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Corresponding author(s): Helliwell, Stephen
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☐ Initial submission ☐ Revised version ☒ Final submission

Life Sciences Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form is intended for publication with all accepted life science papers and provides structure for consistency and transparency in reporting. Every life science submission will use this form; some list items might not apply to an individual manuscript, but all fields must be completed for clarity.

For further information on the points included in this form, see Reporting Life Sciences Research. For further information on Nature Research policies, including our data availability policy, see Authors & Referees and the Editorial Policy Checklist.

Experimental design

1. Sample size

Describe how sample size was determined.

We made no pre-determinations for sample size

2. Data exclusions

Describe any data exclusions.

No data has been excluded.

3. Replication

Describe whether the experimental findings were reliably reproduced.

For non-quantitative experiments (microscopy etc) we note in figure legends the number of times experiments have been repeated with similar observations. For quantitative experiments we generally performed minimally triplicate experiments (biological replicates).

4. Randomization

Describe how samples/organisms/participants were allocated into experimental groups.

n/a

5. Blinding

Describe whether the investigators were blinded to group allocation during data collection and/or analysis.

n/a

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

6. Statistical parameters

For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

n/a	Confirmed	
☐	☒	The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
☐	☒	A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐	☒	A statement indicating how many times each experiment was replicated
☒	☐	The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)
☒	☐	A description of any assumptions or corrections, such as an adjustment for multiple comparisons
☒	☐	The test results (e.g. *P* values) given as exact values whenever possible and with confidence intervals noted
☐	☒	A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
☐	☒	Clearly defined error bars

See the web collection on statistics for biologists for further resources and guidance.

Nature Chemical Biology: doi:10.1038/nchembio.2493

Software

Policy information about availability of computer code

7. Software

Describe the software used to analyze the data in this study.	no specialised software. Mean and standard deviations in excel or tibco spotfire.

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). *Nature Methods* guidance for providing algorithms and software for publication provides further information on this topic.

Materials and reagents

Policy information about availability of materials

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.	no restrictions

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).	methods section

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.	ATCC
b. Describe the method of cell line authentication used.	HeLa snp tested
c. Report whether the cell lines were tested for mycoplasma contamination.	mycoplasma checks were carried out regularly.
d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.	n/a

Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.	n/a

Policy information about studies involving human research participants

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.	n/a

Nature Chemical Biology: doi:10.1038/nchembio.2493

Supplementary Information

Stendomycin selectively inhibits TIM23-dependent mitochondrial protein import

Ireos Filipuzzi[1,3], Janos Steffen[2,3], Mitchel Germain[1], Laetitia Goepfert[1], Michael A. Conti [2], Christoph Potting[1], Raffaele Cerino[1,5], Martin Pfeifer[1], Philipp Krastel[1], Dominic Hoepfner[1], Julie Bastien[1], Carla M. Koehler[2,4], Stephen B. Helliwell[1,4]

[1]Novartis Institutes for BioMedical Research, Basel, Switzerland
[2]Department of Chemistry and Biochemistry, University of California, Los Angeles, CA 90095, USA
[3]equal contributors
[4]corresponding authors
[5]current address: Pharma Research and early Development, Roche, Basel, Switzerland

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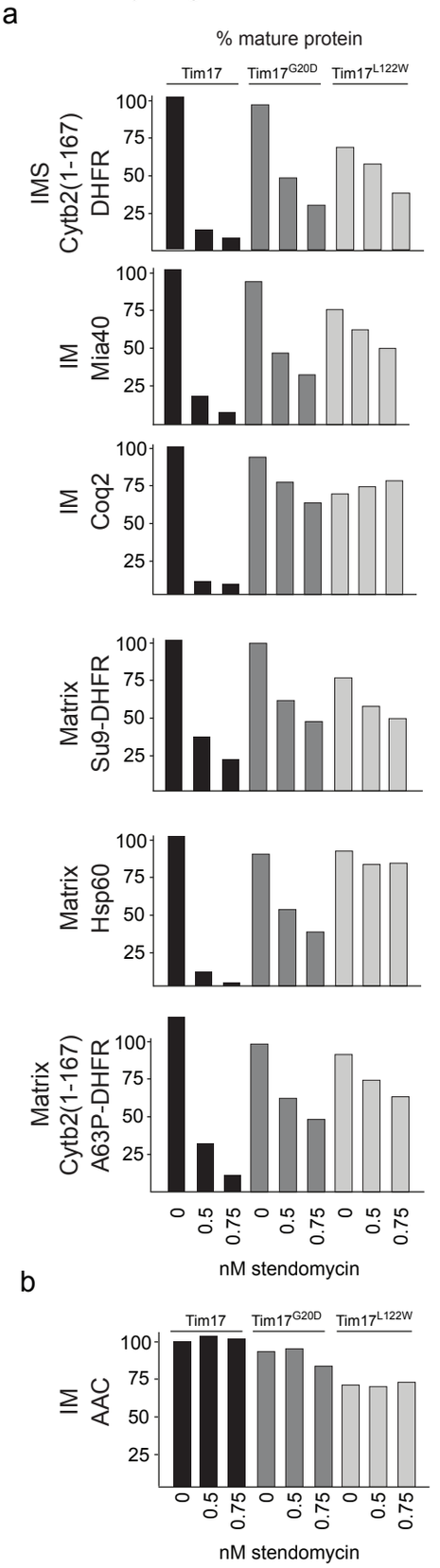
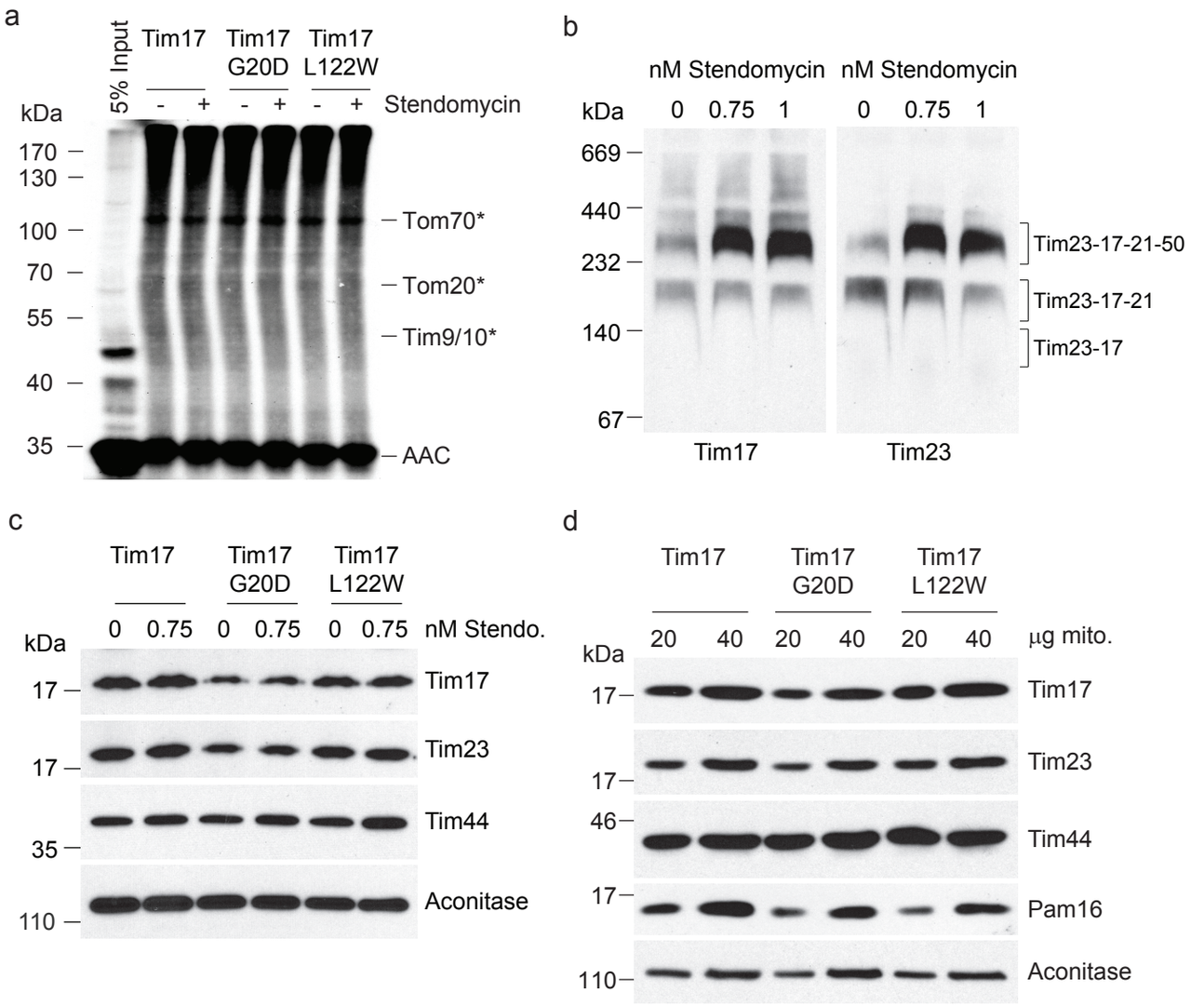
Supplementary Results

Supplementary Figure 1

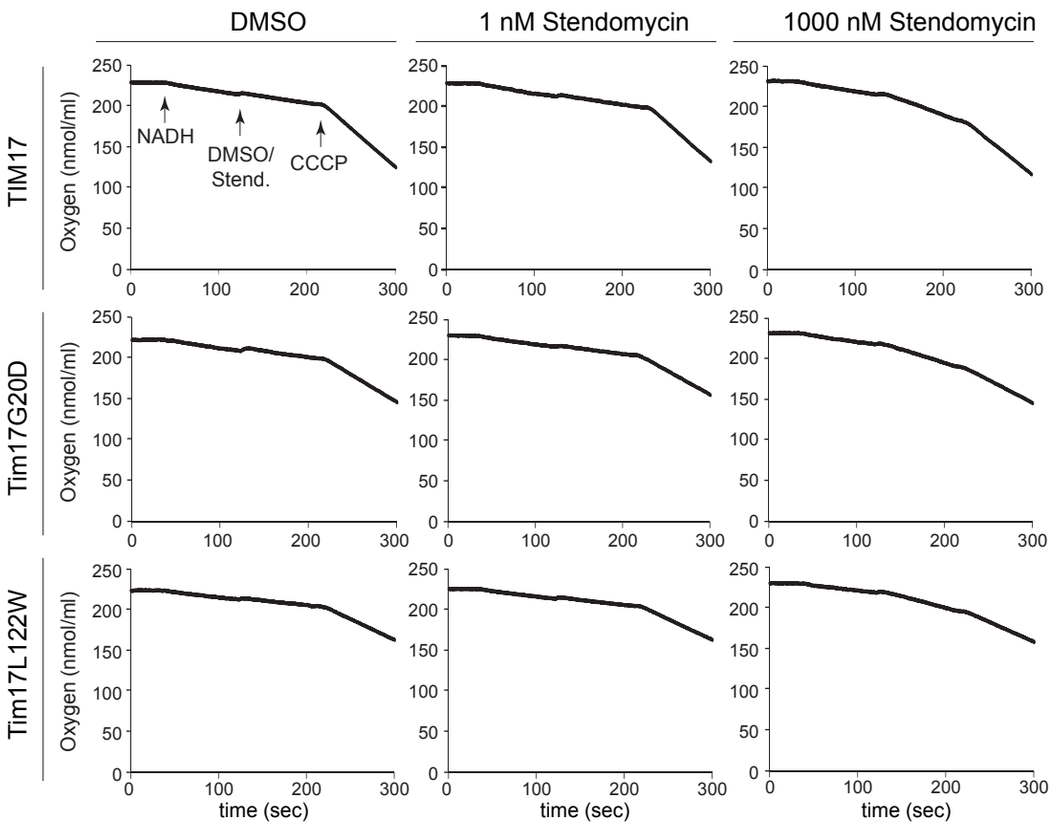


Supplementary Figure 2



Supplementary Fig. 2
Stendomycin alters the TIM23 complex (a) Radiolabeled AAC was imported into isolated yeast mitochondria of indicated strains in the absence of the membrane potential (-ΔΨ) and the presence of either 1% DMSO or 0.5 nM stendomycin. After the import, mitochondria were treated with 1 mM EGS to arrest import intermediates, followed by SDS-PAGE and autoradiography. Proteins crosslinked to AAC are indicated by an asterisk (22). (b) Mitochondria isolated from wild type yeast were treated with 0.75 or 1 nM of stendomycin for 15 min, solubilized with digitonin and separated by BN-PAGE. The TIM23 complex was analyzed by immunoblot using antibodies against Tim23 and Tim17. The locations of the different TIM23 complexes are indicated according to (13)(see also Fig. 3c) (c) Lysates as in (Fig. 3c) were separated by SDS-PAGE and analyzed by immunoblotting using antibodies against Tim17, Tim23, Tim44 and Aconitase as loading control. (d) Mitochondria isolated from wild type and Tim17 mutant yeast solubilized with digitonin and separated by SDS-PAGE. The TIM23 complex was analyzed by immunoblot using antibodies against Tim17, Tim23, Tim44 and Pam16, Aconitase was used as loading control

Supplementary Figure 3

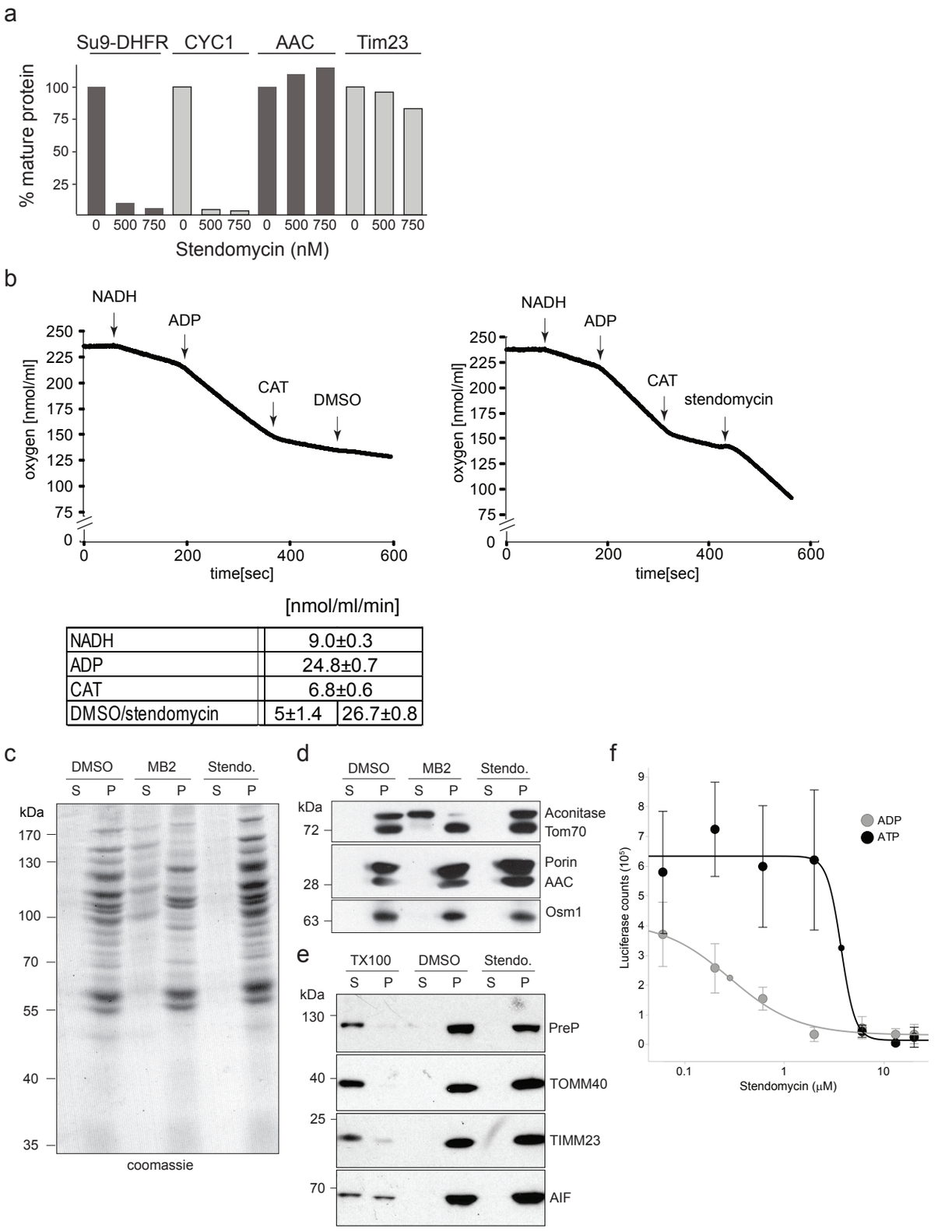


Supplementary Fig. 3
Stendomycin-resistant Tim17 mutations do not alter ΔΨat doses that block TIM23-dependent protein import Oxygen consumption of isolated mitochondria from indicated yeast strains was measured with an oxygen electrode. Respiration was initiated by the addition of NADH. 1 or 1000 nM stendomycin or 1% DMSO was added once a stable respiration had been established. Raw plots are shown, with quantification shown in Fig. 3d.

Supplementary Figure 4

Supplementary Fig. 4
Tim17 is > 45% identical to TIMM17A and TIMM17B. Tim17 was aligned pairwise to TIMM17A, TIMM17B1 and TIMM17B2 using blast (https://blast.ncbi.nlm.nih.gov/BlastAlign.cgi) and the amino acid identity scores are given. The conserved residues, mutated in yeast Tim17 to give resistance to stendomycin, are highlighted.

Supplementary Figure 5

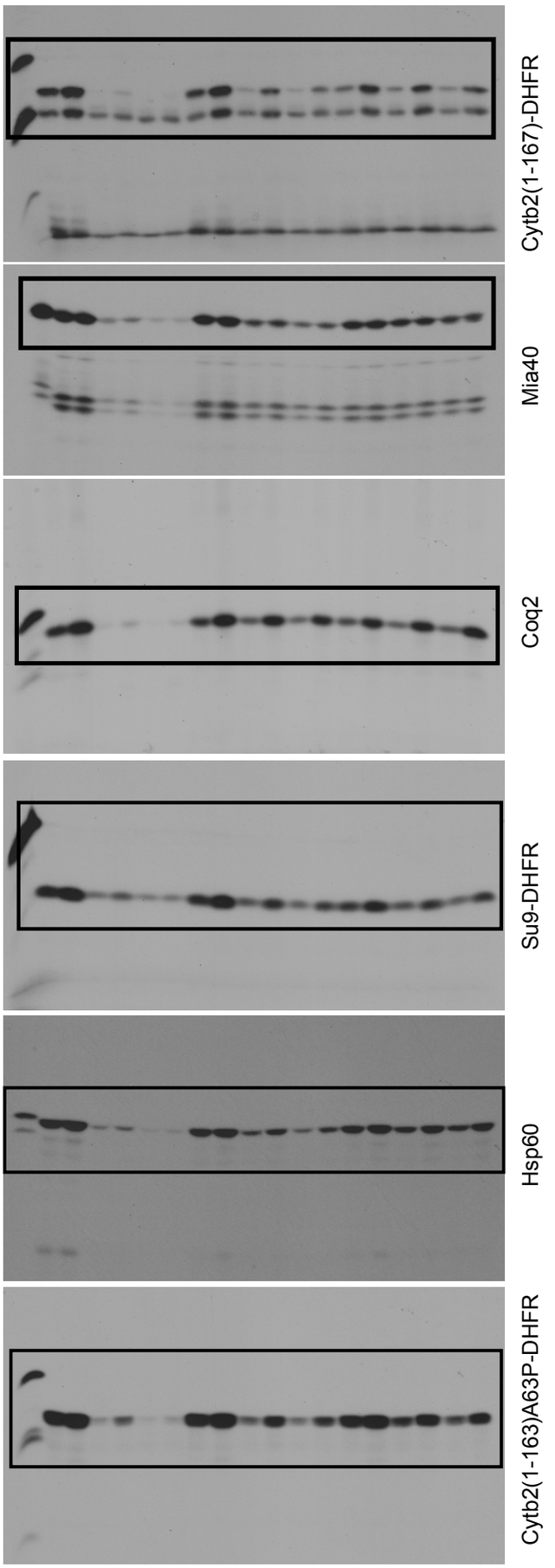


Supplementary Fig. 5

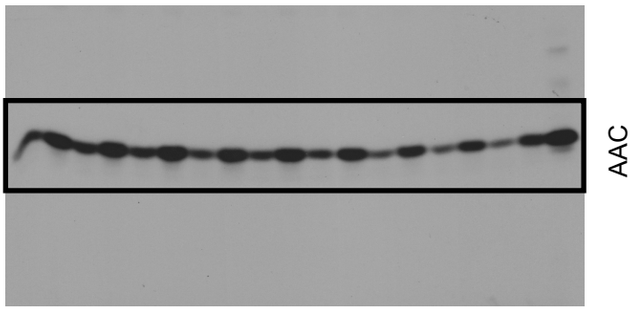
Stendomycin does not uncouple via inhibition of ANT or membrane permeabilzation (a) In vitro import assays were performed as described in Fig. 4. 10 min time points were quantified and are displayed as percentages. (b) Oxygen consumption of isolated mitochondria from wild type yeast strains was measured with an oxygen electrode. Respiration was measured following sequential addition of NADH, ADP, carboxyatractyloside and then either DMSO or stendomycin. Slope rates are given in the table. (c) Yeast mitochondria were incubated with 100 μM stendomycin or 100 μM MB2 for 30 min in import buffer and released proteins (S) were separated from mitochondria (P) by centrifugation. Proteins were visualized by Coomassie staining. (d) As in (c), except immunoblot analysis was performed to determine the fractionation for aconitase, Osm1, AAC, and Tom70. As a control, treatment with the vehicle (1% DMSO) was included. (e) Isolated HeLa cell mitochondria were treated with 0.5% Triton X-100, 1% DMSO, or 1 μM stendomycin for 30 minutes followed by separation of the supernatant (S) and pellet (P) by centrifugation. Samples were subjected to SDS-PAGE and analyzed by immunoblotting for indicated proteins. (f) HeLa cells were incubated with stendomycin doses indicated for 24h and subjected to ADP:ATP level analysis using a luciferase reagent. n = 3 biological replicates; error bars are s.d.

Supplementary note

Gels - Figure 2a

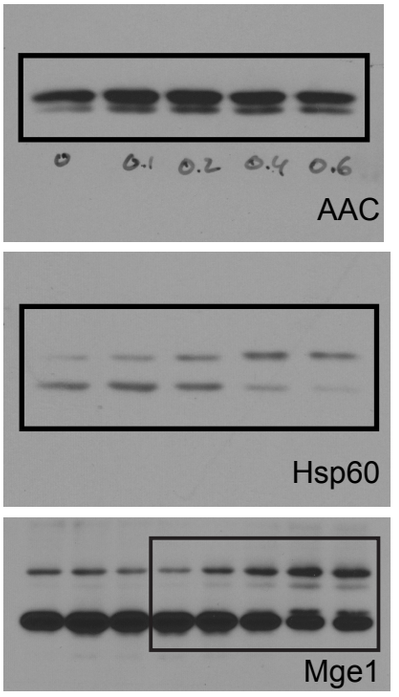


Gels - Figure 2b

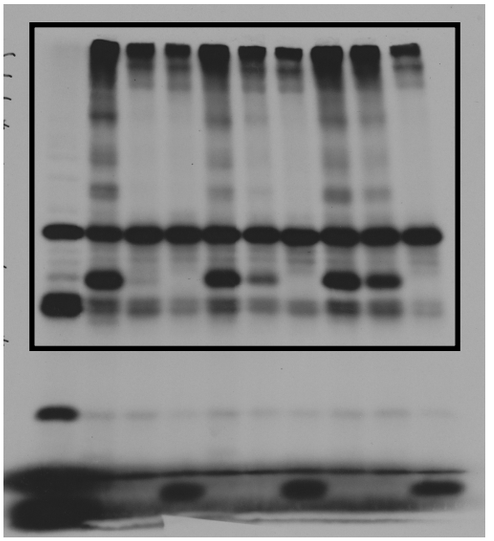


Supplementary note

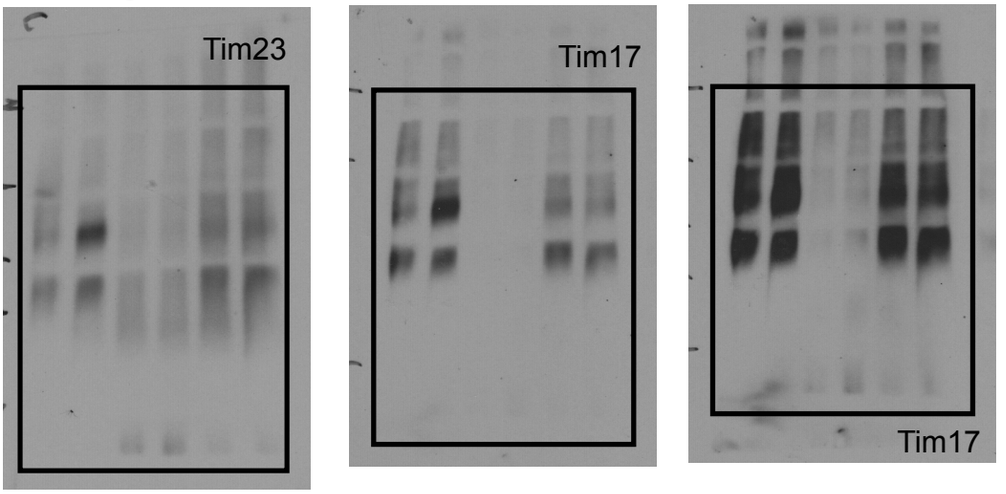
Gels - Figure 3a



Gels - Figure 3b

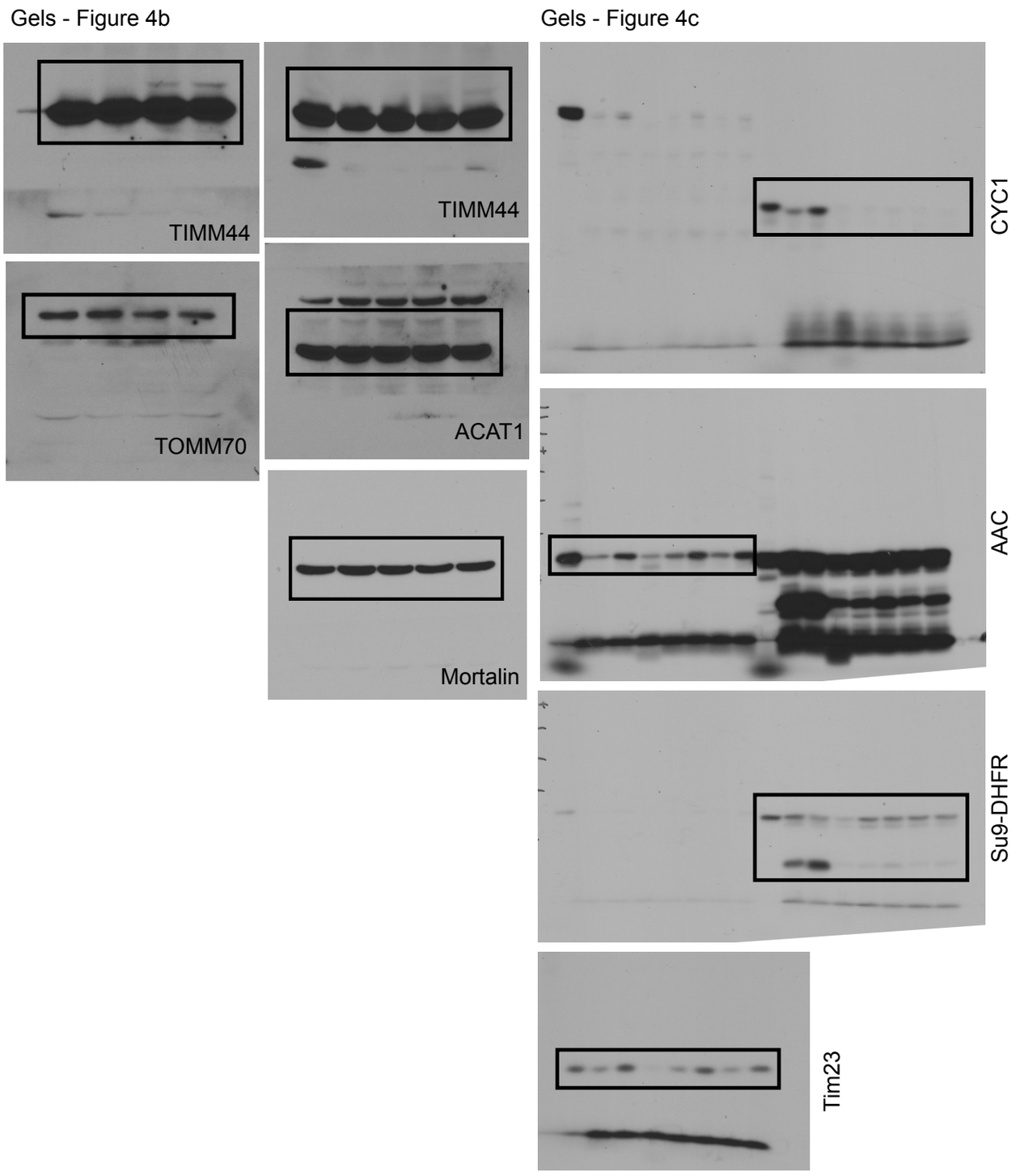


Gels - Figure 3c



Nature Chemical Biology: doi:10.1038/nchembio.2493

Supplementary note

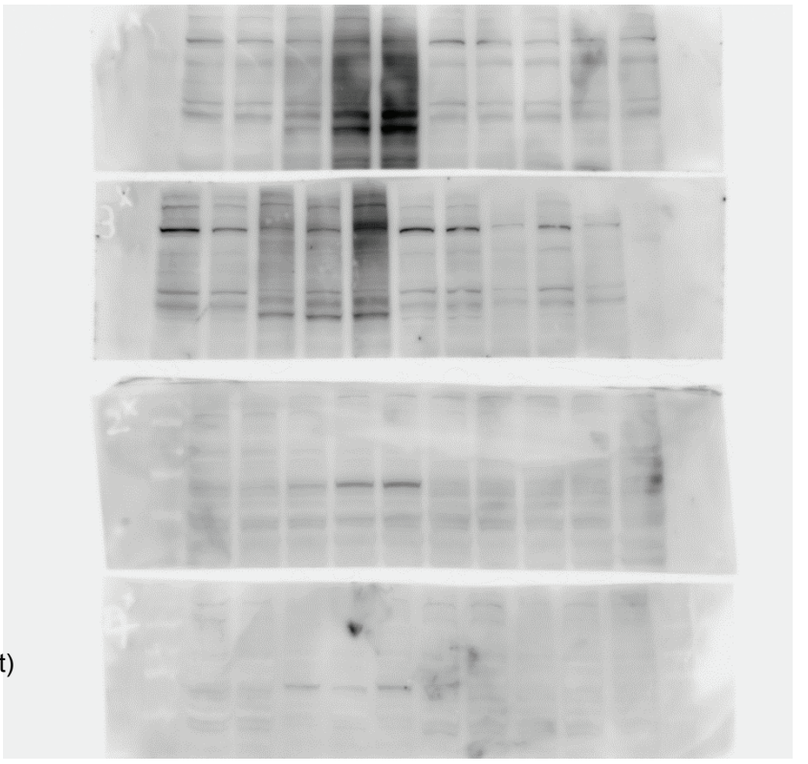


Nature Chemical Biology: doi:10.1038/nchembio.2493

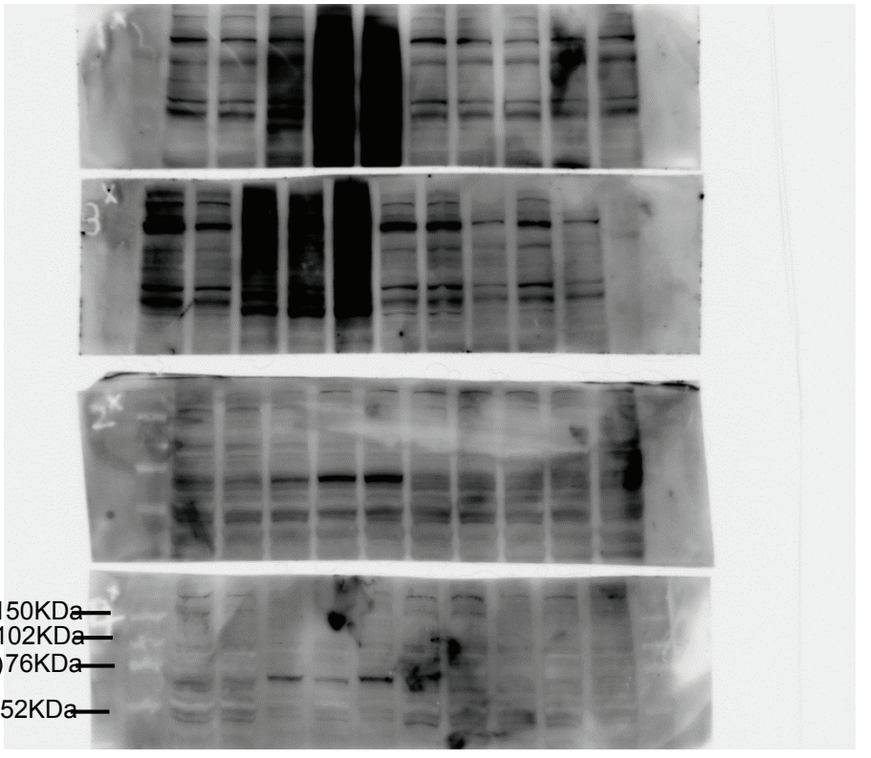
Supplementary note

Gels_Fig5a_one

ECL
Exposure time
2min



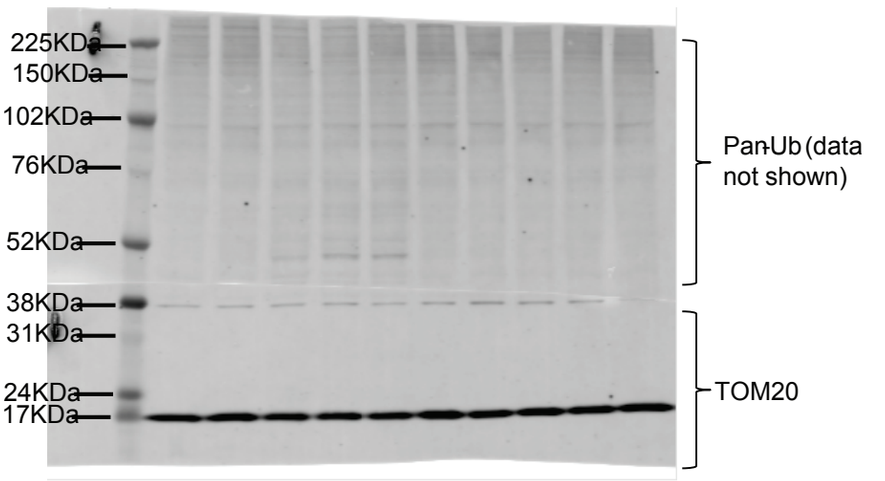
ECL
Exposure time
7min



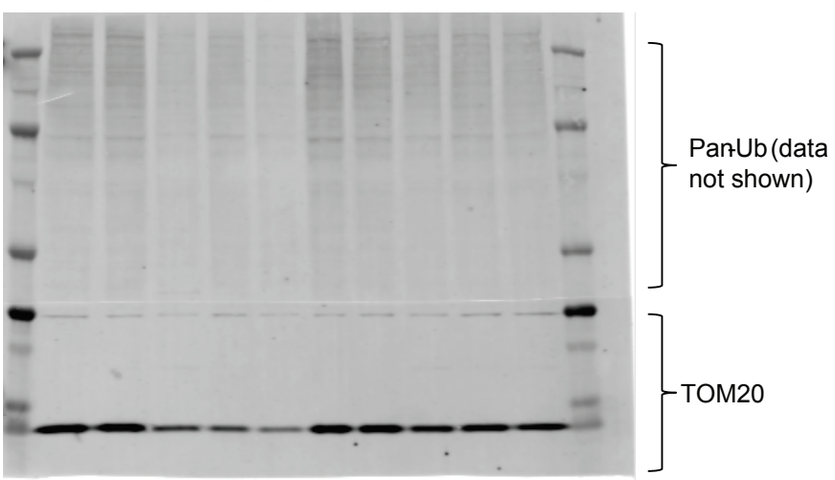
Gels_Fig5a_two

Licorodyssey Scan in 700nM channel,intensity 2

4h treatment

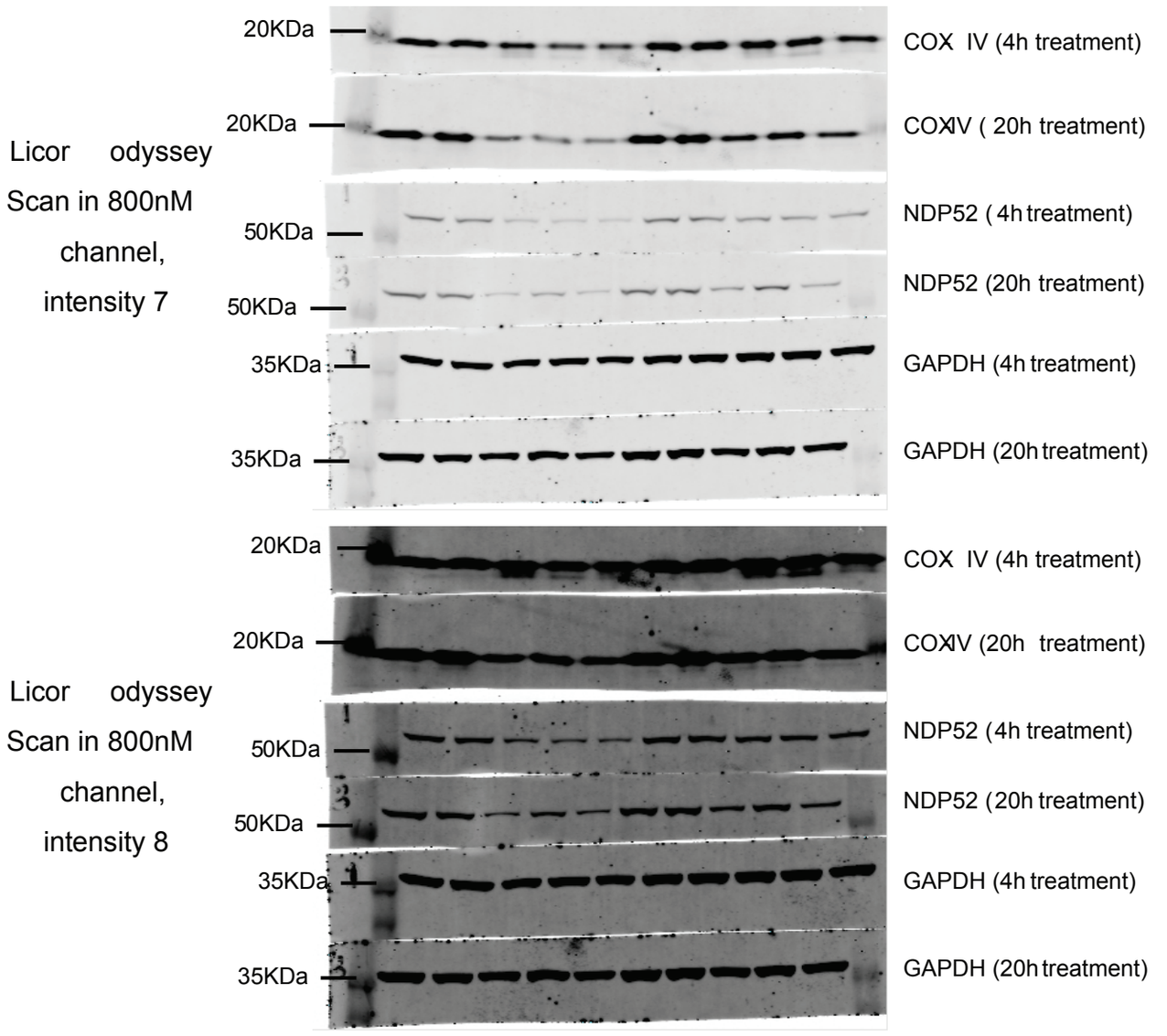


20h treatment



Supplementary note

Gels_Fig5a_three



Supplementary note

Gels_Fig5b_c_d

Figure 5b

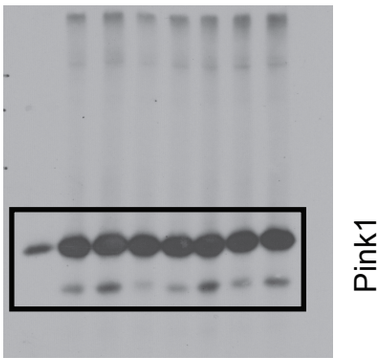


Figure 5c

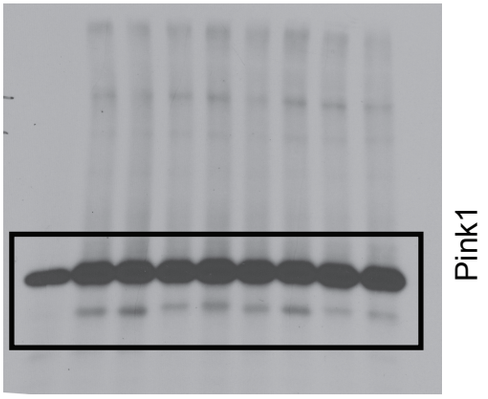
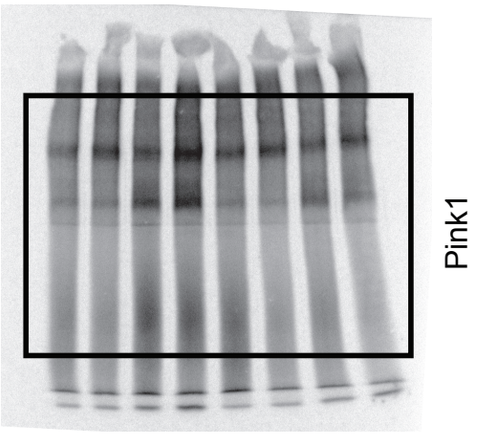
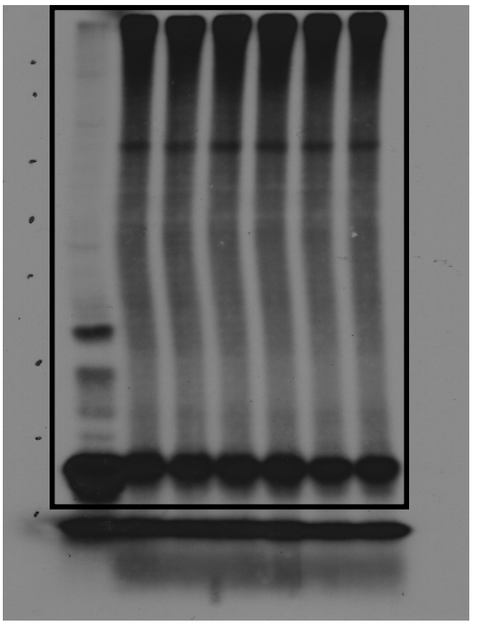


Figure 5d



Supplementary note

Suppl. Figure 2a



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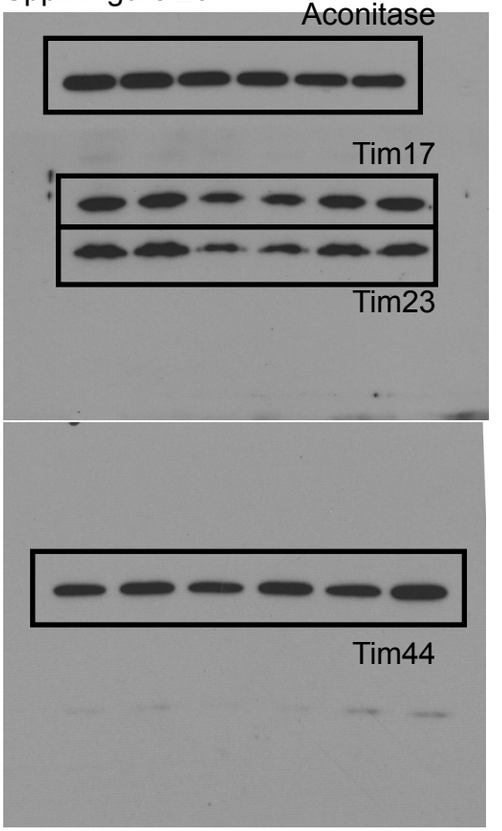

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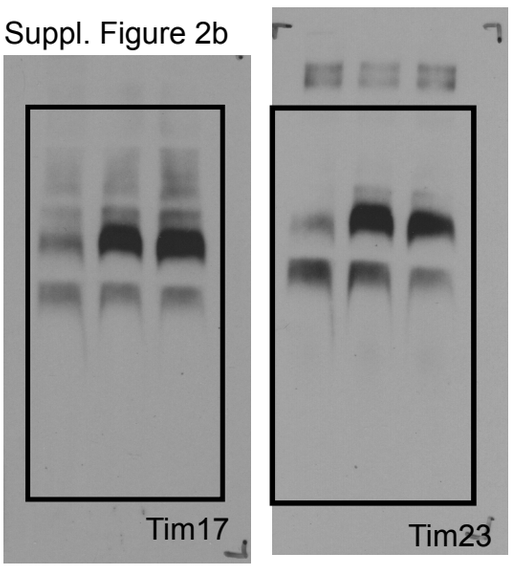

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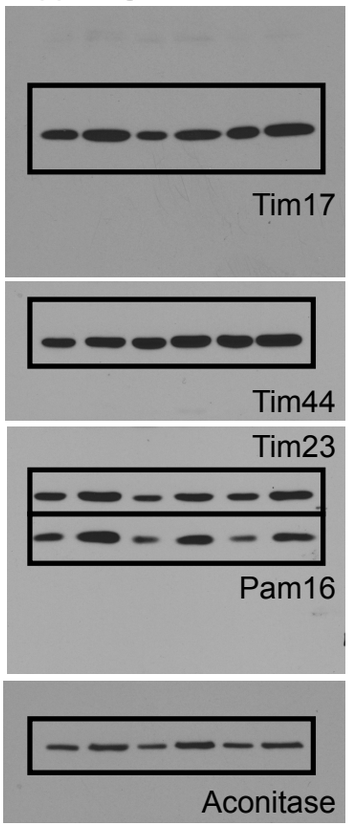

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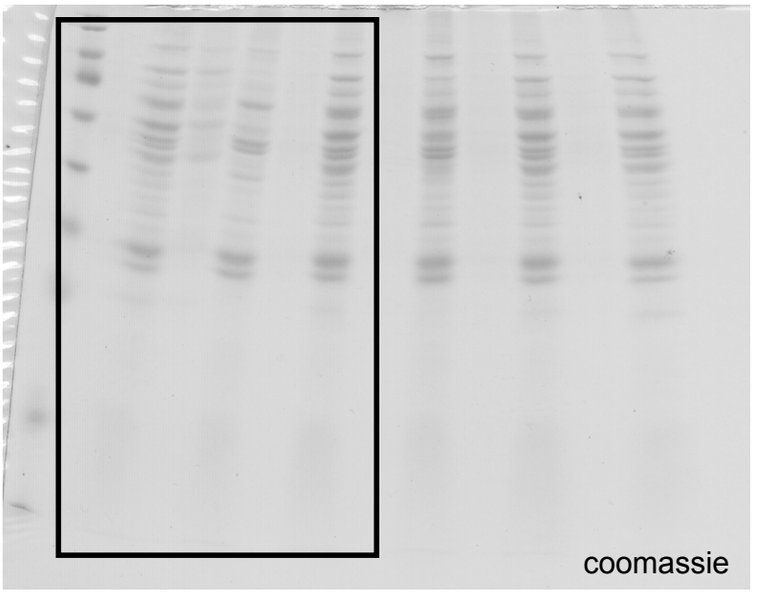
Supplementary note

Suppl. Figure 5c



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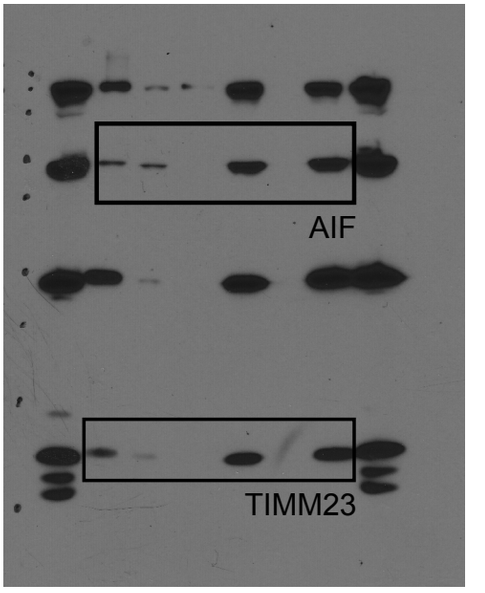

Suppl. Figure 5d