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

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Stable 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}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.

Keywords: stable isotope labeling, metabolic labeling, proteomics, SILAC, *C. elegans*, RNAi, lipid metabolism, nuclear hormone receptors

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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.[1] 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.[2-5] 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.[3] 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 physicochemical properties except from their masses, making distinguishable by mass spectrometry. In vitro labeling includes incorporation of ^{18}O by enzymatic hydrolysis in heavy water and chemical labeling of amino acids containing certain reactive groups.[3] 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.[1] However, quantitative proteomics has only to a limited extent been applied to study signaling events governing metabolism in *C. elegans*. In 2003 Krijgsveld et al. showed that *C. elegans* animals can be metabolically labeled with ^{15}N by feeding them on ^{15}N-labeled *E. coli* for two generations,[6] that, when combined with an ^{14}N-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 *glp-4* animals compared with wild type animals.[6] 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.[7] 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.[8] However, compared with metabolic labeling with ^{15}N, stable isotope labeling by amino acids in cell culture[9,10] (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 in vivo systems like the yeast *Saccharomyces cerevisiae*,[9,11] the fruit fly *Drosophila melanogaster*,[12] the plant *Arabidopsis thaliana*[13] and mice.[14] Recently, our laboratory and others added *C. elegans* to the SILAC zoo (see below).[15,16]

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.[15] 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 $\log_2 = 0.22$), which is comparable to similar approaches applied on the fruit fly *Drosophila melanogaster*.[12] 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.[16] 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.[17]

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)-4α 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.[15] Among the downregulated proteins we identified proteins involved in lipid metabolism to be significantly overrepresented. In particular, we found that the Δ9 fatty acid desaturases FAT-5 and FAT-6, which previously have been shown to be controlled by NHR-49,[18] were significantly downregulated in response to functional loss or knockdown of *nhr-49*. Moreover, we found FAT-1 and FAT-2, an ω3 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.[18] 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*, *cpt-5* and *acs-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 (*gei-7* and *sdha-1*), arguing that NHR-49 is required for fatty acid degradation in *C. elegans*.[18] Accordingly, *nhr-49* animals have enlarged lipid stores.[18] 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.[18] 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.

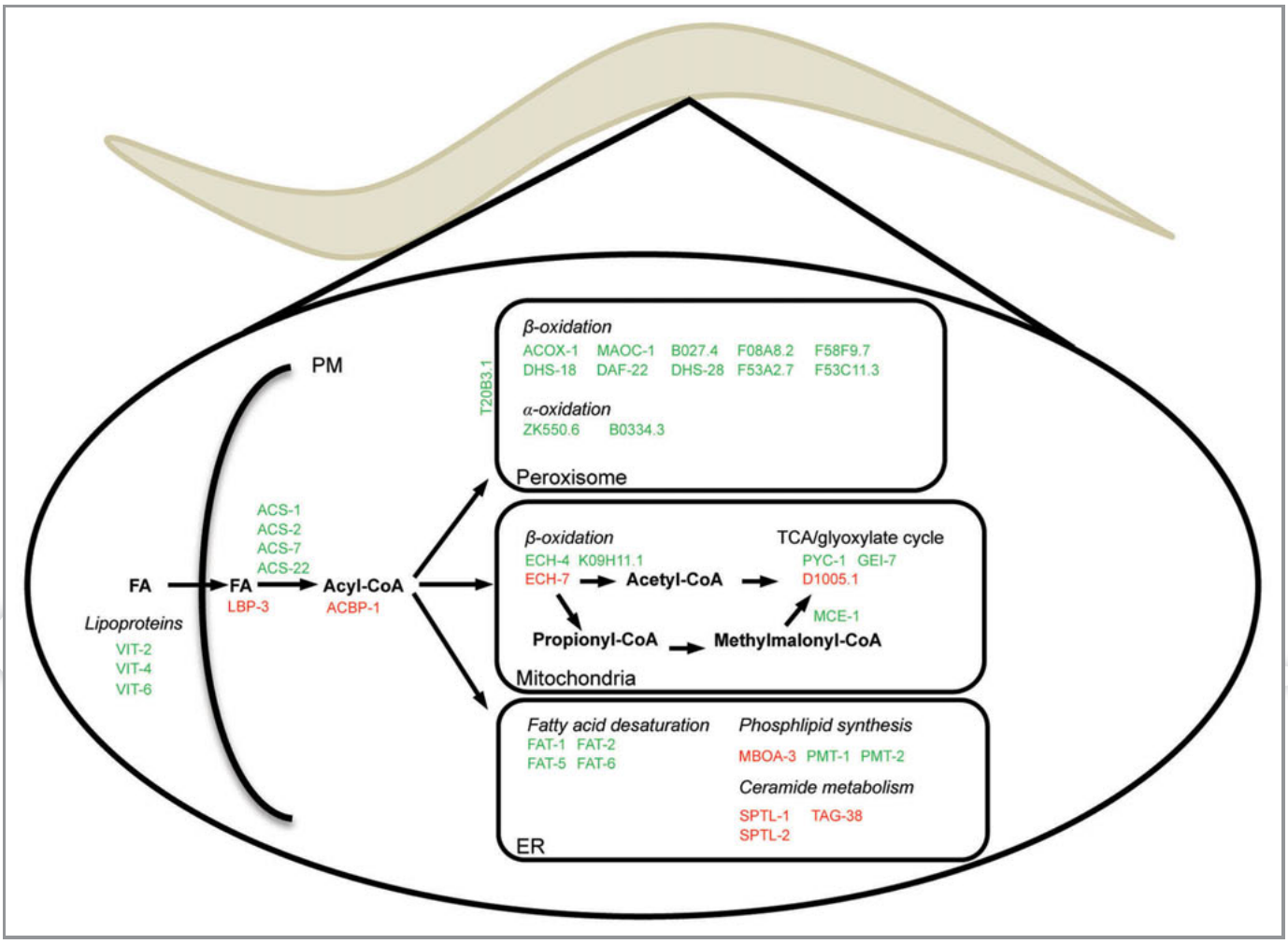

Figure 1. Downregulation of *nhr-49* by RNAi affects the abundance of proteins involved in fatty acid metabolism. Stable amino acid labeling and quantitative proteomics was use to identify the differentially expressed proteins in L4 stage nematodes treated with *nhr-49* RNAi compared with empty vector controls. Among the regulated proteins, enzymes involved in fatty acid metabolism, especially peroxisomal β-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*.

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.[19,20] 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.,[18] the level of some proteins did not correlate with the mRNA level.[15] 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.[21]

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[15,16] 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.

Table 1. NHR-49 affects abundance of metabolic enzymes. Quantitative proteomics was use 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 log_2 ratios indicate less or more abundant proteins after RNAi against NHR-49. See Fredens et al. for details.[15]

Biochemical Process	Worm Protein	Log_2	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	CYS3	CTH
	CTH-2	-0,69	Cystathionine gamma-lyase	CYS3	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		PEPCK1
	FBP-1	-0,34	Fructose 1,6-bisphosphatase	FBP1	FBP2
Mitochondrial energy metabolism	ANT-1.2	-0,70	ADP/ATP translocator	AAC1	SLC25A31
	C44B7.10	-0,35	Acetyl-CoA hydrolase	ACH1	
	MAI-2	-0,41	ATPase inhibitor		ATPIF1
	SUR-5	0,47	Acetoacetyl-CoA synthetase	ACS2	AACS1
	W10C8.5	-0,44	Creatine kinase		CKM
	ZC434.8	-0,41	Creatine kinase		CKM
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	ω3-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	T20B3.1	-1,33	Carnitine O-acyltransferase	CAT2	CROT
	ACS-2	-1,17	Acyl-CoA synthetase	FAA2	ACSF2
	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	LSC1	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	T20B3.1	-1,33	Carnitine O-acyltransferase	CAT2	CROT
	ACS-1	-0,69	Acyl-CoA synthetase	FAT2	ACSF2
	ACS-7	-0,89	Acyl-CoA synthetase	FAT2	ACSF2
	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	Log_2	Function	Yeast homolog	Mouse homolog
	ACOX-1	-1,06	Acyl-CoA oxidase	POX1	ACOX1
	F08A8.2	-0,86	Acyl-CoA oxidase	POX1	ACOX1
	F58F9.7	-1,30	Acyl-CoA oxidase	POX1	ACOXL
	MAOC-1	-1,49	Enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase	FOX2	MFE2
	DHS-18	-1,97	Enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase	FOX2	HSDL2
	DHS-28	-0,62	Enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase	FOX2	HSD17B4
	DAF-22	-1,25	Enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase	FOX2	SCP2
	F53A2.7	-0,39	Acetyl-CoA acyltransferase	ERG10	ACAA2
Lipid synthesis	Y71H10A.2	0,36	Fatty acyl CoA reductase		FAR1
	MBOA-3	0,52	Lysophospholipid 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	ISE1	
	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 (proteinase A)	PEP4	BACE2
	Y16B4A.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 (proteinase A)	PEP4	BACE2
	ASP-1	0,34	Vacuolar aspartyl protease (proteinase A)	PEP4	BACE2
	K12H4.7	0,35	Serine protease		PRCP
	ASP-3	0,40	Vacuolar aspartyl protease (proteinase A)	PEP4	CTSD
	ASP-6	0,41	Vacuolar aspartyl protease (proteinase A)	PEP4	CTSD
	F13D12.6	0,43	Putative serine type carboxypeptidase	YBR139W	CTSA

Table 1. NHR-49 affects abundance of metabolic enzymes. Quantitative proteomics was use 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 log_2 ratios indicate less or more abundant proteins after RNAi against NHR-49. See Fredens et al. for details.[15] (continued)

Biochemical Process	Worm Protein	Log_2	Function	Yeast homolog	Mouse homolog
	C15C8.3	1,18	Vacuolar aspartyl protease (proteinase A)	PEP4	BACE2
	K10B2.2	1,27	Putative serine type carboxypeptidase	YBR139W	CTSA
	Lon protease	-0,60	Serine protease	PIM1	LONP2
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	ABCC12
	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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