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LC/MS BASED DIFFERENTIAL PROTEOMICS OF THE MITOCHONDRIA OF [PSI+] AND [PSI-] *SACCHAROMYCES CEREVISIAE* STRAINS

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OVERVIEW

- Quantitative label-free differential LC/MS^E proteomics analysis has been applied to the study of mitochondria of [PSI+] and [psi-] *Saccharomyces cervisiae* strains.
- In total, 380 proteins from yeast mitochondria were characterized, with 45% showing expression change in the [PSI+] strain.
- Significant prohibitin decrease was measured in the mitochondria of the [PSI+] strain and confirmed by Western blotting.

INTRODUCTION

Proteomics focuses on the high throughput study of the expression, structure, interactions, and, to some extent, function of complex sets of proteins. Differential proteomics aims at finding differences between two or more multi-protein samples, which is imperative for the understanding of many biological problems.

[PSI+] is a protein-based heritable phenotype of the yeast *Saccharomyces cerevisiae*, which reflects the prion-like behavior of the endogenous Sup35 protein release factor. Previous work has shown that the presence of a prion form of this protein in the cytosol can cause respiratory deficiency by decreasing the level of the mitochondrially-encoded Cox2 protein.[1] The goal of the work is to identify proteins that are present at different levels in the mitochondrial fractions of [PSI+] and [psi-] yeast strains.

The latter should allow for the identification of the molecular mechanism of prion-dependent switching between respiratory competence and deficiency.

In this study, a label-free LC/MS based approach was used where data is acquired in an alternating fashion, with low collision energy on the gas cell in the first function, switching to elevated energy in the alternate scan. In neither scan is a precursor ion isolated with the quadrupole, thus providing a parallel approach to ion detection and sequencing. The low energy portion of the obtained data sets is typically used for quantification of the proteins, whereas the combined low and elevated energy information are utilized for qualitative, identification purposes.

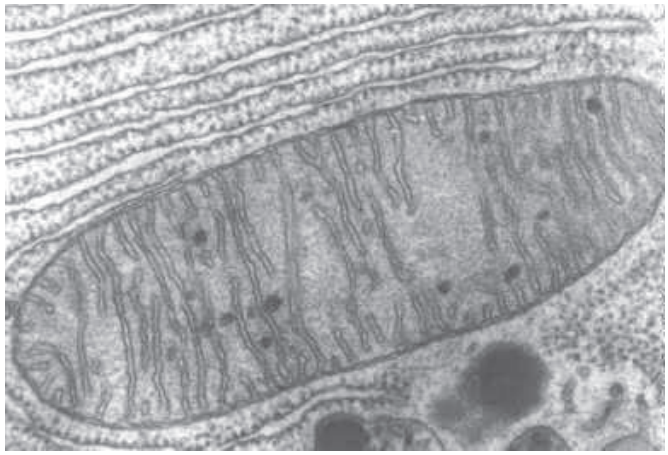

Figure 1. Mitochondria are the cells' power sources and are distinct organelles with two membranes. The key is to get as much energy out of glucose as possible, which is subsequently utilized for various kinds of cellular functions like movement, transport, entry and exit of products, division, etc.

Results obtained from yeast mitochondrial fractions allowed differentiation of proteins originating from [PSI+] vs. [psi-] strains, leading to the identification of a significant decrease of Phb1 and Phb2 (prohibitins) in mitochondria of the [PSI+] strain. The obtained results were confirmed by Western blotting experiments.

EXPERIMENTAL

Sample preparation

Fractionation of yeast cells grown at 30 °C on glycerol medium and purification of mitochondria was performed as previously described.[1] Total protein fractions for Western blotting analysis were received using standard NaOH-TCA precipitation.

The mitochondrial fractions were resolubilized in 50 mM ammonium bicarbonate/0.1% RapiGest™ solution. The proteins were reduced (10 mM DTT) and alkylated (10 mM IAA) prior to enzymatic overnight digestion with trypsin – 1:50 (w/w) enzyme/protein ratio – at 37 °C. RapiGest was removed by the addition of 2 µL conc. HCl, followed by centrifugation, and the supernatant collected. Samples were diluted with 0.1% formic acid to an appropriate final working concentration prior to analysis – corresponding to an 0.3 µg of protein digest on-column load.

LC conditions

LC/MS identification and quantification experiments were conducted using either a 1.5 or 3 hr reversed-phase gradient at 250 nL/min (5 to 40% acetonitrile over 90 or 180 minutes) on the Waters® Identity^E High Definition Proteomics™ System, using as an inlet the nanoACQUITY UPLC® System and a 1.7 µm BEH C_{18} NanoEase™ 75 µm x 20 cm column. Each sample was run in triplicate.

MS conditions

The Identity^E System also included the Q-Tof Premier™ Mass Spectrometer, which was programmed to step between normal (5 eV) and elevated (15 to 40 eV) collision energies on the gas cell, using a scan time of 1.5 s per function over 50 to 1990 m/z, shown in Figure 2.

Data processing and protein identification

Data alignment, protein identifications, and quantitative analysis were conducted with the use of the Identity^E System's dedicated algorithms and peptide ion accounting informatics, as well as searching yeast specific databases. 3D LC/MS^E visualizations were created using development software.

Western blotting

For immunoblotting, proteins were separated by SDS-PAGE gels and transferred electrophoretically to nitrocellulose. After incubation with antibody, visualization was made with anti-rabbit or anti-mouse peroxidase-conjugated antibody. Autoradiograms were quantified using ImageQuant (GE Healthcare Life Sciences) with local average background correction.

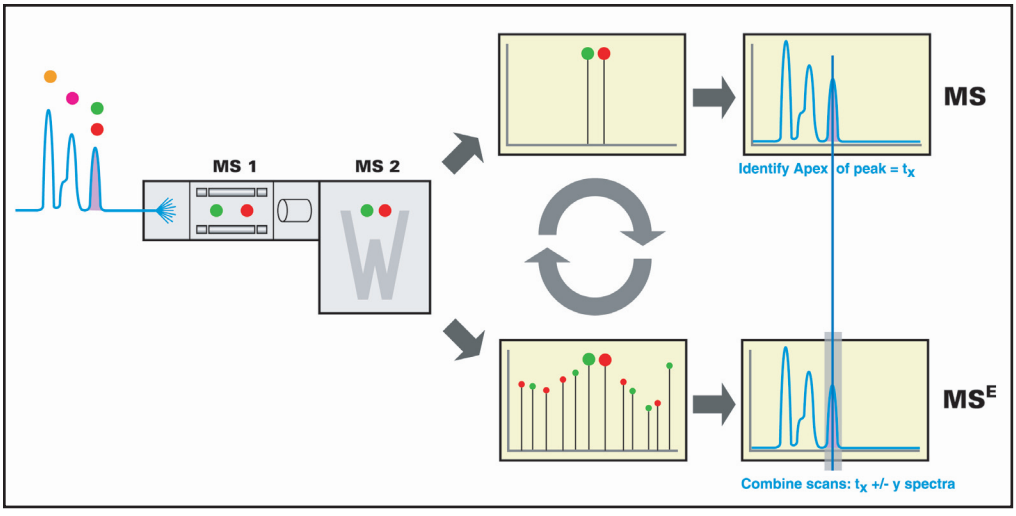

Figure 2. LC/MS^E alternating scanning principle using nanoACQUITY UPLC and Q-Tof Premier and subsequent alignment of the low and elevated energy ions with Identity^E System software.

RESULTS AND DISCUSSION

Alignment

As illustrated in Figure 2, alignment of the low energy precursor ions and elevated energy fragment ions is performed to associate them for identification purposes. However, for quantitative purposes, alignment is also conducted across injections. For a two-condition comparison this translates into the alignment of at least four to six chromatographic data sets – assuming duplicate or triplicate injections, respectively. Hence, both mass accuracy and chromatographic reproducibility are imperative. The latter is demonstrated by the results shown in Figure 3, where expanded portions of the low energy BPI chromatogram are shown for three replicate injections for one of the investigated conditions in this study.

A more comprehensive view of the low and elevated energy chromatograms is shown in Figures 4a and 4b. Figure 4a shows the 3D visualization of a low energy LC/MS^E experiment, with intensity displayed as a function of time and m/z. Figure 4b shows a similar display for the elevated energy information from the same LC/MS^E experiment. Shown inset in Figure 4b is a series of elevated energy ions observed from the time domain direction, which illustrates that high energy fragment ions generated can be time and profile aligned to the correct precursor ion from the low energy data, and subsequently used for identification purposes.

Relative quantification

A binary comparison of the peptide precursor intensity measurements of [PSI+] and [psi-] is discussed in Figure 5. For conditions with identical composition and showing no change in concentration, a 45-degree diagonal line with no variation throughout the detected range would be obtained. This example demonstrates, however, significant deviation from a non-regulated type of distribution, indicating that changes in protein expression occurred between the two investigated conditions of interest.

Displayed (inset) are only those ions that are statistically up- or down-regulated ($p < 0.05$ and $p > 0.95$). For this study, these peptides were subsequently searched utilizing both the peptide accurate precursor mass and accurate mass fragment ion information to identify the parent protein.

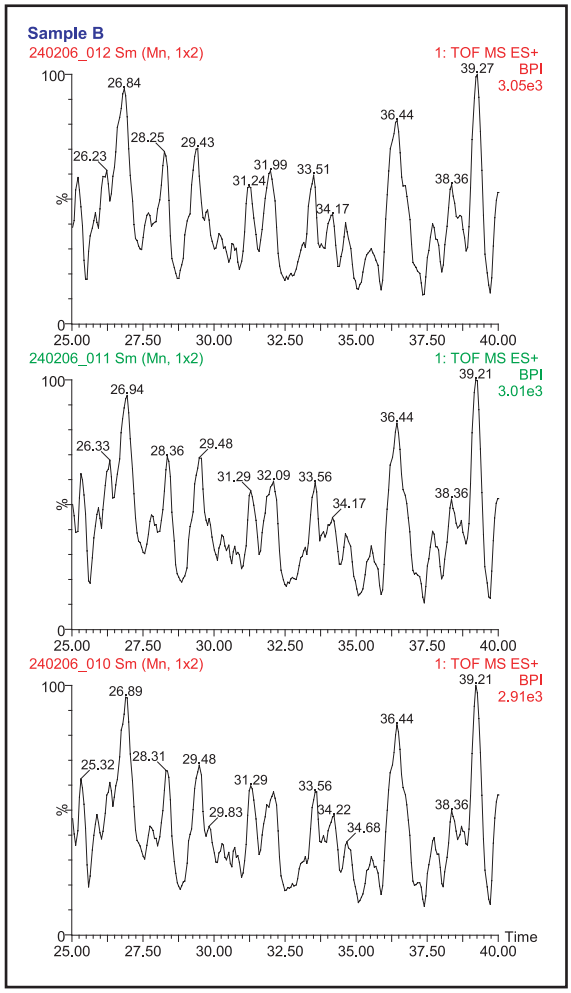

Figure 3. Low energy LC/MS^E chromatogram details for three consecutive injections of condition [psi-], illustrating good quality chromatographic performance and reproducibility.

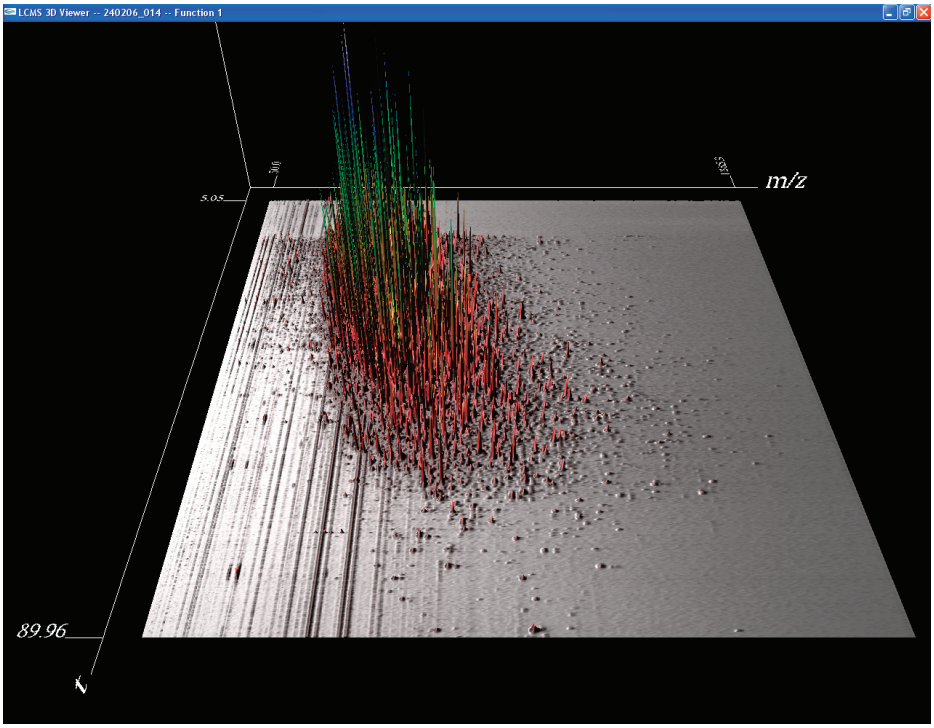

Figure 4a. 3D representation of the low energy chromatogram from the LC/MS^E analysis of the [PRI+] condition.

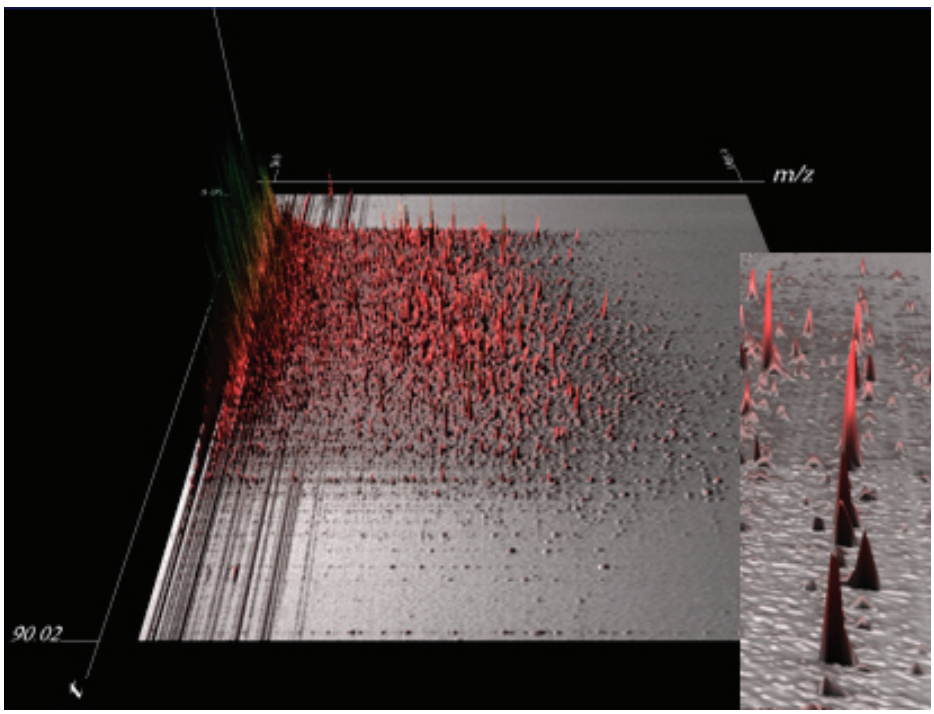

Figure 4b. 3D representation of the elevated energy chromatogram of the [PRI+] condition. The inset shows a series of elevated energy ions with similar profile and elution time from the time domain direction.

In total, 380 proteins were identified, of which approximately 45% were regulated or unique to one of the two conditions. An overview of the number of identified regulated and non-regulated proteins is shown in Figure 6.

Interrogation of the LC/MS^{E} data identified prohibitin 1 (Phb1) and prohibitin 2 (Phb2), which were down-regulated proteins in the mitochondria of the [PSI+] strain. These proteins have been previously reported as membrane bound chaperones stabilizing products of mitrochondiral translation of, for instance, cytochrome c oxidase subunit 2 (Cox2), which catalyses the reduction of oxygen to water.

The regulation of Phb1 and Phb2 was confirmed by means of Western blotting. A comparison of the results in terms of relative amounts is summarized in Figure 7. It can be seen from these results, that good agreement was found using both methods.

Additionally, Phb1, Phb2, and Cox2 were measured in the total fraction of the [PSI+] strain by means of Western blotting. These results showed no significant difference in expression of the targeted proteins occurred within the cytosol. The level of mitochondrically encoded Cox2 remained unchanged, which might imply that the [PSI+] factor can cause delocalization of Phb1 and Phb2.

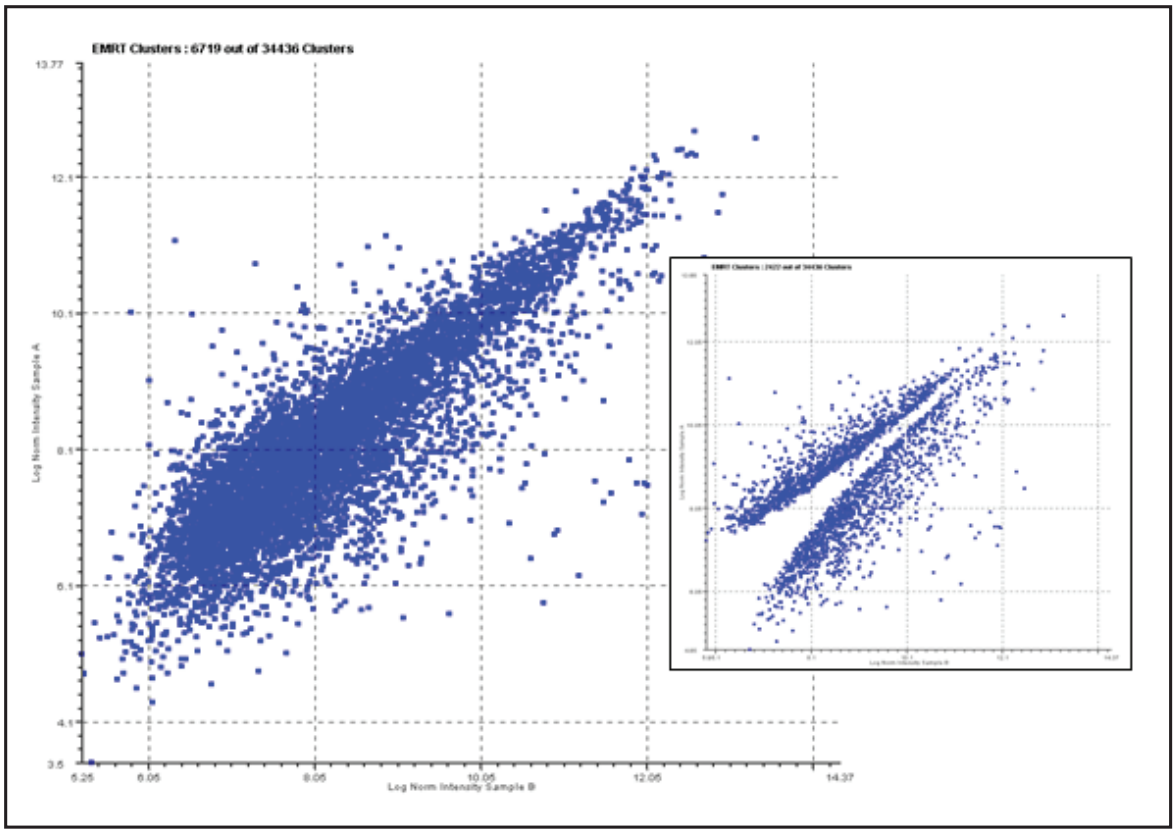

Figure 5. Log intensity accurate mass/retention time clusters [PSI+] vs. log intensity accurate mass/retention time clusters [psi-]. Shown inset are the significantly up- and down-regulated peptides, which can be subsequently used for identification by either using PMF strategies or searches using the elevated energy fragment ion information.

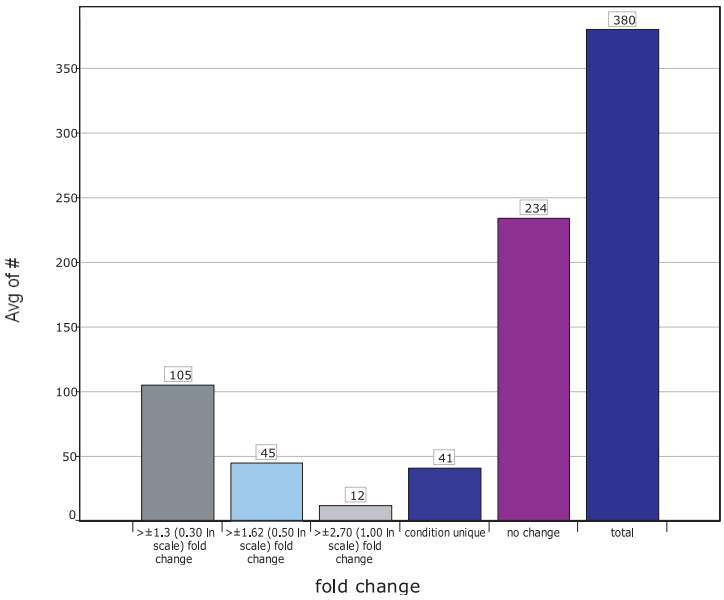

Figure 6. Expression distribution of the proteins regulated and non-regulated mitochondrial proteins for conditions [PSI+] and [psi-].

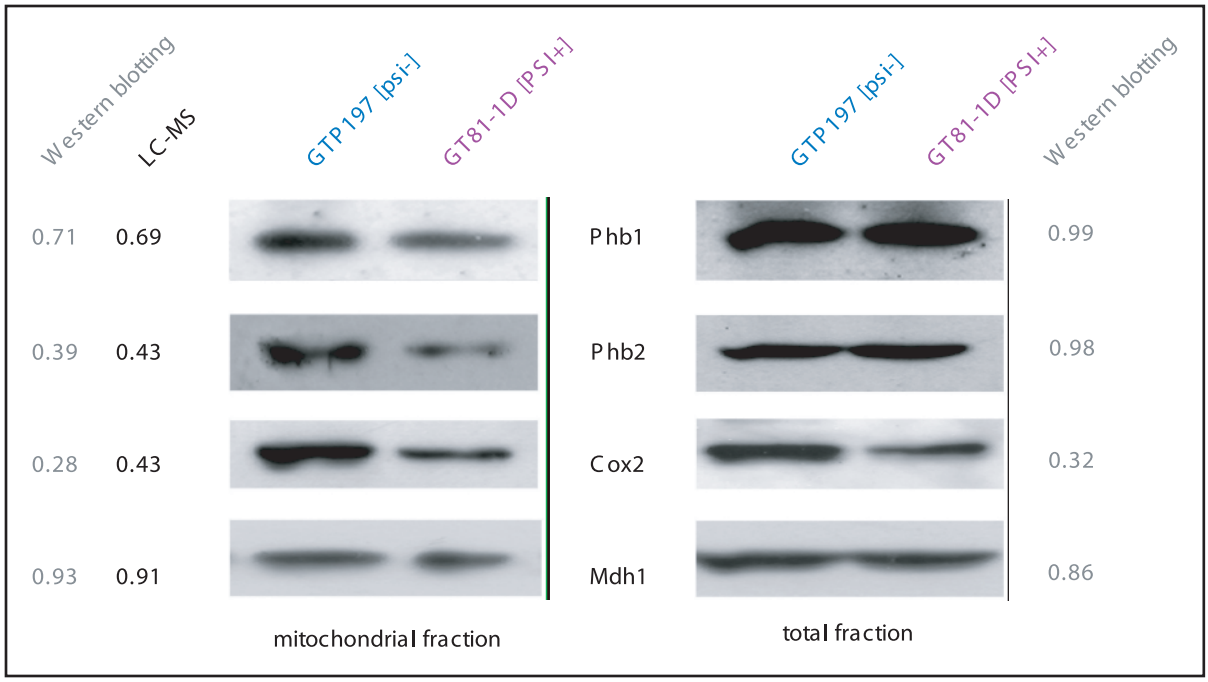

Figure 7. Comparison of Western blotting and nanoscale LC/MS^E for the investigated mitochondrial fractions (left) and the results of Western blotting experiments conducted on the total fractions of yeast strains (right) shows lack of difference in expression of proteins synthesized in cytosol (Phb1 and Phb2), while mitochondrial encoded Cox2 remains decreased in [PSI+] strains. Relative amounts, i.e. [PSI+]/[psi-] are presented for both techniques.

CONCLUSION

- Significant regulation between the investigated strains – [PSI+] and [psi-] – have been identified and quantified.
- Several proteins of interest, found to be down-regulated in the nanoscale LC/MS^E dataset, were validated by biological quantification methods.
- The Western blot analysis showed excellent correlation with the LC/MS^E data.
- Further investigation of the up- and down-regulated proteins from this study is being performed.

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