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Exploring Novel Function of Yeast Ssa1/2p by Quantitative Profiling Proteomics Using NanoESI-LC−MS/MS

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In the present study, we profiled proteins in *ssa1/2* mutant and wild-type using one-dimensional gel electrophoresis coupled with liquid chromatography and mass spectrometry to reveal a total of 322 proteins. Sixty and 84 nonredundant proteins were detected in *ssa1/2* and wild-type, respectively, whereas 178 were common. A quantitative profiling proteomic approach using a modified N-terminal isotope tagging method was undertaken to determine quantitative changes in proteins between mutant and wild-type. Out of 210 identified proteins selected for quantification, 103 propionylated proteins were obtained. Eight only D_0-propionylated protein (wild-type) and 4 only D_5-propionylated proteins (*ssa1/2*) were detected; 90 proteins were overlapped in the *ssa1/2* mutant and wild-type. In the *ssa1/2* mutant, 28 proteins were up-regulated and 26 were down-regulated. The expression levels of the rest of 49 proteins were not changed compared with the wild-type. Furthermore, non-correlation between mRNA and protein expressions was found. Among up-regulated proteins, 19 proteins involved in protein synthesis, chromatin condensation, and silencing showed unchanged mRNA expression levels. Among down-regulated proteins, 21 proteins consisting mainly of transcription factors showed unchanged mRNA expressions. Surprisingly, several proteins involved in protein synthesis were also found among the down-regulated proteins. These results suggested that the proteins showing changed protein expressions and unchanged mRNA expressions were affected by the deletion of *SSA1* and *SSA2* genes at translational efficiency, mRNA degradation, or protein degradation. Moreover, we found the proteins related to chromosomal control were up-regulated in *ssa1/2* mutant, a novel finding of this study, suggesting that the Ssa1/2p might contribute to chromosomal control.

Keywords: cytosolic Hsp70 • N-terminal isotope tagging • tandem mass spectrometry • yeast • transcriptional control • chromosomal control

1. Introduction

Heat shock protein (Hsp) 70 (70 kDa HSPs) family is found in a variety of organisms, including the unicellular model organism yeast, *Saccharomyces cerevisiae*,[1,2] and the cytosolic HSP70 genes, *SSA1* and *SSA2*, are functionally redundant.[1] *SSA1* is involved in protein transport and rescue of denatured proteins,[3−6] and possesses ATPase activity.[7] The cooperation of Hsp26p with Hsp104p/Ssa1p/Ydj1p chaperone system on protein disaggregation in yeast has been shown.[8,9] Deletion of both the *SSA1* and *SSA2* genes in the yeast strain, *ssa1/2*, causes thermotolerance even at 23 °C.[10] Global mRNA expression analysis of the *ssa1/2* deletion mutant (hereafter called *ssa1/2* or the *ssa1/2* mutant) using cDNA microarray revealed that genes involved in protein synthesis were up-regulated in *ssa1/2*, but markedly suppressed in the mildly heat-shocked wild-type, and genes involved in ubiquitin-proteasome protein degradation were also up-regulated in *ssa1/2*, whereas the unfolded protein response (UPR) genes were highly expressed in the mildly heat-shocked wild-type (Figure 1).[11]

Translated proteins from mRNA go through various post-translational modifications (phosphorylation, glycosylation) followed by maturity. Moreover, excess or unnecessary proteins are degraded by the ubiquitin-proteasome system. Therefore, it has been suggested that there is an inconsistent correlation

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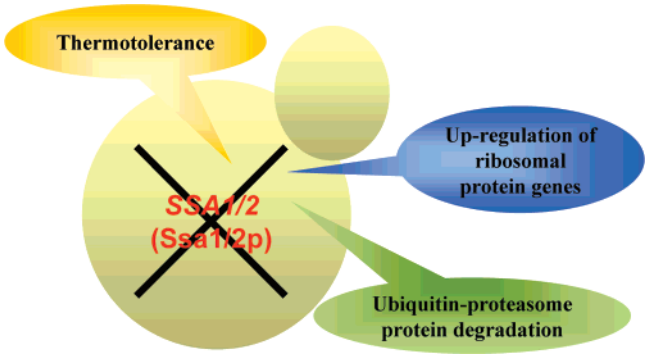

Figure 1. The character of the *ssa1/2* deletion mutant. Deletion of both the *SSA1* and *SSA2* genes in yeast *S. cerevisiae*, *ssa1/2*, causes thermotolerance. Global mRNA expression analysis revealed that genes involved in protein synthesis are up-regulated in *ssa1/2*, and genes involved in ubiquitin−proteasome protein degradation are also up-regulated in *ssa1/2*, whereas the unfolded protein response (UPR) genes are highly expressed in the mild heat-shocked wild-type.

between mRNA and protein expressions. In fact, various studies have shown that there is no correlation between mRNA and protein expressions,[12−14] though it must be remembered that these results depend on methods used, and the number of target proteins are different among them. Keeping the above fact (incomplete correlation between mRNA and protein expressions) in mind, we recently carried out global protein expression analysis in the *ssa1/2* mutant.[15] Two-dimensional gel electrophoresis (2-DGE) was employed to identify protein components whose expression is altered due to deletion of the *SSA1/2* genes in *ssa1/2*. Differentially expressed proteins were identified by nanoelectrospray ionization-liquid chromatography−mass spectrometry (nESI-LC−MS/MS) along with N-terminal amino acid sequencing.[15] In total, 22 unique proteins, among which 9 proteins were up-regulated by 2-fold or more, including many stress response proteins, were identified from the study.[15] Among these, a translation factor, Hyp2p, was found to be induced and highly phosphorylated in *ssa1/2*, suggesting up-regulation of protein synthesis.

Despite its powerful capability of protein profiling, 2-DGE has its limitations for global expression analysis. 2-DGE can separate the numerous proteins into "individual" spots; however, membrane proteins and alkaline p*I* range proteins are difficult to separate on the 2-D gel, thus, reducing the coverage of 2-DGE-based approach. This drawback can be partially overcome using one(1)-DGE. Therefore, in the present study, we applied a more global level protein expression analysis in *ssa1/2* by 1-DGE coupled with nESI-LC−MS/MS (shotgun proteomics/direct MS technique). Furthermore, we also employed a modified N-terminal isotope tagging (NIT) method (uses D_0/D_{10}-propionic anhydride as the label[16]), which is one of the powerful quantitative proteomics tools in profiling proteomics, to reveal quantitative differences in proteins between *ssa1/2* and wild-type. The NIT technology quantifies proteins rapidly and with high sensitivity.[16] The NIT method is a chemical incorporation tagging technique that has advantage in easy accessibility by a simple reaction, sample preservation by nonremoval of peptide which increases the identification possibility, low levels of impurity peaks by small tagging agent, and no multiple-charge overlap by 5 Da difference between native peaks and isotope tagging peaks, which removes quantification errors. Also recently, Reinders and coworkers showed its (NIT) high performance in yeast mitochondrial proteomics using 1D-SDS-PAGE (1-DGE) and LC−MS/MS compared to 2-DGE or multidimensional LC−MS/MS.[17] The operation of 1-DGE is simple, and the protein amounts are enough to combine with the NIT method. As the whole gel slice of a defined width is excised and digested with trypsin, the rate of identification of proteins will be higher than that with 2-DGE.

Our study revealed a total of 322 proteins in the *ssa1/2* mutant and wild-type, out of which 210 proteins were selected for quantification. Present results revealed that the proteins involved in chromatin silencing, chromosome condensing, and telomere maintenance were up-regulated, and several transcription factors were suppressed in the *ssa1/2* mutant.

2. Materials and Methods

2.1. Yeast Strains and Growth Conditions. *S. cerevisiae* JN14 is the *ssa1/2* mutant strain (*MATa his3−11, 3−15 leu2−3, 2−112 ura3−52 trp1-Δ1 lys2? Ssa1−3::HIS3 ssa2−2::URA3*).[18] *S. cerevisiae* JN54 (*MATa his3−11, 3−15 leu2−3, 2−112 ura3−52 trp1-Δ1 lys2?*) is the parent strain (wild-type) of the *ssa1/2* mutant.[18] For protein extraction, the yeast cells were incubated in 100 mL of YPD (1% yeast extract, 2% polypeptone, and 2% glucose) medium at 30 °C to a logarithmic phase ($OD_{660} = 1$) using 500 mL Erlenmeyer flasks, and collected by centrifugation. Cells were washed twice with Milli Q (MQ) water, and stocked in a −80 °C deep freezer until used for protein extraction. Three bioreplicates were performed in the study.

2.2. Preparation of Yeast Crude Protein Extract. Frozen yeast cells (250 mL YPD culture) were ground in liquid nitrogen using a pre-chilled ceramic mortar and pestle. The cell powder was transferred into 5 liquid nitrogen-chilled Eppendorf tubes, followed by extraction of total protein from cells in 650 μL of lysis buffer [LB: 50 mM Tris-HCl, pH 7.4, 300 mM NaCl, 1% Triton X-100, reduced (Sigma, St. Louis, MO), 0.4 mM Na_3Vo_4 (Sigma), 0.4 mM EDTA, 10 mM NaF, 10 mM sodium pyrophosphate (Wako, Tokyo, Japan), 1 mM PMSF (Sigma), 10 μg/mL leupeptin (Sigma), and 10 μg/mL aprotinin, as described in Matsumoto et al.[19]] per tube. Suspended cells in LB were vortexed briefly and sonicated for 1 min. The suspensions were combined into 3 tubes, and then incubated at 4 °C for 20 min with occasional vortexing. Following centrifugation (15 000 rpm, at 4 °C for 15 min), the supernatants were used for protein determination by the method of Bradford,[20] with bovine serum albumin (BSA; Sigma) as standard. Equal amounts of protein for *ssa1/2* mutant and wild-type strains were taken and divided to 2 new eppendorf tubes, followed by addition of 1.6 mL of cold (−20 °C) acetone to 400 μL of protein supernatant per tube, vortexed, and incubated at −20 °C for 1 h. Precipitations were collected by centrifugation (15 000 rpm, at 4 °C for 15 min) and dried at room temperature for 15 min, and tubes were sealed with Parafilm and stored at −80 °C till used for downstream analysis. The workflow is schematically presented in Figure 2.

2.3. 1-DGE, nESI-LC−MS/MS, and Quantification by NIT. Each precipitated protein sample was dissolved in 100 μL of LB, and protein (50 μg per lane) was loaded onto 1-D (sodium dodecyl sulfate-polyacrylamide gel electrophoresis, SDS-PAGE) gels. After SDS-PAGE, gels were stained with Coomassie brilliant blue (CBB) and then cut into 5 slices/lane of same size according to the method reported by Zappacosta and coworkers.[21] Each lane cut into 5 slices had similar protein concentration as determined by the CBB staining results that

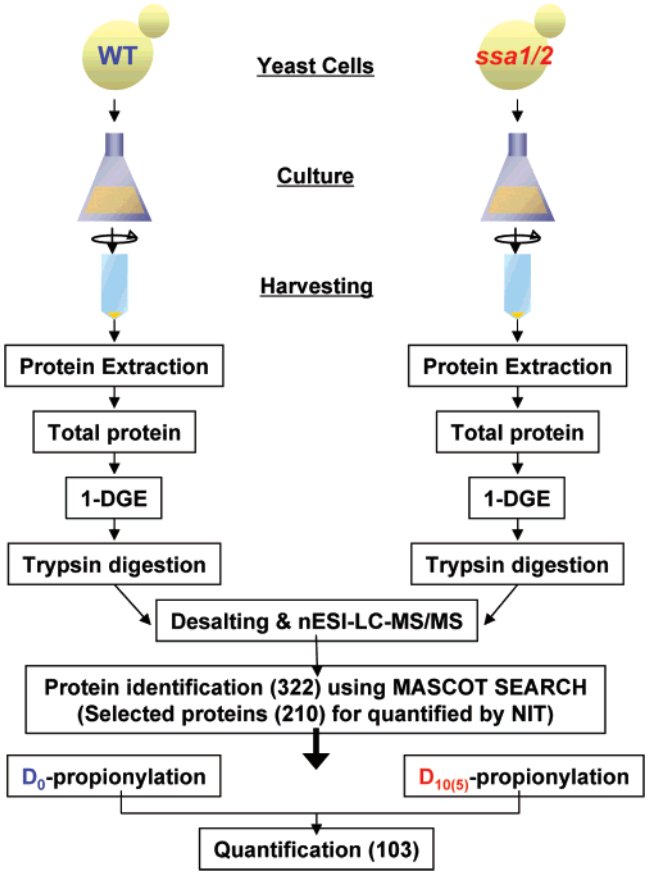

Figure 2. Proteomics workflow for direct MS. *S. cerevisiae* JN14 (*ssa1/2*) and JN54 (wild-type) strains were incubated in YPD medium at 30 °C to logarithmic phase, and then harvested. Total proteins of the *ssa1/2* mutant and wild-type were extracted from 3 independent cultures and placed into independent Eppendorf tubes for each strain. The lysates were precipitated with cold acetone, and precipitated samples were dissolved in lysis buffer. The samples were subjected to 1-DGE, and each lane in gel was divided into 5 regions and sliced out followed by trypsin digestion of the eluted proteins and identification by nESI-LC−MS/MS, MASCOT search engine and NCBInr yeast protein database. For quantification by NIT method, the labeling of wild-type extracts was done with D_0-propionyl anhydride and the *ssa1/2* mutant extracts with D_{10}-propiony anhydride. The labeled and desalted samples were analyzed by LC−MS/MS. For further details see sections 3.2−3.4.

is needed to normalize the digestion efficiency. Approximately, each slice that had 10 μg of protein was treated with trypsin in a ratio (500 ng) of 1/20 to each gel slice, and incubated at 37 °C for 18 h. For protein identification in the each lane, the trypsin-digested samples were analyzed by nESI-LC−MS/MS,[16] and the identified proteins were summarized in supplementary Table 1 in Supporting Information.

For quantification, the digested samples (each sample is from 5 gel slices) were pooled together into 1 tube. From the gel extract, the wild-type proteins were labeled with D_0-propionyl anhydride, and the *ssa1/2* proteins were labeled with D_{10}-propionic anhydride by the procedure of Nam and co-workers.[16] Briefly, for the nESI-LC−MS/MS analysis, samples were separated by C-18 reverse-phase column. An Ultimate nanoLC system, combined with the FAMOS autosampler and Switchos column switching valve (LC-Packings, Amsterdam, Netherlands), was used. The samples were loaded onto precolumn (2 cm × 200 μm i.d.; Zorbax 300SB-C18, 5 μm, Agilent, CA) and washed with the loading solvent (H_2O/0.1% formic acid; flow rate, 4 μL/min) for 10 min to remove salts. Subsequently, a Switchos II column switching device transferred flow paths to the analytical column (15 cm × 75 μm i.d.; Zorbax 300SB C18, 5 μm, Agilent). The nanoflow eluted at a flow rate of 200 nL/min using a 110 min gradient elution from 0% solvent A to 32% solvent B, where solvent A was 0.1% formic acid with 5% acetonitrile, and solvent B was 0.1% formic acid with 90% acetonitrile in water. The column outlet was coupled directly to the high voltage ESI source, which was interfaced to the QSTAR mass spectrometer (Applied Biosystems, Foster city, CA). The nESI-LC−MS/MS running on the QSTAR instrument was acquired in 'Information Dependent Acquisition' (IDA) mode, which allows the user to acquire MS/MS spectra based on an inclusion mass list and dynamic assessment of relative ion intensity. The data acquisition time was set to 3 s per spectrum over m/z range of 400−1500 Da. Acquired data were searched in the National Center for Biotechnology Information (NCBI) protein database for yeast sequence using the MASCOT software package (Version 2.1, Matrix Sciences, U.K.; www-.matrixscience.com). The peptide mass and MS/MS tolerance were 1.0 and 0.8 Da, respectively. The peptides have the allowance of two tryptic miscleavages and also partially modified with oxidation (M) within two charge states (+2, +3). For the compensation of quantificational results, we selected 3-phosphoglycerate kinase (Pgk1p) as an internal standard protein according to a previously published report.[22] To normalize the experimental data based on Pgk1p, we had to use multiplication factor, 1.3, to make equal the level of proteins in *ssa1/2* and wild-type.

2.4. RT-PCR Analysis. Total RNA from *ssa1/2* and wild-type was extracted with the RNeasy Mini Kit (Qiagen, Valencia, CA). RT-PCR was performed using the mRNA Selective RT-PCR Kit (TaKaRa, Shiga, Japan), according to the instructions provided by the manufacturer. The primers used for RT-PCR are described in Table 1, and 0.1 mg of total RNA was used for RT-PCR. After reverse transcription, samples were subjected to a cycling regime of 20−22 cycles (details are mentioned in Table 1). Five to 8 μL of RT-PCR products were loaded into the wells of a 2% Nu-Sieve GTG agarose (Cambrex Bio Science Rockland, Inc., Rockland, ME) gel, and electrophoresis was carried out for 15 min at 100 V. The gels were stained using 10 mg/mL ethidium bromide followed by visualization of the stained bands with an UV-transilluminator (ATTO, Tokyo, Japan).

3. Results

3.1. Proteomics Analysis of *ssa1/2* and Wild-type. In a previous study, we had performed 2-DGE analysis of the *ssa1/2* deletion mutant and compared proteins profiles with the wild-type.[15] Although numerous CBB stained proteins spots were detected on the 2-D gels, only 22 proteins could be identified. Therefore, in order to have a more global view of protein expression change, we carried out 1-DGE and MS protein profiling in the *ssa1/2* mutant and wild-type. Moreover, we performed protein quantification using the NIT method.

3.2. The Experimental Strategy. In Figure 2, the experimental strategy is schematically depicted. *S. cerevisiae* JN14 (*ssa1/2* mutant) and JN54 (wild-type) strains were incubated in YPD medium at 30 °C to a logarithmic phase (OD_{660} = 1), and then harvested. Total proteins were extracted, separated by 1-DGE, stained by CBB, excised, and analyzed by nESI-LC−MS/MS,

Table 1. The RT−PCR Primers Used in the Present Study[a]

ORF code	common name	relative ratio	mRNA ratio*	primers		product size (bp)	number of cycles
				forward	reverse		
YGR214	*RPS0A*	ss	1.3	5′−GAAGCTACTGAATGGGCTGAA−3′	5′−CATGGCTAAATGTGTGACAAGA−3′	196	22
YDR227	*S1R4*	11.1	1.2**	5′−TGAAAATGCGAGAAGTCAAA−3′	5′−GCCAAAAGCCCAACTAATCA−3′	222	22
YDL112W	*TRM3*	20.9	0.6	5′−CCGCAGTTATCGTTCATTCC−3′	5′−AAGCAAGGTCTTGCAATGGT−3′	194	22
YPL104W	*MSD1*	9.3	1.3	5′−GGGCTGATTTGGTTGTCAAG−3′	5′−TCATCTCTTCATATACAAGGCAAA−3′	199	21
YLR086W	*SMC4*	7.0	0.8	5′−CGAATTAGCACAACAGTTAGTTGG−3′	5′−ACTGCTGCCCAAAAGAAGAA−3′	207	22
YNL167C	*SKO1*	ws	−1.3	5′−GCAAATTCCGATACCCATGA−3′	5′−GCGGGAGAATAAAAGGGATT−3′	197	22
YNL178W	*RPS3*	−8.8	−0.9	5′−TCTTGCTCCATCTGTCAAGG−3′	5′−AACGTGCACCCTCCTGACTA−3′	188	22
YMR205C	*PFK2*	−3.6	−1.1	5′−CATTGCTGACCATTTGGTTG−3′	5′−TGGTTTCATGGGGTAGTACTTG−3′	202	22
YLR249W	*YEF3*	−2.7	−1.1	5′−GGAATTGGGTGATGCTTACG−3′	5′−TCTGACTAATGGAACGCTTTTTC−3′	216	22
YDR311	*TFB1*	−2.2	−0.9	5′−CGCCTATTTGAAGCCAGTTT−3′	5′−TCCCGCGTTTTTCTATTGTT−3′	238	22
YLL026W	*HSP104*	37.1	5.5	5′-GGATAAGGAAACTGTCAATGTCG−3′	5′−CTTTTGCTCGGGTGTCAAGT−3′	289	21
YJL130C	*URA2*	ws	nd	5′−CGATCATCGTGCTGCTTACT−3′	5′−AAAACTCGACTTTGGGAAATCA−3′	204	21
YEL030W	*ACT1*	−	1.0	5′−CCTTCCAACAAATGTGGATCT−3′	5′−CAGTGCTTAAACACGTCTTTTCC−3′	200	21

[a] *, mRNA ratio is described in Matsumoto et al.;[11] **, reference data which was determined by single experiment; ss, *ssa1/2* mutant specific; ws, wild-typ specific; nd, not detected.

and protein identification was performed using the NCBInr database on the MASCOT search engine. For quantitative analysis by NIT, total proteins of the *ssa1/2* mutant and wild-type were extracted from 3 independent cultures and placed into independent Eppendorf tubes for each strain. The lysates were precipitated with cold acetone, and precipitated samples were dissolved in LB. The samples were subjected to 1-DGE, and each lane in gel was divided into 5 regions and sliced out followed by trypsin digestion of the eluted proteins, and subsequent labeling of wild-type protein extracts with D_0-propionyl anhydride and the *ssa1/2* mutant protein extracts with D_{10}-propiony anhydride was performed. The labeled and desalted samples were injected into the mass spectrometer (nESI-LC−MS/MS), and peptides were marked for identification.

3.3. 1-DGE and LC−MS/MS Identified a Total of 322 Proteins. Following 1-DGE and MS, we obtained protein profiles of the *ssa1/2* mutant and the wild-type. In the present study, the number of identified proteins was 322 from both the *ssa1/2* mutant and wild-type strain. A total of 60 and 84 nonredundant proteins were detected in *ssa1/2* and wild-type, respectively. Between them, a total of 178 nonredundant overlapped proteins were found (Supplementary Table 1 in Supporting Information). Detected proteins that were found in only *ssa1/2* and wild-type were categorized using GO in SGD (Supplementary Figure 1 in Supporting Information). In the *ssa1/2* mutant, proteins mainly belonged to protein biosynthesis, transport, organelle organization and biogenesis, response to stress, and amino acid and derivative metabolism. On the other hand, in wild-type, the proteins were mainly categorized into protein biosynthesis, organelle organization and biogenesis, ribosome biogenesis and assembly, amino acid and derivative metabolism, and RNA metabolism. Thus, compared to our previous 2-DGE study, a large increment of identified proteins was seen, demonstrating the improved protein coverage using the 1-DGE coupled with LC−MS/MS approach. We next focused on the quantification of the detected and identified overlapped proteins between the *ssa1/2* mutant and wild-type.

3.4. Quantification of Propionylated Proteins in Both the *ssa1/2* Mutant and Wild-type. The quantification of the detected proteins is an important part of any global protein expression analysis, which we performed for the *ssa1/2* mutant. The propionylation of proteins provide more precise differential expression protein profiles. The NIT with propionic anhydride reagents is the acylating reaction in high pH solutions. In high pH solution, the primary amine in the peptide works as a nucleophile to the carbonyl group in the middle of propionic anhydride. Consequently, the fission of carbonyl group in D_{10}-propionic anhydride reagent makes the D_5-propionyl-labeled peptide form as a reaction product. The increments after the proper propionyl-reaction are 56 Da/61 Da (D_0-propinic anhydride/D_{10}-propinic anhydride), which seems to modify with half structure of original reagent. As an example, in Figure 3, we show extracted ion chromatograms (XIC) of target peptide from Ssa1p; it is the unique peptide of Ssa1p. The two XICs represent the Ssa1p in each sample (native versus *ssa1/2* mutant), and the peaks intensity show the amount of protein expression. Among 322 total identified proteins, the number of identified proteins selected for quantification was 210 from both the *ssa1/2* mutant and wild-type strain. Of these, the total number of propionylated proteins was 103. All the identified proteins are described in Supplementary Table 2 in Supporting Information. Ninety-nine D_0-propionylated proteins (wild-type) and 94 D_5-propionylated proteins (*ssa1/2* mutant) were detected, and among them, 90 proteins were overlapped in the *ssa1/2* mutant and wild-type (Figure 4). Only 8 D_0-propionylated proteins (wild-type) (excluding the Ssa2p) and only 4 D_5-propionylated proteins (*ssa1/2* mutant) were detected in this study. All these proteins are described in supplementary Tables 3 and 4 in Supporting Information. Wild-type-specific proteins were Ssd1p, Blm10p, Ura2p, Bck1p, Ssc1p, Ssa2p (deleted in the *ssa1/2* mutant), Sko1, and Tcb1p (Table 3), and these were newly found as the down-regulated proteins by the deletion of Ssa1/2p except Ssa2p. Among the *ssa1/2* mutant-specific proteins were Chs3p, Enp1p, Rps0ap, and Fun30p; these were also newly found as the up-regulated proteins by the deletion of Ssa1/2p except Rps0ap.

3.5. The 28 Up-Regulated and the 28 Down-Regulated Proteins in the *ssa1/2* Mutant and Their Functional Categorization. The expression ratio between the *ssa1/2* mutant and wild-type were calculated from the D_0- and D_5-propionyled peaks area of ion chromatograms, and were then normalized using the experimental data based on Pgk1p (multiplication factor, 1.3). Proteins increased or decreased more than 2-fold in the *ssa1/2* mutant over the wild-type were described as up- and down-regulated proteins, respectively. Among them, the numbers of up-regulated proteins were 28 including 4 *ssa1/2* mutant-specific proteins, and the down-regulated proteins were 26 including 8 wild-type-specific proteins. The expression levels of the rest of 49 proteins were not changed (Figure 4). These proteins were categorized using GO in SGD (Figure 5). Main

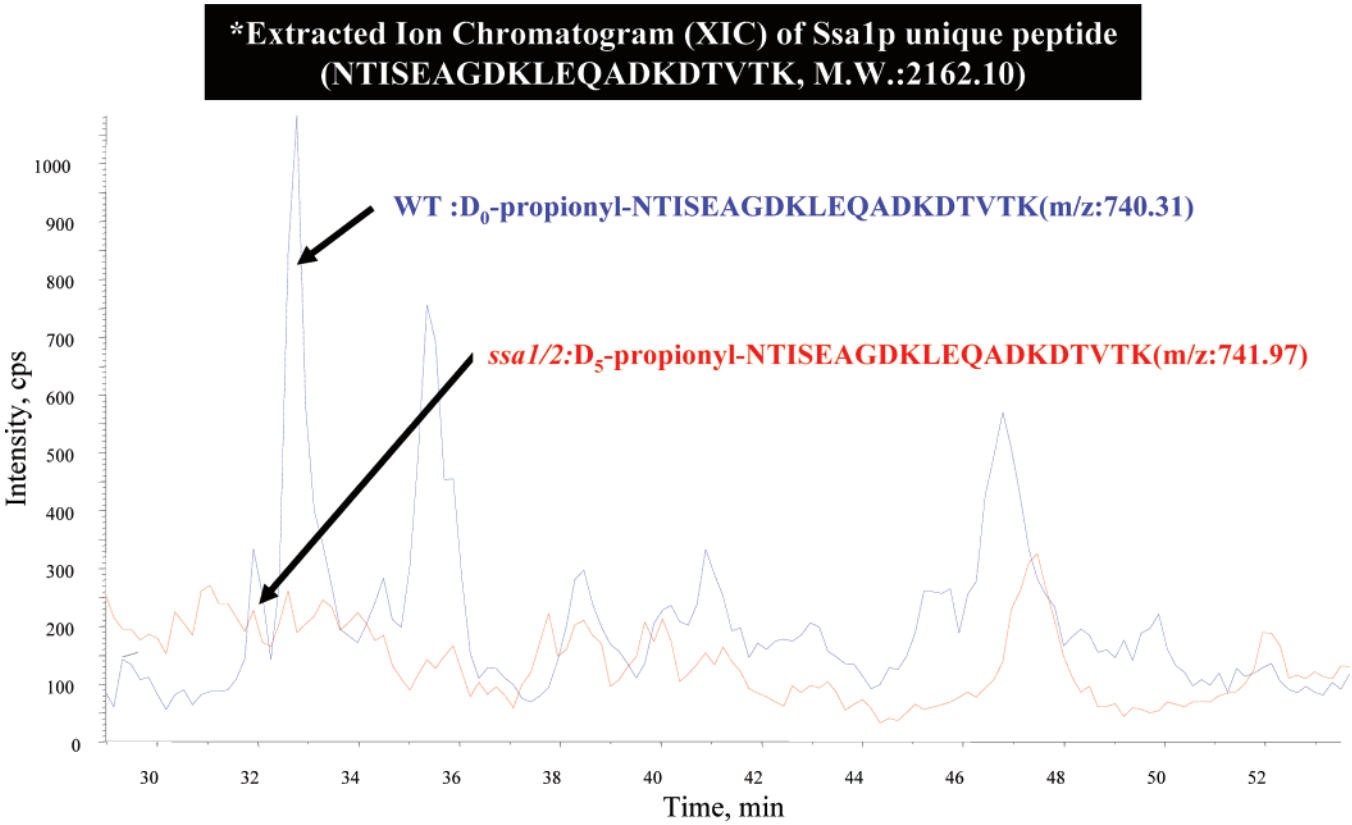

Figure 3. Ion chromatogram of target peptide (in Ssa1p) in the *ssa1/2* mutant. The extracted ion chromatogram (XIC) of Ssa1p unique peptide [NTISEAGDKLEQADKDTVTK; MW, 2162.10; *m*/*z*, 721.7(+3)] is shown. The two XICs represent the Ssa1p in each sample (native versus *ssa1/2* mutant), and the peaks intensity show the amount of protein expression. In the wild-type (WT) sample, this peptide is labeled with D_0-propionylation (*m*/*z*: 740.31) and eluted at 32.93 min. In the *ssa1/2* mutant sample, D_5-propionylated peptide (741.97) was not detected in a nearby retention time.

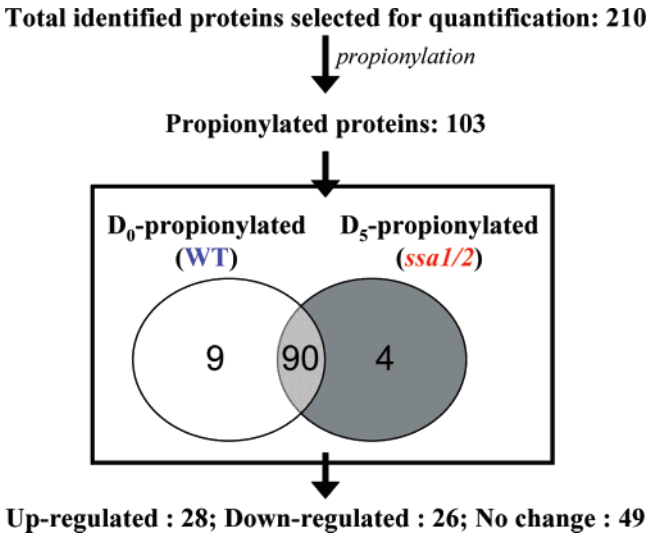

Figure 4. Protein quantification in *ssa1/2* and wild-type using a modified NIT method. The number of selected proteins for quantification was 210 from both the *ssa1/2* mutant and wild-type strains. The total number of propionylated proteins was 103. All the identified proteins are described in Supplementary Table 2 in Supporting Information. Nine only D_0-propionylated proteins (wild-type) and 4 only D_5-propionylated proteins (*ssa1/2* mutant) were detected, and 90 proteins were overlapped in the *ssa1/2* mutant and wild-type.

functional categories that were up-regulated in *ssa1/2* were as follows: organelle organization and biogenesis, response to stress, transport, translation (i.e., protein biosynthesis), and RNA metabolic process. On the other hand, main functional categories that were down-regulated in *ssa1/2* were as follow: translation, response to stress, and organelle organization and biogenesis.

3.6. mRNA Expression Analysis of Selected Proteins. We performed confirmatory RT-PCR for candidates with different expressions of mRNA and proteins. Five up- and down-regulated proteins were chosen for mRNA expression analysis. RT-PCR analysis of *HSP104* was carried out as a positive control, of *URA2* as a negative control, and of *ACT1* as a housekeeping gene. The results presented in Figure 6 showed good correlation to previous global mRNA expressions.[11] It implies that protein expressions are altered even when mRNA expressions are not changed in the *ssa1/2* mutant.

4. Discussion

4.1. 1-DGE Coupled with LC−MS/MS Identified 322 Proteins, whereas Quantification by NIT Revealed 103 Propionylated Proteins. In the present study, 322 proteins were identified from both *ssa1/2* and wild-type comprising 60 and 84 nonredundant proteins, respectively. Compared with 2-DGE, where only 22 proteins were identified,[15] the 1-DGE and MS approach provided an improved protein coverage. Furthermore, these results provide us with new information on protein components of the *ssa1/2* mutant. When the NIT method was employed, 103 propionylated proteins were quantified (Figure 4). In total, 28 up-regulated and 26 down-regulated proteins were found by the deletion of Ssa1/2p in the *ssa1/2* deletion

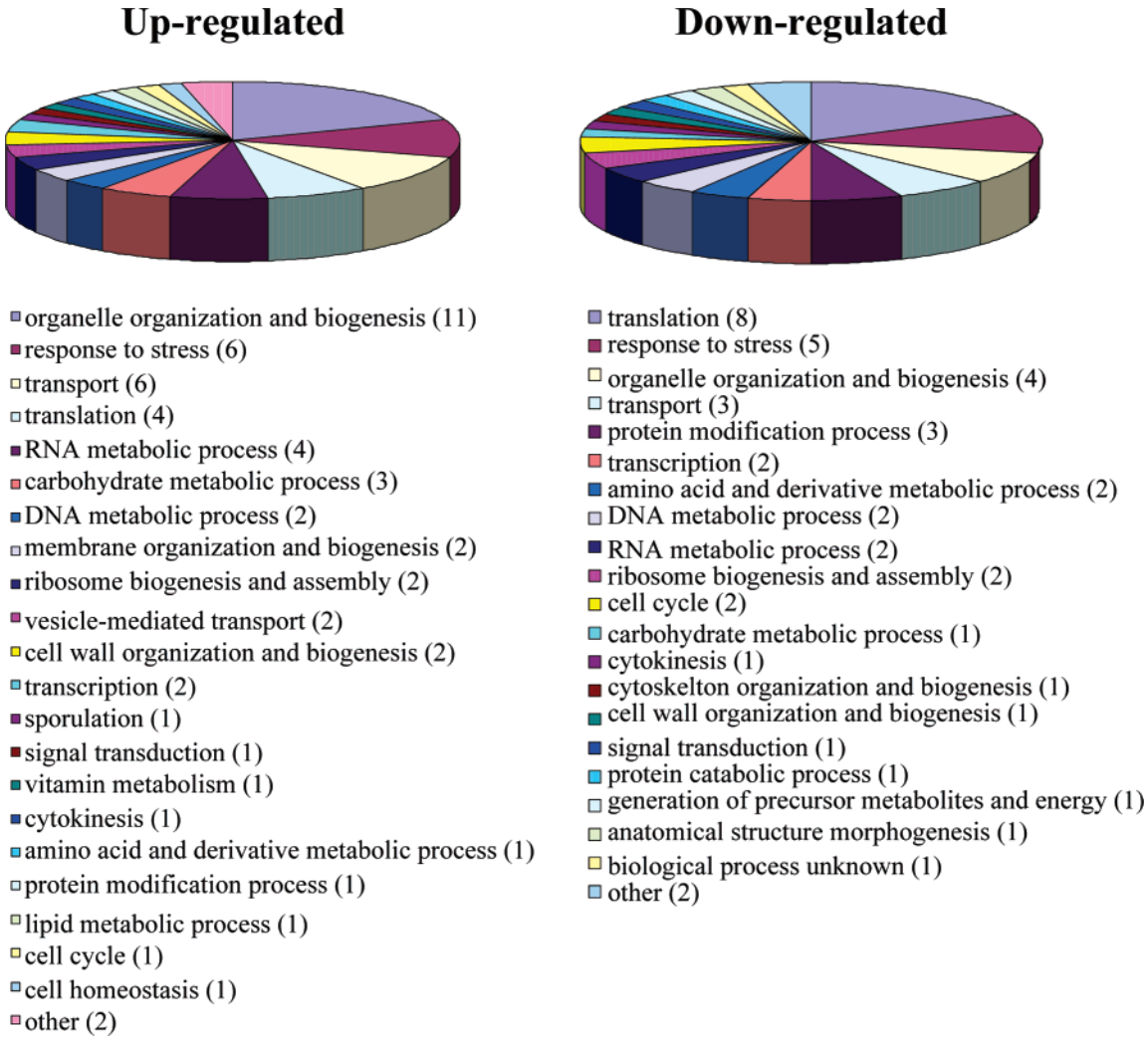

Figure 5. Functional categorization of the up- and down-regulated proteins in the *ssa1/2* mutant. Main functional categories that were up-regulated in the *ssa1/2* mutant were as follows: organelle organization and biogenesis, response to stress, transport, translation (i.e., protein biosynthesis), and RNA metabolic process. On the other hand, main functional categories that were down-regulated in the *ssa1/2* mutant were as follow: translation, response to stress, and organelle organization and biogenesis.

mutant. The expression levels of the rest of 49 proteins were not changed. This shotgun proteomics strategy identified a large number of proteins with high confidence, because we could only find 9 up-regulated and 2 down-regulated proteins from the previous 2-DGE analysis.[15] Moreover, we could get an overview of the mutant functional categories from these results. The main functional categories were similar between *ssa1/2* and wild-type, and therefore, only a detailed analysis of their individual protein components might reveal the character of the *ssa1/2* mutant.

4.2. Overview of the Up- and Down-Regulated Proteins in the *ssa1/2* Mutant. We found that 4 proteins were specific or highly expressed in the *ssa1/2* mutant. Chs3p encodes chitin synthase III,[23,24] which is required for synthesis of cell wall chitin. It has been shown that the intracellular localization of Chs3p changed from ER/golgi to plasma membrane by heat stress.[25] Enp1p is a protein associated with U3 and U14 snoRNA, which is required for pre-rRNA precessing and 40S ribosomal subunit synthesis.[26] Rps0ap is a 40S ribosomal subunit protein.[27] Fun30p is potential Cdc28p substrate,[28] and its overexpression affects chromosome stability.[29] All heat-shock proteins (Hsp104p, Ssa3p, Hsc82p, Hsp60p, and Sse1p) that were detected in this experiment were up-regulated and were correlated with our previous mRNA expression data (Table 1, and see Matsumoto et al.[11]). However, we could not find a correlation with the mRNA expressions of other up-regulated proteins in the *ssa1/2* mutant. These included the proteins involved in protein biosynthesis (Enp1p, Rps0ap, Msd1p, Tef2p, and Rps22bp), chromatin silencing (Sir4p), and chromosome organization (Fun30p and Smc4p).

The Table 3 shows the list of down-regulated proteins in the *ssa1/2* mutant. Among them, Ssd1p, Blm3p, Ura2p, Bck1p, Ssc1p, Sko1p, and Tcb1p were specific in the wild-type or highly suppressed in the *ssa1/2* mutant. This is a new finding of this study. Ssd1p interacts with components of the TOR pathway,[30] and Bck1p is MAPKKK acting in the protein kinase C signaling pathway.[31] Both proteins are involved in cellular integrity.[32−34] Sko1p is a basic leucine zipper (bZIP) transcrition factor and is involved in osmotic and oxidative stress responses.[35] The Ssc1 is a mitochondrial Hsp70.[36] Ura2p is bifunctional carbamoylphosphate synthetase and aspartate transcarbamylase, which catalyzes the first steps in the de novo biosynthesis of pyrimidines.[37,38] Tcb1p is reported as lipid-binding protein; however, its function is unknown. In addition, Blm10p is involved in last

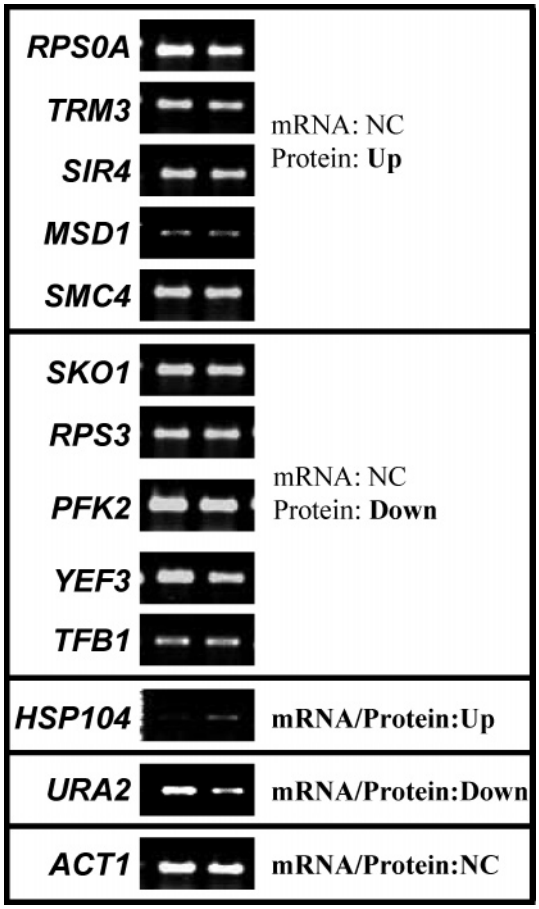

Figure 6. RT-PCR analysis of genes shows incomplete correlation between mRNA and protein expressions in the *ssa1/2* mutant and wild-type. RT-PCR was carried out as described in section 2.4, and primers, product size, and numbers of PCR cycle are described in Table 1.

stage of nuclear proteasome assembly,[39] and it has been reported that Blm10p is a proteasome activator.[40] However, the function of this protein is still controversial. Additionally, the down-regulated proteins contain several transcription factors and transcription-related proteins (Sko1p, Ngg1p, and Tfb1p). The proteins involved in protein biosynthesis (Rps3p, Ssb1p, Rps8bp, Yef3p, Rpl14bp, Rps2p, Rpl19b, and Rpl10p) were also found in the down-regulated group.

4.3. Some Ribosomal Proteins Are Down-Regulated in the *ssa1/2* Mutant. It was surprising to find that several proteins involved in protein synthesis (e.g., ribosomal proteins) were down-regulated in the *ssa1/2* mutant without corresponding down-regulation of mRNA expressions. In the previous global mRNA expression analysis, we did not observe any down-regulation of ribosomal protein genes in the *ssa1/2* mutant,[11] and this contradicts our present study. Many strains lacking individual ribosomal protein genes are lethal; however, the function of each ribosomal protein gene is not exactly clear. We found that Enp1p was up-regulated in *ssa1/2* (Table 2). Enp1p is localized in the nucleus and nucleolus, and it has been recently shown via a large-scale proteomics study that Enp1p interacts with many ribosomal proteins.[41] In fact, Enp1p is required for pre-rRNA processing and the synthesis of 40S ribosome subunit.[26] We also showed the up-regulation of Rps0a and Rps22bp, and down-regulation of Rps3p, Rps8p, and Rps10p (Tables 2 and 3). Thus, various expression levels of ribosomal proteins exist in the Enp1p complex in the *ssa1/2* mutant. This suggests that translational regulation of ribosomal proteins has occurred by the deletion of *SSA1* and *SSA2*, and supports the incomplete correlation between mRNA and protein expression levels.

Meanwhile, a relationship between ribosomal proteins and ubiquitination has also been reported. Rps3p is a component of 40S ribosome subunit and has 66.0% homology with human S3 ribosomal protein. In the HEK293T cells, it has been reported by a large-scale proteomics study of ubiquitinated proteins that S3 ribosomal protein is ubiquitinated.[19] Moreover, it has also been reported that Hsp90 suppresses the ubiquitination of S3.[42] The down-regulation of Rps3p in the *ssa1/2* mutant suggests that Rps3p is degraded by the ubiquitin−proteasome system, that is, ubiquitin−proteasome protein degradation is facilitated by the deletion of *SSA1* and *SSA2*. In the *ssa1/2* mutant, it is speculated that the ubiquitination of Rps3p is facilitated by the deletion of Hsp70 (Ssa1/2p). However, up-regulation of Hsc82p is found in the *ssa1/2* mutant at both mRNA and protein expression levels.[11,15] Thus, it can be hypothesized that, although the level of Hsc82p is increased, its function is inhibited by the deletion of *SSA1* and *SSA2*.

4.4. Is the Down-Regulation of Blm10p Important in the *ssa1/2* Mutant? The down-regulation of Blm10p was found in the *ssa1/2* mutant. Schmidt and co-workers reported a novel proteasome style Blm10p−CP−RP complex by Native PAGE,[40] and it seems that Blm10p has a potential function in degradation of ubiquitinated proteins. However, the deletion of *BLM10* did not result in an increase of ubiquitinated proteins by canavanine treatment,[40] and the authors discussed this reason as follows: "The activity of Blm10-CP-RP complexes is limited to only a specific subset of ubiquitinated proteins, or the effect of *BLM10* deletion might be masked by complemental mechanism".[40] Global mRNA expression analysis and biochemical analysis of the *ssa1/2* mutant revealed the up-regulation of some ubiquitin−proteasome degradation genes along with an increase in the ubiquitinated proteins by the deletion of *SSA1/2*.[11] It seems that the down-regulation of Blm10p is in conflict with the results of global mRNA expression analysis. However, the effect of the down-regulation of Blm10p might be complemented by some other mechanism in the *ssa1/2* mutant; this needs to be investigated in future studies. Therefore, we conclude that the down-regulation of Blm10p does not have any effect on proteolysis in the *ssa1/2* mutant.

4.5. Ssa1/2p Might Contribute to Chromosomal Control. We found three proteins, Smc4p, Fun30p (newly expressed), and Sir4p, to be up-regulated in the *ssa1/2* mutant. They are known Cdc28p-target proteins, and are potentially phosphorylated.[28] Smc4p is a condensin subunit and has a role in chromosome condensation at M-phase in cell cycle (reviewed in Strunnikov et al.[43]).[44,45] Although the function of Fun30p is not well-known, it is speculated that it has a role in chromosome stability.[29] Sir4p is a subunit of silent information regulator[46] and involved in chromatin silencing and the repression of gene expression at the silent mating loci (*HML* and *HMR*), rDNA, and telomeric regions.[47−54] Overexpression of Sir4p reduces the HM loci, telomere, and rDNA silencing called the "anti-*SIR*" effect.[51,54−56] In *ssa1/2*, Sir4p expression was increased by 11-fold over the wild-type (Table 1). It is possible that chromatin silencing is repressed in the *ssa1/2* mutant. In particular, the repression of rDNA silencing can be linked to the up-regulation of genes involved in protein synthesis in the *ssa1/2* mutant at transcriptional level.[11] It must be remembered

Table 2. The Up-Regulated Proteins in the *ssa1/2* Mutant (over 2-fold)[a]

GI acc. no.	ORF code	common name	relative ratio	biological process (GO)	mRNA*
6319497	YBR023C	Chs3p	ss	cell wall chitin biosynthesis cytokinesis spore wall assembly	0.6
6319724	YBR247C	Enp1p	ss	35S primary transcript processing rRNA processing ribosome biogenesis and assembly	1.6
6321653	YGR214W	Rps0ap	ss	translation ribosomal small subunit assembly and maintenance	1.3
6319300	YAL019W	Fun30p	ss	chromosome organization and biogenesis	1
6321895	YHR103W	Sbe22p	45.9	cell wall organization and biogenesis	1.5
6323002	YLL026W	Hsp104p	37.1	chaperone cofactor dependent protein folding response to stress telomere maintenance	5.5
6320091	YDL112W	Trm3p	20.9	tRNA methylation	0.6
6325331	YPR074C	Tk11p	13.9	pentose−phosphate shunt	nd
6319396	YBL075C	Ssa3p	13.9	SRP−dependent cotranslational protein targeting to membrane, translocation protein folding response to stress	3
6320433	YDR227W	Sir4p	11.1	chromatin silencing double−strand break repair via nonhomologous end−joining loss of chromatin silencing during replicative cell aging telomere maintenance	1.2**
6325153	YPL104W	Msd1p	9.3	translation	1.3
6319336	YAR033W	Mst28p	8.3	vesicle organization and biogenesis	1
6319772	YBR295W	Pca1p	8.3	cadmium ion transport iron homeostasis metal ion homeostasis	1
6322631	YKL218C	Sry1p	8.3	amino acid derivative catabolic process	1
6321005	YER157W	Cog3p	7.0	ER to Golgi vesicle-mediated transport intra-Golgi vesicle-mediated transport retrograde transport, vesicle recycling within Golgi	0.6
6323115	YLR086W	Smc4p	7.0	mitotic chromosome condensation mitotic sister chromatid segregation	0.8
6319616	YBR140C	Ira1p	7.0	negative regulation of Ras protein signal transduction negative regulation of cAMP biosynthetic process positive regulation of Ras GTPase activity regulation of adenylatecyclase activity	nd
6323840	YMR186W	Hsc82p	4.5	proteasome assembly protein folding response to stress telomere maintenance	3.1
6323288	YLR259C	Hsp60p	3.8	protein folding protein import into mitochondrial matrix	2.5
6321693	YGR254W	Eno1p	3.5	gluconeogenesis glycolysis regulation of vacuole fusion, non-autophagic	1.6
6320419	YDR213W	Upc2p	3.5	steroid metabolism sterol biosynthesis	0.8
6319594	YBR118W	Tef2p	2.8	tRNA export from nucleus translational elongation	1.5
6322271	YLR367W	Rps22bp	2.8	translation	0.8
6322010	YHR214C−B	Yhr214c−bp	2.8	transposition, RNA-mediated	nd
6325151	YPL106W	Sse1p	2.7	protein folding telomere maintenance	3.1
6321613	YGR174C	Cbp4p	2.6	protein complex assembly	0.9
6321891	YHR099W	Tra1p	2.3	DNA repair histone acetylation regulation of transcription from RNA polymerase II promoter	nd
6321523	YGR086C	Pil1p	2.1	endocytosis protein localization response to heat	1.4

[a] *, mRNA ratio is described in Matsumoto et al.;[11] **, reference data which was determined by single experiment; ss, *ssa1/2* mutant specific; nd, not detected; description of proteins are presented in Supplementary Table 3 in Supporting Information.

that, although some down-regulated ribosomal proteins were found in the *ssa1/2* deletion mutant, this is due to the translational efficiency. Additionally, it has been shown that the Ssa1p and Sir4p exist in the same complex[28,57] and this supports our idea that Ssa1/2p is involved in chromatin silencing control (Figure 7). Taken together with the high expression of Smc4p and Fun30p, it is suggested that Ssa1/2p might contribute to chromosomal control.

4.6. The Correlation between mRNA and Protein Expressions Is Incomplete in the *ssa1/2* Mutant. Different expression between mRNA and protein abundance in the *ssa1/2* deletion mutant was confirmed from proteome and RT-PCR analysis. We also considered the incomplete correlation between mRNA and protein expressions in the *ssa1/2* mutant. One scenario is that protein expression is up-regulated without any change in mRNA expression. It is possible that there may be an increase of translational efficiency or mRNA degradation by the deletion of *SSA1* and *SSA2*. These include proteins involved in protein synthesis, chromatin condensation, and silencing (Table 2). It has been shown that the genes involved in protein synthesis are up-regulated in the *ssa1/2* mutant at the mRNA expression level.[11] From the present study, it can be suggested that some proteins may be up-regulated without corresponding change in mRNA expression. We report here for the first time that the proteins involved in chromatin control are up-regulated by the deletion of *SSA1* and *SSA2*, and it is possible that transcriptional control may have occurred. However, further study is required to prove this hypothesis. The second scenario to be also

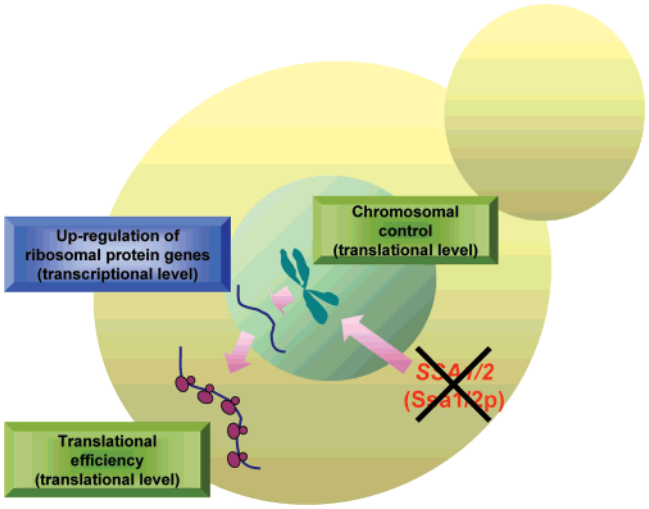

Figure 7. Ssa1/2p might contribute to the chromosomal control. The proteins related to chromosomal control were found to be up-regulated in *ssa1/2* mutant, a novel finding of this study, suggesting that the Ssa1/2p might contribute to chromosomal control. For detailed discussion see section 4.5.

considered here is down-regulation of protein expression with unchanged mRNA expression, due to a possible decrease of translational efficiency or protein degradation by the deletion of *SSA1* and *SSA2*. In particular, with respect to protein degradation, it has been shown that the genes involved in protein degradation are up-regulated, and ubiquitinated proteins increase in the *ssa1/2* mutant.[11] The transcription factors were found in the down-regulated protein group with unchanged mRNA expression in the *ssa1/2* mutant (Table 3). This might be probably related to the up-regulation of chromatin control protein in the *ssa1/2* mutant.

5. Conclusions

Using 1-DGE and LC−MS/MS, the present study identified 322 proteins from both the *ssa1/2* mutant and wild-type strain. By NIT method, 103 proteins were quantified, comprising 8 only D_0-propionylated protein (wild-type) and 4 only D_5-propionylated proteins (*ssa1/2* mutant). From the quantification data, we identified 28 up-regulated and 26 down-regulated proteins in the *ssa1/2* mutant. These results provided new information on the protein components of the *ssa1/2* mutant. Furthermore, an incomplete correlation between mRNA and

Table 3. The Down-Regulated Proteins in the *ssa1/2* Mutant (over 2-fold)[a]

GI acc. no.	ORF code	common name	relative ratio	biological process (GO)	mRNA*
6320499	YDR293C	Ssd1p	ws	cell wall organization and biogenesis replicative cell aging	0.6
37362646	YFL007W	Blm10p	ws	proteasome assembly protein catabolic process	nd
6322331	YJL130C	Ura2p	ws	de novo' pyrimidine base biosynthetic process glutamine metabolic process pyrimidine base biosynthetic process	nd
6322366	YJL095W	Bck1p	ws	establishment of cell polarity protein amino acid phosphorylation protein kinase cascade response to acid response to nutrient unfolded protein response	0.8
6322505	YJR045C	Ssc1p	ws	protein folding protein import into mitochondrial matrix	1.1
6323004	YLL024C	Ssa2p	ws	SRP-dependent cotranslational protein targeting t membrane, translocation protein folding response to stress	nd
6324162	YNL167C	Sko1p	ws	negative regulation of transcription from RNA polymerase II promoter	0.8
6324660	YOR086C	Tcb1p	ws	biological process unknown	0.9
6320381	YDR176W	Ngg1p	−10.5	chromatin modification histone acetylation response to drug	nd
6320598	YDR390C	Uba2p	−9.0	protein sumoylation	0.6
6324151	YNL178W	Rps3p	−8.8	translation response to DNA damage stimulus	1.1
6321812	YHR023W	Myo1p	−4.8	cytokinesis, completion of separation; contractile ring contraction and formation	0.6
6319972	YDL229W	Ssb1p	−4.5	cotranslational protein folding translation regulation of translational fidelity	0.7
6320805	YEL030W	Ecm10p	−4.2	protein refolding protein targeting to mitochondrion	0.5
6323861	YMR205C	Pfk2p	−3.6	glycolysis	0.9
6319399	YBL072C	Rps8ap	−2.9	translation	1.3
6323278	YLR249W	Yef3p	−2.7	ribosome biogenesis and assembly translational elongation	0.9
6321786	YHL001W	Rpl14b	−2.6	translation	1.2
6320936	YER091C	Met6p	−2.4	methionine biosynthetic process	0.7
6321315	YGL123W	Rps2p	−2.4	translation regulation of translational fidelity	1.2
6320432	YDR226W	Adk1p	−2.3	ADP biosynthetic process nucleotide metabolic process	1
6322136	YIL053W	Rhr2p	−2.3	glycerol biosynthetic process response to osmotic stress	1.2
6319444	YBL027W	Rpl19b	−2.2	translation	1.4
6323104	YLR075W	Rpl10	−2.2	translation ribosomal large subunit assembly and maintenance	0.9
6320517	YDR311W	Tfb1p	−2.2	negative regulation of transcription from RNA polymerase II promoter, mitotic nucleotide−excision repair nucleotide−excision repair, DNA duplex unwinding transcription initiation from RNA polymerase II promoter	1.1
6325196	YPL061W	Ald6p	−2.0	acetate biosynthetic process	1.8

[a] *, mRNA ratio is described in Matsumoto et al.;[11] ws, wild-type specific; nd, not detected; description of proteins are presented in Supplementary Table 4 in Supporting informatin.

protein expressions was also shown to exist in the *ssa1/2* mutant. This suggests that a change of translational efficiency or mRNA/protein degradation has occurred by the deletion of *SSA1* and *SSA2*. A notable finding was that proteins related to chromosomal control were up-regulated in the *ssa1/2* deletion mutant, suggesting that the Ssa1/2p might contribute to chromosomal control.

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Supporting Information Available: Figure of the detected proteins that were found only in *ssa1/2* and wild-type categorized using GO in SGD; tables listing the identified proteins by nESI-LC−MS/MS, the quatification of proteins, and description of the up-regulated proteins in the *ssa1/2* mutant. This material is available free of charge via the Internet at http://pubs.acs.org.

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