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Proteomic and Phosphoproteomic Analyses of Yeast Reveal the Global Cellular Response to Sphingolipid Depletion*

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Abstract

Sphingolipids are essential components of eukaryotic cells with important functions in membrane biology and cellular signaling. Their levels are tightly controlled and coordinated with the abundance of other membrane lipids. How sphingolipid homeostasis is achieved is not yet well understood. Studies performed primarily in yeast showed that the phosphorylation states of several enzymes and regulators of sphingolipid synthesis are important, although a global understanding for such regulation is lacking. Here, we used high-resolution mass-spectrometry-based proteomics and phosphoproteomics to analyze the cellular response to sphingolipid synthesis inhibition. Our dataset reveals that changes in protein phosphorylation, rather than protein abundance, dominate the response to blocking sphingolipid synthesis. We identified Ypk signaling as a pathway that is activated under these conditions, and we identified potential Ypk1 target proteins. Our data provide a rich resource for on-going mechanistic studies of key elements of the cellular response to the depletion of sphingolipid levels and the maintenance of sphingolipid homeostasis.

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Sphingolipids, present in all eukaryotes and some prokaryotes, are important cellular lipids enriched in the plasma membrane. They are important for membrane integrity, membrane trafficking and signaling. Thus, maintaining sphingolipid levels at a set point and in tight balance with other lipid species is crucial. Sphingolipid homeostasis involves the post-transcriptional modulation of enzymes and regulators of sphingolipid biosynthesis, as well as altering gene expression to adapt to changing environmental or intrinsic conditions [1].

The yeast *Saccharomyces cerevisiae* is a powerful system to decipher sphingolipid regulation. For instance, inhibition of sphingolipid synthesis by myriocin, inhibiting serine-palmitoyl-transferase, the enzymatic protein complex catalyzing the first and committed step of *de novo* sphingolipid synthesis, has been useful to reveal the response to restore sphingolipid homeostasis [2–6].

Sphingolipid regulatory mechanisms are closely linked to human diseases. For example, a single nucleotide polymorphism (SNP) linked to altered expression of the SPT regulator ORMDL3 is a risk factor for asthma development and other inflammatory diseases [7]. The Golgi Associated Retrograde Protein Trafficking (GARP) complex is required for sphingolipid homeostasis and is associated with progressive cerebello-cerebral atrophy type 2 when mutated (PCCA2; [3, 8]).

To determine the response of *S. cerevisiae* to decreased sphingolipid levels, we used quantitative MS-based proteomics and phosphoproteomics. To capture the response to sphingolipid synthesis inhibition at early and late stages, we treated cells for 30 or 90 minutes with 5 μ M myriocin. This was based on our previous studies showing that treating yeast cells with myriocin for 30 minutes strongly affects the early intermediates of *de novo* sphingolipid synthesis, whereas treatment for longer times results in additional decreases in complex sphingolipids [9].

The experimental design is shown in (Fig 1a). Experiments were performed in triplicates (Fig. 1b, c).

We detected 3,247 proteins (with a minimum of one unique and two peptides identified) and 5,097 phosphorylated sites (with an Andromeda score threshold of 108.57_ in cells treated with myriocin for 30 minutes. 3420 sites were class I sites with a localization probability >0.75 (suppl. Table 1, 2). Among these proteins, we observed significant ($P < 0.05$) changes for 491 proteins. We also noted significant ($P < 0.05$) changes in the abundance of 672 phosphorylated peptides corresponding to 446 proteins (Fig. 1b, d).

For the 90-minute time point, we identified 3,293 proteins and 5,706 phosphorylation sites. The Andromeda score threshold for the phosphorylation sites was 104.72 and 4706 were class I sites (suppl. Table 1, 2). At this time point, the abundance of 399 proteins and 579 phosphorylation sites on 370 proteins were significantly changed according to MaxQuant's significance A value (Fig. 1 f, h).

In an alternative approach for phosphorylated peptides that were identified at least in two replicate experiments, we calculated the p -values with a moderated t-test, Benjamini-Hochberg corrected them for multiple hypothesis testing [10, 11]. These calculations yielded

516 phosphorylated peptides after 30 min myriocin treatment (BH corrected p -value <0.1 ; 463 class I sites) and 2346 phosphorylated peptides after 90 min myriocin treatment (BH corrected p -value <0.1 ; 2123 class I sites; suppl. Table 3).

We tested the reproducibility between replicates by pairwise scatterplots (Fig 1f,g). The Pearson correlation coefficient at 30 min myriocin treatment was ~ 0.6 (Fig 1 f; grey dots) and around 0.75 at 90 min myriocin treatment (Fig 1g; grey dots). The Pearson correlation coefficient increased when we only analyzed significant outliers ($P > 0.05$ according to significance A) calculated on the combined ratio (Fig 1 f,g; orange dots). This correlation is in the range of previously generated phosphoproteomes using SILAC labeling [12–14].

To determine which kinase signaling pathways dominate the response to sphingolipid synthesis inhibition, we analyzed the amino acid sequences around the phosphorylated serine and threonine residues, which constituted the majority of the identified sites at either time (6,165 of 6,190 sites quantified at least at one time; 99.59%). Analyzing the sites with a ratio H/L > 2.5 as a cut-off at 90 minutes of sphingolipid synthesis inhibition by myriocin revealed striking patterns. Among the up-regulated phosphorylation-sites, the likelihood of a proline at the +1 position relative to the S/T is significantly decreased, indicating that MAP kinases (mitogen activated protein kinase) [15] likely do not play a major role in mediating the response (Fig. 2a).

We also found significant ($p < 0.05$; according to iceLogo algorithm [16]) over-representation of arginine at the -3 and -5 positions (Fig. 2a). This RxRxxS/Tp pattern corresponds to the reported consensus motif for yeast protein kinase 1 (Ypk1) and has so far not been observed for any other AGC kinase in yeast (Fig. 2b) [17]. Ypk1 is critically involved in the response to changes in sphingolipid levels [1].

We next extracted all phosphorylation sites that conformed to the RXXRXXS/T motif, which yielded 39 total peptides in our dataset (suppl. Table 4). Of these 39 peptides, 13 showed a minimum twofold increase in phosphorylation after 90 minutes of myriocin treatment (Fig. 2c and suppl. Table 4). Among these, seven had been suggested to be Ypk1 targets (Fig. 2c, d, [18]). In addition, sites on eight different proteins with the similar phosphorylation site motif were upregulated (Fig. 2c, d). These 13 proteins act in multiple cellular processes, including endocytosis (Sla1) and regulation of the cell cycle (Ssd1, Irr1). Within these are sphingolipid metabolism enzymes involved in catalyzing the conversion of the complex sphingolipids inositolphosphorylceramide (IPC) to mannosyl-IPC (Csh1 and Sur1 [19]). 26 additional proteins, including the previously suggested Ypk1-targets Rpl3, Ycg1 and Hal5 contained the RXXRXXpS/pT motif but had a ratio < 2.5 .

To investigate the sphingolipid homeostatic response, we first analyzed changes in the abundance of sphingolipid metabolic enzymes in the proteome and found no marked changes after 30 or 90 min of sphingolipid synthesis inhibition (Fig. 3a). Exceptions at the later time point included upregulation of SPT subunit Tsc3 (1.9-fold), the ceramide synthase component Lac1 (1.4-fold), and the mannosylinositol phosphorylceramide synthase components Sur1 and Csg2 (2.1-fold and 1.4-fold). In addition, the abundance of the SPT

inhibitor Orm1 was decreased (1.7-fold), and Tsc10, involved in phytosphingosine synthesis, were modestly decreased (1.4-fold; Fig. 3b).

Analysis of the phosphoproteome confirmed several previous findings. Elo2 phosphorylation (S336, S338) was decreased [2]. Also, Orm1 and Orm2 phosphorylation increased during the time course [5]. Orm1 and Orm2 are phosphorylated by Ypk1 and Npr1 at different sites [20]. Here, we detected a change in the TORC1-dependent sites of Orm1 phosphorylation (a peptide with S29, S32, S34, S35 and S36) which was mildly but significantly increased after 30 min of myriocin treatment (Fig. 3c), but did not further increase after 90 minutes (Fig. 3d). In contrast, Orm2 phosphorylation was not significantly changed after 30 minutes of myriocin treatment but increased after 90 minutes (Fig 3c, d). We did not detect the Ypk1-responsive Orm1 and Orm2 sites, likely because they are located in a sequence predicted to yield large (49 amino acids for Orm1 and 92 amino acids for Orm2, suppl. Fig. 1) peptides after LysC digestion, complicating their detection.

For Lac1, we identified serine7 as a phosphorylation site downregulated with myriocin treatment. This site is different from the reported Ypk1-responsive site [18]. The observed phosphorylation site contains a proline in the +1 position, matching a MAP kinase motif.

In addition to these phosphorylation sites on proteins known to be important for the response to sphingolipid synthesis inhibition, we found that phosphorylation of Sur1 and Csh1, enzymes catalyzing conversion of IPC to M(IP)₂C, was upregulated and on the long-chain base kinase Lcb5 and the SPT component Lcb1 was downregulated. In addition, Itr1, a plasma membrane transporter for inositol, which is required for the synthesis of the most abundant sphingolipids, IPC, MIPC and M(IP)₂C was more phosphorylated during myriocin treatment.

In summary, we provide proteomic and phosphoproteomics datasets revealing the complex cellular response to sphingolipid synthesis inhibition. We envision it will provide a rich resource for research on sphingolipid homeostasis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

ER	endoplasmic reticulum
GARP	Golgi-associated retrograde protein trafficking complex
IPC	inositol-phosphorylceramide

MIPC	mannosylinositol-phosphorylceramide
M(IP)₂C	mannosyldiinositol-phosphorylceramide
MAP	mitogen-activated protein
MS	mass spectrometry
PCCA2	progressive cerebello-cerebral atrophy type 2
SNP	single nucleotide polymorphism
Ypk1	yeast protein kinase 1

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FIG. 1. Quantitative proteomic and phosphoproteomic analysis after 30 and 90 minutes of myriocin treatment

(a) Experimental outline. Wild-type cells were grown in the presence of “light” lysine and vehicle-treated or “heavy” lysine and treated with 5 μ M myriocin for 30 or 90 minutes. (b,c,d,e) Venn diagrams show the overlap between three biological replicates. Overlap of phosphopeptides quantified after (b) 30 and (d) 90 minutes of myriocin treatment. Overlap of significant ($P < 0.05$; according to significance A) outliers for (c) 30- and (e) 90-minute experiments. (f,g). Pairwise Scatter plots of heavy/light ratios from each experiment after 30 min myriocin (f) and 90 min myriocin treatment (g). The Pearson correlation coefficient is given. Significant outliers ($P < 0.05$; according to significance A) in the combined dataset

from **(f)** 30- and **(g)** 90-min experiments are color coded in orange. Phosphopeptides with a combined ratio >2.5 are color coded in green **(f)**. Violin plots are added to show the density distribution of phosphopeptides for each experiment **(f, g)**

FIG. 2. Ypk1 kinase is activated in myriocin treated cells

(a) RXRXXpS/pT-motif is significantly upregulated ($p < 0.05$) after 90 min of myriocin treatment. (b) Ypk-target motif [18]. (c) Upregulated phosphopeptides (ratio H/L > 2.5) carrying the RXRXXpS/pT motif. Previously predicted Ypk1 target peptides [18] in green. Other peptides carrying the RXRXXpS/pT motif in red. (d) Phosphopeptides carrying the RXRXXpS/pT motif are highlighted (green, red, blue) among all phosphopeptides (grey). Previously predicted target sites of Ypk1 [18] are shown in green (ratio H/L > 2.5) or in steel blue (ratio H/L < 2.5). Phosphopeptides carrying the RXRXXpS/pT motif not predicted as Ypk1 target sites are shown in red (ratio H/L > 2.5) or blue (ratio H/L < 2.5).

FIG. 3. Sphingolipid metabolism enzymes are regulated by myriocin treatment

(a and b) Changes in protein levels of sphingolipid metabolism enzymes in response to myriocin treatment after 30 (a) or 90 minutes (b). Proteins are color coded according to significance. Red ($P < 10^{-11}$), orange ($P < 10^{-4}$), or light blue ($p < 0.05$). Sphingolipid metabolism enzymes are labeled in green. (c and d) Changes in phosphopeptide abundances of sphingolipid enzymes after 30 (c) and 90 minutes (d) of myriocin treatment. Phosphopeptides are color coded according to significance. Red ($P < 10^{-11}$), orange ($P < 10^{-4}$), or light blue ($p < 0.05$). Phosphopeptides of sphingolipid metabolism enzymes are labeled in green. (e) Sphingolipid enzymes are color coded according to their

phosphopeptide heavy/light ratios after 30 and 90 minutes of myriocin treatment (color code in legend; white boxes show proteins with no identified phospho-peptides).

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