nature portfolio

Peer Review File

Global Mitochondrial Connectivity Map Reveals the Landscape of Yeast Functional Assemblies and Conserved Protein Communities

Corresponding Author: Professor Mohan Babu

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)
Jessulat et al. present the interactome of a large set of mitochondrial proteins based on pulldowns of GFP-tagged proteins and size exclusion. A detailed analysis of the mitochondrial interactome is clearly interesting. However, there are previous studies which analyzed the interaction partners of GFP-tagged proteins or untagged proteins by complexome profiling. The presented analysis lacks comparison of the obtained data with previous studies, which would be important to assess the quality of the data set. Moreover, the impact of the used large GFP-tag on the localization should be taken into account. Finally, the manuscript would benefit from streamlining and shortening for better readability.

Specific points:

The authors used two approaches, pulldown of GFP-tagged proteins and co-fractionation via size exclusion. The authors should present the size exclusion data in more detail since this is the new approach for protein interaction studies for mitochondria. For instance, how high was the resolution using different established protein complexes as markers?

The authors did not compare the data with findings of previous interaction studies. How does the interactome compare to the published interactome of the same GFP-tagged strains from Huh et al., 2003 and Michaelis et al. 2023? What is the overlap and what are the differences? The authors should also discuss differences and similarities to the published complexome of mitochondrial proteins (Schulte et al., 2023 Nature).

Figure 2B, why is the overlap between AP-MS and CF-MS so small?

The selected GFP-tag is very large and could potentially affect the import and assembly of mitochondrial proteins. Therefore, it is important to include controls to reveal localization and interaction partners of well-established proteins using the GFP-tagged proteins.

The authors test their pulldown strategy by western blotting only looking at the baits (Extended Data 1f-i) Do they co-purify the known interaction partners with this approach? Western blots for example of other SAM or MICOS subunits is required here to assess the quality of the approach. For other interactions tested by western blotting only “yes/no” is given as a result in a table. The original data should be presented in the supplement.

Figure 4A is not informative. What are these MPCs? Where they suggested based on affinity purification or size exclusion? Can some of them assigned to known protein complexes like respiratory chain complexes?

Supplementary Fig. 4, some blots are empty (S4j). It is not clear to the reviewer what should be shown here. In general, controls should be shown for the pulldowns.

The text and main figures are overloaded with details that should be presented in the extended data (e.g. remove details from Fig. 1 to the supplement). The information gained by the in silico analysis (Fig. 6) should be moved to the Extended

data.

Reviewer #2

(Remarks to the Author)
In their manuscript Jessulat et al. describe a large PPI study of yeast mitochondria which is clearly the result of many years work by multiple groups. Although other studies have done similar using an AP/Co-IP approach, the authors pair this with co-fractionation and additionally use media permissive to mitochondrial respiration to enrich functionally relevant interactions. Overall the study is technically impressive with extensive validation against literature and other databases and using various orthogonal approaches to demonstrate specific novel interactions are real, even in other model systems. This is a useful resource for those interested in mitochondrial biology, and the authors make genuine efforts to improve relevance for those interested in mammalian systems. Methodology is generally sound with good detail. There was some overreach in interpretation (see specific comments for examples).

Major strengths:
PPIs under conditions of essential mitochondrial respiration defined by both epitope tagging pulldown and co-fractionation coupled with MS, orthogonal validation of selected novel interactions using Y2H, co-IP, studies in knockouts (yeast mutants) using RNAseq, Seahorse, lipidomics and other approaches including mammalian systems. Extensive comparisons with literature. Validation of OMM and MICOS interactors were particularly detailed. The authors should be commended for inclusion of essentially all relevant datasets in supplementary data.

Weaknesses:
Reliance on large tag (GFP) which is well known to impact correct mitochondrial localisation and protein complex formation, particularly for small proteins. Dated MS and related methods - comparable studies in mammalian cells (though focusing only on AP-MS and not CF-MS) were published in 2015 using more modern instruments and quantitation approaches than used here (Hein et al. 2015 Cell; Huttlin et al. 2015 Cell).

Specific comments:
- The non mitochondrial OMM interactors described in Fig. 3 are a major novelty. While some are validated in whole cell approaches, the authors should comment on the potential impacts of mitochondrial isolation (all PPI data is from what I assume is crude mitochondria) on validity of the other non-mito proteins novel to this study.
- This manuscript is dense. It is overly reliant on abbreviations, which are introduced as early as the second word in the abstract. In particular I would suggest to use ‘mitochondria’ instead of ‘mt’ throughout, but there are also many that are used a small number of times (or not at all) in text after their original definition eg. UNK, MM, HGS, ROC, PCC. The abstract is very technical and doesn’t speak to the novelty (or does, but not in an accessible way). In many ways the text in the last paragraph of the introduction would be a more appropriate abstract. The authors should also include the URL (or at least a mention) of the web tool in the abstract or end of the introduction. It isn’t introduced until the discussion which in my view is a wasted opportunity! Much of Fig. 1 and various elements in other main text figures describing technical validation could be moved to extended data. Much of the text surrounding said elements could also be moved to supplementary data or online methods section.
- Extended Data Fig. 1A – the legend states ‘cell pellet weights for six GFP-tagged mt proteins’. What do the authors mean by this? I assume the yield of mitos from each strain given same amount of input? If they are referring to raw cell pellet weight then why would that be relevant without stating input eg OD? Please clarify in main text and legend.
- What is the rationale behind using spectral counts for quantification of AP-MS data? This is a rather outdated approach and I note that the CF-MS studies, presumably performed later, used MS1 LFQ quant via MaxQuant (first used in the 2015 studies mentioned above).
- I felt some of the suggested interpretations of the data are overreach unsupported by literature or novel data eg. the novel interaction between FARS2 and MT-ATP6, which while shown to also exist in HEK293s, the authors cannot conclude from this alone that indicates an “interplay between translation and respiration”. The PDHA experiments in HEK293s are also interesting however I don’t understand the conclusion that the interaction of PDH complex with DLST would indicate ‘that physical coupling of these proteins may be relevant to glucose metabolism’ given DLST is a TCA cycle enzyme. There are many such examples of this throughout the manuscript. While I agree speculation is normal for this type of high throughput study I suggest the authors tone some of these down.
- The RCF3 validation part of the study is interesting, particularly the loss and thus implied importance of the residues after the TM in interaction with MICOS complex subunits. For this to be definitive the authors need to show that the truncation mutants correctly localise to the IMM as at present this is not clear and incorrect/no localisation could also equally explain the results in Fig. 5i. I also felt the conclusion that Mic60 is epistatic to Rcf3 is not supported by the data (since the growth is the same in YPEG with or without RCF3 being present).

Minor points
- End of P2 – proteins are not ‘in the nuclear genome’, they are ‘encoded by the nuclear genome’
- ED Fig. 2G: are the expected molecular weights the sum of the complexes or individual proteins? Given lysates are dig solubilised shouldn’t the proteins elute according to complex MW (or really stokes radius)?
- P15 & ED Fig. 6e: The state that RNAseq showed dysregulation of transcripts for various OXPHOS proteins in the orphan knockouts but the steady state levels of corresponding proteins were unchanged. Could this be due to increased/decreased mito biogenesis rather than the genes underpinning specific mito processes?
- Fig. 4C-F: It has very hard to see which MPCs are from literature/annotation and which are novel (I think it is the stroke outline colour?). Can the authors make this more obvious?

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors addressed some of my concerns in their revised version and the manuscript improved. The presented data included a lot of work and provides an interesting asset for analysis of mitochondrial protein-protein interactions. Therefore, the manuscript is after revision a good candidate for Nature Communications.

I have a few points the authors should address to improve clarity of the presented work. First, I recommend to at least cite the previous studies about mitochondrial protein-protein interactions in the main text. Second, I also recommend to improve quality and clarity of Extended Data Fig. 4. Third, it is also important to show some data for the size exclusion experiment. In the current version it is not possible to assess the quality of this data set. Finally, I also recommend to use standard names instead of ORFs for the Supplementary Data tables.

Reviewer #2

(Remarks to the Author)
The authors have addressed my major concens (ie 'specific comments') and from my side I consider their revised manuscript suitible for publication.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors addressed all my concerns in the revised version of the manuscript. The manuscript provides interesting new insights into the molecular organisation of mitochondrial proteins and protein complexes. I recommend publication of the manuscript in Nat. Commun..

Response to Reviewers

We thank the reviewers for their thoughtful and constructive feedback, which has substantially improved the clarity and rigor of our manuscript. In addition to the point-by-point revisions addressing each comment, we have thoroughly revised the manuscript, moving new or less central experimental data to the supplemental section and streamlining the main text where appropriate. We also ensured that the conclusions presented in the main text are fully supported by the data shown in the figures and clarified details that were previously missing in the main text and figure legends. To address specific reviewer concerns, we performed additional experiments showing that truncated mutants of HA-tagged Rcf3 (lacking defined amino acid segments) localize to the inner mitochondrial membrane (IMM). We also provide further evidence supporting the conclusion that Mic60 is epistatic to Rcf3. We believe these revisions present our work more clearly and will make it more accessible to a broad readership. Detailed responses to each reviewer's comments are provided below.

Reviewer #1:

Comment #1: *Jessulat et al. present the interactome of a large set of mitochondrial proteins based on pulldowns of GFP-tagged proteins and size exclusion. A detailed analysis of the mitochondrial interactome is clearly interesting. However, there are previous studies which analyzed the interaction partners of GFP-tagged proteins or untagged proteins by complexome profiling. The presented analysis lacks comparison of the obtained data with previous studies, which would be important to assess the quality of the data set. Moreover, the impact of the used large GFP-tag on the localization should be taken into account. Finally, the manuscript would benefit from streamlining and shortening for better readability.*

Response: We appreciate the reviewer's positive feedback and recognition of our efforts. Regarding the request to compare our dataset with previous studies to assess data quality, we would like to highlight **Extended Data Fig. 3a–3b and 3h,** which include quantitative mapping of mitochondrial protein assemblies based on high-resolution complexome profiling of the yeast mitochondrial proteome[1]. In these analyses, we show that nearly one-third (31%; 4,246 of 13,716) of the predicted mitochondrial protein-protein interactions (mtPPIs) are supported by orthogonal evidence, including alternative methods, small-scale yeast studies curated in BioGRID, experimentally determined interactions from previous large-scale interactome screens[1-7], and physical associations compiled in the STRING database. We also acknowledge the reviewer's valid concern regarding the potential impact of the GFP tag on protein localization. To address this, we evaluated the localization accuracy of the C-terminal GFP strain library[8] used in our study by comparing its single-cell localization measurements with those reported in LOQATE (the localization and quantitation atlas of the yeast proteome)[9, 10]. We found that over two-thirds (~70%; 474 of 681) of the proteins were localized to mitochondria, consistent with their reported endogenous localization[10, 11], and with prior studies showing that C-terminal tagging generally preserves mitochondrial targeting more effectively than N-terminal tagging[12].

Importantly, all GFP-tagged proteins in our study are expressed from their endogenous promoters and chromosomally integrated, thereby avoiding overexpression artifacts and ensuring physiological expression levels, conditions essential for reliable localization and interaction analyses. Nonetheless, we observed cases where the large GFP tag had an effect. As shown in **Extended Data Fig. 2a**, 59 strains exhibited issues such as unsuccessful GFP tagging, missing mitochondria-encoded hydrophobic membrane proteins in the GFP-tagged library, or impaired growth often associated with low protein abundance. Although we attempted to purify 681 proteins, only 543 baits were successfully recovered. Interactions from these proteins were included in downstream analyses only if the corresponding proteins were confirmed to localize to mitochondria. If the GFP tag altered localization, such proteins would not be expected to yield detectable mitochondrial interactions. Additionally, we were unable to obtain usable localization

or interaction data for 197 of the 740 targeted mitochondrial proteins, reflecting the stringency of our workflow in minimizing false-positive mtPPIs caused by tag-induced mislocalization. We have now added these points acknowledging this limitation and clarifying how our analysis framework mitigates the potential impact of tag-induced artifacts. In response to the reviewer's final comment, we have shortened the text where possible to improve readability.

Comment #2: *The authors used two approaches, pulldown of GFP-tagged proteins and co-fractionation via size exclusion. The authors should present the size exclusion data in more detail since this is the new approach for protein interaction studies for mitochondria. For instance, how high was the resolution using different established protein complexes as markers?*

Response: We appreciate the reviewer's comment. The CF-MS data were included primarily as a complementary proteomic approach to support and validate the interactions identified by affinity purification mass spectrometry (AP-MS). For this reason, the CF-MS dataset was integrated with the AP-MS results to generate the final network of 13,716 mtPPIs in YPEG medium (yeast extract, peptone, ethanol, glycerol), rather than presented as a standalone analysis. Regarding the question about resolution, we highlighted the performance of CF-MS using well-established mitochondrial protein complexes in **Extended Data Fig. 2j1–j3**. These examples demonstrate that CF-MS fractions effectively resolve known interactions and assemblies, including succinyl-CoA ligase (Lsc1-Lsc2), mitochondrial processing peptidase (Mas1-Mas2), the TOM import channel (Tom22-Tom40), phosphofructo-1-kinase (Pfk1-Pfk2), F-type ATP synthase subunits (Atp14-Atp3/4/5/7; Atp7-Atp4/5; Atp4-Atp5), coenzyme Q–cytochrome c reductase/complex III (Qcr7-Qcr10), and cytochrome c oxidase/complex IV (Cox4-Cox6; Cox5A-Cox14). Moreover, over one-third (39%; 3,372 of 8,631) of the CF-MS-derived interactions are supported by orthogonal evidence, including previous large-scale interactome screens, small-scale yeast studies curated in BioGRID, the STRING database, CF-MS performed on yeast grown in YPD medium, and reciprocal confirmation (see **Fig. S2i source data**). These results further underscore the resolving power of the size-exclusion-based approach when benchmarked against established mitochondrial markers. Given that the manuscript already contains extensive analyses and figures, adding more detailed CF-MS results would be excessive. However, the complete CF-MS dataset is publicly accessible through our web portal and source data, enabling the community to explore CF-MS-derived PPIs in depth for follow-up analyses.

Comment #3: *The authors did not compare the data with findings of previous interaction studies. How does the interactome compare to the published interactome of the same GFP-tagged strains from Huh et al., 2003 and Michaelis et al. 2023? What is the overlap and what are the differences? The authors should also discuss differences and similarities to the published complexome of mitochondrial proteins (Schulte et al., 2023 Nature).*

Response: The reviewer raises several related points. As noted in our response to **Comment #1,** we have already compared our dataset with previous large-scale interaction studies to assess data quality. Regarding the specific studies mentioned by the reviewer, the GFP-tagged strain collection from Huh et al. (2003) did not generate or deposit pairwise PPI data in BioGRID or any other public repository, and therefore could not be included in our comparative analyses. However, we did compare our interaction network with the interactome reported by Michaelis et al. (2023), which also used the GFP-tagged yeast library. As shown in **Extended Data Fig. 3b** and the corresponding source data, our dataset shares just over one-tenth (14%; 1,937 of 13,761) of the interactions with this study. It is important to note that the two studies differ substantially in experimental conditions and workflows. Michaelis et al. performed AP-MS on cells grown in YPD medium (1% yeast extract, 2% bactopeptone, 2% glucose), whereas our study systematically purified C-terminally GFP-tagged mitochondrial proteins and resolved native mitochondrial assemblies using complementary AP-MS and CF-MS approaches on mitochondria isolated from cells grown in nonfermentable glycerol medium.

With respect to the reviewer's request to discuss similarities and differences between our work and the mitochondrial complexome reported by Schulte et al. (2023), our analyses already show that our approach recovers a significant fraction of previously reported mtPPIs from various large-scale datasets, including those derived from complexome studies (31%; 4,246 of 13,716; **Extended Data Fig. 3h**). The remaining interactions identified in our dataset represent mitochondrial associations not previously observed in YPEG-grown cells, underscoring the ability of our dataset to generate new hypotheses in mitochondrial biology. Because the Schulte et al. complexome study and other related large-scale interactome efforts differ fundamentally from our work in methodology, input material, and experimental conditions, a detailed one-to-one comparison of specific similarities and differences would not be directly informative and is beyond the scope of this manuscript. Nonetheless, the degree of overlap recovered through our quality assessments indicates that our dataset is consistent with established interaction studies while substantially expanding the known landscape of mitochondrial protein associations.

Comment #4: *Figure 2B, why is the overlap between AP-MS and CF-MS so small?*
Response: As shown in **Extended Data Fig. 2i**, approximately one-quarter (23%; 3,110 of 13,716) of the mtPPIs exceed the ΣLLS threshold in at least one of the two proteomic methods. This level of overlap is expected because AP-MS and CF-MS rely on fundamentally different biochemical principles and therefore capture distinct subsets of protein interactions. AP-MS enriches physical binding partners of a tagged bait and thus favors stable interactions, whereas CF-MS identifies proteins that co-elute in size-exclusion fractions, often reflecting membership in larger assemblies. As a result, the two techniques differ in sensitivities, biases, and dynamic ranges, and their computational scoring pipelines also diverge, factors that collectively contribute to limited overlap in the final interaction sets. Importantly, this observation is consistent with previous studies[13],[14], which has reported that AP-MS and CF-MS datasets typically share fewer than 10% of interaction pairs. In that context, the overlap observed in our study is actually higher than historically reported, demonstrating that the two proteomic methods are highly complementary[15]. For this reason, integrating AP-MS and CF-MS is widely recognized as essential for obtaining a more complete representation of a protein interactome[13-15], which is the strategy we adopted here. In the revised manuscript, we have added clarifying text to explain why low overlap is expected based on these methodological and analytical differences.

Comment #5: *The selected GFP-tag is very large and could potentially affect the import and assembly of mitochondrial proteins. Therefore, it is important to include controls to reveal localization and interaction partners of well-established proteins using the GFP-tagged proteins.*
Response: In addition to our response to **Comment #1** regarding the potential impact of a large GFP tag on mitochondrial protein localization, we emphasize that we have implemented a comprehensive set of controls and validation steps that meet or exceed current standards in the field. Together, these measures demonstrate that our dataset is robust and that the use of a GFP tag does not compromise the overall biological accuracy of the detected mtPPIs.

First, we optimized and benchmarked our AP-MS workflow using the MICOS complex, a core component of mitochondrial protein import and inner membrane organization (**Extended Data Fig. 1e**). Because MICOS subunits are particularly sensitive to mislocalization or assembly defects, they serve as a stringent test of whether a large GFP tag affects mitochondrial import or complex stability. Second, we validated a substantial fraction of our network against independent large-scale datasets. Nearly half of all reported mtPPIs (43%, 5,958 of 13,716; **Extended Data Fig. 3h**) are supported by previous proteomics studies, including those focused on mitochondrial protein import and assembly, as well as by reciprocal purification experiments and our newly implemented global reliability assessment using CF-MS. Third, we performed targeted experimental validation of interactions that could be sensitive to tagging. Of the 35 protein pairs examined, 30 were confirmed by co-immunoprecipitation or iMYTH (**Extended Data Fig. 3i**).

These include interactions directly relevant to the reviewer's concern, such as the association between the signal-anchored Om45 protein and the TOM20 import receptor, as well as interactions involving matrix processing peptidases (Mas1-Mas2). These successful validations across diverse classes of import-related proteins strongly support the functional integrity of our GFP-tagged constructs. Fourth, we explicitly differentiate well-established versus previously uncharacterized PPIs using correlation profiles (**Extended Data Fig. 5g-j**) and multimeric complex predictions (**Fig. 4a, c, 5a**). All quantitative data are publicly accessible through the web portal and supplementary/source files, enabling readers to assess specific cases as needed.

To further address the reviewer's request, we now provide in **Supplementary Data 4** a detailed breakdown of PPIs detected within each of the 12 major mitochondrial import and assembly complexes: the TOM complex, small Tim chaperones, TIM23 presequence translocase, PAM, TIM22 carrier translocase, SAM complex, MIA machinery, matrix processing peptidase, AAA proteases, MICOS, ERMES, and associated chaperones or supporting factors. As expected, our AP-MS workflow captured many canonical interactions in these systems. In a minority of instances, known interactions were not detected, likely due to our intentionally stringent scoring thresholds or possible steric effects of the GFP tag. However, this selective sensitivity does not undermine the reliability of the interactions that are recovered. Finally, the overall performance of our PPI network compares favorably with other AP-MS datasets in terms of overlap, reproducibility, and agreement with orthogonal evidence. Together, these multiple, independent, and complementary quality-control measures demonstrate that the vast majority of PPIs identified in our study are accurate, robust, and biologically meaningful. We are confident that the resulting mtPPI resource will be of substantial value to the research community.

Comment #6: *The authors test their pulldown strategy by western blotting only looking at the baits (Extended Data 1f-i) Do they co-purify the known interaction partners with this approach? Western blots for example of other SAM or MICOS subunits is required here to assess the quality of the approach. For other interactions tested by western blotting only "yes/no" is given as a result in a table. The original data should be presented in the supplement.*

Response: We would like to clarify the purpose of the immunoblots shown in **Extended Data Fig. 1f–i**. These panels were included specifically to illustrate the optimization of our AP-MS workflow. Immunoprecipitations of detergent-solubilized mitochondrial extracts from GFP-tagged Mic19, Mic27, Mic26, and Sam35 strains were probed with anti-GFP to confirm efficient recovery of the GFP-tagged bait proteins under different solubilization conditions. The intent of these blots is not to re-validate known MICOS or SAM interactions by Western blot. For this reason, we do not believe additional Western blots of SAM or MICOS subunits are necessary to assess the quality of our approach. The performance of the AP-MS workflow on MICOS components is already demonstrated in **Extended Data Fig. 1e**, where mass spectrometry robustly recovered the GFP-tagged bait proteins together with their known MICOS interaction partners under the optimized conditions. In addition, we performed Western blot validation of several well-established interactions across diverse mitochondrial complexes to further demonstrate the robustness of our workflow. These include outer-membrane proteins Tcd1 and Tcd2 (tRNA base modification), subunits of the matrix processing peptidase (Mas1, Mas2), components of the glycine decarboxylase complex (Gcv1, Gcv3), and elements of the CoQ synthome (Coq5, Coq7, Coq9), as shown in **Extended Data Fig. 4a** and **4f**. Because these are physiologically well-defined complexes with established interactions, successful validation across such diverse systems provides strong evidence for the overall quality and reliability of our AP-MS workflow, making additional SAM or MICOS immunoblotting unnecessary.

Regarding the reviewer's final comment, the table summarizes these validations using "yes/no" entries that correspond directly to the Western blots presented in **Extended Data Fig. 4**. The original immunoblot data are already included in the supplement, and the table is intended solely as an easily readable summary of those results.

Comment #7: *Figure 4A is not informative. What are these MPCs? Where they suggested based on affinity purification or size exclusion? Can some of them assigned to known protein complexes like respiratory chain complexes?*

Response: We thank the reviewer for this helpful comment. Regarding the origin of the multiprotein complexes (MPCs), we noted in the original submission that the ClusterONE algorithm was applied to the final YPEG network. Integrating the AP-MS and CF-MS datasets (see response to **Comment #4**) was essential both to increase mtPPI coverage and to reduce the inherent CF-MS bias toward large, stable complexes over transient interactions. To make this clearer for readers, we have updated the figure legend to state explicitly that the MPCs were predicted from the integrated AP-MS and CF-MS final YPEG network. In line with the reviewer's suggestion to assign clusters to known MPCs, we agree that doing so improves clarity. We have therefore annotated predicted clusters that relate to known complexes, as well as MPCs discussed elsewhere in the manuscript, by marking them with circles in the figure.

Comment #8: *Supplementary Fig. 4, some blots are empty (S4j). It is not clear to the reviewer what should be shown here. In general, controls should be shown for the pulldowns.*

Response: The reviewer appears to have misunderstood **Extended Data Fig. 4j**. These blots represent the negative controls for the pulldown experiments shown in panels a-d, f, and i, and therefore they are expected to be empty. Specifically, in wild-type (no-tag) samples, the pulldowns should not recover any prey proteins, which confirms the specificity of the interactions observed in the tagged strains. As stated in the **Extended Data Fig. 4 legend**, the control co-immunoprecipitations were performed using the same HA-tagged strains as in panels a-d, f, and i, but in the wild-type background, pulled down with calmodulin beads and probed with anti-HA.

Comment #9: *The text and main figures are overloaded with details that should be presented in the extended data (e.g. remove details from Fig. 1 to the supplement). The information gained by the in silico analysis (Fig. 6) should be moved to the Extended data.*

Response: We appreciate the reviewer's suggestion and, as noted in our response to **Comment #1**, we have streamlined the text to improve clarity and reduce unnecessary detail. However, we respectfully disagree with the recommendation to relocate **Figs. 1 and 6** to the Extended Data. **Fig. 1** presents essential experimental results that underpin the main conclusions of the study, and moving it out of the main text would disrupt the logical progression of the manuscript. Likewise, **Fig. 6** provides a key extension of our findings from yeast to humans and other species, making it central to the overall narrative rather than supplemental. Keeping these figures in the main text ensures that the core results and their significance remain clear and coherent for readers.

Reviewer #2:

Comment #1: *In their manuscript Jessulat et al. describe a large PPI study of yeast mitochondria which is clearly the result of many years work by multiple groups. Although other studies have done similar using an AP/Co-IP approach, the authors pair this with co-fractionation and additionally use media permissive to mitochondrial respiration to enrich functionally relevant interactions. Overall the study is technically impressive with extensive validation against literature and other databases and using various orthogonal approaches to demonstrate specific novel interactions are real, even in other model systems. This is a useful resource for those interested in mitochondrial biology, and the authors make genuine efforts to improve relevance for those interested in mammalian systems. Methodology is generally sound with good detail. There was some overreach in interpretation (see specific comments for examples).*

Major strengths:

PPIs under conditions of essential mitochondrial respiration defined by both epitope tagging pulldown and co-fractionation coupled with MS, orthogonal validation of selected novel interactions using Y2H, co-IP, studies in knockouts (yeast mutants) using RNAseq, Seahorse, lipidomics and other approaches including mammalian systems. Extensive comparisons with

literature. Validation of OMM and MICOS interactors were particularly detailed. The authors should be commended for inclusion of essentially all relevant datasets in supplementary data.
Response: We thank the reviewer for their positive evaluation and for recognizing the study's scope, rigor, extensive validation, and practical value as a resource.

Comment #2: *Reliance on large tag (GFP) which is well known to impact correct mitochondrial localisation and protein complex formation, particularly for small proteins. Dated MS and related methods - comparable studies in mammalian cells (though focusing only on AP-MS and not CF-MS) were published in 2015 using more modern instruments and quantitation approaches than used here (Hein et al. 2015 Cell; Huttlin et al. 2015 Cell).*
Response: We thank the reviewer for their thoughtful comments. Regarding the concern about GFP tagging, we agree that a large GFP tag can occasionally affect mitochondrial localization or protein complex formation. As noted in our responses to **Reviewer #1 (Comments #1 and #5)**, a small minority of known interactions were not detected, likely due to our intentionally stringent scoring thresholds or occasional steric hindrance from the GFP tag. However, we also provide extensive orthogonal evidence showing that, in the vast majority of cases, GFP tagging did not disrupt localization or complex assembly. Our co-fractionation data, yeast two-hybrid assays, co-immunoprecipitations, and validations in mammalian systems consistently support the integrity of most tagged proteins and their associated interactions. Given the breadth of these validations, we are confident that GFP tagging does not compromise the main conclusions of the study. We refer the reviewer to our responses to Reviewer #1 (**Comments #1 and #5**) for additional detail.

With respect to the comment that our mass spectrometry methods were "dated," we respectfully note that the studies cited by the reviewer (Hein et al., 2015; Huttlin et al., 2015) are not directly comparable to ours. Those studies performed AP-MS in human cell lines to map the global human interactome using GFP-tagged HeLa or FLAG-HA-tagged HEK293T cells. Their experiments were not designed to characterize the mitochondrial interactome and did not use mitochondria-enriched extracts. Although we compared our dataset to these human resources in **Fig. 6**, this comparison was conceptual, not methodological.

Regarding instrumentation, it is important to clarify that the Orbitrap systems used in our study are not inherently less sensitive than those used in the cited studies. Thermo Fisher Scientific releases new Orbitrap models every 2-3 years, but these updates involve trade-offs among resolution, scan speed, dynamic range, and mass range, rather than simple improvements in sensitivity. For example, the LTQ Orbitrap Elite (released in 2011), used at the start of this project, offers up to 240,000 resolution at m/z 400; the Q Exactive Plus (2013) provides 140,000 at m/z 200; and the Q Exactive HF (2014) again reaches 240,000 at m/z 200. Thus, our instrumentation is fully comparable in resolution and detection capability to those available to Hein et al. and Huttlin et al. In fact, Huttlin et al. used Q Exactive platforms with performance very similar to the Orbitrap Elite, and Hein et al. report using a "Orbitrap instrument," which based on publication timing, would also have been a Q Exactive or LTQ Orbitrap system. Therefore, our instruments are consistent with, and not inferior to, those used in these landmark AP-MS studies. We also emphasize the scope and timeline of this project. We performed 1,121 AP-MS and CF-MS experiments beginning in 2012. Data acquisition and computational analysis were completed by 2020, after which COVID-19-related delays and subsequent extensive validation experiments extended the finalization of this work. Although we now have access to newer Orbitrap Exploris instruments with resolutions approaching 480,000 at m/z 200, repeating more than a thousand experiments on newer platforms is neither technically nor logistically feasible and would not materially alter the study's conclusions.

With respect to quantitation and protein identification methods, our analytical pipeline is directly aligned with, and in several respects more stringent than, those used in the cited human interactome studies. We used SEQUEST for peptide identification, as in Huttlin et al., and additionally incorporated MS-GF+ (2017 release), which postdates that study. For interaction

scoring, we used CompPASS, identical to the Huttlin workflow, and further applied Hypergeometric Spectral Counts, previously validated in *Drosophila melanogaster* (Guruharsha et al., 2011[16]) and *Escherichia coli* (Babu et al., 2018[17]). Using two independent search engines and two distinct scoring frameworks provides greater stringency than either of the cited studies.

While it is true that the newest mass spectrometers (e.g., Orbitrap Fusion Lumos, Orbitrap Excedion Pro) could detect some extremely low-abundance or highly transient PPIs, this does not compromise the value or reliability of our dataset. Very high-resolution modes can introduce their own artefacts, and critically, our extensive orthogonal validation, including yeast two-hybrid, co-IP, genetic perturbations, RNA-seq, lipidomics, Seahorse assays, and confirmation in mammalian-system, provides strong support for the accuracy and robustness of the interactome. In summary, the instrumentation and computational methods used in our study were state-of-the-art at the time of data generation, comparable to those used in the 2015 human interactome maps, and implemented within a workflow that is, in several respects, more stringent. Combined with extensive experimental validation, these choices ensure that our mitochondrial interactome is robust, reliable, and not limited by the instrumentation or quantitation approaches employed.

Comment #3: *The non mitochondrial OMM interactors described in Fig. 3 are a major novelty. While some are validated in whole cell approaches, the authors should comment on the potential impacts of mitochondrial isolation (all PPI data is from what I assume is crude mitochondria) on validity of the other non-mito proteins novel to this study.*

Response: We thank the reviewer for highlighting the novelty of the outer mitochondrial membrane (OMM) interactions with non-mitochondrial proteins shown in **Fig. 3**, and we appreciate the request for clarification regarding how mitochondrial isolation might affect their validity. Our mitochondrial preparations were generated by differential centrifugation, a well-established and widely used method for AP-MS workflows[18-22]. In this approach, low-speed centrifugation removes intact cells, nuclei, and debris, followed by high-speed centrifugation to enrich mitochondria and separate them from most other organelles. While this method produces enriched mitochondrial fractions, we acknowledge they are not fully pure and that some non-mitochondrial material may remain. Achieving higher purity would require sucrose-density gradient ultracentrifugation. However, this was not feasible given the scale of our AP-MS experiments and the variable growth and expression of individual mitochondrial proteins in YPEG media. Processing hundreds of GFP-tagged strains through density gradients would create major bottlenecks, increase technical variability, and risk disrupting peripheral or transient interactors, particularly those at membrane contact sites, that are often lost under more stringent purification conditions. Preserving these interactions was intentional, as they likely reflect physiologically relevant associations that higher-purity methods may systematically eliminate.

Regarding the biological relevance of the non-mitochondrial OMM interactors we report is supported by several lines of evidence. First, we independently confirmed 177 reciprocal interactions between OMM and non-mitochondrial proteins using 77 extended AP-MS baits (**Supplementary Data 4**). Second, many of these proteins are known to localize to inter-organelle contact sites. Indeed, over one-third (35%; 61 of 174) of proteins in our curated contact-site list associate with OMM proteins in the YPEG network. For example, the interaction between the OMM fission GTPase Dnm1 and endoplasmic reticulum (ER)-localized proteins, including the known partners Rtn1/2, is consistent with established models in which mitochondrial division occurs at ER-mitochondria junctions, a finding supported by our data (**Fig. 3i**) and by prior work[23]. Third, we detect 1,648 OMM-non-mitochondrial interactions in both AP-MS and CF-MS datasets (see **Supplementary Data 4**). Although only a subset meets our stringent high-confidence threshold, the substantial overlap supports the robustness of these associations. Fourth, among the 681 GFP-tagged mitochondrial strains, 88 localized to other cellular compartments, resulting in ~3% (468 of 13,716) of all PPIs in the high-confidence network. Notably, 32% (149 of 468) of these interactions are supported by orthogonal evidence from

BioGRID, previous large-scale yeast interactome studies, or the STRING database (**Supplementary Data 4**), a strong validation rate given the scale of the experiment. Together, these observations suggest that most non-mitochondrial proteins identified as OMM interactors are unlikely to arise from contamination and instead represent genuine, biologically meaningful associations. In the revised version, we now explicitly acknowledge in the Results section that we used crude, but intentionally not overpurified, mitochondrial preparations, and we clarify the practical and biological rationale for this choice. While some low-level contamination cannot be ruled out, the extensive orthogonal support and consistency across datasets give us confidence that these interactions reflect authentic cellular biology rather than technical artifacts.

Comment #4: *This manuscript is dense. It is overly reliant on abbreviations, which are introduced as early as the second word in the abstract. In particular I would suggest to use 'mitochondria' instead of 'mt' throughout, but there are also many that are used a small number of times (or not at all) in text after their original definition eg. UNK, MM, HGS, ROC, PCC. The abstract is very technical and doesn't speak to the novelty (or does, but not in an accessible way). In many ways the text in the last paragraph of the introduction would be a more appropriate abstract. The authors should also include the URL (or at least a mention) of the web tool in the abstract or end of the introduction. It isn't introduced until the discussion which in my view is a wasted opportunity! Much of Fig. 1 and various elements in other main text figures describing technical validation could be moved to extended data. Much of the text surrounding said elements could also be moved to supplementary data or online methods section.*

Response: We thank the reviewer for these thoughtful suggestions regarding clarity and accessibility. In response, we have replaced "mt" with "mitochondria" throughout the manuscript and removed acronyms that appeared only once or were not meaningfully reused. These changes reduce reliance on abbreviations and improve overall readability. Regarding the abstract, we appreciate the reviewer's concern and have revised it to be more accessible and to be more clearly emphasize the novelty of the study. Although the abstract and the concluding paragraph of the introduction convey the same scientific message, we have reworked the abstract to highlight the key innovations more explicitly and to reduce technical density where possible. As recommended, we have also added the URL for the web portal at the end of the introduction to ensure the resource is immediately visible to readers.

With respect to the suggestion to move **Fig. 1** and certain technical validation elements to the extended data, we carefully considered this point. As noted in our response to **Reviewer #1** (**Comment #9**), the extended data already contains 68 panels, and relocating additional core elements would disrupt the logical flow of the manuscript and diminish the accessibility of the main findings. We have been intentional in retaining only those figures in the main text that are essential for understanding the experimental framework, validating the approach, and conveying the central biological insights. Because this is a resource-focused study that introduces a new combination of approaches for mtPPI mapping (e.g., CF-MS), some technical context must remain in the main figures to ensure clarity, reproducibility, and proper framing of the resource. That said, we agree with the reviewer on the importance of reducing density where feasible. We have streamlined the text and shortened technical descriptions to improve readability without compromising rigor. Together, these revisions address the reviewer's concerns while preserving the coherence and scientific integrity expected for a resource manuscript of this scale.

Comment #5: *Extended Data Fig. 1A – the legend states 'cell pellet weights for six GFP-tagged mt proteins'. What do the authors mean by this? I assume the yield of mitos from each strain given same amount of input? If they are referring to raw cell pellet weight then why would that be relevant without stating input eg OD? Please clarify in main text and legend.*

Response: We appreciate the reviewer's request for clarification. In **Extended Data Fig. 1a**, our goal was to illustrate the relative mitochondrial yield obtained from GFP-tagged strains grown

under different media conditions. The values originally shown represented the average raw whole cell pellet weights from six GFP-tagged strains. Although both whole cell and mitochondrial pellet weights were included in the source data, we agree that showing only the whole cell pellet weights in the bar graph could be misleading and does not fully convey the purpose of the comparison. To address this, we have revised both the figure and the legend. The updated version now plots mitochondrial pellet weight normalized to the corresponding whole cell pellet weight for each growth condition, providing a clearer and more direct measure of mitochondrial recovery efficiency across media types. Regarding the reviewer's question about culture input and OD measurements, all GFP-tagged strains were cultured under identical conditions: each GFP-tagged strain was grown overnight in the respective medium at 30 °C, back-diluted to an OD_{600} of 0.1, and then cultured for an additional 18 hours before harvesting. This information has now been added explicitly to the figure legend for clarity. We believe these revisions more clearly convey the intended message of **Extended Data Fig. 1a**.

Comment #6: *What is the rationale behind using spectral counts for quantification of AP-MS data? This is a rather outdated approach and I note that the CF-MS studies, presumably performed later, used MS1 LFQ quant via MaxQuant (first used in the 2015 studies mentioned above).*

Response: We thank the reviewer for this important observation. We recognize the concern and have clarified the rationale and corrected a misleading statement in the original text. First, in this study, both the AP-MS and yeast CF-MS datasets were quantified using MS2 spectral counts, with protein identifications assigned using SEQUEST/STATQUEST and MS-GF+/Percolator. Our original wording unintentionally suggested that MS1 intensities were used for these datasets; this has now been corrected. Only the brain-region CF-MS data (**Fig. 6h-k**), taken from our previous study[24], use MS1-based quantification via MaxQuant, as those experiments involved TMT-labeled fractions specifically optimized for accurate MS1/report-ion measurements. In that prior work, we observed that complexes identified by MS2 spectral counts were overwhelmingly recapitulated when reanalyzed using MS1-based quantification, indicating strong agreement between the two approaches. Second, MS2 spectral counts remain widely used and appropriate for AP-MS-based interaction mapping, in part because they integrate directly with established scoring frameworks such as CompPASS and HGS, which we apply here. The choice between MS1- and MS2-based quantification generally depends on instrument type, sample complexity, and study goals. All AP-MS and CF-MS experiments in this manuscript were performed on an Orbitrap Elite, where MS2-based quantification is robust and widely applied in PPI studies.

Finally, MS2-based quantification offers practical advantages for AP-MS workflows: in complex mixtures, MS1 ion intensities can be influenced by co-eluting peptides of similar m/z, whereas MS2 fragmentation provides greater selectivity and improves confidence in peptide-level quantification. Although MS1-based label free quantification has advanced considerably with modern high-resolution instruments and computational tools, MS2 spectral counting continues to provide reliable and appropriate quantification for AP-MS scoring, particularly in data-dependent acquisition workflows. We hope this explanation sufficiently addresses the reviewer's concerns and clarifies the rationale behind our use of MS2-based quantification.

Comment #7: *I felt some of the suggested interpretations of the data are overreach unsupported by literature or novel data eg. the novel interaction between FARS2 and MT-ATP6, which while shown to also exist in HEK293s, the authors cannot conclude from this alone that indicates an "interplay between translation and respiration". The PDHA experiments in HEK293s are also interesting however I don't understand the conclusion that the interaction of PDH complex with DLST would indicate 'that physical coupling of these proteins may be relevant to glucose metabolism' given DLST is a TCA cycle enzyme. There are many such examples of this throughout the manuscript. While I agree speculation is normal for this type of high throughput*

study I suggest the authors tone some of these down.

Response: We thank the reviewer for this thoughtful and constructive comment. We agree that some of our earlier wording may have implied mechanistic conclusions that are not fully supported by the current dataset. For example, while the FARS2–MT-ATP6 interaction is observed in both HEK293 cells and our yeast system, this finding alone does not justify concluding an explicit "interplay between translation and respiration." Our intention was to highlight a potentially interesting association rather than to assert a defined functional relationship. In the revised manuscript, we have therefore tempered this interpretation and now describe the interaction as suggesting a possible coordination between mitochondrial translation and respiration. Similarly, regarding the PDHA-PDHB-DLST interactions, we appreciate the reviewer's clarification. Although these proteins encode essential components of the pyruvate dehydrogenase complex, a key enzyme system linking glycolysis to the mitochondrial TCA cycle, we agree that our previous phrasing suggested a level of functional coupling that cannot be inferred from physical association alone. We have reworded this section to present the observation more cautiously, without implying direct relevance to glucose metabolism. More broadly, we have revisited the manuscript to moderate speculative statements throughout, ensuring a clearer distinction between data-driven observations and hypotheses that remain to be tested. We are grateful to the reviewer for this feedback, which has helped improve the balance and precision of our interpretations.

Comment #8: *The RCF3 validation part of the study is interesting, particularly the loss and thus implied importance of the residues after the TM in interaction with MICOS complex subunits. For this to be definitive the authors need to show that the truncation mutants correctly localise to the IMM as at present this is not clear and incorrect/no localisation could also equally explain the results in Fig. 5i. I also felt the conclusion that Mic60 is epistatic to Rcf3 is not supported by the data (since the growth is the same in YPEG with or without RCF3 being present).*

Response: We thank the reviewer for these insightful comments. Regarding the first point, we agree that verifying the localization of the *rcf3* truncation mutants is essential. As requested, we performed immunoblot analyses of HA-tagged truncated Rcf3 variants using NaCl-washed membrane (P) and soluble (S) fractions, as well as mitochondrial extracts prepared in IPLB or HEPES buffer and treated with or without proteinase K (PK) or PK plus Triton. Immunoblotting with anti-HA and control antibodies (anti-porin or COX3) showed that, similar to full-length Rcf3, all truncation mutants remain membrane-associated under high-salt conditions and are accessible to PK digestion, confirming correct localization to the inner mitochondrial membrane. These results support our conclusion that residues beyond amino acid 44 are critical for Rcf3 binding to the MICOS complex. The new data are now included in **Extended Data Fig. 8a, b.**

Regarding the reviewer's comment on genetic epistasis, we agree that the effect is not clearly discernible in the spot assays, even when performed at multiple time points. To address this, we conducted quantitative growth assays over 72 hours. These experiments show that deletion of *rcf3* or *mic60* significantly impaired growth on glycerol compared with wild-type. Notably, the *rcf3 mic60* double mutant showed no greater defect than either single mutant, indicating that Rcf3 and Mic60 act in the same pathway, with one gene being epistatic to the other in respiratory function. The new growth assay data have been added to **Fig. 5i**, and the original spot assay has been moved to **Extended Data Fig. 8c**.

Comment #9: *End of P2 – proteins are not 'in the nuclear genome', they are 'encoded by the nuclear genome'*

Response: This was an oversight, and we have corrected the phrasing as suggested.

Comment #10: *ED Fig. 2G: are the expected molecular weights the sum of the complexes or individual proteins? Given lysates are dig solubilised shouldn't the proteins elute according to complex MW (or really stokes radius)?*

Response: Thank you for raising this point and for the opportunity to clarify. In **Extended Data Fig. 2g**, the molecular weight categories refer to the individual theoretical molecular weights of

the proteins, not to the expected masses of the complexes they form. For example, the <50 kDa panel illustrates how proteins with monomeric sizes below 50 kDa distribute across the fractions. We agree that, because the lysates are digitonin-solubilized, proteins elute according to the apparent size of the complexes they reside in, their Stokes radii, which is the fundamental principle of CF-MS. The purpose of this figure, however, is not to infer complex sizes but to provide an overview of how proteins with different predicted molecular masses elute across the gradient. In this context, it serves as a quality-control assessment, proving that the fractionation performed as expected and supporting the validity of co-elution–based interaction analyses.

Comment #11: *P15 & ED Fig. 6e: The state that RNAseq showed dysregulation of transcripts for various OXPHOS proteins in the orphan knockouts but the steady state levels of corresponding proteins were unchanged. Could this be due to increased/decreased mito biogenesis rather than the genes underpinning specific mito processes?*

Response: We thank the reviewer for this thoughtful comment. In our original submission, we relied on visual inspection of the immunoblot in **Extended Data Fig. 6e** and mistakenly concluded that steady-state protein levels were unchanged. We have now reanalyzed and quantified band intensities using ImageJ (ten independent measurements of the same blot). This analysis revealed that steady-state levels of coenzyme Q biosynthesis (COQ4, COQ5, COQ9, and COQ11) proteins were altered in the indicated orphan gene deletion strains, showing ~26-81% decreases or ~102-199% increases relative to controls. These percentage changes are now reported in **Extended Data Fig. 6e**, and provided in the source data file. These results suggest that Coq proteins may be less or more stable in the absence of specific orphan genes, and we have updated the text accordingly. We also note that more than one-third of mitochondrial transcripts (37%; 233 of 628) were significantly differentially expressed in one or more orphan mutants, indicating broad effects on mitochondrial biogenesis rather than selective disruption of specific mitochondrial pathways. In **Extended Data Fig. 6e**, we focused on transcripts associated with respiratory complexes and the CoQ synthome because of their direct physical interaction with CoQ proteins (**Fig. 2k**). Together, the transcriptomic and CoQ protein changes suggest that orphan gene deletions broadly affect mitochondrial biogenesis rather than regulating specific mitochondrial processes. We have now revised the main text to reflect this conclusion.

Comment #12: *Fig. 4C-F: It has very hard to see which MPCs are from literature/annotation and which are novel (I think it is the stroke outline colour?). Can the authors make this more obvious?.*

Response: We thank the reviewer for this helpful comment and for prompting us to improve the clarity of **Fig. 4c-f**. The edge color for each subunit of the protein complexes is defined in **Fig. 4b**; however, we inadvertently omitted the edge colors for complex IDs 014, 345, and 555. These have now been added. We also clarify in **Supplementary Data 9** which complex subunits are annotated, novel, or previously assigned to the known complex. Additionally, we have revised the figure legend to explicitly state the meaning of each edge color, making it easier for readers to distinguish between annotated and newly proposed multiprotein complexes.

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Response to Reviewers

We thank the reviewers for their positive evaluation of our revised manuscript. In this second revision, we addressed the remaining points raised by Reviewer #1 in our point-by-point responses, specifically by including two additional analyses related to the size exclusion experiment (SEC). First, we present total MS1 ion current intensity profiles across SEC fractions from two independent biological replicates. Second, we show co-elution profiles for 19 well-characterized mitochondrial protein complexes annotated in the Complex Portal across SEC fractions. We believe these additions further clarity our results and improve the accessibility of the manuscript. Detailed responses to Reviewer #1's comments are provided below.

Reviewer #1:

Comment #1: *The authors addressed some of my concerns in their revised version and the manuscript improved. The presented data included a lot of work and provides an interesting asset for analysis of mitochondrial protein-protein interactions. Therefore, the manuscript is after revision a good candidate for Nature Communications.*

Response: We thank the reviewer for the positive assessment of our revised manuscript and for recognizing the work presented. We appreciate the constructive feedback, which helped improve the manuscript, and we are pleased that the dataset is considered a valuable resource for studying mitochondrial protein assemblies and mitochondrial biology.

Comment #2: *I have a few points the authors should address to improve clarity of the presented work. First, I recommend to at least cite the previous studies about mitochondrial protein-protein interactions in the main text. Second, I also recommend to improve quality and clarity of Extended Data Fig. 4. Third, it is also important to show some data for the size exclusion experiment. In the current version it is not possible to assess the quality of this data set. Finally, I also recommend to use standard names instead of ORFs for the Supplementary Data tables.*

Response: We thank the reviewer for these helpful suggestions to improve the clarity of our work. Regarding the first point, we agree that previous studies on mitochondrial protein-protein interactions are important to cite. In the original version of the manuscript, we included references to previous mitochondrial interactome studies associated with **Extended Data Fig. 3b** and **Fig. 6b**. However, *Nature Communications* limits the number of references for this article type to a maximum of 70, which prevents us from including all relevant studies, specifically 14 references from human interactome analyses (**Fig. 6b**) and three references from yeast interactome studies (**Extended Data Fig. 3b**). To ensure that these studies remain accessible to readers, we have already included the yeast interactome references in **Supplementary Data 4** and the human interactome references in **Supplementary Data 13**.

Regarding the second point, we thank the reviewer for the suggestion to improve the quality and clarity of **Extended Data Fig. 4**. The immunoprecipitation (IP) blots in **Extended Data Fig. 4a, 4c, 4f, and 4i** show clear signals with minimal background. However, we agree that the whole-cell lysates (WCL) blots in **Extended Data Fig. 4b and 4d** display higher background. We repeated these immunoblots twice and observed similar background levels, which is commonly encountered when using HA-tagged strains from the Movable Open Reading Frame collection, as the high-copy plasmid system can lead to elevated protein expression and increased background in immunoblots. We attempted several optimization strategies, including: **(1)** reducing galactose concentration, (2) increasing wash stringency, and (3) lowering the induction temperature; however, the background signal persisted. To improve figure clarity while preserving data integrity, we linearly adjusted exposure and contrast of the original immunoblot images. These adjustments were applied uniformly and are described in the **Supplementary Note** to ensure transparency. Importantly, the background in WCL blots does not affect the interpretation of the results. Notably, 8 of the 35 detected interactions have been previously reported in both small-scale and large-scale interaction studies and have also been observed under

different growth conditions, supporting the validity of our findings. Although five interactions could not be confirmed by co-IP, they have been validated in other studies. To further clarify this point, we have added the relevant supporting publications to the source data for **Extended Data Fig. 3i** and improved the presentation of **Extended Data Fig. 4** in the revised manuscript.

Regarding the third point, we agree that it is important to demonstrate the quality of the SEC experiment. In our previous response, we highlighted that the SEC fractionation profiles of well-established mitochondrial protein complexes (**Extended Data Fig. 2l1–l3** in this revised version) demonstrate that SEC-mass spectrometry (SEC-MS) effectively resolves known protein assemblies. Furthermore, 39% (3,372 of 8,631) of the interactions identified from the SEC-MS dataset are supported by independent evidence, including previous large-scale interactome studies, curated small-scale yeast studies in BioGRID, the STRING database, SEC-MS experiments performed in YPD medium, and reciprocal confirmation within our dataset (see **Fig. S2k** source data in this revised version). To further clarify the quality of the SEC experiment, we have now included additional supporting analyses in the revised manuscript. First, MS1 ion current intensity profiles across SEC fractions from two independent biological replicates grown in YPEG medium show highly consistent patterns over retention time, demonstrating the reproducibility of the chromatographic separation and MS acquisition (**Extended Data Fig. 2e**). Second, we present co-elution profiles for 19 well-characterized mitochondrial protein complexes annotated in the Complex Portal. The subunits of these complexes co-fractionate in the expected SEC fractions from YPEG-grown cells, confirming that the SEC separation preserves native mitochondrial assemblies and enables accurate detection of mitochondrial protein complex membership (**Extended Data Fig. 2f**). In addition, protein co-elution profiles are highly reproducible between biological replicates within each growth condition, while showing clear differences between YPEG and YPD conditions, consistent with biological remodeling of mitochondrial protein assemblies (**Extended Data Fig. 2g** in this revised version). Together, these observations exhibit the biological relevance of the SEC-MS dataset.

Regarding the final point, we agree that using standard gene names improves clarity. In the revised manuscript, we have updated the relevant **Supplementary Data** to include standard gene names alongside ORF identifiers. Specifically, gene names have been added to **Supplementary Data 3, 4, 7, 13, and 14**, facilitating easier interpretation and cross-referencing with existing databases and published studies.

Reviewer #2:

Comment #1: *The authors have addressed my major concerns (ie 'specific comments') and from my side I consider their revised manuscript suitable for publication.*

Response: We thank the reviewer for their positive evaluation and for recognizing that the major concerns have been addressed. We also appreciate the constructive feedback, which helped improve the clarity and quality of the manuscript.

Response to Reviewers

We once again thank the reviewers for their positive feedback on our manuscript, and we have made the remaining revisions to the text (highlighted in yellow) in accordance with the authors' checklist.