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A region-resolved proteomic map of the human brain enabled by high-throughput proteomics

Johanna Tueshaus, Amirhossein Sakhteman, Severin Lechner, Matthew The, Eike Mucha, Christoph Krisp, Jürgen Schlegel, Claire Delbridge, and Bernhard Küster
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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

Dear Bernhard,

Thank you for submitting your manuscript to The EMBO Journal. I am sorry for the delay in getting back to you with a decision. I have now received two reports on your study. I have still not received the report from a third referee who had agreed to review the manuscript. I will therefore go with the two reports on hand.

As you can see from the comments below, both referees find the analysis interesting and insightful, but also that revisions are needed. Should you be able to address the raised concerns in full then I would like to consider a revised version.

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Let me know if we need to discuss anything further

With best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study.

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Referee #1:

The present manuscript by Tüshaus et al. describes the development of an optimized proteomics approach which achieves scalability, high throughput, and high sensitivity in proteomics. Using this setup, the authors performed a proteome analysis of 13 brain regions, constructed a comprehensive human brain proteome atlas, and identified region-specific and cell type-specific proteins. Additionally, they have provided a Shiny APP, which is publicly available to facilitate access to, and deep mining of, the data. Overall, the manuscript is well-conceived and well-written. The established workflow enables high-throughput, large-scale analysis and could serve the community as a valuable tool. Several points that need addressing are listed below; I hope these comments will be helpful.

Major comments:

In Figure 3E, the fresh frozen CC samples showed a deeper (23%) unique peptide level compared to the FFPE samples, while only 7% for the protein groups. It would be helpful to provide more interpretation of this difference. Additionally, could you explain why the brain regions of CC and HPC exhibit relatively larger levels of peptides and proteins compared to other regions?
In Figure 4A, the CBM region contains the most region-enriched proteins compared to other regions. Is there any consistency evidence from the mRNA data analysis to support this observation?
In Figure 4C, why is one CCw sample closer to the CL samples in the tSNE plot? Some clarification on this observation would be valuable.
In Figure 4D, it would be necessary to denote the difference between the solid and dotted lines. Additionally, could you explain why these four proteins (RELN/GRID2/ENO3/GRID2) were selected as examples?
The findings of region-specific and cell type-specific proteins are potentially significant. I wonder if the protein-protein interaction (PPI) network between proteins exclusive to a specific brain region is more densely connected compared to proteins with moderate levels across all brain regions.
In Figure 5, could you perform GO enrichment analysis to compare the region-enriched genes specifically expressed in astrocytes, microglia, oligodendrocytes, and neurons separately? It would be interesting to see if the identified GO terms are relevant to the specific functions of the related brain regions.
It would be helpful if you could provide statistical evidence to compare the optimized set-up with existing workflows in terms of sensitivity, efficiency, and proteomic depth.
How about the detection sensitivity of modified proteins, such as phosphorylated sites? If possible, please provide relative results. Additionally, it would be informative to show the major protein types detected using this method, including receptors, DNA binding proteins, neuropeptides, enzymes, secreted molecules, or others.
Minor comments:

To define the proteomics of distinct cell types, it could be beneficial to combine it with flow cytometry to sort specific subtypes using antibodies before sampling and protein extraction.
"In contrast, many more modifications that can be rationalized by the use of formaldehyde in the fixation process (Metz et al., 2004) were indeed found in the fixed tissue (Fig. 4D)." Here, the citation of Fig. 4D appears to be incorrect.
"Among the top 10 most enriched synaptic proteins in the cerebral cortex are SHISA6 and SHISA7, which control synaptic transmission in excitatory neurons, as well as SYNPO and LRRC7 involved in spine architecture (Fig. 5G)." Here, the citation of Fig. 5G appears to be incorrect. It should be Fig. 5F.
How can CSF from the lateral, third, or fourth ventricle be effectively collected in the postmortem human brain?

Referee #3:

In their manuscript entitled „ A region-resolved proteomic map of the human brain enabled by high- throughput proteomics" Tüshaus et al present the development of a microflow-based high-throughput quantitative proteomics workflow on the timsTOF HT system. The authors conclusively validate the performance of the workflow, capable of identifying >10.000 proteins in the presented study using offline high-pH fractionation, notably with very low variability and long-term stability.
The manuscript is very well written and results are clearly presented. Figure quality is excellent and the shiny app developed by

the group enables researches to easily mine the plethora of data acquired in the study.
Materials and methods are very well described, and all rawdata have been deposited on ProteomeXchange, enabling other researchers to reproduce the findings and make optimal use of the data.
The data presented is of exceptional value for the community.

Minor comments:

a)Please use a colorblind-friendly scheme in Figure 1.

I highly recommend publication of this excellent manuscript.

Point to point response to reviewer comments

The authors thank the reviewers for their careful evaluation of our work. In light of the revisions, the manuscript has become stronger and is hopefully now fit for publication.

Referee #1:

The present manuscript by Tüshaus et al. describes the development of an optimized proteomics approach which achieves scalability, high throughput, and high sensitivity in proteomics. Using this setup, the authors performed a proteome analysis of 13 brain regions, constructed a comprehensive human brain proteome atlas, and identified region-specific and cell type-specific proteins. Additionally, they have provided a Shiny APP, which is publicly available to facilitate access to, and deep mining of, the data. Overall, the manuscript is well-conceived and well-written. The established workflow enables high-throughput, large-scale analysis and could serve the community as a valuable tool. Several points that need addressing are listed below; I hope these comments will be helpful.

Major comments:

1. In Figure 3E, the fresh frozen CC samples showed a deeper (23%) unique peptide level compared to the FFPE samples, while only 7% for the protein groups. It would be helpful to provide more interpretation of this difference.

   Such a percentage difference between the peptide and protein level is observed in almost any mass spectrometry-based proteomic data set and is of technical nature. It stems from the fact that identified proteins are sometimes covered by many peptides (high abundance proteins), sometimes by just few (low abundance proteins or those having few tryptic cleavage sites). Hence, when analyzing a proteome deeper and deeper, it is more likely to identify more peptides of already identified (abundance) proteins before one identifies any peptide of a new low abundance protein.

2. Additionally, could you explain why the brain regions of CC and HPC exhibit relatively larger levels of peptides and proteins compared to other regions?

   We investigated this point in some detail and found that the composition of proteins that make up the bulk of the CC and HPC proteomes are different to the average (see figures below). More specifically, the CC and HPC data contains 20% (4.5%) more peptides (proteins) compared to the average of all other brain regions. Figure 1A shows that the overall dynamic protein expression range is comparable between all brain regions (about 6 orders of magnitude). However, the expression levels of the most abundant proteins (approximated by the measured protein intensity) is more substantially different between CC/HPC and the other regions (Figure 1B). For example, the top 100 most highly expressed proteins in CC or HPC account for 36% of the total protein mass in these regions. For the other regions, the average is 41%. The interpretation of this difference is that the few high abundant proteins detected in the other brain region consume substantially more of the available (fixed) MS data acquisition capacity, which, in turn, hampers the detection of lower abundance peptides (and hence proteins). An extreme case of this phenomenon is human plasma in which about 10 proteins account for >90% of the total

protein mass. Therefore, the MS analysis of neat plasma usually only leads to the identification of a few hundred proteins.

Figure 1: A) Rank plot of proteins quantified in the different brain regions sorted by iBAQ intensity. B) Cumulative iBAQ protein intensity of the proteins quantified in the cortex (CC), hippocampus (HPC) and the average of all brain regions. Proteins are ranked according to their abundance with rank "1" being the most abundant protein in that region. The inset table shows the number of proteins contributing to the intensity quantiles (Q1 being the most abundant proteins) and how much of the total protein intensity are contributed by the top "n" most abundant proteins of cortex (CC), hippocampus (HPC) and the average of all brain regions.

3. In Figure 4A, the CBM region contains the most region-enriched proteins compared to other regions. Is there any consistency evidence from the mRNA data analysis to support this observation?

   Yes, previous mRNA level studies comparing different human brain regions have also identified CBM to contain high levels of region-enriched candidates. The human protein atlas project, for example, discovered the cerebellum to contain the second most regionally enriched genes after the choroid plexus (https://www.proteinatlas.org/humanproteome/brain/human+brain). The choroid plexus was not part of our proteomic analysis.

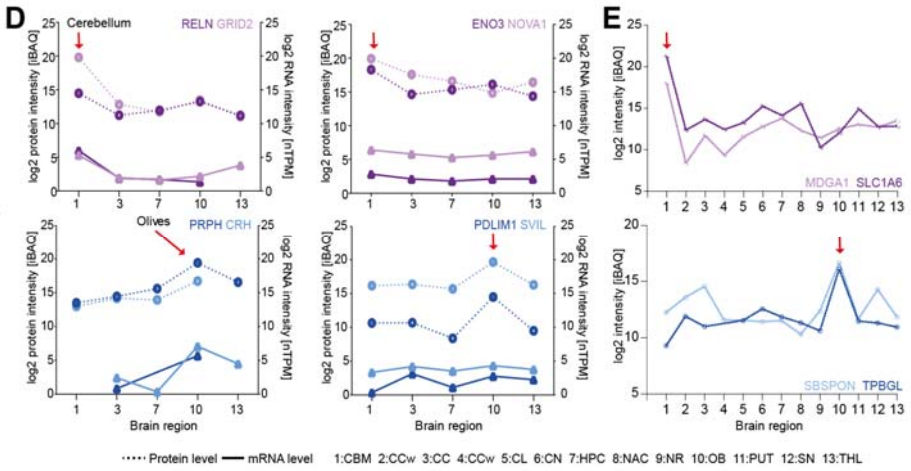
4. In Figure 4C, why is one CCw sample closer to the CL samples in the tSNE plot? Some clarification on this observation would be valuable.

   We do not have a clear answer to this question and can only speculate at this point. For each brain region, three biological replicates (different samples from the same brain region) were analyzed. It is possible that one of the CCw replicates happened to be closer to the anatomically close brain region CL. The proximity of CL and CCw in the tSNE plot indicates this somewhat close relationship of the two brain regions compared to other brain regions. We are confident that the observation is not rooted in a technical bias because all samples were analyzed in a block randomized fashion, i.e. the first biological replicate of all 13 brain regions was analyzed in random order followed by the second and third biological replicates.

5. In Figure 4D, it would be necessary to denote the difference between the solid and dotted lines. Additionally, could you explain why these four proteins (RELN/GRID2/ENO3/GRID2) were selected as examples?

   To clarify, the solid lines indicate mRNA expression results published previously by Sjöstedt *et al.*, 2020 and the dotted line indicates the protein expression results analyzed in the current proteomic study. We have added an explanation to Figure 4D.

   RELN/GRID2/ENO3/NOVA1 were chosen as representative examples of proteins enriched in cerebellum in our proteomic dataset. While the enrichment of RELN and GRID2 in cerebellum was previously detected on mRNA level by Sjöstedt et al., we also detected proteins such as ENO3 and NOVA1 which reveal the enrichment in cerebellum on protein level but not on mRNA level (see figure below).



6. The findings of region-specific and cell type-specific proteins are potentially significant. I wonder if the protein-protein interaction (PPI) network between proteins exclusive to a specific brain region is more densely connected compared to proteins with moderate levels across all brain regions.

   To address this comment, we analyzed our data using STRING (functional protein association network, string-db.org). We first chose 2,000 proteins with moderate expression levels that were identified in all brain regions and showed the least expression differences between brain regions. We then compared their connectivity to that of class 1 enriched proteins of the different brain regions. Proteins with moderate expression levels across brain regions had an average node degree of 15.9, an avg local clustering coefficient of 0.317 and significantly more interactions than expected. Interestingly, none of the class1 enriched proteins of any brain region showed a higher average node degree or average local clustering. It is currently unclear what this observed difference means. Either, class 1 enriched proteins are actually less densely connected than other proteins, or they are simply understudied so that there is less interaction information in STRING.

	Number	Avg. node degree	Avg. local clustering
Moderate	2,000	15.9	0.317
CBM – Class1	254	3.29	0.377
CBw – Class1	14	0.143	0.143
CC – Class1	75	0.427	0.196
CCw – Class1	29	0.0714	0.0714
CL – Class1	12	NaN	NaN
CN – Class1	26	0.0769	0.0769
HPC – Class1	32	0.0645	0.0645
NAC – Class1	18	NaN	NaN
NR – Class1	9	NaN	NaN
OB – Class1	54	0.481	0.253
PUT – Class1	8	NaN	NaN
SN – Class1	27	0.0769	0.0769
THL – Class1	35	0.182	0.121

7. In Figure 5, could you perform GO enrichment analysis to compare the region-enriched genes specifically expressed in astrocytes, microglia, oligodendrocytes, and neurons separately? It would be interesting to see if the identified GO terms are relevant to the specific functions of the related brain regions.

   We have done as requested. Cell type specific functions such as “synapse” and “plasticity” were enriched in neuron enriched proteins or phagocytosis for glial proteins. However, these cell type specific functions were very predominant for multiple brain regions and the additional step of linking these GO terms to brain region specific functions was not possible at this point.

8. It would be helpful if you could provide statistical evidence to compare the optimized set-up with existing workflows in terms of sensitivity, efficiency, and proteomic depth.

   Unfortunately, this is not possible. First, there is no generally accepted standard workflow for any proteomic analysis. This is because a plethora of factors influence the performance of any particular workflow. This starts with the input material used (fresh frozen vs formalin-fixed), carries through the many options for sample preparation, column materials for liquid chromatography as well as the mass spectrometry technique applied. Hence, our workflow was empirically optimized within the possibilities offered by our laboratory. Second, to the best of our knowledge, the current work is the first systematic proteomic analysis of formalin-fixed healthy human brain tissue using LC-MS/MS technology with deep proteome coverage. In our study, we identified, on average, 9,500 proteins per brain region (11,325 total) from 400 µg formalin-fixed brain material by separating peptides into 48 fractions and using 30 min LC-MS/MS each (total of one day of measurement time). For comparison, previously published data of fresh frozen brain material reached comparable proteomic coverage. For example, Wang et al. 2019 identified close to 11,000 proteins in brain using 300 µg of fresh frozen brain material, 36 hSAX fractions requiring three days of measurement time. Carlyle et al. used 200 µg of pooled brain and analyzed 15 peptide fractions using a 180 min gradient each, totaling about two days of measurement time. On average, they detected 7,945 proteins per brain region. In light of the prior literature, it is probably fair to say that our workflow enables similarly deep proteome coverage but is more time-efficient.

9. How about the detection sensitivity of modified proteins, such as phosphorylated sites? If possible, please provide relative results. Additionally, it would be informative to show the major protein types detected using this method, including receptors, DNA-binding proteins, neuropeptides, enzymes, secreted molecules, or others.

   Most PTMs require enrichment prior to analysis because they are often of low stoichiometry. Hence, the coverage of modified peptides in our study is generally low. To address the comment directly, we compared the results of fresh frozen to formalin-fixed cortex by performing an open search using FragPipe. This revealed a reduced number of unmodified PSMs in FFPE material and an increased percentage of modifications such as methylation which can be attributed to the fixation process (main manuscript Figure 3D). Phosphorylation showed up as the top 20 most frequent modification. Specifically, 0.51% of all detected PSMs of the fresh frozen material were annotated to be phosphorylated while only 0.26% of PSMs of the formalin-fixed material were phosphorylated. We cannot ascertain if this difference is due to differences in ischemia

time or the result of the fixation process which is why we do not want to read too much into this data. Still, we have added an extended list of PSM modifications to Expanded View Figure 3.

To address the comment on detected protein classes, we compared the fraction of major protein classes annotated in the canonical human fasta (UniProt keywords) to the proteins identified in this study. Across the board, one would expect about 56% coverage (11,325 proteins identified here vs 20,376 sequences in the fasta). As observed in many studies before, not all cellular localizations or protein classes were equally well covered (see figure below). There is a clear over-representation of cytoplasmic and mitochondrial proteins and an equally clear under-representation of secreted proteins. Similarly, kinases are over-represented (most reside in the cytosol) and a strong under-representation of GPCRs and (secreted) neurotransmitters was observed. We added the figure below to Expanded View Figure 3.

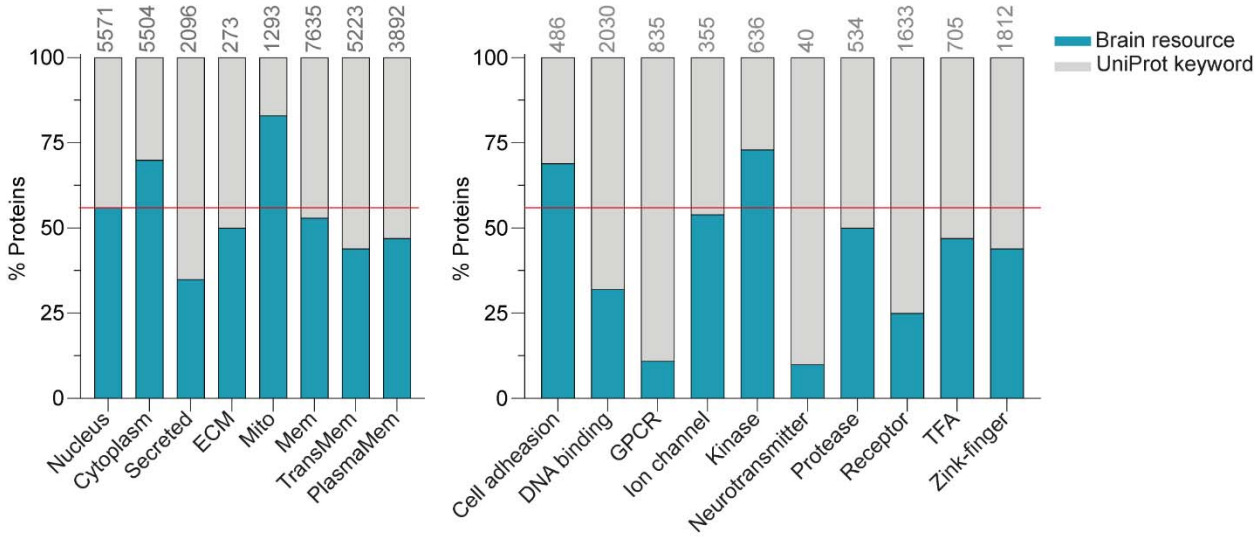


Figure 2: Proteins annotated by class according to UniProt keywords in the canonical human proteome (grey) and the proteins included in our brain region resource (blue). The expected coverage is indicated with the red line. The absolute number of UniProt entries is indicated above the bar chart.

Minor comments:

1. To define the proteomics of distinct cell types, it could be beneficial to combine it with flow cytometry to sort specific subtypes using antibodies before sampling and protein extraction.

   We acknowledge the suggestion and are working on this in the context of a follow-up project to the current story. Defining the proteomes of distinct cell types is a laborious and time-consuming task, especially when working with FFPE material. The combination of laser-capture microdissection with proteomics can be a powerful approach here. These studies are not far advanced. Therefore, we think this is beyond the scope of the current work.

2. "In contrast, many more modifications that can be rationalized by the use of formaldehyde in the fixation process (Metz et al., 2004) were indeed found in the fixed tissue (Fig. 4D)." Here, the citation of Fig. 4D appears to be incorrect.

Thank you for spotting this mistake. It has been corrected.

3. "Among the top 10 most enriched synaptic proteins in the cerebral cortex are SHISA6 and SHISA7, which control synaptic transmission in excitatory neurons, as well as SYNPO and LRRC7 involved in spine architecture (Fig. 5G)." Here, the citation of Fig. 5G appears to be incorrect. It should be Fig. 5F.

   Also corrected.

4. How can CSF from the lateral, third, or fourth ventricle be effectively collected in the postmortem human brain?

   Unfortunately, we are not sure about what this question relates to. To the best of our knowledge, proteome analysis of postmortem CSF samples is not possible due to blood contaminations present in these CSF samples independent of the way the CSF was collected. The CSF used in the technical part of this manuscript was collected conventionally from human donors.

Referee #3:

In their manuscript entitled „ A region-resolved proteomic map of the human brain enabled by high-throughput proteomics" Tüshaus et al present the development of a microflow-based high-throughput quantitative proteomics workflow on the timsTOF HT system. The authors conclusively validate the performance of the workflow, capable of identifying >10.000 proteins in the presented study using offline high-pH fractionation, notably with very low variability and long-term stability.
The manuscript is very well written and results are clearly presented. Figure quality is excellent and the shiny app developed by the group enables researches to easily mine the plethora of data acquired in the study.
Materials and methods are very well described, and all rawdata have been deposited on ProteomeXchange, enabling other researchers to reproduce the findings and make optimal use of the data.
The data presented is of exceptional value for the community.

Minor comments:

1. Please use a colorblind-friendly scheme in Figure 1.

Thank you for the comment – we changed to colors in Figure 1 accordingly.

I highly recommend publication of this excellent manuscript.

Dear Bernhard,

Thank you for submitting your revised manuscript to The EMBO Journal. Referee #1 was not available to review the revised version and I asked referee #3 to look at the introduced changes. I have now heard back from referee #3 and as you can see the referee finds that you have response to the raised concerns in a good way. I am therefore very pleased to let you know that we will accept the manuscript for publication here.

Before sending you, the formal acceptance letter there are just a few editorial points to sort out.

- For resources we require a structured Material and Methods section. See also guidelines for authors.

- We can only have 5 keywords. You have now 7.

- COI title needs renaming to "DISCLOSURE AND COMPETING INTERESTS STATEMENT"

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- Supplementary Table 1 should be renamed to Dataset EV1 with the corresponding callout

- Appendix figures should be compiled in one Appendix PDF, with ToC with page numbers. The nomenclature should be Appendix Figure S1-S13 (not Figure S1.1, S1.2, S2.1, S2.2...), with the appropriate callouts, and the legends should be removed from MS file and uploaded below each figure respectively.

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- Please make sure that the deposited datasets are open and available.

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When you submit the revised version, please also submit a point-by-point response to the editorial points.

Congratulations on a super nice MS - I hope that this a good reason for a nice celebration in the lab!

With best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
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Guide For Authors: https://www.embopress.org/page/journal/14602075/authorguide

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Referee #3:

I have read through the comments of reviewer 1 and the responses of the authors.
In my opinion, the authors have addressed the concerns and comments of reviewer 1 thoroughly.

I fully recommend this manuscript for publication.

Point-by-point response to the editorial points

1. For resources we require a structured Material and Methods section. See also guidelines for authors.
   Done.

2. We can only have 5 keywords. You have now 7.
   Done.
   Keyword: High-throughput proteomics, Brain proteome atlas, Brain regions, micro-flow LC, timsTOF

3. COI title needs renaming to "DISCLOSURE AND COMPETING INTERESTS STATEMENT"
   Done.

4. Please remove the Authors Contributions from the manuscript. The 'Author Contributions' section is replaced by the CRediT contributor roles taxonomy to specify the contributions of each author in the journal submission system. Please use the free text box in the 'author information' section of the manuscript submisssion system to provide more detailed descriptions (e.g., 'X provided intracellular Ca++ measurements in fig Y')
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   Uploaded

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   Uploaded

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    Done.

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Dear Bernhard,

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Congratulation on a super nice paper!!

with best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
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The data shown in figures should satisfy the following conditions:
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- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data

2. Captions
Each figure caption should contain the following information, for each panel where they are relevant:
- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ^2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

Materials

Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Not Applicable	

Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Not Applicable	

DNA and RNA sequences	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Short novel DNA or RNA including primers, probes: provide the sequences.	Not Applicable	

Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Not Applicable	
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Not Applicable	
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Not Applicable	

Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Laboratory animals or Model organisms: Provide species, strain, sex, age, genetic modification status. Provide accession number in repository **OR** supplier name, catalog number, clone number, **OR** RRID.	Not Applicable	
Animal observed in or captured from the field: Provide species, sex, and age where possible**.**	Not Applicable	
Please detail **housing and husbandry conditions**.	Not Applicable	

Plants and microbes	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Plants: provide species and strain, ecotype and cultivar where relevant, unique accession number if available, and source (including location for collected wild specimens).	Not Applicable	
Microbes: provide species and strain, unique accession number if available, and source.	Not Applicable	

Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Yes	Material and Methods

Core facilities	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Not Applicable	

Design

Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Not Applicable	
Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

Laboratory protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Not Applicable	

Experimental study design and statistics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about **sample size** estimate even if no statistical methods were used.	Yes	Figures
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Yes	Material and Methods - order of measuring the samples using LC-MSMS
Include a statement about **blinding** even if no blinding was done.	Not Applicable	
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Material and Methods

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	Figures
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	Figures, Material and Methods, Main text

Ethics

Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	
Studies involving **human participants**: Include a statement confirming that **informed consent** was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Not Applicable	
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approva**l (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Not Applicable	
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Dual Use Research of Concern (DURC)	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm	Not Applicable	
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
If a study is subject to dual use research of concern regulations, is the name of the **authority granting approval and reference number** for the regulatory approval provided in the manuscript?	Not Applicable	

Reporting

The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

Adherence to community standards	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Not Applicable	
For **tumor marker prognostic studies,** we recommend that you follow the **REMARK** reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	Not Applicable	
For **phase II and III randomized controlled trials**, please refer to the **CONSORT** flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	Not Applicable	

Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Yes	Material and Methods
Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective **data citations in the reference list**.	Yes	Figures, Main Text, Material and Methods