteome as a background to focus on fraction-specific signatures. The resulting heatmap (Figure 4D, Table S6) revealed a functional divergence between the canonical membrane fractions (IE, thy) and the lightThy intermediate density fraction…" In brief, again the lightThy stands out from other fractions as a metabolic hub that *includes* enrichment of lipid metabolism and transport proteins among other functions. However, to avoid any concern regarding

overstatement, we have changed the language of the title and abstract to note that lipid metabolism is “present” and “contained” in the lightThy fraction.

Reviewer 1. In addition, key enzymes of chloroplasts lipid metabolism, including MGD1 and SQD1 are apparently not find in the proteomic data (Table 1), which is highly surprising.

Response. We agree with the reviewer that MGD1 absence is notable. MGD1, a highly hydrophobic inner-envelope enzyme, can be under-detected in LC–MS/MS; additionally, MGD1 may preferentially function in contexts that channel MGDG toward outer envelope processes to make DGDG (Kelly et al., 2016), which may further reduce recovery in the fractions analyzed here. With respect to SQD1, this is a soluble enzyme that produces UDP-6-sulfoquinovose (Mulichak et al., 1999), a precursor to lipid SQDG synthesis by SQD2, and we would not expect it to be preferentially retained in our membrane-enriched fractions. For these reasons, we emphasize fraction-proteome-wide statistical patterns (Figure 4; Table S6) rather than drawing conclusions from the presence/absence of individual enzymes.

Combes, F., Loux, V., Vandenbrouck, Y., 2021. GO Enrichment AnalysisGO enrichment analysis for Differential Proteomics Using ProteoREProteoRE, in: Cecconi, D. (Ed.), Proteomics Data Analysis. Springer US, New York, NY, pp. 179–196. https://doi.org/10.1007/978-1-0716-1641-3_11

Kelly, A.A., Kalisch, B., Holzl, G., Schulze, S., Thiele, J., Melzer, M., Roston, R.L., Benning, C., Dormann, P., 2016. Synthesis and transfer of galactolipids in the chloroplast envelope membranes of Arabidopsis thaliana. Proc Natl Acad Sci U S A 113, 10714–9. https://doi.org/10.1073/pnas.1609184113

Mulichak, A.M., Theisen, M.J., Essigmann, B., Benning, C., Garavito, R.M., 1999. Crystal structure of SQD1, an enzyme involved in the biosynthesis of the plant sulfolipid headgroup donor UDP-sulfoquinovose. Proc.Natl.Acad.Sci.U.S.A. 96, 13097–13102.

Reviewer 1. 2. Data Analysis and Objectivity
The proteomic dataset requires a comprehensive reanalysis with an unbiased approach. The current interpretation appears selective and insufficiently supported by quantitative comparisons. In its present form, the study does not reach the analytical

rigor expected for publication in Nature Communications.

Response: We thank the reviewer for helping maintain solid analysis criteria in published work. While the proteomic dataset itself was generated and analyzed using standard, quantitative best practices, we have revised the manuscript to improve objectivity, transparency, and balance in how the results are presented. Briefly, protein identification used stringent criteria (≥2 peptides, peptide-level FDR 0.1%, protein-level FDR 1%*), followed by analysis in Perseus using normalized spectral abundance factors (NSAF), (Rudolph and Cox, 2019; Tyanova et al., 2016). Proteins were required to be detected in at least three biological replicates. Multivariate and statistical analyses (including PCA and enrichment testing) were performed with permutation-based FDR correction. Downstream annotation and interpretation relied on established databases, including SUBA5 and PPDB for subcellular localization, Metascape for functional enrichment, the AraLip database for a second confirmation of lipid metabolism, and the MCS database for contact site homology.

In our revision efforts, we did completely reanalyze the proteomics to ensure its accuracy, and revised the related results and methods sections completely to improve clarity. We apologize that we accidentally reported a more stringent statistical cutoff to define proteins abundant in specific fractions in the original functional enrichment analysis and database searches (-logPadj of 1.5 instead of 1.3 as reported in the Methods) for our submitted manuscript, which made numbers different in Tables S4 versus S6. We have rectified this in the current version, and all analyses are based on the reported1.3 cutoff now. The change in cutoff changed the precise statistical values, but not the conclusions. Nevertheless, we apologize for this error, which may have caused confusion for the reviewer.

* We note these cutoffs are more stringent than those required by ProteomeXchange, which follows HUPO 3.0 guidelines which are 1% for both peptide and protein-level FDR (https://hupo.org/HPP-Data-Interpretation-Guidelines).

Rudolph, J.D., and Cox, J. (2019). A Network Module for the Perseus Software for Computational Proteomics Facilitates Proteome Interaction Graph Analysis. J Proteome Res 18, 2052-2064.

Tyanova, S., Temu, T., Sinitcyn, P., Carlson, A., Hein, M.Y., Geiger, T., Mann, M., and Cox, J. (2016). The Perseus computational platform for comprehensive analysis of (prote)omics data. Nat Methods 13, 731-740.

Reviewer 1. 3. Purification Strategy

The strategy employed for MCS purification is not clearly described and may not be optimal. The fractionation scheme omits several potentially informative intermediate fractions, which could result in the loss of key candidate proteins. The rationale for the chosen fractionation workflow should be explicitly justified, and methodological details (e.g., sample volumes, gradients, and fraction boundaries) should be clarified.

Response: We thank the reviewer for giving us this opportunity to explain our purification rationale. Our goal was not to purify membrane contact sites (MCSs) as a discrete organelle, but rather to enrich for tightly associated membrane subdomains of intermediate buoyant density that could plausibly contain components of thylakoid–inner envelope contact sites (TIES). We certainly agree that our dataset must fall short of complete candidate capture. As MCSs may be transient, small, and not biochemically isolatable as pure entities, our approach was intentionally designed to capture intermediate-density membrane populations rather than to fractionate exhaustively across all gradient regions. Similar approaches have "…been successfully applied to characterize contact sites between the mitochondrial inner and outer membranes (Hermann et al., 1998; Pon et al., 1989), mitochondria and the ER (Rusiñol et al., 1994; Vance, 2014), and bacterial inner and outer envelope membranes (Ishidate et al., 1986; Tefsen et al., 2005)." As we note in our results.

Specifically, intact chloroplasts were first separated into crude outer envelope, inner envelope (IE), and thylakoid fractions using a discontinuous sucrose gradient, following established envelope isolation protocols. These membrane fractions were then subjected to a second round of lysis and homogenization to disrupt weak associations (membrane contact sites should be quite strong as shown in Andersson et al., 2007), after which membranes were resolved on a continuous sucrose gradient to allow finer separation by density (Figure 3A; Methods). This two-step strategy prioritized recovery of stably associated membrane subdomains, which we expected to migrate at densities intermediate between canonical IE and thylakoid membranes.

We agree that additional intermediate fractions could contain additional candidate proteins. However, in practice, broad sampling across the gradient resulted in insufficient reproducibility across biological replicates due to varying protein levels, which can be observed in Figure 3B and especially in our new supplemental figure (Figure S4) showing A280 levels of fractions. It clearly indicates the strong differences between thylakoid and inner envelope protein levels, and shows how quickly protein levels dropped at fractions further from the peak protein of IE and thylakoid membranes. We therefore focused our proteomic analyses on reproducible fractions—IE, heavyIE, thylakoid, and light thylakoid

(lightThy)—that showed consistent banding patterns (Figure 3B), reproducible clustering by PCA (Figure 4A), and distinct proteomic signatures (Figure 4C). Importantly, the lightThy and heavyIE fractions occupied intermediate positions between IE and thylakoid in multivariate space, supporting their interpretation as discrete membrane populations rather than contamination artifacts.

In response to the reviewer's request for greater clarity, we have expanded the Methods and revised Figure 3 and its legend to explicitly describe gradient compositions, fraction volumes, boundaries, and the rationale for fraction selection, and to clearly indicate which fractions were used for downstream proteomics. We also respond to the reviewers additional request for information below with our methodological development. We believe these clarifications help to make the workflow and its underlying logic fully transparent and reproducible.

Andersson, M.X., Goksör, M., Sandelius, A.S., 2007. Optical manipulation reveals strong attracting forces at membrane contact sites between endoplasmic reticulum and chloroplasts. The Journal of Biological Chemistry 282, 1170–1174. https://doi.org/10.1074/jbc.M608124200

Reviewer 1. 4. Data Presentation and Transparency

The presentation of proteomic data is insufficiently transparent. Table S4 should be reformatted for clarity, with proteins sorted according to their most enriched compartment and their relative abundance compared with total chloroplast proteins. This would facilitate discrimination between genuine enrichment and potential contamination. Moreover, the criteria used to derive Table S5 from Table S4 are not described beyond homology to known MCS components, making the selection process opaque.

Response: As stated above, we have completely revised the results and methods sections involving proteomics data. We have also sorted Tables S4 and S5 (now S6) based on enrichment. However, we sorted based on enrichment in relative fractions (e.g., heavy IE vs IE), since these are the analyses used in the work. We note that in methods development, relative enrichment to whole chloroplasts was somewhat uninformative, as all envelope proteins were dramatically enriched relative to whole chloroplasts, which have far more thylakoidal protein (now observable in our fractions in Figure S4), and directly addressed in the results, "We performed pairwise comparisons of IE versus heavyIE and lightThy versus Thy, as these represent the most closely related membrane populations and this strategy avoids the dynamic range compression typically seen when comparing to intact chloroplast

proteomes (Bouchnak et al., 2019; Rolland et al., 2012)."

Finally, we have updated the Methods to clarify how Table S6 was derived: "To assess similarity to known membrane contact site (MCS) proteins, non-redundant sequences of Arabidopsis homolog proteins uniquely enriched in each fraction, or co-enriched in the heavyIE and lightThy fractions were queried against the MCSdatabase v 1.7 (Pan et al., 2024) using blastp with standard settings. Proteins with bit scores over 50 were considered to share homology with MCS components, and are indicated in Table S6." During the process of revision, we checked all proteomics data tables thoroughly and found that in our original submission of Table S5, a more stringent fold change cutoff had been applied when generating the original table. We apologize if this caused the reviewer confusion, and thank them for their thoroughness. We now have a supplemental table (S6) that is consistent with cutoffs used in the methods. The corresponding results section has been slightly updated to reflect the new values.

Reviewer 1: 5. Candidate Selection and Validation
The rationale for selecting proteins for localization studies and knockout mutant generation is unclear. The localization in the different sub-fractions of the candidate identified by microscopy is not discussed. The exclusion of other potentially interesting candidates should be explained. The analysis of the two chosen mutants is limited to ultrastructural observations, which alone do not provide evidence for a role in MCS or lipid metabolism. Additional phenotypic, physiological, and metabolic analyses are required to substantiate these claims.

Response: We thank the reviewer for highlighting the need to clarify candidate selection and to strengthen functional validation. We have revised the manuscript to make the rationale for candidate prioritization explicit and to expand validation beyond ultrastructural observations.

We now clarify that practical considerations (availability of mutants and lack of prior functional characterization) contributed to candidate selection. The results now states that TVPFP and PMFP "… were selected for additional characterization based on their predicted roles, unstudied nature, and the availability of T-DNA insertion lines. " We also clarify for the reviewer that other candidates were not excluded for lack of interest but rather deferred for future study, as validation work is necessarily time intensive. In our opinion, it does not make sense to restrict these datasets, which could be worked on by

many, for the amount of time that investigation of all candidates would take.

We agree that ultrastructural analysis alone is insufficient to support functional relevance. In response, we have added physiological and biochemical analyses of *tvpfp* and *pmfp* mutants, including chlorophyll fluorescence measurements and pigment quantification. As stated above, these data reveal altered photosynthetic performance and pigment accumulation in both mutants relative to wild type and are presented in Figures S6 and S7, with associated statistical supplemental tables S8, S9, and S10. Together with the observed perturbations in thylakoid–inner envelope contact architecture (Figure 6), these results provide functional support for roles of TVPFP and PMFP in chloroplast membrane organization.

Finally, we have revised the manuscript text to avoid definitive claims that any candidate is localized to MCSs. Instead, we frame the data as localizing chloroplast lipid transport proteins concentrated in sub-organellar compartments, and identifying candidate proteins associated with intermediate-density membrane subdomains, whose disruption affects chloroplast membrane architecture and function, consistent with a structured and functionally specialized intermembrane zone.

Reviewer 1. 6. Interpretation of Localization Data

Localization analyses were performed in transient overexpressing system that is known to produce artifacts. Thus, this should be done in stable lines where the proteins are expressed under their own promotor. In addition, the imaging was not performed at a sufficient resolution to assess clearly sub-chloroplastic localization. Therefore, the statement that "the lightThy" fraction is enriched with chloroplast proteins distributed at discrete locations within the chloroplast and distinct from typical envelope localization patterns or chlorophyll fluorescence of thylakoid membranes" is not fully supported by the data. The observed discrete localization patterns could equally correspond to other chloroplast subdomains (e.g., nucleoids, sites of chlorophyll synthesis, or sugar transport regions). Without direct colocalization with envelope, thylakoids… markers, and higher resolution microscopy, no definitive conclusions can be drawn regarding MCS localization Correlative-light electron microscopy is stable lines expressing the candidates under their own promotor will be the best evidence of localization of these candidates at MCSs.

Response: We agree with the reviewer that localization data obtained from transient overexpression systems must be interpreted cautiously and that higher-resolution imaging in stable lines under native promoters would be required to definitively assign proteins to thylakoid–inner envelope contact sites (TIES). We also fully agree that stable lines expressing candidates under their native promoters, combined with colocalization to envelope/thylakoid markers or correlative light–electron microscopy (CLEM), represent the most definitive strategy for assigning proteins to MCSs. However, generating and validating such lines and implementing CLEM or super-resolution approaches in the highly autofluorescent thylakoid environment would constitute a substantial, standalone study. Such work is beyond the scope of the manuscript, which already provides multiple lines of evidence to support sub-chloroplastic concentration, functional relevance, and refrains from claiming definitive MCS localization. Instead, we use transient expression as an initial screening tool to assess chloroplast targeting and suborganellar distribution patterns. We note that we believe the distinction between envelope control proteins (Figure 1B) and these non-uniformly distributed proteins (Figure 5A) is fair, as both tests were consistently performed under the same transient expression systems.

In the revised manuscript, we have clarified the interpretation of all localization data. Specifically, we avoid statements implying that punctate fluorescence corresponds directly to MCSs. Instead, we describe these signals as chloroplast subdomains, acknowledging clearly that, "These subdomains are not exclusive to lipid transport. Instead, our data and the Chlamydomonas data (Sun et al., 2025) suggest the presence of multiple functions, potentially at separate subdomains."

Reviewer 1. Minor Comments

• Table 1: The protein LACS9 appears twice. Since the proteomic analysis was performed in pea, the corresponding pea protein identifiers should be indicated.

Response: Thank you for catching this. The duplication of LACS9 was our mistake, and it has been removed from Table 1. The legend of Table 1 has been updated to note that these are "non-redundant" Arabidopsis homologs of lipid metabolism proteins. Finally, the pea identifiers are indicated in Tables S3 and S4.

Reviewer 1. • Figure 4: The fraction used for the final ultracentrifugation step is not clearly specified. It remains unclear why proteomic analysis was not conducted on the intermediate fraction between the inner envelope (IE) and thylakoid fractions from

the initial centrifugation. Alternatively, a continuous gradient from intact chloroplasts might have provided a more comprehensive view of subchloroplast compartments.

Response: The fractions used for final ultracentrifugation and subsequent proteomics analysis are now indicated with arrowheads in Figure 3, and corresponding supplemental Figure S4. They were 2 fractions away from the first fraction (thylakoid) or last fraction (IE) representing the bulk of the membrane. As needed, an additional heavy or light fraction 3 fractions away from the indicated IE or thylakoid fractions were grouped with the one 2 fractions away to allow analysis of equal total proteins. We selected these fractions after multiple practice runs.

We explored both alternatives suggested by the reviewer during method development. First, when using a single continuous sucrose gradient directly from intact chloroplasts, fractions between the IE and thylakoid bands were dominated by thylakoid membranes and were not readily distinguishable from the bulk thylakoid fraction by proteomic composition. We attribute this to the large disparity in abundance between thylakoid and IE membranes, with thylakoids greatly exceeding the IE in both lipid and protein content. Only after an initial discontinuous fractionation step were we able to more effectively separate the bulk IE and thylakoid membranes and recover a reproducible intermediate-density fraction suitable for downstream analysis.

Second, we initially surveyed multiple intermediate fractions between the IE and thylakoid bands by proteomics. Fractions farther from the bulk membrane bands yielded substantially lower total protein, limiting quantitative comparability across samples, whereas fractions closer to the thylakoid band were dominated by photosynthetic proteins. Based on these empirical assessments, we selected the intermediate fractions shown in Figure 3 and S4, which provided the best balance of reproducibility, protein yield, and compositional distinction for comparative proteomic analysis.

Based on the reviewer's interest, we have updated our Methods and results sections to better reflect the rationales behind our decisions.

Reviewer 1. • Figure 4 Overlap: Only one protein appears to be shared between the heavy IE and lightThy fractions. The identity of this protein should be specified.

Response: When the proteomic data (Table S4) is filtered for significant enrichment and identification ($\log_2$ fold change threshold of ±0.5 and an adjusted p-value cutoff of $-\log_{10}(p) \geq 1.3$ as stated in the methods) for the heavy IE and light Thy fractions, there are 16 proteins enriched in both the heavyIE and lightThy fractions.

While this information was always available, we appreciate that it was not easily accessible, and based on the mutual interest of both reviewers, we have added this table to the results as "Table 2", included a brief description of it, and added a column in Table S4 "Enriched in both Heavy IE and light Thy fractions" that is easily sortable to return the above table integrated with other relevant data (Pea identifiers, abundances, etc.). We also integrate the "overlap" fraction into analyses for lipid and MCS enrichment, though due to its very small number of proteins, we draw cautious conclusions from its high levels of both MCS homologs and lipid metabolic proteins. The relevant results paragraph is below:

"Although the heavyIE fraction appeared dominated by photosynthetic functions in the broad GO enrichment analysis (Figure 4D), we reasoned that proteins co-enriched in both the heavyIE and lightThy fractions would still represent high-priority candidates for TIES. By filtering for Arabidopsis homologs that were enriched in the intermediate density fractions in both directions, we identified a discrete set of 16 proteins (Table 2). A feature of this overlapping set is the presence of the complete plastidial pyruvate dehydrogenase (PDH) complex—including PDH E1α, PDH E1β, LTA2 (E2), and LPD1 (E3)—which provides the acetyl-CoA precursor required for de novo fatty acid synthesis (Li-Beisson, et al., 2013). The co-enrichment of these complex components, alongside regulatory proteins like the PSII accumulation factor LPA1 and the metalloproteases FTSH1 and FTSH2, suggests that the heavyIE fraction, despite its large photosynthetic signature, contains a protein subset likely present at envelope-thylakoid interfaces."

Reviewer 1. • Data Completeness: The relationship between Figures 4, Table S4, and Table S5 should be explicitly described to allow reproducibility of the selection pipeline.

Response: Yes, as stated above, we have revised both the results and methods sections for clarity on the proteomic data analysis. The relevant methods section now reads, "All proteins meeting quality control and statistical criteria are reported in Table S4, which serves as the complete source table underlying Figure 4A-E. Table S4 includes normalized abundance values (NSAF), log□ fold changes for all pairwise fraction comparisons, adjusted p-values, enrichment direction, and curated subcellular and suborganellar localization assignments used for the analyses shown in Figure 4A–C, E. Proteins meeting enrichment thresholds in each fraction were used to generate the fraction-specific functional enrichment analyses shown in Figure 4D, F, and G."

Also as indicated above, the contents of Table S5 (now S6) have been clarified to allow reproducibility of the selection pipeline in the methods, which now states, "To assess similarity to known membrane contact site (MCS) proteins, non-redundant sequences of Arabidopsis homolog proteins uniquely enriched in each fraction, or co-enriched in the heavyIE and lightThy fractions were queried against the MCSdatabase v 1.7 (Pan et al., 2024) using blastp with standard settings. Proteins with bit scores over 50 were considered to share homology with MCS components, and are indicated in Table S6."

Reviewer 1. Overall Assessment
The study addresses an important question regarding the molecular composition of chloroplast membrane contact sites and their potential role in lipid metabolism and thylakoid development. However, the current dataset and analyses do not yet support the major conclusions. The manuscript would benefit from (i) a clearer methodological description, (ii) a more objective and transparent proteomic analysis, and (iii) additional experimental validation of candidate proteins.
With substantial revision and additional data, the study could make a meaningful contribution to the understanding of chloroplast MCS. In its current form, however, it falls short of the analytical depth and experimental rigor required for publication in Nature Communications.

Response. As suggested, we have added (i) a clearer methodological description of the fractionation and (ii) proteomics workflows including a transparent treatment of the proteomic analysis, and (iii) provided additional experimental validation of candidate proteins. We believe these changes have substantially improved the manuscript and we thank the reviewer for their assistance in doing so.

Reviewer #2. LaBrant et al. describes the identification of putative protein players involved in inner envelope-thylakoid contact sites, a little studied type of membrane contact site (MCS) found within plant chloroplasts and hypothesized to mediate lipid and fatty acid trafficking and thylakoid membrane biogenesis. How lipids move within the chloroplast and facilitate thylakoid biogenesis has been a long-standing and important question, but for which little headway has been made thus far.

The authors describe a two-pronged effort to identify putative mediators of IE-thy MCSs to address this long-standing question: i) a bioinformatics search for MCS /transporter homologs (followed by confocal validation of chloroplast localization) and ii) biochemical fractionation and proteomics analysis of light fractions among the

thylakoid and heavier fractions among the IE. The resulting lists of candidates should provide a rich set of proteins for functional characterization in IE-thy MCSs. Importantly, the authors selected two candidate proteins and provide experimental support (cellular ultrastructure anomalies from electron micrographs) for roles in IE-thy MCSs, most notably the TVPFP protein which shows a greatly expanded length of MCSs in young chloroplasts of the cotyledon, providing experimental support for their lists. It is notable that, despite the clear impact on ultrastructure caused by disruption of *tvpfp* (Figure 6), the plant growth and development does not appear to be disturbed. Some discussion of why this may be true should be included in the Discussion.

Response. Thank you for your appreciation of our research topic. As suggested by the first reviewer, we have expanded the number of physiological experiments done on *pmfp* and *tvpfp*, and accordingly expanded the discussion to include the phenotype severity. We thank the reviewer for this opportunity, which is likely to improve reader understanding. The reviewer is correct that the *tvpfp* mutant exhibits a striking ultrastructural phenotype (expanded MCS length) without severe developmental defects under standard growth conditions. We now know it has photosynthetic defects that are detectable, though moderate (Figures S6 and S7 and corresponding statistical tables S8, S9, and S10). This "lack" of significant growth defects—is a well-documented phenomenon in the study of membrane contact sites due to redundancy, as we now note clearly in the following discussion paragraph.

"The significant expansion of MCS length in *tvpfp* mutants (Figure 6C-D, despite the moderate developmental and photosynthetic phenotypes (Figures 6B, S6 and S7), aligns with established paradigms in MCS biology. Lipid transfer at membrane interfaces is often governed by functionally redundant protein families. A classic example is the yeast OSH family; while the septuple mutant is inviable, single or even sextuple mutants exhibit few growth defects, suggesting overlapping roles in lipid homeostasis (Beh et al., 2001). Furthermore, the loss of primary tethers often triggers compensatory morphological changes. In mammalian cells, the loss of E-Syt tethers does not disrupt basic cellular viability, likely due to the presence of secondary tethers that maintain membrane proximity (Sclip et al., 2016). The expanded MCSs observed in *tvpfp* plastids (Figure 6C-D) may therefore reflect a compensatory response, where physical contact area is increased to facilitate lipid flux via parallel, perhaps less efficient, transport pathways. It is also possible that the physiological impact of *tvpfp* disruption is conditional, causing significant phenotypes under specific conditions that demand high-flux lipid transport or in

combination with other mutations, a common feature of MCS proteins in other organisms (Manford et al., 2012). "

Reviewer 2. The science is sound, though there are several concerns to raise and suggestions to strengthen the impact and enhance the clarity:

Concerns -

1. An extensive proteomics dataset was developed as part of this work though I don't see any reference to its availability or way for reviewers to review the raw/metadata. In my experience, it is standard practice to make proteomics data available for review of a manuscript.

Response. Our deep apologies for this oversight. The dataset is at PRIDE, and you can access it by logging in to the PRIDE website using the following details:

Project accession: PXD065686

Token: KtwaOR0o4cAX

Alternatively, reviewers can access the dataset by logging in to the PRIDE website using the following account details:

Username: reviewer_pxd065686@ebi.ac.uk

Password: 9cl0p1u321sk

Again, our apologies for omitting this from our letter in the first round. We incorrectly assumed it was accessible to reviewers from the submission portal.

Reviewer 2. 2. I am confused with the statement that banding patterns of certain lanes are "characteristic" of IE or thy. Characteristic based on what? At the least, some references to previous papers with a descriptive comparison seems warranted for a reader to judge if this is indeed the case. And, the asterisks that are supposed to highlight such characteristic banding patterns mark only a few selected bands within a lane. Such a pattern doesn't sound very "characteristic" of any sub-compartment fraction. Furthermore, the asterisks are marking a few proteins in lane 11 (IE) and lane 19 (thy), even though these are not annotated as the genuine IE and thy fractions marked in lane 12 (IE) and lane 16 (thy). I question how appropriate is the execution of the biochemical fractionation without understanding correctly what was done or being able to judge if the thy and IE fractions are indeed what they are claimed to be.

Response. We absolutely take your point. Yes, there are a fairly large number of papers from different labs from the 80s and 90s in which the three large molecular weight bands can be observed as unique to the inner envelope membrane of pea (Cline et al., 1985; Keegstra and Cline, 1982; Miquel and Dubacq, 1992; Waegemann et al., 1992; Waegemann and Soil, 1991). This is also true in spinach (Heemskerk et al., 1985). Although these papers do not identify these bands, by comparison to our proteomic abundances, it seems likely that they are PICC, Tic110, and Hsp93. In contrast, thylakoid fractions lack these three densely-silver-stained bands, and have intense staining between 25 and 35 kDa, which the inner envelope lacks (Heemskerk et al., 1985; Keegstra and Cline, 1982). Again, these papers do not identify the bands but looking at our proteomics they are likely to be a group of LHCBs, LHCAs, and OE33. We selected those gel regions because they made distinguishing between the fractions routine, and they had strong previous evidence for them from pea. Most proteomic studies have used Arabidopsis, making similar 1:1 comparisons more challenging. The release of the pea genome allowed us to use pea effectively, which was critical for isolation of sufficient quantities of these intermediate density regions. Regarding your concern for the fractionation process, we have improved the clarity of our description in Figure 3, its associated legend and results text, including new supplemental Figure S4 and methods text. We reiterate that our analysis (Figure 4) shows the profile of proteins associated with each fraction clearly shows the heavyIE is distinct from IE and the lightThy from Thy.

Cline, K., Keegstra, K., Staehelin, L.A., 1985. Freeze-fracture electron microscopic analysis of ultrarapidly frozen envelope membranes on intact chloroplasts and after purification. Protoplasma 125, 111–123. https://doi.org/10.1007/BF01297356

Heemskerk, J.W.M., Bögemann, G., Wintermans, J.F.G.M., 1985. Spinach chloroplasts: localization of enzymes involved in galactolipid metabolism. Biochim. Biophys. Acta BBA - Lipids Lipid Metab. 835, 212–220. https://doi.org/10.1016/0005-2760(85)90275-9

Keegstra, K., Cline, K., 1982. Evidence that Envelope and Thylakoid Membranes from Pea Chloroplasts Lack Glycoproteins. Plant Physiol. 70, 232–237. https://doi.org/10.1104/pp.70.1.232

Miquel, M., Dubacq, J.-P., 1992. In Situ Incorporation of Fatty Acids into Lipids of the Outer and Inner Envelope Membranes of Pea Chloroplasts. Plant Physiol. 100, 472–481. https://doi.org/10.1104/pp.100.1.472

Waegemann, K., Eichacker, S., Soll, J., 1992. Outer envelope membranes from chloroplasts are isolated as right-side-out vesicles. Planta 187, 89–94. https://doi.org/10.1007/BF00201628

Waegemann, K., Soil, J., 1991. Characterization of the protein import apparatus in isolated outer envelopes of chloroplasts. Plant J. 1, 149–158. https://doi.org/10.1111/j.1365-313X.1991.00149.x

Reviewer 2. 3. Why do the authors not address possible overlap of protein identifications between the bioinformatics search and biochemical fractionation? It seems like an obvious question for a reader whether there are proteins that come from the first-prong and the second, and if there such overlapping proteins it seems that these would be especially high-priority candidates to act in MCSs.

Response. Thank you for this suggestion, shared with Reviewer 1. Although these proteins were present in Table S4 they were not highlighted in the text. We have added Table 2, which describes these 16 proteins, as well as relevant descriptive text in the results and discussion.

Results text, "Although the heavyIE fraction appeared dominated by photosynthetic functions in the broad GO enrichment analysis (Figure 4D), we reasoned that proteins co-enriched in both the heavyIE and lightThy fractions would still represent high-priority candidates for TIES. By filtering for Arabidopsis homologs that were enriched in the intermediate density fractions in both directions, we identified a discrete set of 16 proteins (Table 2). A feature of this overlapping set is the presence of the complete plastidial pyruvate dehydrogenase (PDH) complex—including PDH E1α, PDH E1β, LTA2 (E2), and LPD1 (E3)—which provides the acetyl-CoA precursor required for de novo fatty acid synthesis (Li-Beisson et al., 2013). The co-enrichment of these complex components, alongside regulatory proteins like the PSII accumulation factor LPA1 and the metalloproteases FTSH1 and FTSH2, suggests that the heavyIE fraction, despite its large photosynthetic signature, contains a protein subset likely present at envelope-thylakoid interfaces."

Reviewer 2. 4. In the introduction, I believe another relevant citation for MCSs in ER-chloroplast lipid transfer would be: DOI: 10.1038/s41467-024-50425-7 and for cryo-ET tomography would be: DOI: 10.7554/eLife.105496

Response. Thank you, we have incorporated the suggested references.

Reviewer 2. 5. There seems to be some disconnect between the description of biochemical fractionation in the main text on one-hand and the M&M and Figure 3 legend on the other hand. Specifically, how was the homogenization for the second homogenization performed? hypertonic lysis? or freeze/thaw dounce

homogenization? Or perhaps both? At any rate, it should be made clear and consistent between the different locations.

Response. Our apologies for the misunderstanding. The exact same process was used for homogenization in the first and second steps: Freeze/thaw and Dounce homogenization of 30 strokes with a tight pestle. We have updated the methods text and the Figure 3 legend to clarify this, incorporating a double arrow for Figure 3 to clarify that the fractions are separate when homogenized.

Reviewer 2. 6. There is a reference to enrichment of proteins in various biochemical fractions but the percentage of what? Percentage of number of proteins? Percentage of protein abundance ie NSAFs? Or something else?

Response. We thank the reviewer for this helpful request for clarification. We have revised both the results and methods for clarity for the entire proteomic analysis. The specific section of the methods reads, "Unless otherwise indicated, percentages reported for categorical data (e.g., text regarding Figures 4B–C, F-H) refer to the fraction of proteins by count within a given enriched set assigned to each category, while NSAF values were used for all differential abundance calculations, statistical enrichment assessment, and for volcano plots (Figure 4E)."

We hope this clarification resolves the ambiguity regarding the basis of the reported percentages.

Reviewer 2. 7.The authors describe an enrichment of lipid-related enzymes in lightThy of 3-fold compared to IE fraction. However, there are also about 3x as many identified proteins in the lightThy fraction as IE fraction, so I question if there really is this described enrichment of lipid related proteins in lightThy.

Response. We thank the reviewer for pointing out that the lightThy fraction contains more total proteins than IE, which complicates interpretation of raw "3 fold more lipid enzymes" statements. To address this, we now analyze proportions of MCSdb & lipid metabolism proteins and explicitly treat the heavyIE–lightThy overlap as a distinct interface-enriched subset.

The updated methods section now reads, "We next applied a conservative approach to test whether lipid metabolism and MCS homologs were representative of the intermediate-density fractions. By focusing on Arabidopsis homologs enriched uniquely in one fraction—or in the discrete set co-enriched in heavyIE and lightThy—we aimed to identify the most reliable signatures for each membrane domain, a strategy previously used for high-

confidence marker generation (Ferro et al., 2010). Among these, the lightThy fraction contained the highest proportion of acyl-lipid enzymes (9.4%) compared with IE (3.6%) and heavyIE (3.1%) and Thy (0%, Figure 4F). Fisher's exact tests confirmed a significant enrichment of AraLip proteins in lightThy relative to the thylakoid ($p < 0.05$), where the absence of lipid metabolic enzymes aligns with longstanding observations that thylakoid membranes are not sites of lipid biosynthesis (Douce, 1974; Seifert and Heinz, 1992). Notably, the "overlapping" set of proteins co-enriched in both heavyIE and lightThy contained the highest relative abundance of acyl-lipid enzymes (25%, the PDH complex).

Finally, we used the same conservative strategy to ask if MCS homologs were representative of the intermediate density fractions by calculating the proportion of proteins in each fraction with homology to MCS database proteins using a high stringency filter for homology (Figure 4G, Table S6 (Pan et al., 2024). Consistent with the trends observed for acyl lipid metabolism, the lightThy fraction contained the highest proportion of MCS homologs (43%), followed by the IE (37%) then the heavyIE (28%) and thylakoid (20%) fractions. Fisher's exact tests confirmed a significant enrichment in MCSdb hits for the lightThy relative to the thylakoid fraction ($p < 0.01$). Again, the "overlapping" set of proteins co-enriched in both heavyIE and lightThy fractions had the highest proportion of MCS homologs (69%), consistent with these intermediate-density bands including an MCS-rich interface. Together, the synchronized enrichment of functional specialization (Figure 4D), including lipid-metabolic machinery (Figure 4D-F) and contact site homologs (Figure 4G) suggests the presence of a distinct membrane zone with a unique enzymatic signature that is largely absent from canonical envelope or thylakoid membranes."

Reviewer 2. 8. In the M&M, IE and thylakoid fractions are said to be "diluted" and pelleted (130,000 xg, 30 mins). What were they diluted in?

Response. They were diluted in TE buffer with 0.2 M sucrose as referenced previously in Keegstra and Yousif (1986). The methods have been updated to clarify this buffer in the text.

Reviewer 2. 9. In Figure 1 it seems the partial and none categories are statistically significantly different from the full category but no asterisks are present. Is this correct?

Response. Asterisks are present in Figure 1, and full statistical information is available in Table S2.

Reviewer 2. 10. In Figure 3 - panel A, the arrows seem to suggest (incorrectly) that the IE and thy were subsequently pooled before the second separation step. The authors could also mark the lower density broken chloroplast band in the initial centrifugation, along with the intact chloroplast band. Also, is 'M' indicating the protein marker lane? And finally, the legend stats, "These fractions were homogenized again", however from what I can tell only IE and thy were homogenized again, not OE as indicated in the legend. Finally, on this point, it would be very helpful for the reader if the approximate level/position of those fractions selected for proteomics were marked alongside the centrifuged samples. It seems that there is space within the figure to a blow-up of the tubes perhaps, which may be an effective and simple way to achieve this?

Response. We appreciate these very helpful suggestions and have revised Figure 3 and the legend for clarity as follows:

We have redrawn the arrows so that the inner envelope (IE) and thylakoid (Thy) fractions are clearly shown as being processed in parallel, rather than pooled, for the second continuous sucrose gradient separation. The accompanying text in the legend now explicitly states that "IE and Thy fractions from the discontinuous gradients were each re-homogenized by freeze/thaw and Dounce, then finely resolved on separate continuous sucrose gradients to isolate core IE, Thy, and intermediate density heavyIE and lightThy fractions, with approximate locations indicated by arrowheads."

We have added labels indicating the intact chloroplast band and the lower-density broken chloroplast band in the initial gradient tube schematic, so that the origin of the vesicle fractions is unambiguous.

We now state in the legend that, "M" indicates the molecular weight marker lane."

The legend previously read "These fractions were homogenized again …" which could be interpreted as including OE. We have revised this sentence to begin with, "The IE and Thy fractions…" as indicated above.

We have added arrowheads and labels alongside the tubes in panel A and corresponding open arrowheads in panel B to indicate the approximate positions of the fractions selected for proteomic analysis (IE, heavyIE, lightThy, and Thy).These labels match the fraction numbers shown in panel B and referenced in the text, providing a direct visual link between the physical gradients and the proteomics samples. Further, we reflected on the inherent inaccuracies of these labels, as fraction collection is not 1:1 with position. Therefore we have added Figure S4, which is the A280-based protein concentration of the same representative

samples as shown in Figure 3B, and clearly indicates both the high protein abundance of Thylakoids relative to IE, a challenge of chloroplast proteomics, and the exact fractions used for proteomic analyses. We used the peak protein content fraction to indicate the core IE or Thy fractions, then moved 2 fractions toward the other to collect the heavy IE and light Thy fractions.

We believe these changes resolve the ambiguities you highlighted and make the fractionation workflow and sample selection much clearer to the reader.

Reviewer 2. 11. Figure 4F and G - I believe there are supposed to be colors associated with the FDR that are missing, correct?

Response. Thank you for your concern, due to the request of the other reviewer, we have revised our enrichment analysis, and now present results more clearly in a heatmap in Figure 4D.

Reviewer 2. 12. Figure 4C legend - what are the fold-change and p-value respective to? is this relative to all other sub-compartments, or ...?

Response. We apologize for our previous lack of clarity, we have now revised our results and methods text to be more clear. In Figure 4C (and throughout Figure 4 and Table S4 and S5), these values are calculated from pairwise comparisons between IE vs heavyIE and Thy vs lightThy, respectively, rather than relative to all other sub-compartments or to whole chloroplasts. We chose these comparison pairs because IE and heavyIE, and Thy and lightThy, represent closely related membrane populations; additionally, whole-chloroplast proteomes are dominated by highly abundant thylakoid proteins and Rubisco. The thylakoid membrane is exceptionally protein dense, and chloroplast envelopes typically account for only 1 – 2 % of the total chloroplast protein mass (Figure S3; Lindquist and Aronsson, 2018; Zoschke and Barkan, 2015; Bouchnak et al., 2019). The presence of so many highly abundant proteins causes masking that limits the dynamic range of mass spectrometry, making identification of low-abundance components challenging in whole-chloroplast samples (Jarvis, 2007). Enrichment of the envelope proteome is dependent on rigorus sub-organellar fractionation (Ferro et al., 2010; Simm et al., 2013).

To avoid further reader confusion, we have now clarified our choice to do pairwise comparisons in the results section, stating, "We chose to perform pairwise comparisons of IE versus heavyIE and lightThy versus Thy, because these pairs represent the most closely related membrane populations. This strategy also avoids comparisons to whole chloroplasts, which exhibit a skewed protein abundance distribution that restricts the dynamic range of detection; in such samples, hyper-abundant photosynthetic complex

proteins and stromal Rubisco typically mask the signal of low-abundance regulatory and envelope-associated proteins (Rolland et al., 2012; Bouchnak et al., 2019).”

References

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Lindquist, E., & Aronsson, H. (2018). Chloroplast vesicle transport. *Photosynthesis Research*, *138*(3), 361–371. https://doi.org/10.1007/s11120-018-0566-0

Zoschke, R., & Barkan, A. (2015). Genome-wide analysis of thylakoid-bound ribosomes in maize reveals principles of cotranslational targeting to the thylakoid membrane. *Proceedings of the National Academy of Sciences*, *112*(13), E1678–E1687. https://doi.org/10.1073/pnas.1424655112

Reviewer 2. Figure 6 legend - some detail is needed where it is said that plants were grown under "standard conditions". For example, was this continuous light as for the TEM in panel C?

Response: We appreciate the reviewer’s suggestion to clarify the growth conditions referred to as “standard conditions” in Figure 6. We have now explicitly defined these conditions in both the figure legend and the Methods.

In the Figure 6 legend, we revised panel B to read, “(B) Growth phenotypes of wild-type and mutant plants under standard conditions (22°C/18°C, 18 h/6 h light/dark cycle at 120 µmol m−2 s−1 light intensity).” We also specified for panel C, “(C) Transmission electron micrographs of chloroplast ultrastructure in cotyledons of seedlings germinated with continuous light (scale = 1 µm).”

In the Plant Materials and Growth Conditions section of the Methods, we also clarified our default conditions and how exceptions are indicated, “Plants were grown in a 22°C/18°C, 18 h/6 h light/dark cycle at 120 µmol m−2 s−1 light intensity unless otherwise indicated in the figure legend.”

We hope these additions resolve the ambiguity regarding growth conditions used for the experiments in Figure 6.

RESPONSE TO THE REVIEWER

Dear Reviewers,
This is a complex paper with dozens of analyses. I really appreciate that you took the time to carefully consider each and to help us improve the paper. This is getting more and more rare these days and yet is so critical to pursuit of good science. Thank you for your effort and for helping us improve the manuscript.
Sincerely,
Rebecca Roston

Reviewer: This study addresses an important question about the molecular composition of chloroplast membrane contact sites and their potential role in lipid metabolism and thylakoid development. However, this article attempts to combine two independent stories that are only thematically linked. The first story attempts to identify chloroplast-localized lipid transporters using a bioinformatic approach. The researchers selected 19 candidates to verify their localizations and identified five with strong chloroplast localizations, including TVPFP and PMFP.
The second approach is subfractionation, in which the authors isolated membrane contact sites from the inner envelope of the chloroplast or the thylakoid. This approach, combined with proteomics, is now well described and explained, yielding 16 interesting candidates. However, nothing has been done with these candidates except localizing three other proteins that aren't even among the 16 candidates.

For publication, at least the interconnection of the two stories and the function of one candidate are needed.

Response:
Thank you for this thoughtful critique. We agree that, as originally presented, the manuscript could read as two parallel “stories.” In the revised Results and Discussion, we have clarified that the two approaches are complementary in two specific and intentional ways to strengthen the connection between them.

1) Complementarity by design: lipid transporters at contact sites are often underrepresented in MS, motivating a targeted candidate approach.
Our first strategy (homology-based selection followed by imaging) was specifically intended to capture candidate lipid transport and membrane-shaping proteins at chloroplast membrane contact sites. These are frequently missed or weakly detected in LC–MS/MS datasets due to their high hydrophobicity and poor accessibility for protease digestion. We now explicitly state this rationale in the last paragraph of the introduction and emphasize that the bioinformatic candidate screen provides a targeted route to identify and validate chloroplast proteins with punctate, suborganellar distributions consistent with specialized domains (e.g., TVPFP, Fig. 1), including proteins that are not expected to be robustly recovered by proteomics alone. (Notably, peptides from TVPFP were not recovered in any fraction, Supplemental Data 4).

2) Complementarity in scope: proteomics expands beyond transport to the broader metabolic environment of the same domain.
Our second strategy (membrane subfractionation + proteomics) was designed to provide an unbiased, systems-level view of proteins enriched in intermediate-density membrane fractions that are consistent with an interface zone relevant to lipid exchange and thylakoid biogenesis. This approach revealed that the lightThy fraction is enriched not only in acyl-lipid enzymes but also in proteins annotated for lipid/ion transport and other processes (Fig. 4D–F; Table 2),

thereby expanding the candidate space from individual transporters to the broader metabolic and organizational context in which transport must operate.

In response to your point that “nothing has been done” with the 16 co-enriched candidates, we have revised the text to make clear that this overlapping set (16 proteins co-enriched in heavyIE and lightThy) was used as a high-confidence signature of the intermediate-density interface, but that it did not contain lipid transporter candidates. We therefore revisited the lightThy fraction—the intermediate-density fraction most enriched for transport-related functions—and added a second line of evidence by demonstrating that representative, well-characterized proteins implicated in lipid trafficking and thylakoid biogenesis (TGD3, VIPP1) show non-uniform suborganellar distributions consistent with subdomain organization (Fig. 5). This addition explicitly links the proteomic identification of a transporter-rich fraction to imaging-based validation of spatially restricted localization patterns, thereby tying the two approaches to the same biological inference: specialized chloroplast membrane regions that support lipid exchange and thylakoid biogenesis.

Finally, we agree that functional evidence is essential and thus provided in our previous revision functional analysis of two chloroplast-localized candidates, TVPFP and PMFP, showing that loss of either in Arabidopsis perturbs chloroplast membrane ultrastructure (Fig. 6), photosynthetic capacity (Supplementary Fig. 6), and thylakoid lipid pigments (Supplementary Fig. 7). We now explicitly note that PMFP is enriched in the heavyIE fraction by proteomics and found in our localization-based screen when introducing the functional experiments in the results. Therefore, the functional consequences of its loss may be considered an answer to the reviewer’s request for the function of a candidate in both screens.

Reviewer: The researchers analyzed the ultrastructure of the chloroplasts by electron microscopy (EM) and the photosynthetic activity of the KO mutant of these two proteins, observing minor defects. However, there is no evidence that these two proteins are involved in lipid transport.

Response: We agree with the reviewer that there is not yet direct evidence that TVPFP and PMFP are directly involved in lipid transport. We agree that further characterization of lipid transport of TVPFP and PMFP is needed. Typically, this would be done with labeling and is quite technically challenging, and is beyond the scope of this work which is primarily focused on identification of interesting functional candidates. Therefore, the manuscript refrains from stating that TVPFP or PMFP have a direct role in lipid transport. We do, however, remark upon the identification of all subunits of the well-studied lipid transport complex TGD1, 2, and 3 in the lightThy.

Reviewer: In Table S5, there are some empty lines with logP. What is the meaning of logP, and how is it calculated?

Response: In our functional enrichment output, -logP refers to the negative logarithm of the enrichment p-value as reported by Metascape. Metascape calculates P values using the hypergeometric test[1], which measures the probability that the observed overlap between the fraction and a specific Gene Ontology (GO) term occurs by chance, given the total number of proteins identified in the study as the background. We did not customize any of the standard Metascape analyses, and Metascape is referenced in the Methods. The “blank” cells are a result of the table integrating all fractions, and our inclusion of values only above the Metascape p-value threshold[2], resulting in a number of blank cells. We note that integrating all fractions in a single table allows for a clear comparison of biological processes between them.

1. For more information on how Metascape calculates p-values, please see https://metascape.org/blog/?p=122#:~:text=Enrichment%20Factor,our%20own%20publications%20for%20years:
2. For more information on p-value cut-off strategies used in Metascape, please see https://metascape.org/blog/?p=163.

Reviewer: I do not understand how the pairwise analyses in Table S4 are done if the number of analyses is not the same. For instance, there are four thy fractions versus six lightthy fractions. The fold change should be determined experimentally because each fractionation has its own biological variability. In Table S4, what is the difference between "is enriched" and "is more"?

Response: Thank you for the opportunity to clarify the statistical approach. The pairwise comparisons were performed using a two-sided t-test within the Perseus environment, corrected by permutation based false discovery rate as reported in the Methods. This statistical test is robust to unequal sample sizes because it calculates the mean and variance for each group independently to determine the T-statistic and the associated degrees of freedom. It is standard in proteomics to account for biological and technical variability between different experimental fractions. (Please note these are not *paired* tests between individual replicates.)

Regarding the “enriched” and “more” criteria in Table S4 (now Supplementary Data 4), proteins were considered significantly differentially abundant (“enriched”) if they met a $\log_2$ fold change threshold of ±0.5 and an adjusted p-value cutoff of $-\log_{10}(p) \geq 1.3$ (FDR ≤ 5%). The “more” category is provided as a convenience to the reader, as the values of log2 fold changes are arbitrary.

Reviewer: I still don't understand the meaning of the enrichment in Figure 4C. I understand that it represents the redistribution of the localization of the identified proteins in the fraction. However, I don't understand what they are enriched against. The answer to reviewer 2.6 is unclear to me.

Response: Figure 4C does not represent the entire identified proteome. Instead, each bar represents the subcellular composition of only the significantly enriched proteins for that fraction. This is the same calculation referenced in the previous comment, with pairwise t-tests with permutation-based FDR control between heavyIE and IE, or lightThy and Thy. Proteins included in the plot are only those that are significantly more abundant relative to the matched comparator fraction (e.g., enriched in lightThy relative to Thy; enriched in Thy relative to lightThy), using the stated fold-change and adjusted p-value criteria.
By focusing only on these differentially abundant proteins, we can visualize the functional specialization of each fraction. For example, the IE bar shows that among the proteins that specifically partitioned into the inner envelope (relative to the heavyIE), 21% are known envelope proteins.

To help avoid similar confusion for readers, we revised the Figure 4C legend to read: “Sub-organellar summary of the proportion of proteins enriched in each fraction relative to its respective pairwise counterpart (IE vs. heavyIE and lightThy vs. Thy).” We similarly revised the methods to read: “To identify proteins partitioning specifically into related membrane populations, statistical comparisons were performed in a pairwise manner (IE vs. heavyIE and lightThy vs. Thy) ...”

Reviewer: On line 297, it says that TGD3 is enriched in heavyIE and lightThy. In this case, it should be present in Table 2, yet Table S4 states that IE is more.

Response: Thanks, great catch. That's our fault and a leftover from a previous analysis/draft that used a lower-stringency peptide-identification criterion. Our current analysis shows TGD3 enrichment only in the lightThy. We have removed the offending sentence and checked other reported values for accuracy.