Quantitative proteomics by amino acid labeling identifies novel NHR-49 regulated proteins in *C. elegans*

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Table isotope labeling by amino acids combined with mass spectrometry is a widely used methodology to quantitatively examine metabolic and signaling pathways in yeast, fruit flies, plants, cell cultures and mice. However, only metabolic labeling using $^{15}\text{N}$ has been applied to examine such events in the nematode *Caenorhabditis elegans*. We have recently shown that *C. elegans* can be completely labeled with heavy-labeled lysine by feeding worms on prelabeled lysine auxotroph *Escherichia coli* for just one generation. We applied this methodology to examine the organismal response to functional loss or RNAi mediated knock down of the transcription factor NHR-49, and found numerous proteins involved in lipid metabolism to be down-regulated, which is consistent with its previously proposed function as a transcriptional regulator of fatty acid metabolism. The combined use of quantitative proteomics and selective gene knockdown by RNAi provides a powerful tool with broad implications for *C. elegans* biology.

Quantitative proteomics is increasingly being applied to examine and understand how cells and organisms regulate their metabolism in order to support growth, proliferation, differentiation, development and survival. Several different strategies for quantitative proteomics have been developed including label-free quantification, chemical labeling e.g., iTRAQ and dimethyl labeling or metabolic labeling using heavy isotopes. The simplest strategy is label-free quantification, where signal intensities of a given peptide in a number of spectra are used as an estimate of the abundance of the sample protein. Such an approach does not require any sample preparation prior to analysis, thus this approach is applicable to all kinds of samples and an indefinite number of experiments can be compared. However, this methodology suffers from sensitivity to variations in sample composition that can easily affect ionization of the relevant peptide and hence alter its apparent abundance. Consequently, as sample complexity grows the utility of such strategy declines. Instead, several labeling approaches have been developed that benefit from stable isotopes having the same physico-chemical properties except from their masses, making distinguishable by mass spectrometry. In vitro labeling includes incorporation of $^{18}\text{O}$ by enzymatic hydrolysis in heavy water and chemical labeling of amino acids containing certain reactive groups. The latter features isotope-coded affinity tags to facilitate purification and isobaric tags for relative and absolute quantitation (iTRAQ). iTRAQ are isobaric tags that, upon fragmentation, release reporter ions of unique masses. The advantage of these in vitro approaches is that any sample or tissue can be labeled. However, the samples undergo a number of preparation steps before labeling and mixing, which increases the risk of introducing a quantitative bias in the samples and thereby decreasing the quantitative accuracy. Particularly, metabolic labeling with stable isotopes has become the prevailing strategy to quantitatively compare proteomes of cells and organisms. *C. elegans* has during the past decade proven to be a powerful model in identification and characterization of novel genes and signaling pathways regulating cell division,
development, aging, apoptosis and metabolism. However, quantitative proteomics has only to a limited extent been applied to study signaling events governing metabolism in *C. elegans*. In 2003 Kruijsgeld et al. showed that *C. elegans* animals can be metabolically labeled with 15N by feeding them on 15N-labeled *E. coli* for two generations, that, when combined with an 14N-labeled worm population, could be used to determine the relative protein abundance among the two populations by mass spectrometry. Henceforth, they applied this strategy to identify differentially expressed proteins in *egl-4* animals compared with wild type animals. Analogously, Yates and coworkers examined how loss of functional insulin receptors affects the proteome of *C. elegans*, and identified novel key regulators of insulin regulated metabolic outputs. Recently, Simonsen et al. used a similar approach to identify differentially expressed proteins in *C. elegans* in response to short- and long-term infection by a pathogenic adherent-invasive strain of *E. coli*, that were isolated from patients suffering from the inflammatory bowel disease Crohn disease. However, compared with metabolic labeling with 15N, stable isotope labeling by amino acids in cell culture (SILAC) provides a more precise mass spectrometry-based quantitative strategy, as it provides a defined number of labels per identified peptide and therefore enables easier and more comprehensive peptide identification and data analysis. Such methodologies have proven to be a highly valuable tool for studies in vivo systems like the yeast *Saccharomyces cerevisiae*, the plant *Arabidopsis thaliana* and mice. Recently, our laboratory and others added *C. elegans* to the SILAC zoo (see below).

We have shown that *C. elegans* can be completely labeled by stable isotope labeled lysine by feeding animals on a lysine auxotroph *E. coli* strain for a single generation. Moreover, following protein extraction from light and heavy labeled *C. elegans* we showed that peptides can be identified and quantified with high accuracy (standard deviation of log2 = 0.22), which is comparable to similar approaches applied on the fruit fly *Drosophila melanogaster*. SILAC based quantitative proteomics is typically based on labeling with both arginine and lysine, which provides one label per peptide after trypsin digestion, and hence improved proteome coverage. Although we only labeled *C. elegans* with lysine, and subsequently digested with lysyl endopeptidase (Lys-C) resulting in longer peptides, we were able to identify and quantify a vast number of proteins due to intensive peptide fractionation prior to mass spectrometry analysis. Moreover, arginine to proline conversion imposes a major challenge in peptide identification and quantification. To this end, Larance et al. recently showed that up to 20% of the proline become labeled when *C. elegans* is labeled with arginine, which can be prevented by RNAi mediated knock down of ornithine transaminase, *orn-1*, required for the conversion. However, labeling with lysine combined with extensive peptide fractionation may be advantageous as *orn-1* knock down may interfere with the metabolism of the nematode, and prevents that other genes are efficiently knocked down by RNAi.

One of the major advantages by *C. elegans* as a model organism is the unprecedented applicability of RNA interference to systematically study gene functions. We therefore rendered the lysine auxotroph *E. coli* strain RNAi compatible by modifying it to express the T7 RNA polymerase from an IPTG-inducible promoter and by eliminating RNaseIII to prevent degradation of dsRNA in *E. coli*. Subsequently, to validate the use of lysine labeling of *C. elegans* in quantitative proteomics studies we aimed to identify differentially expressed proteins in response to functional loss or RNAi mediated knock down of the nuclear hormone receptor NHR-49. The expression of genes involved in lipid metabolism in *C. elegans* is coordinately controlled by a number of transcription factors including the NHR-49, which is a hepatocyte nuclear factor (HNF)-4x ortholog and has a function analogous to that of peroxisome proliferator-activated receptor α, PPARα, in mammals. By quantitative proteomics we identified 3949 and 4627 proteins, respectively, of which 143 and 330 proteins were differentially expressed in response to disruption or knock down of NHR-49 function. Among the down-regulated proteins we identified proteins involved in lipid metabolism to be significantly overrepresented. In particular, we found that the D9 fatty acid desaturases FAT-5 and FAT-6, which previously have been shown to be controlled by NHR-49, were significantly downregulated in response to functional loss or knockdown of *nhr-49*. Moreover, we found FAT-1 and FAT-2, an α3 fatty acid desaturase and a Δ12 fatty acid desaturase, respectively, to be downregulated. This observation supports the notion that loss of NHR-49 function impedes on fatty acid desaturation leading to accumulation of saturated fatty acids. Besides *fat-5, fat-6* and *fat-7*, van Gilst et al. also found three genes involved in mitochondrial β-oxidation of fatty acids (*ech-1*, *ep-5* and *aβ-2*), three genes involved in peroxisomal β-oxidation (two putative acyl-CoA oxidases and *ech-9*), two genes involved in fatty acid binding/transport (*lbp-7* and *lbp-8*) and two genes involved in the glyoxylate pathway (*glt-7* and *sdha-1*), arguing that NHR-49 is required for fatty acid degradation in *C. elegans*. Accordingly, *nhr-49* animals have enlarged lipid stores. Consistently, we find an array of proteins (Fig. 1 and Table 1) to be downregulated in *nhr-49* animals that either have been shown, or based on sequence similarities to functionally characterized gene products from other model organisms like *Saccharomyces cerevisiae* and mice, are predicted to be involved in β-oxidation of fatty acids or metabolism of acetyl-CoA. Since these proteins contain a C-terminal peroxisomal targeting signal or that their mammalian counterpart previously has been identified to the peroxisomes, our observations suggest that NHR-49 primarily regulates peroxisomal β-oxidation rather than mitochondrial β-oxidation, as suggested by van Gilst et al. The inability to degrade long-chain fatty acids would consequently result in increased intracellular levels of unbound fatty acids and fatty acyl-CoA esters. Consistent with this notion, we find the predicted fatty acid binding protein LBP-3 and acyl-CoA binding protein ACBP-1 to be upregulated in response to loss of NHR-49 function.
These binding proteins may bind, sequester and hence protect cells from detrimental effects of large increases in free fatty acid and acyl-CoA levels, respectively. The fact that we also find glutathione and xenobiotic metabolism to be upregulated may also reflect an increased cellular stress response in response to loss of NHR-49 function.

Gene expression levels are often interpreted based on mRNA levels, yet, it is increasingly recognized that the mRNA and protein levels may not correlate. While the abundance of the majority of the proteins, we identified to be regulated upon impaired NHR-49 function, correlated well with the mRNA levels previously reported by van Gilst et al., the level of some proteins did not correlate with the mRNA level. This may be due to alternative RNA splicing, differences in the half-lives of mRNAs and proteins, as well as rates of transcription and translation.

**Conclusion**

Stable isotope labeling of *C. elegans* with lysine and/or arginine provides a simple and straightforward approach for in vivo incorporation of a label into proteins for mass spectrometry-based quantitative proteomics. We anticipate that the recently described labeling methodologies greatly will facilitate characterization of gene functions in the multicellular organism *C. elegans* and become a widely used technique in all areas of *C. elegans* biology. In contrast to metabolic labeling with $^{15}$N, stable isotope labeling with amino acids provides an in vivo strategy to label proteins with different stable isotopic forms of the amino acids (e.g., lys0, lys4, lys8, Arg4 or Arg10), making it possible to monitor differences at the protein level between multiple conditions or over time in a quantitative manner. Thus, stable isotope labeling with amino acids can be used to monitor how genetic, chemical or environmental perturbations affect the proteome of *C. elegans* over time. Combined with enrichment of posttranslational modified peptides, e.g., phosphopeptides, this approach can also delineate how various signaling cascades are affected in response to a specific perturbation.

*Figure 1.* Downregulation of *nhr-49* by RNAi affects the abundance of proteins involved in fatty acid metabolism. Stable amino acid labeling and quantitative proteomics was used to identify the differentially expressed proteins in L4 stage nematodes treated with *nhr-49* RNAi compared with empty vector controls. Among the regulated proteins, enzymes involved in fatty acid metabolism, especially peroxisomal $\beta$-oxidation, are significantly overrepresented. The indicated protein is known or predicted, based on sequence homology to yeast or mouse orthologs, to be involved in the indicated biochemical pathway. Green and red indicate proteins that become less and more abundant, respectively, in response to RNAi mediated knock down of *nhr-49*. 
Table 1. NHR-49 affects abundance of metabolic enzymes. Quantitative proteomics was used to identify the differentially expressed proteins in L4 stage nematodes treated with nhr-49 RNAi compared to empty vector controls. Among the total number of identified regulated proteins a subset is shown. The log₂ ratios indicate less or more abundant proteins after RNAi against NHR-49. See Fredens et al. for details.¹⁵

| Biochemical Process          | Worm Protein     | Log₂  | Function                              | Yeast homolog | Mouse homolog |
|------------------------------|------------------|-------|---------------------------------------|---------------|---------------|
| **Amino acid metabolism**    |                  |       |                                       |               |               |
|                              | K10H10.2         | -1.28 | Cysteine synthase                     | YGR012W       | CBS           |
|                              | F26H9.5          | -0.59 | Phosphoserine aminotransferase        | SER1          | PSAT1         |
|                              | C31C9.2          | -0.35 | 3-phosphoglycerate dehydrogenase     | SER33         | 3-PGDH        |
|                              | R102.4           | 0.35  | Threonine aldolase                    | GLY1          | THA1          |
|                              | M02D8.4          | -0.91 | Asparagine synthetase                 | ASN2          | ASNS          |
|                              | Y51H4A.7         | -0.62 | Urocanate hydratase                   | UROC1         |               |
|                              | CTH-1            | -1.37 | Cystathionine gamma-lyase             | CY53          | CTH           |
|                              | CTH-2            | -0.69 | Cystathionine gamma-lyase             | CY53          | CTH           |
|                              | R12C12.1         | 0.19  | Glycine decarboxylase                 | GCV2          | GLDC          |
|                              | DDO-2            | -0.89 | D-aspartate oxidase                   | DDO           |               |
| **Carbohydrate metabolism**  |                  |       |                                       |               |               |
|                              | W02H5.8          | -0.52 | Dihydroxyacetone kinase               | DAK1          | DAK           |
|                              | F53B1.4          | 0.35  | UDP-glucose-4-epimerase               | GAL10         | TGDS          |
|                              | R11A5.4          | -0.34 | Phosphoenolpyruvat carboxykinase      | PEPC1         |               |
|                              | FBP-1            | -0.34 | Fructose 1,6-bisphosphatase           | FBP1          | FBP2          |
| **Mitochondrial energy metabolism** |           |       |                                       |               |               |
|                              | ANT-1.2          | -0.70 | ADP/ATP translocator                  | AAC1          | SLC2A31       |
|                              | C44B7.10         | -0.35 | Acetyl-CoA hydrolase                  | ACH1          |               |
|                              | MAI-2            | -0.41 | ATPase inhibitor                      | ATP1F1        |               |
|                              | SUR-5            | 0.47  | Acetoacetyl-CoA synthetase            | ACS2          | AACS1         |
|                              | W10C8.5          | -0.44 | Creatine kinase                       | CM            |               |
|                              | ZC434.8          | -0.41 | Creatine kinase                       | CM            |               |
| **FA transport**             |                  |       |                                       |               |               |
|                              | ACS-22           | -0.46 | Fatty acid transport protein (FATP)   | FAT1          | SLC27A4       |
|                              | ACBP-1           | 0.27  | Acyl-CoA-binding protein              | ACB1          | L-ACBP         |
|                              | LBP-3            | 0.45  | Fatty acid binding protein (FABP)     | FABP4         |               |
| **FA desaturation**          |                  |       |                                       |               |               |
|                              | FAT-1            | -0.51 | α3-desaturase                         |               |               |
|                              | FAT-2            | -0.50 | Δ12-desaturase                        |               |               |
|                              | FAT-5            | -2.31 | Δ-9 desaturase                        | OLE1          | SCD1          |
|                              | FAT-6            | -1.31 | Δ-9 desaturase                        | OLE1          | SCD1          |
| **Mitochondrial FA metabolism** |             |       |                                       |               |               |
|                              | T20B8.3.1        | -1.33 | Carnitine O-acyltransferase           | CAT2          | CROT          |
|                              | ACS-2            | -1.17 | Acyl-CoA synthetase                   | FAA2          | ACF2          |
|                              | MCE-1            | -0.44 | Methylmalonyl CoA epimerase           | MCEE          |               |
|                              | PYC-1            | -0.52 | Pyruvate carboxylase                  | PYC1          | PCX           |
|                              | D1005.1          | 0.31  | ATP-citrate synthase: succinyl-CoA to succinate | LSC           | ACLY          |
|                              | GEI-7            | -0.84 | Malate synthase                       | MLS1          |               |
|                              | ECH-4            | -0.40 | Enoyl-CoA hydratase/Acyl-CoA binding protein | ECI1          | ECI2          |
|                              | K09H11.1         | -0.93 | Acyl-CoA dehydrogenase                | ACAD12        |               |
|                              | ECH-7            | 0.28  | Enoyl CoA hydratase                   | EHD3          | ECHS1         |
| **Peroxisomal FA metabolism** |             |       |                                       |               |               |
|                              | T20B8.3.1        | -1.33 | Carnitine O-acyltransferase           | CAT2          | CROT          |
|                              | ACS-1            | -0.69 | Acyl-CoA synthetase                   | FAT2          | ACF2          |
|                              | ACS-7            | -0.89 | Acyl-CoA synthetase                   | FAT2          | ACF2          |
|                              | ZK550.6          | -1.49 | Converts phytanoyl-CoA to 2-hydroxyphytanoyl-CoA | PHYH         |               |
|                              | B0334.3          | -0.78 | 2-hydroxyacyl-CoA lyase               | YEL020C       | HACL1         |
|                              | B0272.4          | -1.18 | Enoyl-CoA hydratase/isomerase         | ECI1          | PECI          |
|                              | F53C11.3         | -0.69 | 2,4-dienoyl-CoA reductase             | SPS19         | DECR1         |
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| Biochemical Process | Worm Protein | Log₂ | Function | Yeast homolog | Mouse homolog |
|---------------------|--------------|------|----------|---------------|---------------|
| Lipid synthesis     | Y71H10A.2    | 0.36 | Fatty acyl CoA reductase | FAR1          |               |
|                     | MBOA-3       | 0.52 | Lysoospholipid acyltransferase | ALE1          | MBOAT1        |
|                     | SPTL-1       | 0.26 | Serine palmitoyltransferase | LCB1          | SPTLC1        |
|                     | SPTL-2       | 0.49 | Serine palmitoyltransferase | LCB2          | SPTLC3        |
|                     | TAG-38       | 3.45 | Sphingosine phosphate lyase | DPL1          | SGPL1         |
|                     | PMT-1        | -0.52| Phosphoethanolamine N-methyltransferase |               |               |
|                     | PMT-2        | -0.45| Phosphoethanolamine N-methyltransferase |               |               |
|                     | R06C1.2      | 0.32 | Farnesyl diphosphate synthetase | ERG20         | FDPS          |
| Cholesterol transport| VIT-6       | -2.38| Cholesterol transport |               |               |
|                     | VIT-2        | -2.09| Cholesterol transport |               |               |
|                     | VIT-4        | -1.36| Cholesterol transport |               |               |
| Other groups, dehydrogenases | F54F3.4 | -1.24 | short-chain dehydrogenases/reductases family | SPS19         | DHRS4         |
|                     | DHS-9        | -0.72 | short-chain dehydrogenases/reductases family | YMR226C       | DHRS1         |
|                     | DHS-15       | 0.38 | short-chain dehydrogenases/reductases family | YMR226C       | DHRS4         |
|                     | DHS-20       | -0.96| Mitochondrial short-chain dehydrogenase | YMR226C       | HSD16B6       |
|                     | DHS-22       | 0.30 | Mitochondrial short-chain dehydrogenase | ENV9          | RDH12         |
| Cytochrome P450      | CYP-25A2     | -1.02| Cytochrome P450 | ERG11         | CYP3A11       |
|                     | CYP-29A2     | -0.63| Cytochrome P450 | ERG11         | CT033759.1    |
|                     | CYP-33A1     | 0.48 | Cytochrome P450 | ERG5          | CYP17A1       |
|                     | CYP-35C1     | 0.54 | Cytochrome P450 | ERG11         | CYP17A1       |
|                     | CYP-33C7     | 0.73 | Cytochrome P450 | ERG5          | CYP17A1       |
|                     | CYP-13A5     | 0.99 | Cytochrome P450 | DIT2          | CYP46A1       |
|                     | CYP-13A4     | 1.72 | Cytochrome P450 | DIT2          | CYP46A1       |
| Proteases            | F21F8.4      | -0.53| Vacuolar aspartyl protease (proteasome A) | PEP4          | BACE2         |
|                     | Y1684A.2     | 0.24 | Putative serine type carboxypeptidase | YBR139W       | CPVL          |
|                     | Y40D12A.2    | 0.32 | Putative serine type carboxypeptidase | YBR139W       | CTSa          |
|                     | ASP-2        | 0.33 | Vacuolar aspartyl protease (proteasome A) | PEP4          | BACE2         |
|                     | ASP-1        | 0.34 | Vacuolar aspartyl protease (proteasome A) | PEP4          | BACE2         |
|                     | K12H4.7      | 0.35 | Serine protease | PRCP           |               |
|                     | ASP-3        | 0.40 | Vacuolar aspartyl protease (proteasome A) | PEP4          | CTSa          |
|                     | ASP-6        | 0.41 | Vacuolar aspartyl protease (proteasome A) | PEP4          | CTSa          |
|                     | F13D12.6     | 0.43 | Putative serine type carboxypeptidase | YBR139W       | CTSa          |
Table 1. NHR-49 affects abundance of metabolic enzymes. Quantitative proteomics was used to identify the differentially expressed proteins in L4 stage nematodes treated with nhr-49 RNAi compared to empty vector controls. Among the total number of identified regulated proteins a subset is shown. The log2 ratios indicate less or more abundant proteins after RNAi against NHR-49. See Fredens et al. for details.15 (continued)

| Biochemical Process | Worm Protein | Log2 | Function | Yeast homolog | Mouse homolog |
|---------------------|--------------|------|----------|---------------|---------------|
| Lon protease        | C15C8.3      | 1.18 | Vacuolar aspartyl protease (protease A) | PEP4 | BACE2 |
|         | K10B2.2      | 1.27 | Putative serine type carboxypeptidase | YBR139W | CTSA |
| ATP-binding cassette (ABC) transporter | HAF-4 | 0.23 | ATP-binding cassette transporter | MDL1 | TAP2 |
|         | ABT-4        | 0.36 | ATP-binding cassette transporter | YOL075C | EP300 |
|         | MRP-2        | 0.38 | ATP-binding cassette transporter | YCF1 | ABCC1 |
|         | PGP-6        | 0.56 | ATP-binding cassette transporter | STE6 | ABCB11 |
|         | MRP-5        | 0.59 | ATP-binding cassette transporter | YOR1 | ABCB12 |
|         | PGP-9        | 0.82 | ATP-binding cassette transporter | STE6 | ABCB11 |
| Glutathione S-transferase | GST-6 | 0.73 | Glutathione S-transferase | HPGDS |
|         | GST-7        | 0.38 | Glutathione S-transferase | HPGDS |
|         | GST-38       | 1.01 | Glutathione S-transferase | HPGDS |

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