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Protocol for differential analysis of Trim71-associated protein complexes in mouse embryonic stem cells by mass spectrometry using Perseus

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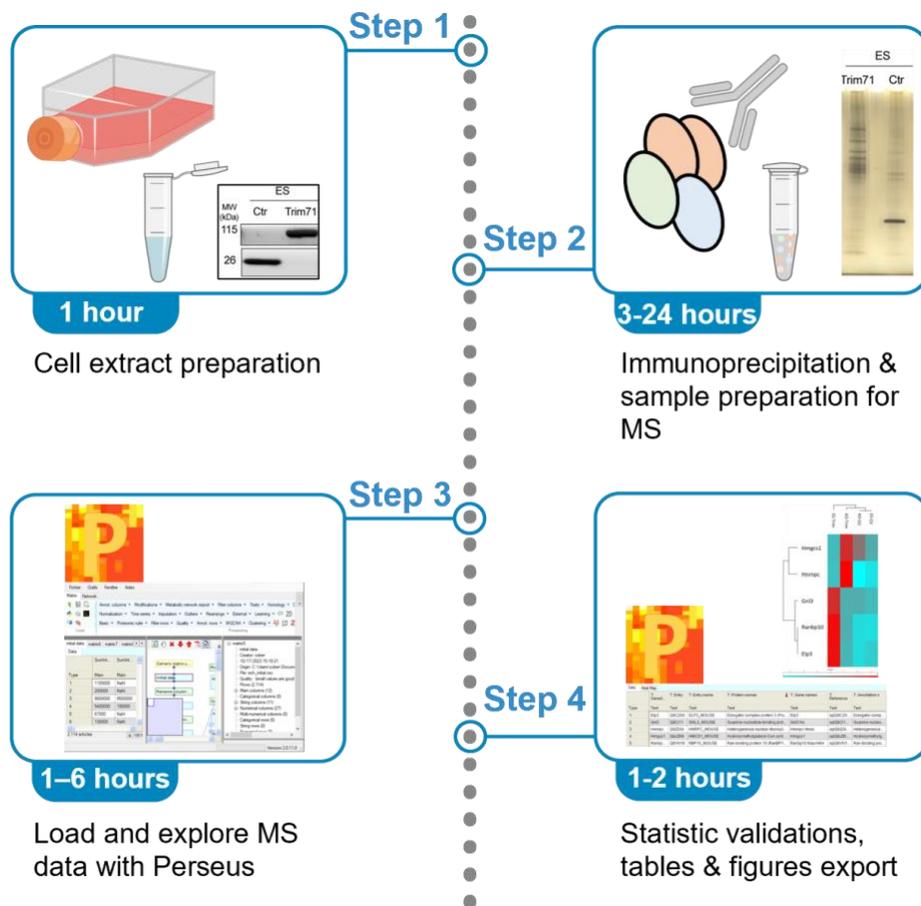
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SUMMARY

Characterizing multi-protein complexes using mass spectrometry is crucial for understanding complex biochemical activities that govern cell fate. Investigating dynamic protein-protein interactions requires diverse qualitative and quantitative approaches. We present a protocol for differential analysis of Trim71-associated proteins in mouse embryonic stem cells under two conditions. We describe steps for cytoplasmic extraction, protein immunoprecipitation, sample preparation for mass spectrometry and data analysis with Perseus. Our versatile tool enables in-depth exploration of protein complex compositional changes, contributing to a deeper understanding of cellular dynamics, suitable for various research domains.

For complete details on the use and execution of this protocol, please refer to Rapone et al. ¹.

Graphical abstract



Before you begin

The provided protocol outlines a comprehensive procedure for:

1. preparing cytoplasmic extracts from mouse embryonic stem cells (mESCs) cultured in feeder-free condition,
2. purifying Trim71-associated complexes,
3. submitting them for mass spectrometry (MS) analysis,
4. bioinformatic exploration of the results.

The primary focus is a comparative study of Trim71-associated proteins under two conditions: acute lysine methyltransferase (KMT) *Setdb1* depletion and basal wild-type (WT) mESCs. This investigation provides insights into alterations in Trim71-associated complexes following *Setdb1* depletion. We recommend using at least 3 replicates for each condition, more replicates will further improve the reliability and robustness of the results. Notably versatile, the protocol can be adapted for analyzing diverse protein complex changes in various cell types

that occur in response to specific environmental or experimental conditions, making it a valuable tool for exploring protein dynamics in different biological contexts. It can be applied for example when assessing the impact of contrasting conditions such as stress *versus* normal growth scenarios, or the investigation of cellular responses in cancer cells under treatment *versus* untreated states, to understand how a new anticancer drug affects the protein interaction network of a key signaling protein in cancer cells.

Before the experiment, mESCs were transiently transfected with a Trim71-GFP expressing vector, allowing for subsequent Trim71 immunoprecipitation using the GFP-Trap approach. As a control, GFP expressing cells were processed in parallel to monitor the background level during the immunoprecipitation. Details on plasmid construction and transfection are omitted here, given their well-documented nature in existing literature. It's important to highlight that the experimental setup can alternatively begin with immunoprecipitation of the endogenous protein, if a suitable high-quality antibody is accessible.

Download and installation of Perseus² (PC only)

Timing: 0.5 hour

1. Connect to https://maxquant.net/download_asset/perseus/latest.
2. Fill the form to receive by mail the download link for Perseus.
3. Follow the link to download the zipped file.
4. Unzip the file and click on "perseus.exe".
5. Backup the installation files (troubleshooting 1).

CRITICAL: This program described in Tyanova et al.² is available only for PC computers with Windows operating system.

Key resources table

See Key Resource Table.docx

Materials and equipment setup

- Buffers for cytoplasmic extraction and immunoprecipitation

Buffer A for the lysis

Reagent	Final concentration	Amount
HEPES pH 7.0	20 mM	2 mL from 1 M
EDTA	0.15 mM	30 µl from 0.5 M
EGTA	0.15 mM	30 µl from 0.5 M

KCl	10 mM	330 µl from 3 M
ddH ₂ O	N/A	Up to 100 mL
Total	N/A	100 mL

Do not store the buffer.

Buffer SR for the lysis

	Final concentration	Amount
HEPES pH 7.0	50 mM	5 mL from 1M
EDTA	0.25 mM	50 µl from 0.5 M
KCl	10 mM	330 µl from 3 M
Sucrose	70%	70 g
ddH ₂ O	N/A	Up to 100 mL
Total	N/A	100 mL

Do not store the buffer.

Wash buffer for IP

Reagent	Final concentration	Amount
Tris pH 7.5	50 mM	25 ml from 1 M
NaCl	150 mM	15 mL from 5 M
Igepal CA-630	0.5%	12.5 mL from 20%
ddH ₂ O	N/A	Up to 500 mL
Total	N/A	500mL

Do not store the buffer.

Dilution buffer for IP

Reagent	Final concentration	Amount
Tris pH 7.5	10 mM	1 mL from 1 M
EDTA	0.5 mM	2.5 mL from 0.5 M
NaCl	150 mM	3 mL from 5 M
ddH ₂ O	N/A	Up to 100 mL
Total	N/A	100 mL

Do not store the buffer.

CRITICAL: Prepare protease inhibitors by dissolving the tablets in H₂O, following the manufacturer's instructions. Protease and phosphatase inhibitors should be added to the buffers extemporaneously.

- Supplementary Table: Table S1 (TableS1.csv), related to step 15, contains the raw data from our mass spectrometry analysis, including the sum of peptide peak intensities (beginning with 'Sumintensity'), numerical data in other columns, and additional information presented in text format.

Step-by-step method details

Cell harvesting from cell plate, cell lysis and cytoplasmic extraction

This section involves detaching and collecting adherent cells from culture plates, disrupting the cell membranes to release intracellular components, and subsequently isolating the cytoplasmic fraction through differential centrifugation for downstream analyses.

Timing: 1 hour

1. Transfer your cell plates to ice.
 - a. Wash the cells with cold 1% PBS and then harvest them by scraping on ice.
 - b. Collect everything in a 15 mL tube.
 - c. Centrifuge it for 10 min at 200 x g at 4°C.

Note: You can continue the protocol or snap-freeze the pellets and keep them at -80°C.

2. Estimate the volume (V) of the pellet.
3. Cell lysis
 - a. Resuspend the pellet with 3V of buffer A.
 - b. Add 0.44 V of 10% IGEPAL CA-630 (final concentration 3 %) and mix by tube inversion.
 - c. Add quickly 0.88 V (8V/9) of buffer SR and mix by tube inversion.
4. Centrifuge cell lysate at 2,000 x g for 5 min at 4°C.
5. Recover the supernatant which corresponds to the *cytoplasmic fraction*.
6. Ultra-centrifuge the cytoplasmic fraction at 100,000 x g for 30 min at 4°C to get rid of the insoluble material.
7. Recover the supernatant which corresponds to the clean cytoplasmic fraction.
8. Quantify protein amount, using Pierce™ BCA™ kit.

Note: we recommend using 2 plates of 15 cm, corresponding to 1.10^7 cells

Optional: detect proteins of interest in the cell extract by western blot (timing: 1 day)

CRITICAL: Add fresh protease and phosphatase inhibitors to all buffers and keep the buffers on ice.

Immunoprecipitation to purify the protein complexes

This section concerns the purification of the protein and its complexes by a definite antibody which selectively binds and isolates the target protein. This is followed by the analysis of the purified proteins through mass spectrometry.

Timing: 3 hours or 2 hours and overnight incubation

9. Beads equilibration: take 25 μ L of GFP-Trap beads slurry per sample and wash them quickly 3 times with wash buffer.
10. Carry out the immunoprecipitation using GFP-Trap beads.

- a. Add 1 mg of proteins (from step 7) to the beads and fill the tube up to 500 μ L with the dilution buffer, using low-binding 1.5 mL tubes.
- b. Incubate for 1 h (or overnight) in rotation at 4°C.
11. Remove the supernatant and wash the beads 5 times using 1 ml of wash buffer. For each wash, rotate the tubes for 10 min at 4°C.
 - a. Change all tubes before starting the washes and before the last wash.
12. To verify that in your samples you have a good enrichment of co-immunoprecipitated proteins and a different profile of co-immunoprecipitated proteins in the different conditions, perform a western blot and / or a silver staining of your samples (see Expected outcomes, Figure 1), following the manufacturer's instructions (ThermoFischer Scientific). Troubleshooting 2 and 3.

CRITICAL: Add fresh protease and phosphatase inhibitors to all buffers and keep the buffers on ice.

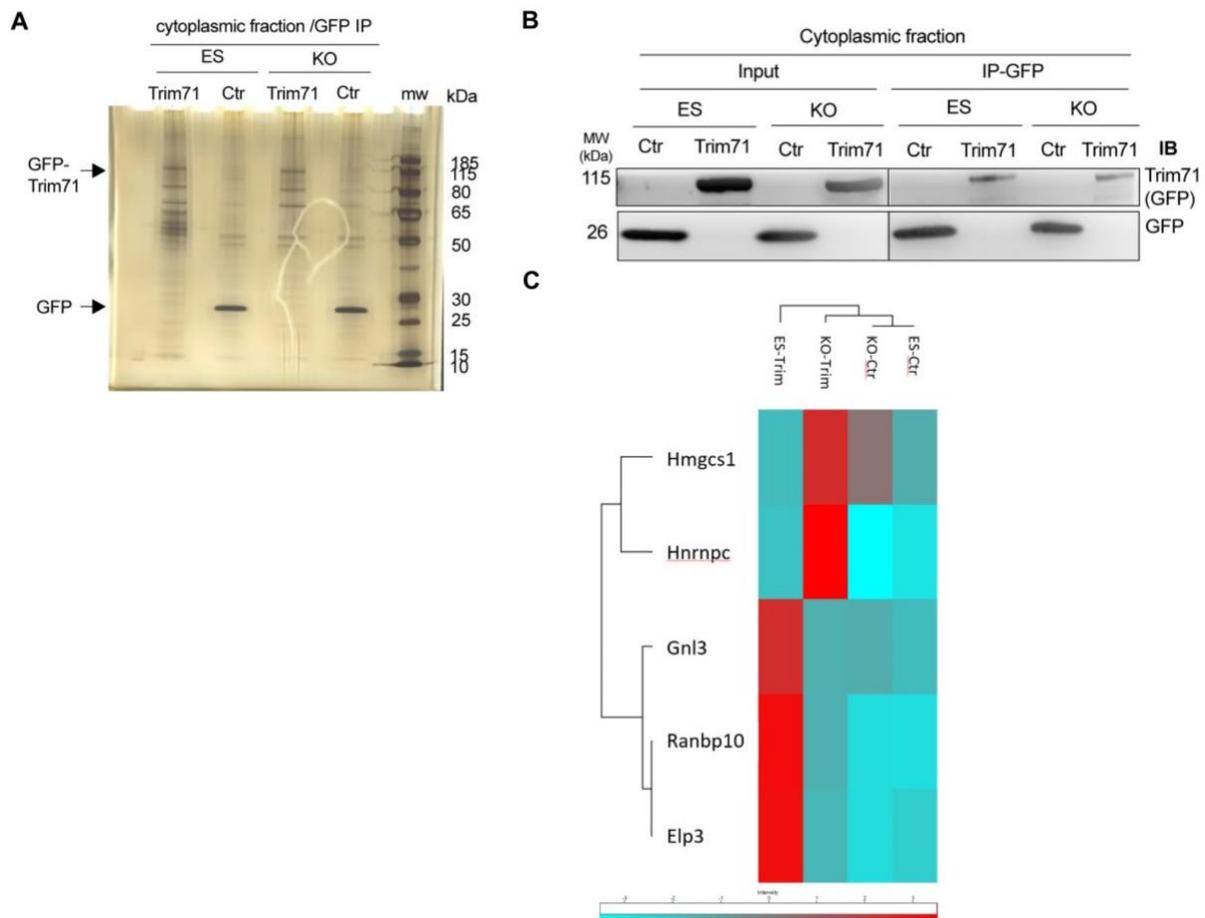


Figure 1. GFP-Trim71 (Trim71) was immunoprecipitated from the cytoplasmic fractions of control or Setdb1 KO mESCs. GFP (Ctrl) was used as control of immunoprecipitation. (A) Silver staining to detect immunoprecipitated proteins. (B) The amount of immunoprecipitated proteins Trim71 and GFP in ES or KO cells was checked by western blot. (C) Heatmap after clustering of the significant proteins with the score obtained by the Tukey's honestly significant difference with an FDR < 0.05 (THSD) after ANOVA test with log-transformed and scaled data.

Sample preparation for the mass spectrometry platform

This section investigates the compatibility and optimization for mass spectrometric analysis.

Timing: from 5 minutes to 3 hours

Depending on the mass spectrometry platform instructions, to perform label-free quantitative analysis of protein complex abundance using liquid chromatography coupled with tandem MS (LC-MS/MS):

13. Provide the beads to the platform or the soluble eluted material, in the *ad hoc* buffer specified by the MS facility.
14. Or provide gel cuts after loading the samples on a polyacrylamide gel:
 - a. Load 50 μ L of sample in 4-12% NUPAGE gels in MES buffer (Invitrogen) for few minutes until 1.5 cm running from the very top of the wells.
 - b. Put the gel in a clean 15 cm petri dish and fix the gel in 10% acetic acid, 50% methanol and 40% H₂O in slight rotation for 25 min.
 - c. Wash the gel 5 times with clean H₂O.
 - d. Cut the gel to separate the samples with a new clean scalpel and put all gel pieces in different 2 mL low binding tubes.
 - e. Add 1 mL of clean water to each tube.
 - f. Sealed the tubes with parafilm and send to the mass spectrometry platform.

CRITICAL: Do not touch the gel with hands or gloves.

Note: Proteins will be processed on a mass spectrometry platform, that will return a data file containing detailed information on identified peptides and proteins, presenting the distribution and the number of unique peptides and intensities obtained by label-free quantification. This file contains information about different peptides identified in a sample, including their abundance (intensities) and how they are distributed across various conditions or experiments. Each line typically represents a protein, with accompanying data on the intensities of various peptides that map to each protein.

Loading data on Perseus

This section concerns the import of processed and formatted datasets from mass spectrometry into the Perseus software platform, which is designed for the analysis and visualization of omics data.

Timing: 10 min

Caution: Regularly save your work as Perseus does not register temporary files (Troubleshooting 4).

15. Open your data file in a tab delimited viewer and save them in .txt or .csv format file (with comma separated values). For our example, open the TableS1.csv file. Identify the columns with the sum of peptide peak intensities (starting with "Sumintensity" in our example), the other columns with numerical data, and the ones with text.
16. Click the green "Generic matrix upload" arrow, then the "select" one.

17. In the pop-up window, navigate and select the .csv or .txt file. In our example, select the TableS1.csv file.
18. Select all the columns :
 - a) with the sum of intensities and transfer them to the “Main” window using the “>” button.
 - b) with numerical data to “numerical” window (in our example, “MWT.kDa.x” and “MWT.kDa.y”),
 - and c) transfer all the other columns to the “Text” window.
19. Validate by clicking on the “OK” button. You have created the first matrix, right click on it and insert as alternative name “initial data”.

Rename, rearrange the columns, and create groups

This section includes the adjustment of the dataset structure to enhance clarity and facilitate analysis.

Timing: 10 min

20. Verify in the “Type” row that each column corresponds to the main/numerical/text categories that you would like to fix, you can modify the category in the “rearrange” menu, “change column type”.
21. To shorter the column names :
 - a) click on “Rearrange”,
 - b) select columns [Reg. ex.],
 - c) insert “SumIntensity” in pattern and “SI” in “Replace”,
 - d) click “OK”.

Alternatively, you can rename the columns by clicking on “rearrange”, “Rename columns”, select “Edit” and modify the text.
22. To group the columns :
 - a) Select “Rearrange”, “Reorder/remove columns”,
 - b) group the columns of the main category by sample groups (SIGFPx, SITGFPx, SITrimx, SITTrimx) using the arrows on the right.
23. Click on “Annot. Rows”, “Categorical annotation rows”, choose “create”, select the 3 first samples and click on the check symbol “✓” to set group names by removing the last character (SIGFP1 - > SIGFP).

Note: At any time, you can delete nodes by selecting them and clicking on the red cross button “remove selected nodes”.

Filter, log transformation, data imputation for missing values, summary statistics

This section explores data analysis. These steps are crucial in data preprocessing and analysis to improve the robustness, accuracy, and interpretability of the analysis, leading to more reliable and insightful conclusions.

Timing: 1 hour

24. Click on "Filter rows" and select "filter rows based on valid values", "percentage", "Minimum of percentage values" : "50". Observe that from 2082 rows, only 815 rows are conserved.
25. Click on "Basic", "Transform", "log₂(x)", and verify that all the Main columns are represented in the right window. Thanks to log transformation, the effects of large data values are moderated, and thus the variance is stabilized, and data are more closely conformed to a normal distribution.
26. Click on "Basic", "Summary statistics (columns) and explore the results.
27. Scatter plot :
 - a) Select the node with the latest matrix (Matrix7), and in the Analysis part of the upper menu, click on "Scatter Plot", and observe the distribution between replicates changing "X" and "Y" in the second window. In the scatter plot comparison of replicates, the expected global distribution tends to cluster near the XY diagonal.
 - b) In the same menu, click on "Histogram" and observe that most of the elements in the data set are close to a distribution symmetric, unimodal, bell-shaped curve, that is typical of a normal distribution.
28. Replace missing values :
 - a) Click on "Imputation", Replace missing values with normal distribution, using default parameters, down shift can be slightly increased but the distribution should remain close to a normal distribution.
 - b) With this new matrix, click on "Histogram" in the upper menu, to create a new "Histogram" tab. In the "points" tab, select from the drop-down menu "Selection from imputation" to observe the distribution of the points that have been added. You can also compare this histogram with the one of the previous matrix.

Optional: Based on the data profile observed in the scatter plot, a "scale to interval" normalization could be used to bring all variables into the same scale without distorting differences in the ranges of values. Scaling data with the default interval [0,1] can make results more interpretable and easier to visualize. Click on "normalization", "scale to interval", Matrix access : "Columns". Create a histogram and observe the transformation. As seen by comparing the new histogram with the previous one, this scaling allows for the direct comparison of variables that follow different nearly normal distributions by bringing them onto a common scale. This subsequent scaling is particularly useful when combining features with different units or scales of measurement, ensuring that this transformed data is on a consistent scale. Scaling after log transformation can further enhance interpretability by putting these patterns on a consistent, understandable scale, especially when visualizing the data.

Multiple sample Anova test, Post hoc Tukey's HSD test.

This section concerns statistical tests. These tests are important for understanding detailed relationships between groups.

Timing: 30 min

29. An analysis of variance (ANOVA test) will be performed to find significant differences between groups, and then a Post-hoc test to determine specifically which groups differ from each other. Click on “Tests”, “Multiple sample tests”, and for our example fix the “FDR” to 0.12.
30. Click on “Filter rows”, “Filter rows based on categorical column”, “column” : “ANOVA significant”, “keep matching rows”. Observe the number of rows of the new matrix.
31. Click on “Tests”, “Post hoc Huckey’s HSD”. Observe the new last column on the right of the table “Significant pairs”.
32. To be able to select specific pairs, click on “Rearrange”, “Change column type”, select “Text” and move “Significant pairs” on the right window, and choose “Categorical” as “Target Type”. Observe that this column has moved close to the other “Categorical” column.
33. Click on “Filter rows”, “Filter rows on categorical rows”, “Significant pairs”, in “Values”, select “SITrim-SITTrim” and “SITTrim_SITTrim” and move them on the right window, select “Keep matching rows”. Observe the rows that are conserved.

Export of tables and figures

This section investigates the export of tables and figures. Saving and exporting data tables and graphical representations into external files ensure that the data are accessible, interpretable, and usable for various purposes.

Timing: 10 min

34. Figures adjustment:
 - a) Click on the last matrix created, and in the “analysis” menu, click on “Hierarchical clustering”.
 - b) Click on the “Heat Map” tab and if you want to change the format of the elements of the figure, click on the “properties” button.
 - c) Click on the colored disk “change color gradient” to change the colors and their intensities.
35. Select the matrix that you want to save, in the menu “export”, click on the floppy disk “Generic matrix export”, and click on select to choose the directory.

Expected outcomes

The outlined protocol facilitates the identification of proteins associated with Trim71, offering insights into potential Trim71 partners. It is essential to note that the composition of Trim71 complexes may vary under different biological conditions (*i.e.* WT versus *Setdb1* KO mESCs), leading to changes in the co-precipitated proteins.

A successful immunoprecipitation should show, by silver staining and/or western blot, a clear enrichment of the immunoprecipitated proteins (in our experiment GFP-Trim71 and GFP, Figure 1 A and B). A silver-stained gel of the immunoprecipitates should exhibit also a specific profile of the co-immunoprecipitated proteins in the two conditions (Figure 1A). For any troubleshooting, see the dedicated section below.

An example of output is presented in Figure 1C, with 2 proteins that bind Trim71 more efficiently (Hmgcs1) and 3 less efficiently (Gnl3, Ranbp10, Elp3) upon *Setdb1* depletion (KO).

Limitations

IP-MS may not accurately reflect the stoichiometry of protein complexes. The technique identifies the presence of proteins in a complex but does not necessarily provide quantitative information about the quantity of each protein subunit within the complex. In interpreting data, complexities arise from the potential identification of proteins that belong to different complexes. These proteins may not interact directly with the bait protein or be part of the same complex.

One of the major challenges is the non-specific binding of proteins to the antibody, beads, or other components of the assay. This can lead to the identification of proteins that are not true interactors of the bait protein. Also, some protein-protein interactions are transient or occur under specific conditions or in specific cellular compartments. A particular attention must therefore be paid to IP conditions.

Optimizing immunoprecipitation is also crucial due to the common issue of obtaining low protein yields, which can hinder comprehensive analysis via mass spectrometry. This limitation often results in a significant number of missing values, thereby negatively impacting the overall analysis quality. To address this challenge, a strategic enhancement may involve the increase of the number of replicates to 4 or 5 per experimental condition, a modification that is likely to yield substantial benefits.

Therefore, for filtering peptides in step 25, it is imperative for users to carefully consider the number of unique peptides identified by mass spectrometry used for determination of peak intensities. Alternatively, in scenarios where the number of unique peptides is low, users may judiciously assess this factor towards the conclusion of the analysis to validate candidate proteins.

Troubleshooting

Problem 1:

When reopening a .sps Perseus file, you obtain the message ("before you begin" section):

"Failed to read session potentially due to version mismatch:

1.6.13.0 Saved

2.0.11.0 Current".

This message means that the Perseus version does not correspond to the one used when you have done your analysis (Step 15 Loading data on Perseus)

Potential solution:

Reopen the version corresponding to the one that you used for your analysis. The version number is indicated before "Saved" in the error message, i.e., 1.6.13.0 in our example.

Problem 2:

The test to verify a successful immunoprecipitation of your protein by western blot and a satisfactory co-immunoprecipitated protein profile by silver staining is a key step for the experiment's success. Silver staining, known for its high sensitivity in detecting proteins, can reveal subtle differences in band patterns, providing valuable insights into protein interactions, modifications, and potential contaminants.

Co-immunoprecipitated protein profile is not satisfactory: low amount of immunoprecipitated and/or co-immunoprecipitated proteins (Step 12).

Potential solution:

- Ensure that you have in the plate a homogenous expression of your protein after transfection, for example by immunofluorescence staining followed by fluorescence microscopy or by flow cytometry to assess the distribution and intensity of protein expression across the cell population.
- Selection of the transfection method, optimization of the transfection conditions, usage of stable cell lines, modification of the promoter that control transgene expression are among the solutions for improving the expression of the protein of interest.
- You can also adjust the IP incubation time, ranging from 1 hour to overnight.

Problem 3:

Co-immunoprecipitated protein profile is not satisfactory: too many bands and no difference between conditions (Step 12).

Potential solution:

It is crucial to interpret the results within the context of the experiment, considering factors such as the expected size of the target protein, known post-translational modifications, and potential protein-protein interactions. Furthermore, implementing appropriate controls and comparing findings with established literature or databases can validate the specificity and significance of the detected bands. When the number of observed bands significantly exceeds the expected profile based on these considerations, it indicates potential issues with nonspecific binding or other experimental artifacts that need to be addressed through optimization of the IP protocol. It can also be due to the presence of heavy and light chains of the antibody used in the IP, degradation of the target protein, or the presence of multiple isoforms or post-translationally modified forms of the protein. Several optimizations can be considered.

- Firstly, decreasing the IP incubation time may minimize nonspecific interactions without compromising target protein binding.
- Additionally, adjusting the stringency of the lysis buffer is crucial; increasing salt concentration or adjusting detergent levels can reduce nonspecific protein binding. Ensuring the lysis buffer contains an optimal mix of fresh protease and phosphatase inhibitors is essential to preserve the integrity and post-translational state of the target protein.
- The choice of antibody is pivotal for successful IP. Highly specific antibodies known for their efficacy in IP experiments should be utilized to target the protein of interest, as specificity is key in reducing background noise from nonspecifically bound proteins.
- Increasing both the number and stringency of washes following IP can eliminate nonspecifically bound proteins, leading to cleaner samples. This may involve using wash buffers with higher salt concentrations or different detergents, tailored to the specific system and target protein.

If the results exhibit significant variability, increase the number of replicates to 4-5 and attempt to perform sample preparation, immunoprecipitation, and mass spectrometry for all samples together.

Problem 4 :

Perseus crashes frequently, causing loss of work as it does not save temporary files. Additionally, the file you are working on can become corrupted after a crash.

Potential solution:

- Regularly save your work
- Modify the file name each time you save to maintain a complete backup of all versions
- If the crashes occur frequently, consider switching to a computer with more RAM

Resource availability

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Slimane Ait-Si-Ali, slimane.aitsiali@u-paris.fr.

Technical contact

Technical questions on executing this protocol should be directed to and will be answered by the technical contact, Bertrand Cosson, bertrand.cosson@u-paris.fr

Materials availability

This study did not generate new unique reagents. All reagents generated in this study are available from the lead contact with a completed Materials Transfer Agreement.

Data and code availability

The published article includes all datasets and code generated or analyzed during this study.

All original data for Figure 1 from Rapone et al.¹ has been deposited at Zenodo and is publicly available as of the date of publication. DOIs are listed in the key resources table.

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Author contributions

Conceptualization, S.A., B.C. and R.R.; Methodology, R.R., L.D.M., B.C., and S.A.; Software, B.C.; Formal Analysis, B.C.; Investigation, R.R., L.D.M., S.A. and B.C.; Data Curation, B.C.; Writing – Original draft, R.R., B.C.; Writing – Review and Editing, R.R., B.C., and S.A.; Visualization, R.R., B.C., and S.A.; Supervision, B.C. and S.A.; Funding Acquisition, S.A; Project Administration, B.C. and S.A.

Declaration of interests

The authors declare no competing interests.

Figure legend

Figure 1: GFP-Trim71 (Trim71) was immunoprecipitated from the cytoplasmic fractions of control or *Setdb1* KO mESCs. GFP (Ctr) was used as control of immunoprecipitation. **(A)** Silver staining to detect immunoprecipitated proteins. **(B)** The amount of immunoprecipitated proteins Trim71 and GFP in ES or KO cells was checked by western blot. **(C)** Heatmap after clustering of the significant proteins with the score obtained by the Tukey’s honestly significant difference with an FDR < 0.05 (THSD) after Anova test with log-transformed and scaled data.

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