Title:
Systematic lipidomic analysis of yeast protein kinase and phosphatase mutants reveals novel insights into regulation of lipid homeostasis

Author names and affiliations:
Aline Xavier da Silveira dos Santos*,‡, Isabelle Riezman*, Maria-Auxiliadora Aguilera-Romero*,‡, Fabrice David†, Manuele Piccolis§, Robbie Loewith*,‡, Olivier Schaad* and Howard Riezman*,‡

*Dept.Biochemistry, University of Geneva, Geneva Switzerland
†École Polytechnique Fédérale de Lausanne, Lausanne, Switzerland;
‡NCCR Chemical Biology, University of Geneva, Geneva Switzerland;
§Dept.Molecular Biology, University of Geneva, Switzerland.

Supplementary Material

Supplementary Tables

Table S1. List of strains used in this study. Available in xls format.

Table S2. List of lipids monitored in this study. Available in xls format.
For each lipid, the name and features were classified into different categories, such as (i) lipid class (CER - ceramides, IPC – inositol phosphorlyceramide, MIPC - mannosylinositol-phosphorylceramide, M(IP)2C - mannosyl-(inositol phosphoryl)2 ceramide, PC – phosphatidylcholine, PE – phosphatidylethanolamine, PI – phosphatidylinositol, PS – phosphatidylerine, ERG – sterol), (ii) lipid subclass (DHC - dihydroceramide, PHC - phytoceramide, IPC, MIPC, M(IP)2C, LysoPC - lysophosphatidylcholine, PC, LysoPE - lysophosphatidylethanolamine, PE, LysoPI - lysophosphatidylinositol, PI, LysoPS - lysophosphatidylerine, PS, ergosterol and ergosterol ester), (iii) fatty acid chain length (short: 14 carbons (C) to 16C, long: 18C to 22C, very long: 24C to 26C) and (iv) unsaturation index of fatty acids (0, 1 or 2 - sum of double bonds in the fatty acids of glycerophospholipids). n.a=non-applicable.

Table S3. Descriptive statistics of lipids and lipid categories analyzed in this study. Available in xls format.
For each lipid class or subclass, basic statistics were calculated based on the normalized (log2 transformed data). Threshold values were established (mean +/- SD). Samples were classified as a hit when passed the log2 threshold filter and the SSMD criteria (SSMD values < -1.28 and >1.28).

Table S4. Complete dataset. Data from all lipids analyzed. Available in xls format.
Lipid profiles of all strains with all lipids are presented as (i) hits, (ii) quantities (expressed as arbitrary units - a.u.), (iii) fold-change and (iv) SSMD scores.

Table S5. Complete data set. Data from lipid categories. Available in xls format.
Lipid profiles of all strains grouped into different categories of biological interest are presented as (i) hits, (ii) quantities (expressed as arbitrary units - a.u.), (iii) fold-change and (iv) SSMD scores.

Table S6. Relative impact of a missing kinase or phosphatase on the lipidome. Available in xls format.
The total number of hits was calculated for each strain and divided by the number of lipids considered for that strain.

**Table S7.** Sphingolipid profile of *pho85Δ, kcs1 Δ and vip1 Δ*. Available in xls format.
| Systematic Name | Standard Name | Systematic Name | Standard Name |
|-----------------|---------------|-----------------|---------------|
| YGL021W         | ALK1          | YBL009W         | ALK2          |
| YFR014C         | CMK1          | YOL016C         | CMK2          |
| YML057W         | CMP2          | YLR433C         | CNA1*         |
| YGR092W         | DBF2          | YPR111W         | DBF20         |
| YCL024W         | KCC4*         | YDR507C         | GIN4\(^{(na)}\) |
| YDR122W         | KIN1          | YLR096W         | KIN2          |
| YPL141C         | FRK1          | YOR233W         | KIN4          |
| YNR047W         | FPK1          | YCR091W         | KIN82         |
| YJL165C         | HAL5          | YKL168C         | KKQ8          |
| YGR188C         | BUB1          | YJL013C         | MAD3\(^{(na)}\) |
| YOR231W         | MKK1          | YPL140C         | MKK2          |
| YIL113W         | SDP1          | YNL053W         | MSG5          |
| YDL214C         | PRR2          | YNL183C         | NPR1          |
| YDR490C         | PKH1          | YOL100W         | PKH2          |
| YDL134C         | PPH21*        | YDL188C         | PPZ2\(^{(na)}\) |
| YML016C         | PPZ1          | YDR436W         | PSK2*         |
| YNL020C         | ARK1          | YIL095W         | PRK1          |
| YAL017W         | PSK1          | YOL045W         | PSR2          |
| YLL010C         | PSR1          | YLR019W         | PSR2          |
| YBL056W         | PTC3          | YER089C         | PTC2\(^{(na)}\) |
| YKL198C         | PTK1          | YJR059W         | PTK2          |
| YGL158W         | RCK1          | YLR248W         | RCK2          |
| YDL079C         | MRK1*         | YMR139W         | RIM11         |
| YER027C         | GAL83*        | YGL208W         | SIP2\(^{(na)}\) |
| YNL298W         | CLA4\(^{(na)}\) | YOL113W         | SKM1          |
| YKL161C         | KDX1          | YHR030C         | SLT2          |
| YNR031C         | SSK2          | YCR073C         | SSK22         |
| YER129W         | SAK1          | YGL179C         | TOS3          |
| YJL164C         | TPK1          | YKL166C         | TPK3*         |
| YPL026C         | SKS1          | YDR247W         | VHS1          |
| YHR135C         | YCK1          | YNL154C         | YCK2          |
| YNL307C         | MCK1          | YOL128C         | YGK3          |
| YKL126W         | YPK1*         | YMR104C         | YPK2*         |

\(^(*)\) Deletion caused high impact on the lipidome. \(^{(na)}\)=not analyzed in this study.
**Table S9. List of primers used for gene expression analysis by Real-Time PCR.**

| Systematic Name | Standard Name | Forward sequence (5'-3') | Reverse sequence (5'-3') |
|-----------------|---------------|--------------------------|--------------------------|
| YFL039C         | ACT1          | TTTGCCGGTGACGACGCTCC     | CGTCCCAGTTGGTGACAATACCGT |
| YKL004W         | AUR1          | AAGCGACTGGGTGCATTACA     | GTAATAGGAGGCCCTGGGTC     |
| YPL057C         | SUR1          | CCTGAGCAGTGAAAGAGGG      | TAGTTTTCTTTCGAGCGGT      |

**Table S10. LipidX and CheBi Identifiers of lipids analyzed in this study. Available in xls format.**

Lipids are identified by their LipidX ID, name, chemical formula and CheBI annotation.

**Table S11. Lipid profile of CED and PPA strains. Available in xls format.**

Lipid profile of wild-type, CED (*cki1Δ, eki1 Δ, dpl1 Δ*) and PPA (*psd1 Δ, psd2 Δ, ale1 Δ*) strains are presented.
Supplementary information

Yeast strains. Yeast knockout strains for protein kinases and phosphatases were obtained from Claudine Kraft and Matthias Peter (Bodenmiller et al., 2010). The following strains were considered dubious or did not have their KanMX cassette location confirmed in their study: dbf2Δ, fpk1Δ, ire1Δ, mih1Δ, yak1Δ, ssk22Δ, rck1Δ and yck3Δ. Knockout strains were plated in YPD plates and isolated colonies were used for the two independent cell cultures. Complementation of snf1Δ was done with SNF1 wild type gene expressed on a centromeric plasmid obtained from the lab of Marian Carlson. Strains for experiments in figure 6 were obtained from Günther Daum’s lab (TU Graz, Austria) (Schuiki et al., 2010). SNF1 deletion was done with standard yeast gene deletion procedures using the plasmid pRS406.

MS data acquisition and processing

Lipid analysis was performed by multiple-reaction monitoring mass spectrometry (MRM-MS) in a TSQ Vantage Triple Quadrupole equipped with a robotic nano-flow injection system (detailed information in methods’ section). The list of lipids analyzed is available in Supplementary Table S2 (N = 377).

The screening was conducted in 12 batches of lipid analysis. Each batch contained a wild-type strain and up to 23 mutants. For each biological replicate (BR), the MS analysis was done up to six sequential repetitions, herein called technical replicates (TR). The signal intensities from the detected lipids in each TR were obtained from the raw files as previously described (Epstein et al., 2012) and quantified according to standard curves from the internal standards. From that, we computed the median of the values obtained in the TRs for each lipid in each sample.

Data normalization and quality control procedures

Interplate correction. In order to avoid inaccuracies arising from batch effect, we standardized the values of each lipid across the different batches. First, we calculate the median of each lipid in each batch:

\[
\text{median} (X)_{(\text{batch} \, 01)} = \text{median of lipid}(X) \text{ in batch } 01
\]

Then, for every lipid, we calculate a master median (MM) across the batches:

\[
\text{MM lipid}(X) = \text{median lipid}(X) \text{ in batches 01 to 12}
\]

The MM values are used to compute a correction factor (CF) to correct the original values as follows:

\[
\text{CF (lipid } X \text{ in batch } 01 = \frac{\text{median lipid } X \text{ in batch } 01}{\text{MM (lipid } X)}
\]

\[
\text{Corrected lipid}(X) = \frac{\text{value (lipid } X)}{\text{CF (lipid } X)}
\]
The corrected values are herein referred as “Quant”.

**Quality control (QC) based on frequency of lipids.** During the course of analysis, we noticed that not all lipids were detected in all batches. These low frequency lipids were of low abundance and their quantities were highly variable. In order to homogenize the datasets for further multiple comparisons, we filtered the data to include only lipids that appeared at least once in every batch of analysis ($N_{\text{filtered}}=174$).

**Quality control based on the variability of the lipids.** We computed the coefficient of correlation (CV) for each lipid in each pair of BR (BR1 and BR2). For each lipid, calculated the median of CV values and we established a cutoff value of 0.4 ($N_{\text{robust}}=159$).

**Removal of lipids considered artifacts.** Lipids with conflicting detection masses and lyso-phospholipids with double unsaturations were manually removed ($N_{\text{final}}=146$).

**Quality control based on the biological replicates.** To assure reproducibility of the strains used in the screening, we computed the correlation coefficient ($r$) of all the biological duplicates (Supplementary Figure S2). Average $r$ was higher than 0.9, which validates the overall quality of the lipidomic approach. A cutoff $r=0.7$ was established and two strains were excluded from the analysis (cla4Δ and ptc7Δ).

**Multiple comparison analysis.** To improve precision and reduce error propagation, we chose to normalize the values of each lipid by the median of all the values of this lipid in each batch.

$$\text{Normalized lipid}(x) = \left[ \frac{\text{Quant lipid}(x)}{\text{Median lipid}(x)} \right]$$

**Analysis of significance.** Hit selection was based on a combination of threshold of (i) fold-change and (ii) strictly-standardized mean difference (SSMD) ([Zhang et al., 2007](#)). The fold-change is the log2 transformation of normalized values. For each lipid, we computed the mean and SD considering all strains. The upper and lower thresholds were then established as mean ± SD. Threshold values of fold-change are available in Table 3. The SSMD is the mean of the fold-change divided by the standard deviation of fold-change of normalized values.

$$\text{SSMD lipid}(x) = \frac{\text{mean}\log2(\text{normalized lipid }(x)|\text{BR1, BR2})}{\text{SD}\log2(\text{normalized lipid }(x)|\text{BR1, BR2})}$$

A cutoff was established for moderately strong hits using absolute values of SSMD>1.28.

A lipid was considered as a hit, if it passed both criteria of fold-change and SSMD threshold.

**Impact of the deletion of protein kinases and phosphatases on the lipidome.** The relative impact of each mutant on the lipidome of yeast was computed as the normalized number of lipid hits to all lipids detected on that strain. The mutants were ranked according to their relative impact. We considered a high impact $>0.22$. 

37
Supplementary Figure S1. Workflow of data analysis. After acquisition, data was normalized and corrected for interplate variability. Quality control (QC) measurements were applied for lipid detection based on frequency of detection and on average reproducibility (CV<0.4) and biological replicate reproducibility (threshold r>0.7). Data which passed QC were then analyzed and scored as a hit according to fold-change and SSMD thresholds (details in supplementary information).
Supplementary Figure S2. Comparative lipid analysis by high resolution mass spectrometry. Lipid extracts were diluted in 5mM ammonium acetate in chloroform:methanol:water (2:7:1, v/v/v) for both positive and negative ion mode mass analysis by direct infusion on a Q Exactive Mass Spectrometer (Thermo Fisher Scientific). Lipid species were identified according to their m/z and their abundance was calculated by their signal intensities relative to internal standards. (A-D) Relative signal intensities of major glycerophospholipids in snfΔ strain showing prevalence of lipid species with longer chain length. (E) Relative signal intensities of major ceramides confirm the phenotype of cka2Δ mutant. (F) Major complex sphingolipid analysis confirms the decrease of major IPC species in ypk1Δ and accumulation of major MIPC in pho85Δ strains. Values are mean and standard deviation of two independent biological replicates.
Supplementary Figure S3. Overview of lipidomic data. (A) Illustrative heat map of the screening. Fold-change data for each lipid in each mutant is shown. (B) The amplitude of the changes in different lipid classes as expressed by the variance of the relative amounts show that mutant cells show larger changes in sphingolipids than in glycerophospholipids.
Supplementary Figure S4. Schematic representation of sphingolipid and glycerophospholipid metabolism in yeast. Major metabolic steps are represented. Black arrows indicate synthesis and dashed arrows indicate degradation (most of degradation routes are omitted for clarity). Lipids analyzed in the study are highlighted in black boxes. Enzyme names (blue) are noted by correspondence to genes and can be found in the text.
Supplementary Figure S5. Correlation between PC-PE and PI-PS changes in all the mutants. (A) Strong correlation between phosphatidylcholine (PC) and phosphatidylethanolamine (PE) levels suggest that similar regulatory mechanisms are employed to control their homeostasis. (B) Phosphatidylinositol (PI) and phosphatidylserine (PS) levels are less well correlated. Strains with unbalanced PI/PS ratio might reveal novel candidates for the switch between Pis1p and Cho1p. Correlation coefficient (r) is shown.
Supplementary Figure S6. Fatty acid chain length in glycerophospholipids was affected by mutants in nutrient-sensing pathways. Sorting of mutants according to the relative content of short and long fatty acids revealed several mutants from the glucose sensing pathway (left panel) with relatively longer chain length fatty acids: snf1Δ, pph21Δ, gal83Δ and elm1Δ; while amongst mutants with relatively shorter chain length fatty acids (right panel) there were a some members of amino acid sensing pathway, for instance tor1Δ and gcn2Δ. Summarized data can be found in text and Table S4.
Supplementary Figure S7. Interaction between CTK1 and YPK1. (A) Phosphorylation of Ypk1 is impaired in ctk1Δ. (B) Phosphoproteomics data revealed a strong correlation in the relative amounts of phosphopeptides in ypkiΔ and ctk1Δ (Data from Bodenmiller et al, 2012).
Supplementary Figure S8. Mutants of the osmotic sensing machinery present alterations in sphingolipid levels. Cell wall stress and HOG pathways are shown. Hits for high levels of ceramides or complex sphingolipids and highlighted in red. Data from Table S5.
Supplementary Figure S9. Reduced levels of sphingolipids are compensated by increase in C26-based PI species. Some strains with low relative amounts of the major sphingolipid IPC-C t44:0 have increased amounts of C26-PI (PI42:1 and PI44:1), considered as compensatory glycerophospholipids, as described in the text.
Supplementary references

Bodenmiller, B., Wanka, S., Kraft, C., Urban, J., Campbell, D., Pedrioli, P.G., Gerrits, B., Picotti, P., Lam, H., Vitek, O., Brusniak, M.Y., Roschitzki, B., Zhang, C., Shokat, K.M., Schlapbach, R., Colman-Lerner, A., Nolan, G.P., Nesvizhskii, A.I., Peter, M., Loewith, R., von Mering, C., and Aebersold, R. (2010). Phosphoproteomic Analysis Reveals Interconnected System-Wide Responses to Perturbations of Kinases and Phosphatases in Yeast. Science Signaling 3, rs4-rs4.

Epstein, S., Kirkpatrick, C.L., Castillon, G.A., Muniz, M., Riezman, I., David, F.P., Wollheim, C.B., and Riezman, H. (2012). Activation of the unfolded protein response pathway causes ceramide accumulation in yeast and INS-1E insulinoma cells. J Lipid Res 53, 412-420.

Schuiki, I., Schnabl, M., Czabany, T., Hrastnik, C., and Daum, G. (2010). Phosphatidylethanolamine synthesized by four different pathways is supplied to the plasma membrane of the yeast Saccharomyces cerevisiae. Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids 1801, 480-486.

Zhang, X.D., Ferrer, M., Espeseth, A.S., Marine, S.D., Stec, E.M., Crackower, M.A., Holder, D.J., Heyse, J.F., and Strulovici, B. (2007). The Use of Strictly Standardized Mean Difference for Hit Selection in Primary RNA Interference High-Throughput Screening Experiments. Journal of Biomolecular Screening 12, 497-509.
**Highlights**

We developed an unbiased mass spectrometry-based lipidomic screening method and analyzed the major membrane lipids of 129 yeast deletions in protein kinase/phosphatase genes. Our approach is a new, rich source of biological insight. It has uncovered new players in lipid homeostasis and provides a useful dataset to further the understanding of lipid regulation by phosphorylation-based signaling networks.