

Reporting Summary

Nature Portfolio wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Portfolio policies, see our [Editorial Policies](#) and the [Editorial Policy Checklist](#).

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a	Confirmed
☐	☒ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
☐	☒ A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐	☒ The statistical test(s) used AND whether they are one- or two-sided <i>Only common tests should be described solely by name; describe more complex techniques in the Methods section.</i>
☒	☐ A description of all covariates tested
☐	☒ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
☐	☒ A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☐	☒ For null hypothesis testing, the test statistic (e.g. F , t , r) with confidence intervals, effect sizes, degrees of freedom and P value noted <i>Give P values as exact values whenever suitable.</i>
☒	☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☒	☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☒	☐ Estimates of effect sizes (e.g. Cohen's d , Pearson's r), indicating how they were calculated

Our web collection on [statistics for biologists](#) contains articles on many of the points above.

Software and code

Policy information about [availability of computer code](#)

Data collection	SerialEM 4.0, Cryo AutoTEM, LAS AF, BD FACSDiva, TVIPS EM-Menu
Data analysis	Relion 4.0, Dynamo 1.1.532, IMOD 4.11, MODELLER 10.4, PyMOL 2.5, GROMACS, GROMaps, IsoNet 0.2, Excel 2016, Blender 4.2

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio [guidelines for submitting code & software](#) for further information.

Data

Policy information about [availability of data](#)

All manuscripts must include a [data availability statement](#). This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our [policy](#)

Data will be available under accessions EMD-19459 (EMDB) and PDB ID 8RRH (PDB). The mass spectrometry data used for analysis in this study was cited at multiple occasions for the readers to follow. Source data have been provided in Source Data. All other data supporting the findings of this study are available from the corresponding author on reasonable request.

Research involving human participants, their data, or biological material

Policy information about studies with [human participants or human data](#). See also policy information about [sex, gender \(identity/presentation\), and sexual orientation](#) and [race, ethnicity and racism](#).

Reporting on sex and gender	N/A
Reporting on race, ethnicity, or other socially relevant groupings	N/A
Population characteristics	N/A
Recruitment	N/A
Ethics oversight	N/A

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	All experiments were conducted at least in triplicate using independent sample preparations. Sample size were chosen based on previous experience and standards in the field. For cryoET data sets the maximum available samples were used for data acquisition.
Data exclusions	No data was excluded to ensure a comprehensive representation of our findings.
Replication	All attempts to replication were successful. Samples were replicated in at least three independent wells.
Randomization	In this study, controlling for covariates was not directly feasible due to the structure of the experimental design. Specifically, the samples were systematically arranged on culture plates and cryo EM grids without employing randomization thereby ensuring equal representation of all conditions. This specific organization minimized the impact of potential unforeseen variables resulting in reliable and consistent data for analysis.
Blinding	Blinding was not possible in this study, as one individual conducted most of the light microscopy analysis and another handled the cryo-ET analysis. However, to minimize potential bias additional authors participated in validating the analysis and thereby ensuring the objectivity of the results.

Behavioural & social sciences study design

All studies must disclose on these points even when the disclosure is negative.

Study description	Briefly describe the study type including whether data are quantitative, qualitative, or mixed-methods (e.g. qualitative cross-sectional, quantitative experimental, mixed-methods case study).
Research sample	State the research sample (e.g. Harvard university undergraduates, villagers in rural India) and provide relevant demographic information (e.g. age, sex) and indicate whether the sample is representative. Provide a rationale for the study sample chosen. For studies involving existing datasets, please describe the dataset and source.
Sampling strategy	Describe the sampling procedure (e.g. random, snowball, stratified, convenience). Describe the statistical methods that were used to predetermine sample size OR if no sample-size calculation was performed, describe how sample sizes were chosen and provide a rationale for why these sample sizes are sufficient. For qualitative data, please indicate whether data saturation was considered, and what criteria were used to decide that no further sampling was needed.
Data collection	Provide details about the data collection procedure, including the instruments or devices used to record the data (e.g. pen and paper, computer, eye tracker, video or audio equipment) whether anyone was present besides the participant(s) and the researcher, and whether the researcher was blind to experimental condition and/or the study hypothesis during data collection.

Timing	Indicate the start and stop dates of data collection. If there is a gap between collection periods, state the dates for each sample cohort.
Data exclusions	If no data were excluded from the analyses, state so OR if data were excluded, provide the exact number of exclusions and the rationale behind them, indicating whether exclusion criteria were pre-established.
Non-participation	State how many participants dropped out/declined participation and the reason(s) given OR provide response rate OR state that no participants dropped out/declined participation.
Randomization	If participants were not allocated into experimental groups, state so OR describe how participants were allocated to groups, and if allocation was not random, describe how covariates were controlled.

Ecological, evolutionary & environmental sciences study design

All studies must disclose on these points even when the disclosure is negative.

Study description	Briefly describe the study. For quantitative data include treatment factors and interactions, design structure (e.g. factorial, nested, hierarchical), nature and number of experimental units and replicates.
Research sample	Describe the research sample (e.g. a group of tagged <i>Passer domesticus</i> , all <i>Stenocereus thurberi</i> within Organ Pipe Cactus National Monument), and provide a rationale for the sample choice. When relevant, describe the organism taxa, source, sex, age range and any manipulations. State what population the sample is meant to represent when applicable. For studies involving existing datasets, describe the data and its source.
Sampling strategy	Note the sampling procedure. Describe the statistical methods that were used to predetermine sample size OR if no sample-size calculation was performed, describe how sample sizes were chosen and provide a rationale for why these sample sizes are sufficient.
Data collection	Describe the data collection procedure, including who recorded the data and how.
Timing and spatial scale	Indicate the start and stop dates of data collection, noting the frequency and periodicity of sampling and providing a rationale for these choices. If there is a gap between collection periods, state the dates for each sample cohort. Specify the spatial scale from which the data are taken
Data exclusions	If no data were excluded from the analyses, state so OR if data were excluded, describe the exclusions and the rationale behind them, indicating whether exclusion criteria were pre-established.
Reproducibility	Describe the measures taken to verify the reproducibility of experimental findings. For each experiment, note whether any attempts to repeat the experiment failed OR state that all attempts to repeat the experiment were successful.
Randomization	Describe how samples/organisms/participants were allocated into groups. If allocation was not random, describe how covariates were controlled. If this is not relevant to your study, explain why.
Blinding	Describe the extent of blinding used during data acquisition and analysis. If blinding was not possible, describe why OR explain why blinding was not relevant to your study.

Did the study involve field work? ☐ Yes ☐ No

Field work, collection and transport

Field conditions	Describe the study conditions for field work, providing relevant parameters (e.g. temperature, rainfall).
Location	State the location of the sampling or experiment, providing relevant parameters (e.g. latitude and longitude, elevation, water depth).
Access & import/export	Describe the efforts you have made to access habitats and to collect and import/export your samples in a responsible manner and in compliance with local, national and international laws, noting any permits that were obtained (give the name of the issuing authority, the date of issue, and any identifying information).
Disturbance	Describe any disturbance caused by the study and how it was minimized.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

Methods

n/a	Involved in the study
☐	☒ Antibodies
☐	☒ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☐	☒ Animals and other organisms
☒	☐ Clinical data
☒	☐ Dual use research of concern
☒	☐ Plants

n/a	Involved in the study
☒	☐ ChIP-seq
☐	☒ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used

anti-PHB1 (EP2803Y, 1:2000, Abcam, Cambridge, UK), anti-PHB2 (EPR14523, 1:5000, Abcam, Cambridge, UK), anti-GFP (JL-8, 1:3000, Clontech, Saint-Germain-en-Laye, France), anti-Actin (AC74, 1:3000, Sigma-Aldrich, St. Louis, MO, USA), HRP-conjugated anti-rabbit or anti-mouse secondary antibodies (Dianova, Hamburg, Germany), rabbit anti-GFP (ab290, Abcam, Cambridge, UK), sheep anti-mouse and goat anti-rabbit (all Dianova, 1:5000, Hamburg, Germany) coupled to KK114 or Alexa 594 (Atto-Tec, Siegen, Germany), anti-COX2 (ab203912, 1:500, Abcam, Cambridge, UK), anti-ATP5A (ab14748, 1:1000, Abcam, Cambridge, UK), anti-tubulin (ab15246, 1:1000, Abcam, Cambridge, UK), anti-ESR1 (sc-8005, 1:Santa Cruz Biotechnology, Texas, USA).

Validation

All antibodies were validated by us on KO/or knockdown cells.

In addition:

anti-PHB1 validated by Abcam (<https://www.abcam.com/en-us/products/primary-antibodies/prohibitin-antibody-ep2803y-ab75766>), anti-PHB2 validated by Abcam (<https://www.abcam.com/en-us/products/primary-antibodies/rea-antibody-epr14523-ab182139>), anti-GFP validated by Clontech (<https://www.takarabio.com/products/antibodies-and-elisa/fluorescent-protein-antibodies/green-fluorescent-protein-antibodies>), anti-Actin validated by Sigma Aldrich (<https://www.sigmaaldrich.com/DE/de/product/sigma/a2228#product-documentation>), rabbit anti-GFP validated by Abcam (<https://www.abcam.com/en-us/products/primary-antibodies/gfp-antibody-ab290>), anti-COX2 validated by Abcam (<https://www.abcam.com/en-us/products/primary-antibodies/mtco1-antibody-epr19628-ab203912>), anti-ATP5A validated by Abcam (<https://www.abcam.com/en-us/products/primary-antibodies/atp5a-antibody-15h4c4-mitochondrial-marker-ab14748>), anti-tubulin validated by Abcam (<https://www.abcam.com/en-us/products/primary-antibodies/alpha-tubulin-antibody-microtubule-marker-ab15246>), anti-ESR1 validated by Santa Cruz Biotechnology (<https://www.scbt.com/de/p/estrogen-receptor-alpha-antibody-d-12>).

Eukaryotic cell lines

Policy information about [cell lines and Sex and Gender in Research](#)

Cell line source(s)

U2OS (Cat. No.: HTB-96, ATCC) Cos-7 (Cat. No.: 87021302, Sigma Aldrich)

Authentication

All by supplier: STR profiling (U2OS), karyotyping (U2OS), morphology (U2OS, Cos-7)

Mycoplasma contamination

negative; cells were tested regularly using extraction-free PCR.

Commonly misidentified lines
(See [ICLAC](#) register)

none

Palaeontology and Archaeology

Specimen provenance

Provide provenance information for specimens and describe permits that were obtained for the work (including the name of the issuing authority, the date of issue, and any identifying information). Permits should encompass collection and, where applicable, export.

Specimen deposition

Indicate where the specimens have been deposited to permit free access by other researchers.

Dating methods

If new dates are provided, describe how they were obtained (e.g. collection, storage, sample pretreatment and measurement), where they were obtained (i.e. lab name), the calibration program and the protocol for quality assurance OR state that no new dates are provided.

☐ Tick this box to confirm that the raw and calibrated dates are available in the paper or in Supplementary Information.

Ethics oversight

Identify the organization(s) that approved or provided guidance on the study protocol, OR state that no ethical approval or guidance was required and explain why not.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Animals and other research organisms

Policy information about [studies involving animals](#); [ARRIVE guidelines](#) recommended for reporting animal research, and [Sex and Gender in Research](#)

Laboratory animals	Wistar rats
Wild animals	N/A
Reporting on sex	N/A
Field-collected samples	N/A
Ethics oversight	All procedures with live animals were conducted in the animal facility at the Max-Planck-Institute for Multidisciplinary Sciences, Göttingen. According to the German Animal Welfare Law, killing is not an experiment on animals. All requirements of § 4 TierSchG together with § 2 Satz 2, Anlage 1, Abschnitt 2 and Anlage 2 TierSchVersV were implemented. The facility is conducted under all aspects of animal welfare. The facility is headed by a veterinarian with special education in laboratory animal science as well as gene technology and molecular genetics. Only professionally educated animal technicians are in charge of animal husbandry and care. The facility is registered according to §11 Abs. 1 TierSchG (Tierschutzgesetz der Bundesrepublik Deutschland, Animal Welfare Law of the Federal Republic of Germany) as documented by 33.23-42508-066-§11, dated Nov 16th, 2023 ("Erlaubnis, zum Halten von Wirbeltieren zur Versuchszwecken", "Permission to keep vertebrates for experimental purposes") by the Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit (Lower Saxony State Office for Consumer Protection and Food Safety). According to the Animal Welfare Law of the Federal Republic of Germany (TierSchG) and the Regulation about animals used in experiments, dated 20th Dec 2022 (TierSchVersV) an animal welfare officer (specialized veterinarian in laboratory animal science) and an animal welfare committee for the institute is established.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Clinical data

Policy information about [clinical studies](#)

All manuscripts should comply with the ICMJE [guidelines for publication of clinical research](#) and a completed [CONSORT checklist](#) must be included with all submissions.

Clinical trial registration	Provide the trial registration number from ClinicalTrials.gov or an equivalent agency.
Study protocol	Note where the full trial protocol can be accessed OR if not available, explain why.
Data collection	Describe the settings and locales of data collection, noting the time periods of recruitment and data collection.
Outcomes	Describe how you pre-defined primary and secondary outcome measures and how you assessed these measures.

Dual use research of concern

Policy information about [dual use research of concern](#)

Hazards

Could the accidental, deliberate or reckless misuse of agents or technologies generated in the work, or the application of information presented in the manuscript, pose a threat to:

No	Yes
☒	☐ Public health
☒	☐ National security
☒	☐ Crops and/or livestock
☒	☐ Ecosystems
☒	☐ Any other significant area

Experiments of concern

Does the work involve any of these experiments of concern:

No	Yes
☒	☐ Demonstrate how to render a vaccine ineffective
☒	☐ Confer resistance to therapeutically useful antibiotics or antiviral agents
☒	☐ Enhance the virulence of a pathogen or render a nonpathogen virulent
☒	☐ Increase transmissibility of a pathogen
☒	☐ Alter the host range of a pathogen
☒	☐ Enable evasion of diagnostic/detection modalities
☒	☐ Enable the weaponization of a biological agent or toxin
☒	☐ Any other potentially harmful combination of experiments and agents

Plants

Seed stocks	N/A
Novel plant genotypes	N/A
Authentication	N/A

ChIP-seq

Data deposition

- ☐ Confirm that both raw and final processed data have been deposited in a public database such as [GEO](#).
- ☐ Confirm that you have deposited or provided access to graph files (e.g. BED files) for the called peaks.

Data access links <i>May remain private before publication.</i>	<i>For "Initial submission" or "Revised version" documents, provide reviewer access links. For your "Final submission" document, provide a link to the deposited data.</i>
Files in database submission	<i>Provide a list of all files available in the database submission.</i>
Genome browser session (e.g. UCSC)	<i>Provide a link to an anonymized genome browser session for "Initial submission" and "Revised version" documents only, to enable peer review. Write "no longer applicable" for "Final submission" documents.</i>

Methodology

Replicates	<i>Describe the experimental replicates, specifying number, type and replicate agreement.</i>
Sequencing depth	<i>Describe the sequencing depth for each experiment, providing the total number of reads, uniquely mapped reads, length of reads and whether they were paired- or single-end.</i>
Antibodies	<i>Describe the antibodies used for the ChIP-seq experiments; as applicable, provide supplier name, catalog number, clone name, and lot number.</i>
Peak calling parameters	<i>Specify the command line program and parameters used for read mapping and peak calling, including the ChIP, control and index files used.</i>
Data quality	<i>Describe the methods used to ensure data quality in full detail, including how many peaks are at FDR 5% and above 5-fold enrichment.</i>
Software	<i>Describe the software used to collect and analyze the ChIP-seq data. For custom code that has been deposited into a community repository, provide accession details.</i>

Flow Cytometry

Plots

Confirm that:

- ☒ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- ☒ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
- ☒ All plots are contour plots with outliers or pseudocolor plots.
- ☒ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation

U2OS cells were transfected with the bicistronic nuclease plasmids and the corresponding donor plasmids using FuGENE HD transfection reagent (Promega, Mannheim, Germany). To this end, 20.000 cells per well were seeded in a 6-well plate with supplemented DMEM. The following day, transfection was carried out using a reagent to DNA ratio of 3.5 to 1 and a total DNA amount of 3 µg. Subsequently, the cells were further incubated at 37°C, 5% CO₂. After seven days, cells were inspected by fluorescence microscopy. Wells containing cells exhibiting the expected sub-cellular localization of the Dreiklang fusion protein were subjected to single cell sorting into 96-well plates.

Instrument

FACSAria II (BD Biosciences, Heidelberg, Germany)

Software

FACSDiva 6.0 software

Cell population abundance

4.3% for PHB1-Dreiklang knockin cells (Fig. S2E, left); 10% for PHB2-Dreiklang knockin cells (Fig. S2E, right)

Gating strategy

We have identified Dreiklang-expressing knock-in cells by plotting the side scatter area versus fluorescence intensity after excitation with a 488 nm laser (Fig. S2D-E). We have sorted knock-in cells displaying a fluorescence signal that was about 5-fold higher compared to U2OS wildtype cells.

- ☒ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.

Magnetic resonance imaging

Experimental design

Design type

Indicate task or resting state; event-related or block design.

Design specifications

Specify the number of blocks, trials or experimental units per session and/or subject, and specify the length of each trial or block (if trials are blocked) and interval between trials.

Behavioral performance measures

State number and/or type of variables recorded (e.g. correct button press, response time) and what statistics were used to establish that the subjects were performing the task as expected (e.g. mean, range, and/or standard deviation across subjects).

Acquisition

Imaging type(s)

Specify: functional, structural, diffusion, perfusion.

Field strength

Specify in Tesla

Sequence & imaging parameters

Specify the pulse sequence type (gradient echo, spin echo, etc.), imaging type (EPI, spiral, etc.), field of view, matrix size, slice thickness, orientation and TE/TR/flip angle.

Area of acquisition

State whether a whole brain scan was used OR define the area of acquisition, describing how the region was determined.

Diffusion MRI

☐ Used

☐ Not used

Preprocessing

Preprocessing software

Provide detail on software version and revision number and on specific parameters (model/functions, brain extraction, segmentation, smoothing kernel size, etc.).

Normalization

If data were normalized/standardized, describe the approach(es): specify linear or non-linear and define image types used for transformation OR indicate that data were not normalized and explain rationale for lack of normalization.

Normalization template

Describe the template used for normalization/transformation, specifying subject space or group standardized space (e.g. original Talairach, MNI305, ICBM152) OR indicate that the data were not normalized.

Noise and artifact removal

Describe your procedure(s) for artifact and structured noise removal, specifying motion parameters, tissue signals and physiological signals (heart rate, respiration).

Volume censoring

Define your software and/or method and criteria for volume censoring, and state the extent of such censoring.

Statistical modeling & inference

Model type and settings

Specify type (mass univariate, multivariate, RSA, predictive, etc.) and describe essential details of the model at the first and second levels (e.g. fixed, random or mixed effects; drift or auto-correlation).

Effect(s) tested

Define precise effect in terms of the task or stimulus conditions instead of psychological concepts and indicate whether ANOVA or factorial designs were used.

Specify type of analysis: ☐ Whole brain ☐ ROI-based ☐ Both

Statistic type for inference

Specify voxel-wise or cluster-wise and report all relevant parameters for cluster-wise methods.

(See [Eklund et al. 2016](#))

Correction

Describe the type of correction and how it is obtained for multiple comparisons (e.g. FWE, FDR, permutation or Monte Carlo).

Models & analysis

n/a | Involved in the study

☐ ☐ Functional and/or effective connectivity☐ ☐ Graph analysis☐ ☐ Multivariate modeling or predictive analysis

Functional and/or effective connectivity

Report the measures of dependence used and the model details (e.g. Pearson correlation, partial correlation, mutual information).

Graph analysis

Report the dependent variable and connectivity measure, specifying weighted graph or binarized graph, subject- or group-level, and the global and/or node summaries used (e.g. clustering coefficient, efficiency, etc.).

Multivariate modeling and predictive analysis

Specify independent variables, features extraction and dimension reduction, model, training and evaluation metrics.

Figure S6, Panel B

α PHB1



α PHB2

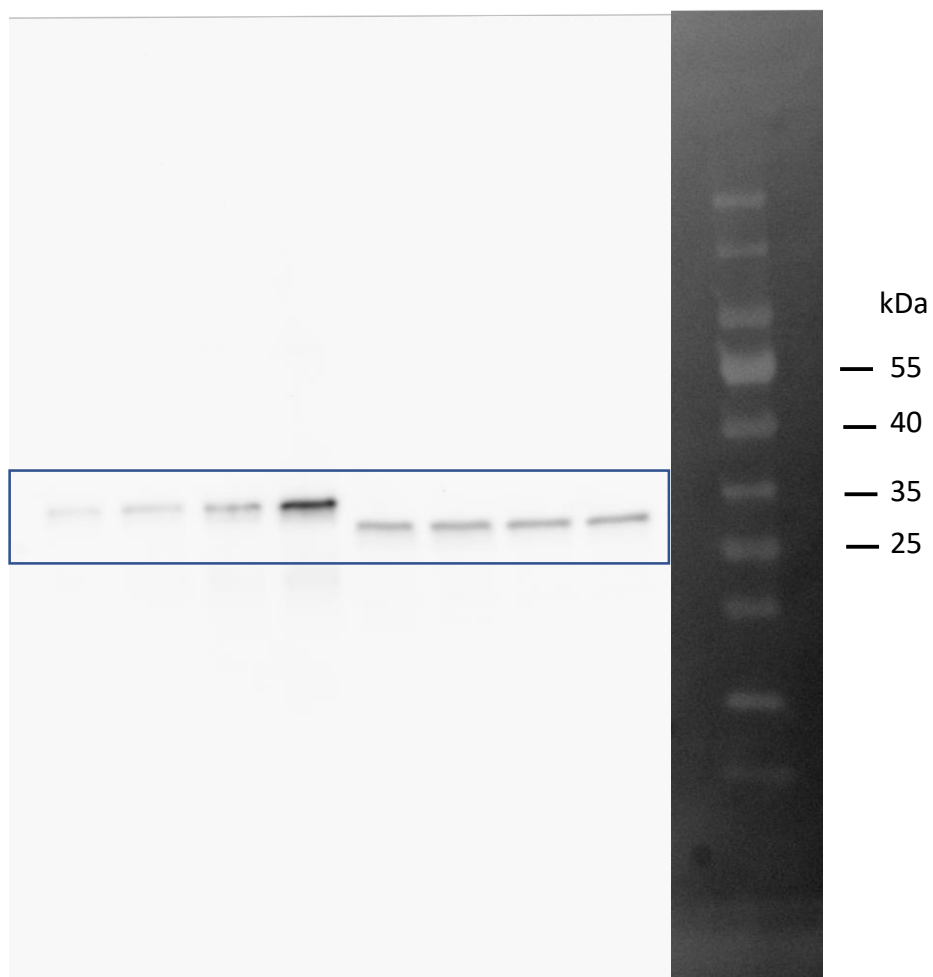
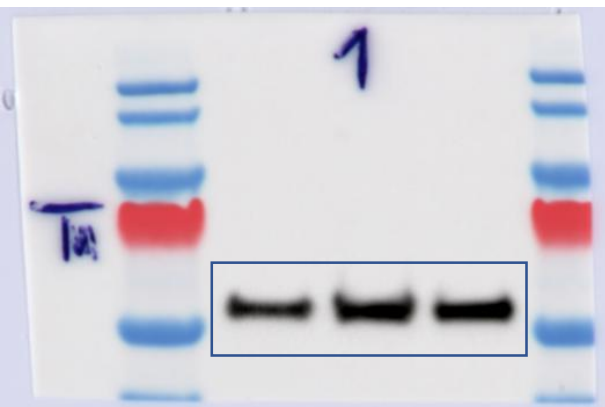


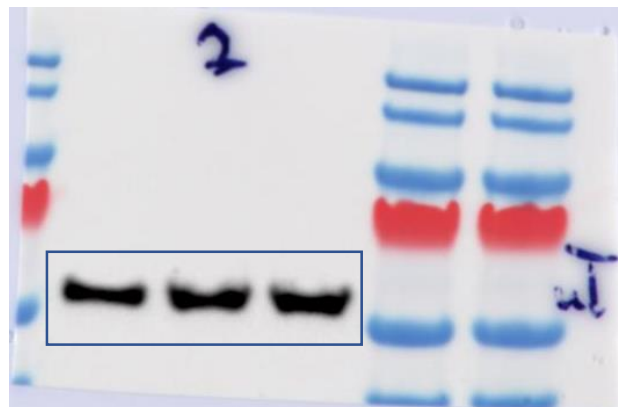
Figure S7, Panel B

PHB knockdown

α Tubulin

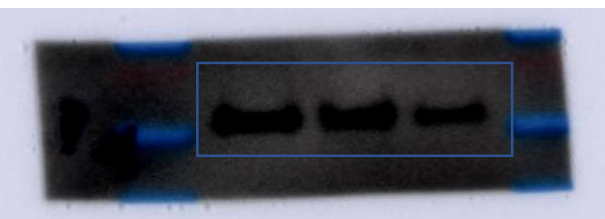


α Tubulin

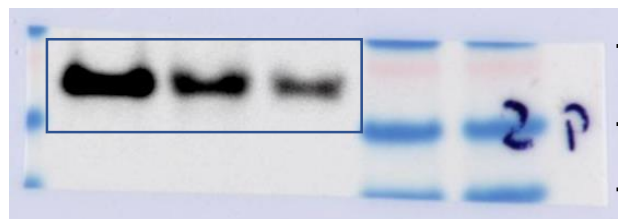


kDa
— 70
— 55
— 40

α PHB1

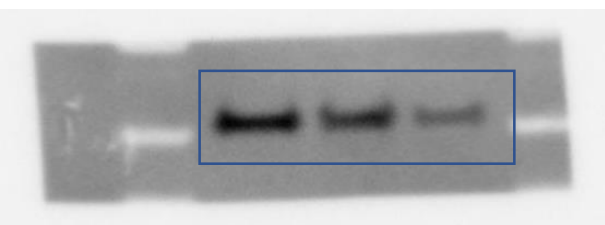


α PHB2



kDa
— 40
— 35
— 25

α PHB1 (greyscale)



In-situ architecture of the human prohibitin complex

Corresponding Author: Professor Stefan Jakobs

Version 0:

Decision Letter:

*Please delete the link to your author homepage if you wish to forward this email to co-authors.

Dear Stefan,

Thank you again for submitting your manuscript, "In-situ architecture of the human prohibitin complex", to Nature Cell Biology and I am very sorry for the delay in sharing our decision with you, as one reviewer needed an extension. The manuscript has now been seen by 3 referees, who are experts in mitochondrial biology (Referee #1); cryoET (Referee #2); and mitochondria, quality control (Referee #3). As you will see from their comments (attached below), they found the work of potential interest but have raised substantial concerns, which in our view would need to be addressed with considerable revisions before we can consider publication in Nature Cell Biology.

As per our standard process, we have now discussed the referee reports in detail within the editorial team to identify key referee points that should be addressed with priority to strengthen the analyses and core conclusions. To guide the scope of the revisions, I have listed these points below. Our standard revision period is six months, and we are committed to providing a fair and constructive peer-review process, so please feel free to contact me if you would like to discuss any of the referee comments further or if you anticipate any issues addressing the reviews.

In particular, it would be essential to address the following points:

1- The reviewers had concerns related to the use of the fusion proteins and interpretation of the cryoET data that need to be addressed:

Rev#2 "- On lines 176-178, the authors should try to assign the MW to the DK densities. If you have 5-6, you would have 150KDa, does this approach the extra density they are seeing? Is it present in the terminus they tagged the protein with? From the nomenclature it should be the C-terminus, at the top of the bell shape, but the authors should specify. A schematic in the supplementary would be needed to rationalize this finding.

- While their KD data strongly suggests that the complex is not formed in the absence of one of the proteins, it is also common that knockdowns have other effects on protein complexes that do not contain the KD protein. The putative presence of DK densities is also somewhat satisfying, but a confidential discussion in my lab left some wanting clearer evidence. Some suggestions include:
- Would it be possible to predict a mutation at the interface between PHB1 and PHB2 and look for the complex not being present, or not pulled down?
- Is there an anti-DK nanobody that could be used to increase the size of the DK density? (I am not sure if the nano body would enter the mitochondria though). Alternative, can a larger tag be added? Can tags be added to both monomers?"

Rev#3 points #1-2-3-4

2- Please also address their questions about strengthening and clarifying existing analyses, minor points, technical comments, requests for text/figure edits or discussion.

3- Finally, please pay close attention to our guidelines on statistical and methodological reporting (listed below) as failure to do so may delay the reconsideration of the revised manuscript. In particular, please provide:

- a Supplementary Figure including unprocessed images of all gels/blots in the form of a multi-page pdf file. Please ensure that blots/gels are labeled and the sections presented in the figures are clearly indicated.

- a Supplementary Table including all numerical source data in Excel format, with data for different figures provided as different sheets within a single Excel file. The file should include source data giving rise to graphical representations and statistical descriptions in the paper and for all instances where the figures present representative experiments of multiple independent repeats, the source data of all repeats should be provided.

We would be happy to consider a revised manuscript that would satisfactorily address these points, unless a similar paper is published elsewhere or is accepted for publication in Nature Cell Biology in the meantime.

When revising the manuscript please:

- ensure that it conforms to our format instructions and publication policies (see below and www.nature.com/nature/authors/).

- provide a point-by-point rebuttal to the full referee reports verbatim, as provided at the end of this letter.

- provide the completed Editorial Policy Checklist (found here <https://www.nature.com/authors/policies/Policy.pdf>), and Reporting Summary (found here <https://www.nature.com/authors/policies/ReportingSummary.pdf>). This is essential for reconsideration of the manuscript and these documents will be available to editors and referees in the event of peer review. For more information see <http://www.nature.com/authors/policies/availability.html> or contact me.

Nature Cell Biology is committed to improving transparency in authorship. As part of our efforts in this direction, we are now requesting that all authors identified as 'corresponding author' on published papers create and link their Open Researcher and Contributor Identifier (ORCID) with their account on the Manuscript Tracking System (MTS), prior to acceptance. ORCID helps the scientific community achieve unambiguous attribution of all scholarly contributions. You can create and link your ORCID from the home page of the MTS by clicking on 'Modify my Springer Nature account'. For more information please visit <http://www.springernature.com/orcid>.

Please submit the revised manuscript files and the point-by-point rebuttal to the referee comments using this link:

Link Redacted

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We would like to receive a revised submission within six months. We would be happy to consider a revision even after this timeframe, however if the resubmission deadline is missed and the paper is eventually published, the submission date will be the date when the revised manuscript was received.

We hope that you will find our referees' comments and editorial guidance helpful. Please do not hesitate to contact me if there is anything you would like to discuss. Thank you again for considering NCB for your work.

Best wishes,

Melina

Melina Casadio, PhD
Senior Editor, Nature Cell Biology
ORCID ID: <https://orcid.org/0000-0003-2389-2243>

Reviewers' Comments:

Reviewer #1:

Remarks to the Author:

This is an interesting, quantitative and rigorously performed study of mammalian prohibitin complexes in situ. Prior work indicated ~12-20 subunits of PHB1/PHB2 dimers form a ring in the inner mitochondrial membrane. The current cryo ET data define a discrete 11 subunit bell shaped complex resembling somewhat, to my eye, half of the major vault protein complex. Structural studies of a distantly related bacterial protein, HflK/C, that shares some domains with prohibitin, also reveals a similar bell shaped structure in a cell free reconstituted system. What this new work also brings is the structure of mammalian prohibitin in the native mitochondrial context. This new structural model differs substantially from the main current ring centric model of prohibitin oligomers. This new cage-like dome structure is also interesting because the center under the dome is empty, apparently not occupied by other proteins such as m-ATPases, and will likely inform/transform currently quite vague models for prohibitin function. The authors also find the prohibitin complex concentrated in cristae in nice quantitative analyses – in contrast to prior reports on the distribution of prohibitin in yeast. Overall, this is a highly rigorous advance into our understanding of prohibitin structure that will likely accelerate the understanding of function. The prior related structure of the bacterial protein HflK/C marginally decreases the novelty.

Specific comments:

On line 106 –State where the immunogold data is shown – Fig 1C-D.

In Fig 2D – please denote in panel 3 which face of the prohibitin bell faces the matrix and which side faces the intermembrane space.

Some of the valuable supplemental data could be incorporated into the main figures.

Reviewer #2:

Remarks to the Author:

The manuscript by Lange et al has a lot of qualities: the authors use state of the arts methods and quantitative calculations to design a project in which they can pursue the in situ structure of the elusive prohibitin complexes.

I really enjoyed following through the path of calculating the feasibility of the data acquisition by calculating mitochondrial surfaces, location of the complexes and endogenous copy numbers in cells, etc. I could only hope on a slam-dunk example like this when we wrote a recent Annual Reviews trying to make this very point.

The authors do everything I would have imagined that could be done for this project, and take advantage of AlphaFold to produce a model of the complex. The result is satisfying, and the first molecular model of prohibitin complexes. I think this is a fine example of the application of the cryo-tomography in situ (in cell) workflow, and it was fun to review. On the other hand, the project doesn't offer a mechanism or describes the context of prohibitins while performing their function. Nevertheless, I think this work is very well executed and I recommend it for publication in NCB.

I have a few minor and not-so minor comments that in my opinion will make the project more well rounded and will hopefully finish convincing a skeptical reviewer/reader.

Major:

- On lines 176-178, the authors should try to assign the MW to the DK densities. If you have 5-6, you would have 150KDa, does this approach the extra density they are seeing? Is it present in the terminus they tagged the protein with? From the nomenclature it should be the C-terminus, at the top of the bell shape, but the authors should specify. A schematic in the supplementary would be needed to rationalize this finding.

- While their KD data strongly suggests that the complex is not formed in the absence of one of the proteins, it is also common that knockdowns have other effects on protein complexes that do not contain the KD protein. The putative presence of DK densities is also somewhat satisfying, but a confidential discussion in my lab left some wanting clearer evidence. Some suggestions include:
 - Would it be possible to predict a mutation at the interface between PHB1 and PHB2 and look for the complex not being present, or not pulled down?
 - Is there an anti-DK nanobody that could be used to increase the size of the DK density? (I am not sure if the nano body would enter the mitochondria though). Alternative, can a larger tag be added? Can tags be added to both monomers?

- The confidence of the AlphaFold models should be included, and if not high in any area, the ability to use it should be justified. Molecular dynamics simulations from models typically have lower confidence than those from structural biology data.
 - It was not clear to me how the authors decide on which of PHB1 or PHB2 should have 5 vs 6 monomers. Is this based on the copy numbers found in Figure 3? If so, it should be specified in the text.
 - How do the authors reconcile the positive/negative alternating monomers with the C11 symmetry? How does the negative-negative or positive-positive interaction work? Why do the authors think about a mismatch from 11 to 12 compared to the bacterial system?
 - It seems like the molecular dynamics model was membrane anchored at the bottom as it exists in the mitochondria, but this is only briefly described in the methods section but not on the figures. The termini needed to be contained in z, the authors should explain if the model is not enough to remain tethered to the membrane.
 - Also, no information of on the reported stability of the molecular dynamics simulation is described. How was it assessed a feasible model?

Minor:

- Fluorescence images should be color-blind friendly
- For figure 1C, I would recommend that the squares around the gold labels are colored whether the gold is localized in the CM or IBM, preferably matching the colors of the bars in Fig. 1D.
- In line 122, is it 3.1×10^6 PHB1 and 9.8×10^6 PHB2 molecules per cell? The way it is written is confusing.
- Fig S7 - remove imod controls in A, caption should specify how particles in D were selected
- Fig 3. The numbers in D should have citations and original of computations in the caption.

Congratulations on the beautiful work, I hope our comments are helpful and constructive.

Elizabeth Villa and trainee

Reviewer #3:

Remarks to the Author:

The study by Lange, Ratz et al. reports on the first in situ cryo-EM structure of the human prohibitin complex. The authors use a combination of methods and an integrative structural approach to come up with a very interesting model on the human prohibitin complex. The data suggests that it forms a bell-shaped structure with around 20 nm diameter and 9 nm height which is consisting of 11 monomers of PHBs.

Overall this is a very interesting study and model of this complex which will be important in the mitochondrial field. It extends our knowledge on known 3D structures of this protein family considerably. I still feel that the refined 3D model after molecular modelling of 11 subunits of PHB into the bell-shaped complex is not substantiated sufficiently and the authors need to strengthen their model further.

Major issues:

1. Can the authors provide additional evidence of the proposed orientation and stoichiometry of 11 PHB subunits? Does their model fit to the density observed they claim to be caused by DK (Fig. S7D-E). What would MD simulations show in this case? The authors might consider to similar to their earlier approach also try to model the DK-containing fusion proteins into the observed densities.
2. Linked to this: Is it possible that these are actually 11 heterodimers fitting or can the authors exclude this with high certainty?
3. The functionality of the DK fusion constructs needs to be shown. The heterotypic interaction with endogenous PHBs is not sufficient in my opinion. A phenotypic rescue compared to the PHB knockout situation would be one possibility to show the functionality of the fusion protein.

Minor issues:

4. The authors have two arguments that the structure they observe is indeed PHB, namely the quantification of the PHB structures after downregulation and the additional density in the DK-containing complex (Fig. S7D-G). The latter is more convincing as the number of bell-shaped structures can result from indirect effects. Can the authors discuss whether the location of DK fits to the prediction where DK was integrated? Where is DK actually integrated exactly? Was this at the N- or C-terminus or internal? Please show a scheme where DK is relative to other PHB domains.
5. lines 98-101. The authors state that concentration of prohibitions is 3 to 5 times higher in the CM compared to the IBM. The calculation appears not to be correct when considering the ratio 0.64 to 1 (IBM to CM membrane length). According to my rough estimate the concentration (not amount) is rather 2 to 3.5 times higher.
6. lines 103-105. missing reference to Fig. 1CD. Please add.
7. Fig. 2D, 3AB, and Fig. S7F. These are very nice in situ images of the putative PHB complex. I suggest to include scale bars/size markers here to see the dimensions also in the figure.
8. Fig. S7C. The control with anti-PHB1 in Wt is missing. Please show
9. Fig S3D, right panel: mislabelling of x-axis as it should read "PHB2-DK". Please correct
10. Fig. S5. To ensure that this heterotypic interaction is indeed specific and not due to an unspecific "stickiness" of PHBs, it is needed to show that another inner membrane protein (e.g. CIV or CV subunit) is not interacting with PHB1-DK or PHB2-DK. It would further be good to show the fraction of unbound proteins. Please state how much was loaded of input and IP fractions.

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AUTHOR AFFILIATIONS – should be denoted with numerical superscripts (not symbols) preceding the names. Full addresses should be included, with US states in full and providing zip/post codes. The corresponding author is denoted by: "Correspondence should be addressed to [initials]."

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ACKNOWLEDGEMENTS – should be kept brief. Professional titles and affiliations are unnecessary. Grant numbers can be listed.

AUTHOR CONTRIBUTIONS – must be included after the Acknowledgements, detailing the contributions of each author to the paper (e.g. experimental work, project planning, data analysis etc.). Each author should be listed by his/her initials.

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Figures should be provided at approximately the size that they are to be printed at (single column is 86 mm, double column is 170 mm) and should not exceed an A4 page (8.5 x 11"). Reduction to the scale that will be used on the page is not necessary, but multi-panel figures should be sized so that the whole figure can be reduced by the same amount at the smallest size at which essential details in each panel are visible. In the interest of our colour-blind readers we ask that you avoid using red and green for contrast in figures. Replacing red with magenta and green with turquoise are two possible colour-safe alternatives. Lines with widths of less than 1 point should be avoided. Sans serif typefaces, such as Helvetica (preferred) or Arial should be used. All text that forms part of a figure should be rewritable and removable.

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Version 1:

Decision Letter:

Our ref: NCB-LE53553A

15th November 2024

Dear Dr. Jakobs,

Thank you for submitting your revised manuscript "In-situ architecture of the human prohibitin complex" (NCB-LE53553A) and for your patience with the process. It has now been seen by the original Referees #2-3 and their comments are below. The reviewers find that the paper has been strengthened in revision, and therefore we'll be happy in principle to publish it in Nature Cell Biology, pending minor revisions to comply with our editorial and formatting guidelines.

We are now performing detailed checks on your paper and will send you a checklist detailing our editorial and formatting requirements in about 1-2 weeks. Please do not upload the final materials and make any revisions until you receive this additional information from us.

Thank you again for your interest in Nature Cell Biology. Please do not hesitate to contact me if you have any questions.

Sincerely,

Melina

Melina Casadio, PhD
Senior Editor, Nature Cell Biology
Consulting Editor, Nature Structural & Molecular Biology
ORCID ID: <https://orcid.org/0000-0003-2389-2243>

Reviewer #2 (Remarks to the Author):

The authors address all the points raised by the reviewers. This is a great manuscript that we recommend for publication.

Reviewer #3 (Remarks to the Author):

The authors have well answered the open questions and addressed all concerns nicely. It is a nice and interesting study important to the field.

Version 2:

Decision Letter:

Dear Dr Jakobs,

I am pleased to inform you that your manuscript, "In-situ architecture of the human prohibitin complex", has now been accepted for publication in Nature Cell Biology.

Thank you for sending us the final manuscript files to be processed for print and online production, and for returning the manuscript checklists and other forms. Your manuscript will now be passed to our production team who will be in contact with you if there are any questions with the production quality of supplied figures and text.

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Please feel free to contact us if you have any questions.

With kind regards,

Melina Casadio, PhD
Senior Editor, Nature Cell Biology
Consulting Editor, Nature Structural & Molecular Biology
ORCID ID: <https://orcid.org/0000-0003-2389-2243>

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Dear Reviewers, dear Dr. Casadio,

Thank you for your constructive comments and the positive views on our manuscript. We have taken the reviewers' comments very seriously and believe that with the revised version of our manuscript we are able to address the raised concerns appropriately.

We added an entirely new dataset of crosslinking mass spectrometry analysis and a new set of cryo electron tomography data. Thereby, we added two entirely new figures (Main Fig. 4 and Suppl. Fig. 10), re-wrote the manuscript and made extensive adjustments and additions to existing figures.

We are convinced that the revised version of our manuscript is now suitable for publication in NCB.

Please find below a detailed point-by-point response to the referee reports.

Yours sincerely,

Reviewers' Comments:

Reviewer #1:

Remarks to the Author:

This is an interesting, quantitative and rigorously performed study of mammalian prohibitin complexes in situ. Prior work indicated ~12-20 subunits of PHB1/PHB2 dimers form a ring in the inner mitochondrial membrane. The current cryo ET data define a discrete 11 subunit bell shaped complex resembling somewhat, to my eye, half of the major vault protein complex. Structural studies of a distantly related bacterial protein, HflK/C, that shares some domains with prohibitin, also reveals a similar bell shaped structure in a cell free reconstituted system. What this new work also brings is the structure of mammalian prohibitin in the native mitochondrial context. This new structural model differs substantially from the main current ring centric model of prohibitin oligomers. This new cage-like dome structure is also interesting because the center under the dome is empty, apparently not occupied by other proteins such as m-ATPases, and will likely inform/transform currently quite vague models for prohibitin function. The authors also find the prohibitin complex concentrated in cristae in nice quantitative analyses – in contrast to prior reports on the distribution of prohibitin in yeast. Overall, this is a highly rigorous advance into our understanding of prohibitin structure that will likely accelerate the understanding of function. The prior related structure of the bacterial protein HflK/C marginally decreases the novelty.

We thank reviewer #1 for his/her positive view on our manuscript and the constructive comments and suggestions.

Specific comments:

On line 106 –State where the immunogold data is shown – Fig 1C-D.

Thank you for pointing to this. We corrected the text accordingly.

In Fig 2D – please denote in panel 3 which face of the prohibitin bell faces the matrix and which side faces the intermembrane space.

We thank the reviewer for this suggestion. We modified the respective figure panel (Fig. 2D3) and denoted the respective mitochondrial compartments as *mitochondrial matrix* and *IMS/crista lumen*, respectively.

Some of the valuable supplemental data could be incorporated into the main figures.

This is an interesting suggestion. We feel that the manuscript is concise in its current form and would prefer to keep it that way. It is also within the length guidelines of NatCellBiol. We would prefer to leave it to the discretion of the journal editors to decide whether further supplementary data should be included in the main figures.

Reviewer #2:

Remarks to the Author:

The manuscript by Lange et al has a lot of qualities: the authors use state of the arts methods and quantitative calculations to design a project in which they can pursue the in situ structure of the elusive prohibitin complexes.

I really enjoyed following through the path of calculating the feasibility of the data acquisition by calculating mitochondrial surfaces, location of the complexes and endogenous copy numbers in cells, etc. I could only hope on a slam-dunk example like this when we wrote a recent Annual Reviews trying to make this very point.

The authors do everything I would have imagined that could be done for this project, and take advantage of AlphaFold to produce a model of the complex. The result is satisfying, and the first molecular model of prohibitin complexes. I think this is a fine example of the application of the cryo-tomography in situ (in cell) workflow, and it was fun to review. On the other hand, the project doesn't offer a mechanism or describes the context of prohibitins while performing their function. Nevertheless, I think this work is very well executed and I recommend it for publication in NCB.

We thank reviewer #2 for her positive view on our manuscript and the constructive comments and suggestions.

I have a few minor and not-so minor comments that in my opinion will make the project more well rounded and will hopefully finish convincing a skeptical reviewer/reader.

Major:

- On lines 176-178, the authors should try to assign the MW to the DK densities. If you have 5-6, you would have 150KDa, does this approach the extra density they are seeing? Is it present in the terminus they tagged the protein with? From the nomenclature it should be the C-terminus, at the top of the bell shape, but the authors should specify. A schematic in the supplementary would be needed to rationalize this finding.

As suggested by the reviewer, we added a schematic in the supplementary to rationalize the orientation of the prohibitins in the inner membrane (Fig. S7E). This figure highlights that DK fused to the C-terminus of PHB1 or PHB 2 is located in the inter membrane space.

The question on how many DKs we do see in the densities is a difficult one: Because both tagged cell lines (PHB1-DK and PHB2-DK) were heterozygous, we expect various numbers of DK molecules in the single complexes. Indeed, in the tomograms we could distinguish 1, 2 and 3 DK molecules and the maximum we were able to see was 5 molecules on top of a complex.

This is now explained in the revised version of the manuscript. It reads:

"We also observed one to three additional densities at most (> 90%) convex structures identified in the tomograms of U2OS PHB1-DK and PHB2-DK cells (Fig. S7E-H). These extra densities sat at the top of the convex structure but were absent in wildtype cells, thereby indicating that they originate from the DK fluorescent protein fused to the C-termini of the PHB1 and PHB2 proteins." (page 5)

- While their KD data strongly suggests that the complex is not formed in the absence of one of the proteins, it is also common that knockdowns have other effects on protein complexes that do not contain the KD protein. The putative presence of DK densities is also somewhat satisfying, but a confidential discussion in my lab left some wanting clearer evidence. Some suggestions include:

- Would it be possible to predict a mutation at the interface between PHB1 and PHB2 and look for the complex not being present, or not pulled down?

We thank the reviewer for this interesting idea. To the best of our knowledge no mutations to that effect have been described. We are not able to make a prediction for such a mutation based on the structure. For these reasons we could not follow this suggestion.

- Is there an anti-DK nanobody that could be used to increase the size of the DK density? (I am not sure if the nano body would enter the mitochondria though).

We thank the reviewer for this interesting idea. We thought about it carefully. Several studies address the live cell delivery of binding molecules (e.g. antibodies) as demonstrated in Kai W. Teng et al.; eLife 2016 or pre-embedding labelling of cytoplasmic targets as demonstrated in Elena V. Polishchuk et al.; Tissue and Cell 2019. While the former would technically be compatible with vitrification and near-native structural preservation the latter is likely not. These techniques have in common that they work on cytoplasmic proteins and studies showing this principle within mitochondria are lacking. To the best of our knowledge there are currently no tools available to facilitate temporary permeabilization or other micropores of the mitochondrial membranes in live cells. We therefore are not able to pursue this idea.

- Alternative, can a larger tag be added?

We thank the reviewer also for this interesting suggestion. Indeed, the identification of novel protein complexes poses a challenge for *in-situ* structural biology studies. Recent developments to identify proteins of interest in cryo electron tomograms include the GEM tag from the Mahamid lab (Herman K. H. Fung et al. 2023; Nature Methods) and the FerriTag from the Royle lab (Nicholas I. Clarke et al. 2018; Nature Communications). As of now, none of these tags were shown to work in mitochondria. Further, both tags require a substantial number of monomers (60 monomers for GEM, 24 monomers for ferritin). Hence, it remains unclear whether such a large particle (>15 nm in diameter) could be assembled within the narrow space of the crista lumen or the intermembrane space of mitochondria. Hence, we doubt that this approach would work with the prohibitin complex. As described in the manuscript, we decided instead to tag also PHB1 in order to provide additional proof that the observed complexes are indeed prohibitin complexes.

- Can tags be added to both monomers?

For the revised version of the manuscript, we tagged both prohibitins (PHB1 and PHB2) individually with DK. For the revision, we also imaged PHB1-DK and found extra densities on top of the prohibitin complexes. Comparable to the complexes in PHB2-DK cells, we recorded populations of the complexes showing 1, 2 or 3 densities that represent individual DK molecules (of about 3 nm size). This data is now included in Figure S7F.

- The confidence of the AlphaFold models should be included, and if not high in any area, the ability to use it should be justified. Molecular dynamics simulations from models typically have lower confidence than those from structural biology data.

We thank the reviewer for this comment. We added additional panels to Figure S9A to highlight the AlphaFold-predicted structures of PHB1 and PHB2 colored according to the pLDDT. Due to the structural similarities among SPFH protein family members and the availability of in-vitro structures for the bacterial homolog HflK/C, the pLDDT scores are high for the majority of the two prohibitins. . pLDDT values < 50 are only present in the flexible part of the C-terminus. To our understanding, the current state of subtomogram averaging approaches is insufficient in capturing small, unstructured and potentially very flexible peptides of a protein and therefore we did not draw any conclusions for this part of the PHB complex.

- It was not clear to me how the authors decide on which of PHB1 or PHB2 should have 5 vs 6 monomers. Is this based on the copy numbers found in Figure 3? If so, it should be specified in the text.

We believe that both options (5 PHB1 vs 6 PHB2, or 6 PHB1 vs 5 PHB2) exist. This is now clearly stated in the manuscript and shown in the new figures Figure 3A and Figure S10B. This is also stated in the text. It reads:

“The final molecular model comprises 11 monomeric PHB molecules forming a bell-shaped assembly (Fig. 3A1, 2, Fig. S9B, C). Obviously, the uneven symmetry implies that one pair of adjacent subunits comprises a repeat of the same molecule (PHB1-PHB1 or PHB2-PHB2).” (page 7)

- How do the authors reconcile the positive/negative alternating monomers with the C11 symmetry? How does the negative-negative or positive-positive interaction work? Why do the authors think about a mismatch from 11 to 12 compared to the bacterial system?

We thank the reviewer for this comment.

To address the question on how the negative-negative or positive-positive interactions work, we analysed for the revised version crosslinking mass spectrometry data (new Fig. 4 and Fig. S10A). The data demonstrate that PHB1-PHB1 as well as PHB2-PHB2 interactions exist, although PHB1-PHB2 interactions are much more abundant, fully supporting the proposed model of a prohibitin structure consisting of an 11-mer of alternating PHB1 and PHB2 molecules.

The C11 symmetry comes out of the data and has not been imposed by us.

This is stated in the revised manuscript:

“We proceeded to extract the CTF corrected subtomograms in Warp for subsequent automated 3D refinement in Relion4.0 with no initial symmetry implied^{25, 26}. Initially, we aligned all particles at 5 Å pixel size. The resulting cryo EM map revealed 11 densities in the top view likely representing subunits of the prohibitin complex (Fig. S8C).” (page 6)

And

“Pose-optimized particles were extracted in Warp at a 5 Å pixel size and aligned in Relion4.0 through 3D auto-refinement, revealing an 11-fold symmetry of the prohibitin complex.” (page 30)

In fact, we do not consider the stoichiometry of the complex to be a mismatch to the bacterial homologue: While the monomers of the SPFH members share substantial structural similarities their multimeric complexes show strikingly different dimensions. The RNA-vault for example comprises of 39 molecules, the bacterial HflK/C contains 24 molecules and two recent studies addressing the structure of flotillin in-vitro and in-situ suggested a range of 42 to 44 molecules per complex. All of these examples result in protein complexes that are substantially larger compared to the PHB complex.

Therefore, the stoichiometry and dimension of the complex is possibly be dictated by the specific function as well as the available space, which is limited in the case of prohibitins compared to cytoplasmic complexes such as the RNA-vault and flotillin. Because this is rather speculative, we prefer not to address this issue in the manuscript.

- It seems like the molecular dynamics model was membrane anchored at the bottom as it exists in the mitochondria, but this is only briefly described in the methods section but not on the figures. The termini needed to be contained in z, the authors should explain if the model is not enough to remain tethered to the membrane.

We thank the reviewer for this suggestion. The molecular dynamics simulation did not include any restraints or anchoring. The restraints on the N-termini position in the Z-axis were only applied during the initial modelling phase, prior to embedding the protein complex in the membrane and preparing it for molecular dynamics. These initial restraints were used to ensure that the newly modelled N-termini were not positioned in the region where the lipid bilayer would later be constructed. Indeed, the model by itself remained in the lipid bilayer during the MD simulation without any enforcements. These points are now explained in the manuscript:

“Ultimately, because biochemical evidence suggests a complex assembly with alternating PHB1 and PHB2 molecules³, successive PHB1 and PHB2 molecules were fitted into the cryo-EM map with the N-terminal transmembrane domains embedded in the lipid bilayer³ and the PHB domains perpendicular to the lipid bilayer.” (page 7)

- Also, no information of on the reported stability of the molecular dynamics simulation is described. How was it assessed a feasible model?

The reviewer is right. In the initial submission we only touched on the stability briefly. We added the missing information to the manuscript in Figure S9F and modified the text accordingly. It now reads:

“The molecular model remained stable over 100 ns of MD simulation without major alterations, suggesting that it represents a plausible model for the molecular structure of the prohibitin complex in human cells.” (page 8)

Minor:

- Fluorescence images should be color-blind friendly

The reviewer is right. To aid readability of the manuscript and figures we changed the lookup tables of the dual-color fluorescence microscopy images to green/magenta.

- For figure 1C, I would recommend that the squares around the gold labels are colored whether the gold is localized in the CM or IBM, preferably matching the colors of the bars in Fig. 1D.

Done. We applied this change and the boxes in Figure 1 C now match the color of the bars shown in Figure 1D.

- In line 122, is it 3.1×10^6 PHB1 and 9.8×10^6 PHB2 molecules per cell ? The way it is written is confusing.

The reviewer is right. We clarified this statement. It now reads:

“To this end, recombinant His-tagged PHB1 and PHB2 proteins were purified from Escherichia coli and used as a reference in quantitative Western blotting (Fig. S6B). It was determined that on average each U2OS cell contains approximately $3.38 \pm 0.23 \cdot 10^6$ molecules of PHB1 and $3.46 \pm 0.15 \cdot 10^6$ molecules of PHB2. These numbers are in good agreement with previous mass spectrometry studies using various human cell lines^{17, 18}.” (page 4)

- Fig S7 - remove imod controls in A, caption should specify how particles in D were selected
Done. In the revised manuscript we removed the IMOD controls in Fig. S7A and indicated the change of perspective with an arrow in Panel A2. Further, the sub-title of Fig. S7D now reads *"Manual annotation of prohibitin complexes"*.
- Fig 3. The numbers in D should have citations and original of computations in the caption.
Done. In the revised manuscript we modified the caption of Fig. 5 which now reads **"B: Mitochondrial key numbers and prohibitin abundance determined in this study. Mitochondrial network length determined in Fig. S6C, inter-cristae distance determined in Fig. S6D-F, PHB molecules per cell determined in Fig. S6B."**

Congratulations on the beautiful work, I hope our comments are helpful and constructive.

Elizabeth Villa and trainee

Reviewer #3:

Remarks to the Author:

The study by Lange, Ratz et al. reports on the first in situ cryo-EM structure of the human prohibitin complex. The authors use a combination of methods and an integrative structural approach to come up with a very interesting model on the human prohibitin complex. The data suggests that it forms a bell-shaped structure with around 20 nm diameter and 9 nm height which is consisting of 11 monomers of PHBs.

Overall this is a very interesting study and model of this complex which will be important in the mitochondrial field. It extends our knowledge on known 3D structures of this protein family considerably. I still feel that the refined 3D model after molecular modelling of 11 subunits of PHB into the bell-shaped complex is not substantiated sufficiently and the authors need to strengthen their model further.

We thank reviewer #3 for his/her positive view on our manuscript and the constructive comments and suggestions.

Major issues:

1. Can the authors provide additional evidence of the proposed orientation and stoichiometry of 11 PHB subunits? Does their model fit to the density observed they claim to be caused by DK (Fig. S7D-E). What would MD simulations show in this case? The authors might consider to similar to their earlier approach also try to model the DK-containing fusion proteins into the observed densities.

The C11 symmetry comes out of the data and has not been imposed by us. We just do not see any possible way to provide additional evidence for a C11 symmetry.

In order to provide additional evidence for the proposed stoichiometry of the PHB subunits we analysed for the revised version crosslinking mass spectrometry data (new Fig. 4 and Fig. S10A). The data demonstrate that PHB1-PHB1 as well as PHB2-PHB2 interactions exist, although PHB1-PHB2 interactions are much more abundant, fully supporting the proposed model of a prohibitin structure consisting of an 11-mer of alternating PHB1 and PHB2 molecules.

Concerning the proposed orientation in the mitochondrial inner membrane: Our initial model design was based on the bacterial homologue HflK/C, which demonstrated N-terminal anchoring of the individual molecules in the lipid bilayer. Indeed, the predicted transmembrane domain (TMD) of prohibitins is also at the N-terminus. The final cryoEM-map of the prohibitin structure reveals a large domain close to the lipid bilayer which indicates the location of the PHB-domain. Finally, our tomography data of PHB1-DK and PHB2-DK cells, both tagged at the C-terminus demonstrated the additional densities at the top of the convex structures indicating that the prohibitin C-termini form the top of the structures. To clarify the orientation, we added an additional panel to Fig. S7 (Fig. S7E) to illustrate the location of DK fused to PHB2.

Both cell lines (PHB1-DK and PHB2-DK) were heterozygous. Therefore, we do expect various numbers of DK molecules being present on top of the complexes. This is exactly what the tomograms show. We identified typically one to three extra densities on top of the prohibitin complexes in the PHB1-DK and PHB2-DK heterozygote cell lines. Due to the flexibility of the C-terminal tag (linker and DK), a sharp density is not expected (and not observed) upon subtomogram averaging. Consequently, the density achieved in the simple subtomogram averaging approach presented in Fig. S7G is only slightly larger than a single DK-molecule. To present this finding clearer we

changed the simple subtomogram average to a transparent surface and fitted the structure of DK into the density (New panel in Fig. S7G).

Because of the variable number of DK-molecules and their flexibility it was possible to achieve a sharpened, higher-resolution cryo-EM map of the DK-PHB complexes. Consequently, we did not consider MD simulations.

This is also better explained in the text of the revised version.

It now reads: *"We also observed one to three additional densities in most (> 90%) convex structures identified in the tomograms of U2OS PHB1-DK and PHB2-DK cells (Fig. S7E-H). These extra densities sat at the top of the convex structure but were absent in wildtype cells, thereby indicating that they originate from the DK fluorescent protein fused to the C-termini of the PHB1 and PHB2 proteins."* (page 5)

2. Linked to this: Is it possible that these are actually 11 heterodimers fitting or can the authors exclude this with high certainty?

We can exclude with high certainty the existence of 11 heterodimers: Heterodimers just do not fit into the high-resolution EM-Map. To explain the conclusion on the C11 symmetry, we also added new panels to Fig. S9 that highlight the rationale of the stoichiometry and the initial molecule placement in more detail (Fig. S9C).

This is now clearly stated in the revised version:

"Numerous possible arrangements were tested but we found that each of the 11 densities in the cryo-EM map can accommodate only one prohibitin molecule. Therefore, the entire complex consists of 11 prohibitin molecules in total." (page 6)

3. The functionality of the DK fusion constructs needs to be shown. The heterotypic interaction with endogenous PHBs is not sufficient in my opinion. A phenotypic rescue compared to the PHB knockout situation would be one possibility to show the functionality of the fusion protein.

Here we respectfully disagree with the reviewer. In this study, we did not draw any functional conclusions from the knock-in cell lines other than the mobility of the complexes (see also Figure 1E and Figure 1F).

We use the tagged versions only as part of a line of evidence to demonstrate that the bell-shaped structure is indeed the prohibitin complex. We believe that our line of evidence is exhaustive.

We therefore argue that a more detailed functional analysis is not necessary at this stage of the work.

Minor issues:

4. The authors have two arguments that the structure they observe is indeed PHB, namely the quantification of the PHB structures after downregulation and the additional density in the DK-containing complex (Fig. S7D-G). The latter is more convincing as the number of bell-shaped structures can result from indirect effects. Can the authors discuss whether the location of DK fits to the prediction where DK was integrated? Where is DK actually integrated exactly? Was this at the N- or C-terminus or internal? Please show a scheme where DK is relative to other PHB domains.

We thank the reviewer for this suggestion. We agree with the reviewer that both experiments, PHB knock-down and cryo-ET imaging of PHB-DK complexes, are necessary. DK was added after a short GSGSG linker to the C-terminus of PHB1 and PHB2. Therefore, the location of the extra densities in cryo-ET corresponds exactly to the expected position, taking into account the N-terminal integration of the prohibitin molecules into the lipid bilayer. In the revised manuscript, we have added the panel Fig. S7E to illustrate this rationale more clearly.

5. lines 98-101. The authors state that concentration of prohibitins is 3 to 5 times higher in

the CM compared to the IBM. The calculation appears not to be correct when considering the ratio 0.64 to 1 (IBM to CM membrane length). According to my rough estimate the concentration (not amount) is rather 2 to 3.5 times higher.

We thank the reviewer for this comment. The analysis of immunogold EM data presented in Fig. 1D was already adjusted for the IBM to CM ratio. Therefore, and to make this section clearer we rephrased the section which now reads:

“For PHB1-DK and PHB2-DK we found 84.9% and 90.6% of the gold particles at the crista membranes, respectively; with antibodies directed against endogenous PHB1 and PHB2 we found 88.6% and 88.9% of the gold particles at the crista membranes, respectively (Fig. 1C). When considering that the ratio of IBM to CM is 0.64 to 1 in the U2OS cells (Fig. S6A), it can be calculated that the concentration of prohibitins is three to five times higher in the crista membranes than in the IBM (Fig. 1D).” (page 3)

6. lines 103-105. missing reference to Fig. 1CD. Please add.

Thank you for pointing to this. We corrected the text accordingly.

7. Fig. 2D, 3AB, and Fig. S7F. These are very nice in situ images of the putative PHB complex. I suggest to include scale bars/size markers here to see the dimensions also in the figure.

We thank the reviewer for this suggestion. We added scale bars to Fig. 2D1,2 and Fig. S7G to highlight the dimensionality of the complexes better.

8. Fig. S7C. The control with anti-PHB1 in Wt is missing. Please show

The reviewer is right. We added the missing control which is now incorporated into Fig. S7C.

9. Fig S3D, right panel: mislabelling of x-axis as it should read “PHB2-DK”. Please correct

The reviewer is right. We changed the labelling of the x-axis which now reads “PHB2-DK clone #”

10. Fig. S5. To ensure that this heterotypic interaction is indeed specific and not due to an unspecific “stickiness” of PHBs, it is needed to show that another inner membrane protein (e.g. CIV or CV subunit) is not interacting with PHB1-DK or PHB2-DK. It would further be good to show the fraction of unbound proteins. Please state how much was loaded of input and IP fractions.

The reviewer is right and we thank for this suggestion. In the revised version we shown two other inner membrane proteins (ATP5A und COX2) and also show the fraction of unbound proteins (Fig. S5B). We also state how much was loaded of input and IP fractions.

In the figure legend to Fig. S5 it reads: “10 µg of sample was loaded for all samples of Input and flow through, respectively.”

From the editor:

1- The reviewers had concerns related to the use of the fusion proteins and interpretation of the cryoET data that need to be addressed:

Rev#2

- On lines 176-178, the authors should try to assign the MW to the DK densities. If you have 5-6, you would have 150KDa, does this approach the extra density they are seeing? Is it present in the terminus they tagged the protein with? From the nomenclature it should be the C-terminus, at the top of the bell shape, but the authors should specify. A schematic in the supplementary would be needed to rationalize this finding.

As suggested by the reviewer, we added a schematic in the supplementary to rationalize the orientation of the prohibitins in the inner membrane (Fig. S7E).

In the revised version of the manuscript, we further modified line 174 to 178. It reads: *"We also observed one to three additional densities at most (> 90%) convex structures identified in the tomograms of U2OS PHB1-DK and PHB2-DK cells (Fig. S7E-H). These extra densities sat at the top of the convex structure but were absent in wildtype cells, thereby indicating that they originate from the DK fluorescent protein fused to the C-termini of the PHB1 and PHB2 proteins."*

- While their KD data strongly suggests that the complex is not formed in the absence of one of the proteins, it is also common that knockdowns have other effects on protein complexes that do not contain the KD protein. The putative presence of DK densities is also somewhat satisfying, but a confidential discussion in my lab left some wanting clearer evidence. Some suggestions include:

- Would it be possible to predict a mutation at the interface between PHB1 and PHB2 and look for the complex not being present, or not pulled down?

To the best of our knowledge no mutations to that effect have been described. We are not able to make a prediction for such a mutation based on the structure. For these reasons we could not follow this suggestion.

- Is there an anti-DK nanobody that could be used to increase the size of the DK density? (I am not sure if the nano body would enter the mitochondria though). Alternative, can a larger tag be added? Can tags be added to both monomers?

We thought about it carefully but to the best of our knowledge there are currently no tools available to facilitate temporary permeabilization or other micropores of the mitochondrial membranes in live cells. We therefore are not able to pursue this idea.

Rev#3 points #1-2-3-4

1. Can the authors provide additional evidence of the proposed orientation and stoichiometry of 11 PHB subunits? Does their model fit to the density observed they claim to be caused by DK (Fig. S7D-E). What would MD simulations show in this case? The authors might consider to similar to their earlier approach also try to model the DK-containing fusion proteins into the observed densities.

In order to provide additional evidence for the proposed stoichiometry of the PHB subunits, we analysed for the revised version crosslinking mass spectrometry data (new Fig. 4 and new Fig. S10A). The data demonstrate that PHB1-PHB1 as well as PHB2-PHB2 interactions exist, although PHB1-PHB2 interactions are much more abundant, fully supporting the proposed model of a prohibitin structure consisting of an 11-mer of alternating PHB1 and PHB2 molecules.

Concerning the proposed orientation in the mitochondrial inner membrane: To clarify the orientation, we added an additional panel to Fig. S7 to illustrate the location of DK fused to PHB2 (Fig S7E).

Because of the variable number of DK-molecules it is not possible to achieve a sharpened, higher-resolution cryo-EM map of the DK-PHB complexes. Consequently, we did not consider MD simulations. This is also better explained in the text of the revised version. It now reads:

"We also observed one to three additional densities at most (> 90%) convex structures identified in the tomograms of U2OS PHB1-DK and PHB2-DK cells (Fig. S7E-H). These extra densities sat at the top of the convex structure but were absent in wildtype cells, thereby indicating that they originate from the DK fluorescent protein fused to the C-termini of the PHB1 and PHB2 proteins." (Line 174 to 178)

To present this finding clearer we changed the simple subtomogram average to a transparent surface and fitted the structure of DK into the density (New panel in Fig. S7G).

2. Linked to this: Is it possible that these are actually 11 heterodimers fitting or can the authors exclude this with high certainty?

We can exclude with high certainty the existence of 11 heterodimers: Heterodimers just do not fit into the high-resolution EM-Map. To explain the conclusion on the C11 symmetry, we also added new panels to Fig. S9 that highlight the rational of the stoichiometry and the initial molecule placement in more detail (Fig. S9C).

This is now clearly stated in the revised version:

"Numerous possible arrangements were tested but we found that each of the 11 densities in the cryo-EM map can accommodate only one prohibitin molecule. Therefore, the entire complex consists of 11 prohibitin molecules in total." (Line 215 to 217)

3. The functionality of the DK fusion constructs needs to be shown. The heterotypic interaction with endogenous PHBs is not sufficient in my opinion. A phenotypic rescue compared to the PHB knockout situation would be one possibility to show the functionality of the fusion protein.

In this study, we did not draw any functional conclusions from the knock-in cell lines other than the mobility of the complexes.

We use the tagged versions only as part of a line of evidence to demonstrate that the bell-shaped structure is indeed the prohibitin complex. We believe that our line of evidence is exhaustive. We therefore argue that a more detailed functional analysis is not necessary at this stage of the work.

4. The authors have two arguments that the structure they observe is indeed PHB, namely the quantification of the PHB structures after downregulation and the additional density in the DK-containing complex (Fig. S7D-G). The latter is more convincing as the number of bell-shaped structures can result from indirect effects. Can the authors discuss whether the location of DK fits to the prediction where DK was integrated? Where is DK actually integrated exactly? Was this at the N- or C-terminus or internal? Please show a scheme where DK is relative to other PHB domains.

We agree with the reviewer that both experiments, PHB knock-down and cryo-ET imaging of PHB-DK complexes, are necessary. DK was added after a short linker to the C-terminus of PHB1 and PHB2. Therefore, the location of the extra densities in cryo-ET corresponds exactly to the expected position, taking into account the N-terminal integration of the prohibitin molecules into the lipid bilayer. In the revised manuscript, we have added the panel Fig. S7E to illustrate this rationale more clearly.

2- Please also address their questions about strengthening and clarifying existing analyses, minor points, technical comments, requests for text/figure edits or discussion.

We are convinced that we addressed all questions appropriately.

3- Finally, please pay close attention to our guidelines on statistical and methodological reporting (listed below) as failure to do so may delay the reconsideration of the revised manuscript. In particular, please provide:

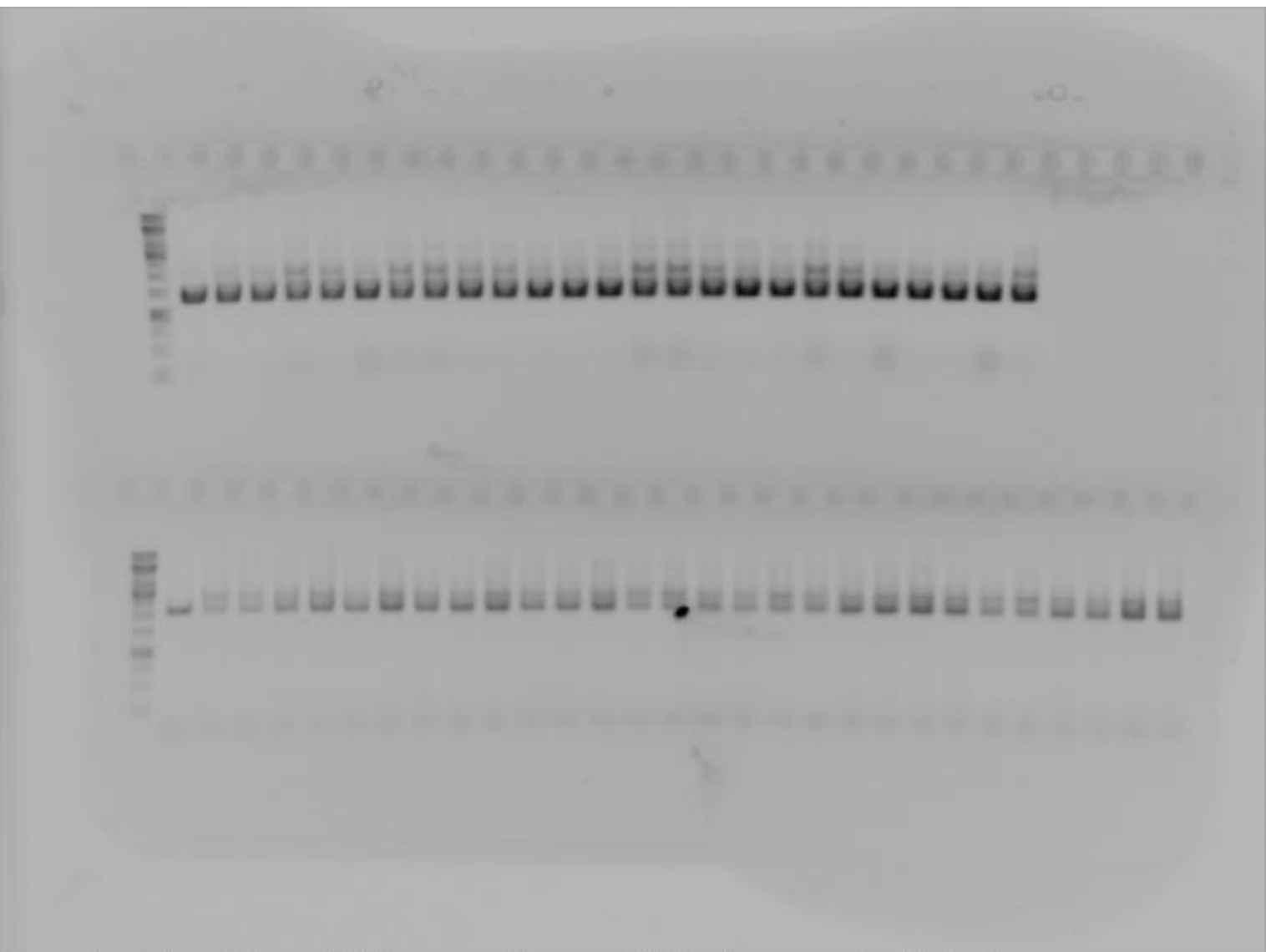
- a Supplementary Figure including unprocessed images of all gels/blots in the form of a multi-page pdf file. Please ensure that blots/gels are labeled and the sections presented in the figures are clearly indicated.

Done.

- a Supplementary Table including all numerical source data in Excel format, with data for different figures provided as different sheets within a single Excel file. The file should include source data giving rise to graphical representations and statistical descriptions in the paper and for all instances where the figures present representative experiments of multiple independent repeats, the source data of all repeats should be provided.

Done.

Figure S3, Panel A and B



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Date: 12.03.2016 Time: 20:54:54

Figure S3, Panel C and D

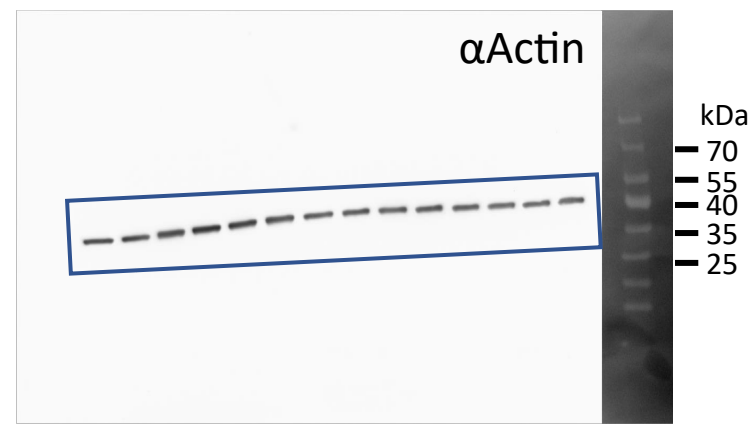
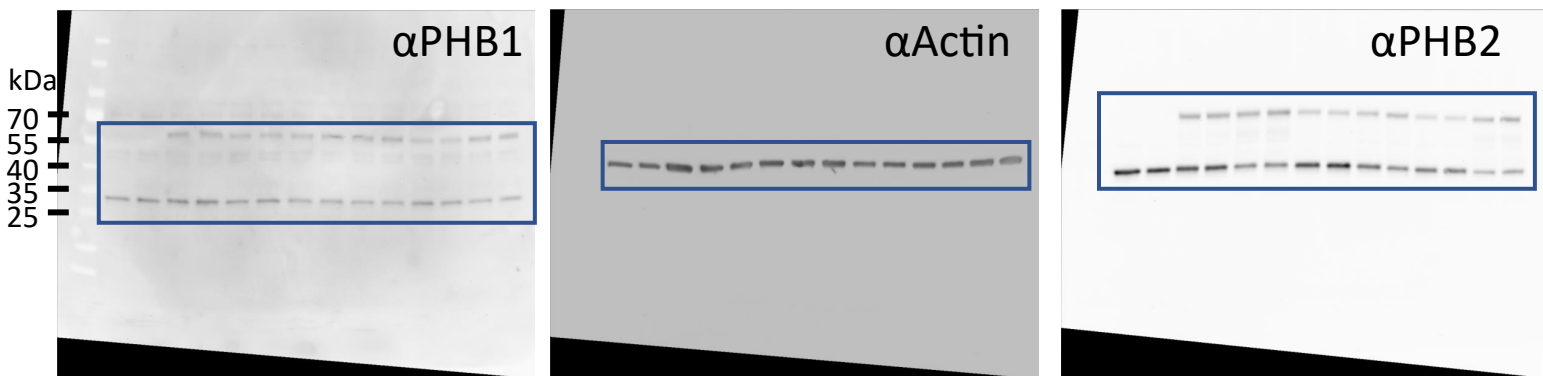
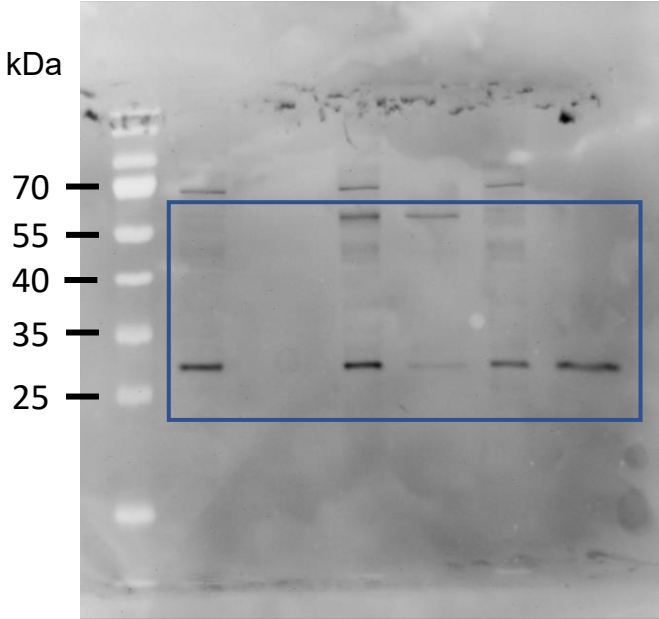


Figure S5, Panel A and B

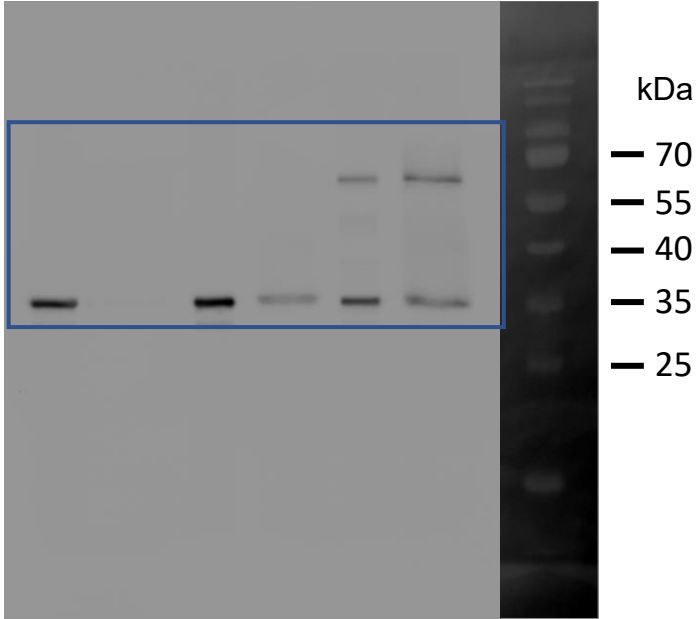
PHB1

Input & IP



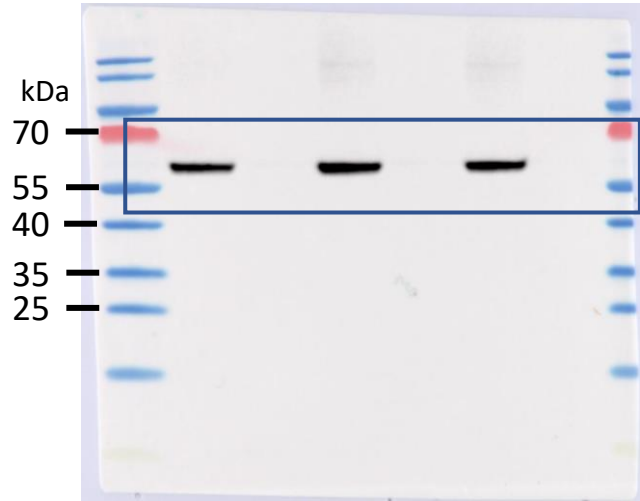
PHB2

Input & IP

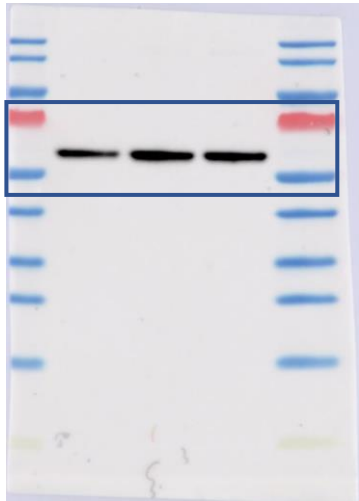


ATP5A

Input & IP

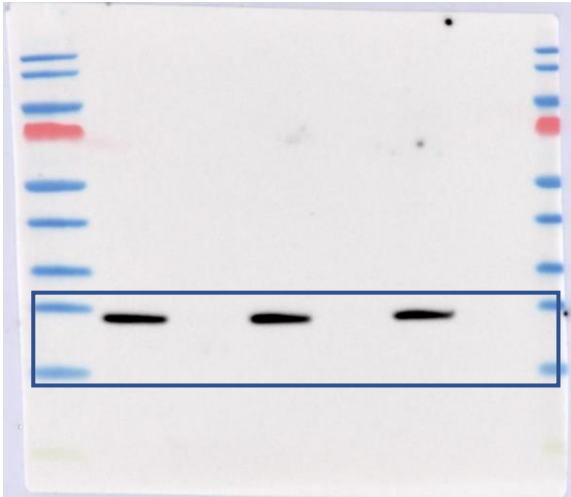


Flowthrough



COX2

Input & IP



Flowthrough

