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A QUANTITATIVE AND TEMPORAL MAP OF PROTEOSTASIS DURING HEAT SHOCK IN *SACCHAROMYCES CEREVISIAE*

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quantification, HSP70 chaperone*

ABSTRACT

Temperature fluctuation is a common environmental stress that elicits a molecular response in order to maintain intracellular protein levels. Here, for the first time, we report a comprehensive temporal and quantitative study of the proteome during a 240-minute heat stress, using label-free mass spectrometry. We report temporal expression changes of the hallmark heat stress proteins, including many molecular chaperones, tightly coupled to their protein clients. A notable lag of 30 to 120 minutes was evident between transcriptome and proteome levels for differentially expressed genes. This targeted molecular response buffers the global proteome; fewer than 15% of proteins display significant abundance change. Additionally, a parallel study in a Hsp70 chaperone mutant (*ssb1Δ*) demonstrated a significantly attenuated response, at odds with the modest phenotypic effects that are observed on growth rate. We cast the global changes in temporal protein expression into protein interaction and functional networks, to afford a unique, time-resolved and quantitative description of the heat shock response in an important model organism.

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Temporal changes in the yeast proteome under heat stress are mapped and integrated to protein networks to reveal cognate groups of chaperones (orange and blue circles) acting on coherent groups of substrate proteins (red and green).

43 **Introduction**

44 All organisms are potentially exposed to adverse environmental conditions at some point
45 during their development. Much effort has therefore been devoted to elucidating
46 mechanisms of stress response in both unicellular and multicellular eukaryotes¹⁻⁴. The
47 budding yeast *S. cerevisiae* has provided an important model organism contributing to our
48 understanding of environmental stress responses at a molecular level for many decades⁵⁻
49 ⁹. Among different types of stresses, heat stress is generally very well understood¹⁰⁻¹².
50 Caused by sudden temperature shifts of as little as a few degrees, it can have many
51 damaging effects and results in pronounced physiological and metabolic changes^{10, 11}. For
52 example: increased protein unfolding and aggregation, destabilisation of cell wall or
53 membranes, metabolic reprogramming, or cell cycle arrest^{10, 11}. In response, a widespread
54 reorganisation of gene expression has been observed. In cases of general cellular stress
55 (proteotoxic, osmotic, starvation, etc.) it is termed the environmental stress response
56 (ESR)^{8, 9, 13} and in the specific case of heat stress, it is simply called the heat shock
57 response (HSR)^{10, 11}.

58 The key protein players in HSR are transcription factors, Hsf1p and the partially redundant
59 Msn2p/Msn4p pair, in addition to various downstream heat-shock effector proteins which
60 mainly act as molecular chaperones. Hsf1 binds to heat shock elements (HSE) in the
61 promoter region of its target genes and activates their transcription^{12, 14, 15}. Hsf1 targets
62 include many chaperones, genes of the protein transport and degradation machinery, cell
63 signalling or transcription^{16, 17}. Msn2/4 on the other hand binds to a stress responsive
64 element (STRE) promoter motif and is also a regulator of multiple stresses¹⁸. As noted, the
65 best known heat-induced proteins are molecular chaperones, initially discovered and
66 characterised as heat shock proteins (Hsps). Chaperones are necessary for protein
67 biogenesis in normal conditions by facilitating and/or assisting *de novo* folding or protein
68 translocation, and in stress conditions (as well as in normal conditions) they prevent
69 aggregation and assist in the refolding of misfolded proteins¹². Hsp70s (for example, the
70 constitutive Ssa1p/Ssa2p and stress-inducible Ssa3p/Ssa4p) and Hsp90 chaperones
71 (Hsp82p redundant in function with its paralogue Hsc82p) with its cofactors, e.g. Sti1p, are
72 all well documented to be induced in heat stress^{10, 13}. Incidentally, it has been proposed
73 that Hsp70 and Hsp90 chaperones are also involved in heat stress sensing by being
74 titrated away from Hsf1p upon accumulation of misfolded proteins¹⁰. Other proteins are
75 also known to be upregulated in heat stress, including the disaggregase Hsp104p,

76 antiaggregases Hsp26p and Hsp42p, and membrane stabilising Hsp12p¹⁰. In addition to
77 induction of heat shock genes, some gene expression is also transcriptionally repressed.
78 These include growth-related and protein biosynthetic genes like ribosomal components
79 and metabolic enzymes⁹.

80 Interestingly the mRNAs encoding two ribosomally-associated Hsp70 family members,
81 Ssb1/2, were also reported to be rapidly reduced in abundance upon shift from 23°C to
82 37°C¹⁹. Ssb1p and Ssb2p are chaperones associated with actively translating ribosomes
83 and, together with Ssz1p and co-chaperone J-domain partner Zuo1p, form the ribosome-
84 associated complex (RAC). Ssb1/2p bind and assist the folding of newly synthesised
85 polypeptide chains, possibly by assisting its transport through the ribosome tunnel or by
86 protecting it from aggregating until other chaperones actively take over the folding²⁰⁻²².
87 Ssb1/2p have also been found to be necessary to maintain translational fidelity in yeast²³.
88 Moreover, despite being 99% identical, differential function of Ssb1p and Ssb2p has been
89 proposed in the literature²⁴. For example, only Ssb1p was able to rescue a phenotype
90 observed in Tim18p mutant cells which are unable to live without mitochondrial DNA.
91 Additionally, in the context of this study, the lack of Ssb1p would be expected to
92 exacerbate the problem of thermal protein misfolding and deleteriously affect translation
93 during heat stress. This presents Ssb1p mutant cells in particular, as an interesting target
94 for deeper investigations of their unique heat stress response.

95 Evidently, HSR is a complex process that requires an integrative system-wide view in
96 order to be fully understood. However, while HSR has been very well characterised on the
97 global transcriptome level^{8, 9, 25}, perhaps surprisingly, no large-scale temporal proteome
98 studies have so far been reported. Importantly, although no HSR proteomics surveys with
99 temporal resolution have been performed, two relevant studies are available: a study by
100 Nagaraj & Kulak et.al²⁶, and another one by Shui & Xiong et.al²⁷. Nagaraj & Kulak et.al²⁶
101 used a quadrupole Orbitrap mass spectrometer and a spike-in SILAC approach to quantify
102 protein abundance changes in a wild type laboratory S288C yeast strain grown at 24°C
103 and transferred to 37°C for half an hour. They achieved an impressive coverage of just
104 over 3100 proteins and found many heat shock proteins to be up-regulated and ribosome
105 biogenesis proteins down-regulated in response to elevated temperature. Additionally,
106 evidence of regulation of translation through down-regulation of proteins implicated in
107 translation initiation and elongation was highlighted in this study. Interestingly, the proteins
108 found to be induced in heat shock were often highly up-regulated; for example Ssa4p had

109 a log₂ fold change of almost 8 and Hsp12p close to 4. In contrast, the down-regulated
110 proteins had much smaller fold changes, often below 2 fold.

111 In a more recent study, Shui & Xiong et.al²⁷ used iTRAQ and 2D reversed phase
112 fractionation to investigate the proteome response to prolonged heat exposure in two
113 thermotolerant industrial strains: ScY01 and its parental strain ScY. Comparisons with the
114 Nagaraj et.al²⁶ study supported the differentiation of the thermotolerant response (TR)
115 from the heat shock response (HSR). Central carbon metabolism and lipid metabolism
116 were decreased in thermotolerance but increased in heat shock response; for example
117 Cyb2p, Gph1p, Faa1p, Hor2p, Cct1p, Aro10p were up-regulated in HSR but down-
118 regulated in TR.

119 Those studies however examined only a single time point during HSR. Here we use a
120 label-free quantitative proteomics approach to determine temporal changes in the yeast
121 proteome at different times during heat stress induced by a mild temperature shift, from
122 30°C to 37°C; a first such study. We measured proteome-level expression for the haploid,
123 a wild type (WT) strain and an isogenic strain lacking the Ssb1p chaperone (*ssb1Δ*). The
124 resulting temporal profiles confirmed many of the HSR hallmark characteristics and were
125 in excellent agreement with the literature. They also revealed additional insights not
126 available from earlier microarray-based efforts or proteomics studies lacking temporal
127 resolution. Of particular interest is that while heat stress response on the mRNA level
128 appears to be almost immediate (minutes) and with large amplitude or distinct temporal
129 patterns, a steady and uniform response (0.5 - 1 hour) on the protein level is visible, at
130 least during the mild heat stress considered here. Additionally, we analyse the temporal
131 profile of an *ssb1Δ* mutant in the same heat stress conditions and characterise at the
132 molecular level how the deletion produces a markedly attenuated response compared to
133 the WT cells. We interpret both data sets in conjunction with known chaperone-target
134 protein interactions, to reveal a global picture of the entire temporal heat shock response,
135 which demonstrates a systems-wide view of proteostasis in action.

136

137 **Experimental**

138 **Yeast growth and proteomics sample preparation**

139 The wild-type BY4742 (*Matα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) and *ssb1* mutant (*ssb*

140 1::kanMX4) yeast strains used in this study were purchased from the Thermo Fisher
141 Scientific Yeast Knockout (YKO) Collection. Batch cultures were grown in Yeast Nitrogen
142 Base medium (supplemented with 10 g/L of glucose, 10 ml/L Arg, 20 ml/L Leu, 10 ml/L
143 Ura, 3 ml/L His and 10 ml/L Lys) in biological quadruplicates. For the heat stress
144 experiment, WT and *ssb1Δ* cells were grown at 30°C in glass conical flasks until they
145 reached mid-exponential growth phase ($OD_{600} \sim 1.9$) and were then moved to a large
146 rotating water bath pre-heated to 37°C. In order to ensure the temperature equilibration
147 inside the flasks was rapid, the water level in the bath was maintained above the culture
148 level and the bath lid was closed. A 5 ml sample of unstressed cells was removed at time
149 0 min (T0) and then at 10 min (T10), 30 min (T30), 60 min (T60), 120 min (T120) and 240
150 min (T240) after transfer to 37°C. For each time point four biological replicates were
151 collected and cells were counted using a Cellometer cell counter (Cellometer AUTOM10
152 by Nexcelom. <http://www.nexcelom.com>). Cell pellets were collected by centrifugation at
153 4000 rpm, for 10 minutes at 4°C and stored at -80°C until use.

154 Cell lysis was performed according to an earlier described protocol²⁸ with minor
155 modifications. Briefly, the cell pellet was re-suspended in ice-cold 50 mM NH_4HCO_3 with
156 added ROCHE Complete mini protease and phosphatase inhibitor cocktails (Roche
157 Diagnostics Ltd, West Sussex, UK), 1 tablet of each/10 ml of buffer, and washed twice by
158 centrifugation (at 4000 rpm for 5 min at 4 °C) to remove any contaminants. Cell lysis was
159 achieved by automated glass bead-beating using the MINILYS® homogenizer (Precellys,
160 UK), 10 x 1 minute cycles at 4°C with 2 min break between each cycle, when lysates were
161 cooled on ice. The lysate was centrifuged (13,000 rpm, 4°C for 10 minutes) and protein
162 collected from supernatant. The remaining cell debris was re-suspended in lysis buffer,
163 collected by centrifugation (10 min, 16 000 rpm, 4°C) and combined with the protein
164 supernatant.

165 For protein digestion, a volume equivalent to 25 million cells (~100 - 150 µg protein) was
166 diluted to 160 µL with 25 mM ammonium bicarbonate. Proteins were denatured with
167 RapiGest™ detergent (10 µL of 1% (w/v), 80°C for 10 minutes), reduced using 60 mM
168 dithiothreitol (10 µL, 60°C, 400 rpm shaking for 10 minutes), cooled on ice and alkylated
169 180 mM iodoacetamide (10 µL, incubation at room temperature in the dark for 30 minutes).
170 Digestion was performed by addition of sequencing grade porcine trypsin (Promega, UK)
171 in a 1:50 enzyme to protein ratio (10 µL of 0.2 µg/µL trypsin), incubation for 4.5 h followed
172 by further trypsin addition (10 µL of 0.2 µg/µL trypsin), and overnight incubation at 37°C.

173 Trifluoroacetic acid (TFA) was added to the reaction mixture (1% (v/v) final concentration)
174 to stop the digestion and hydrolyse *RapiGest*[™] detergent. To aid peptide solubilisation,
175 2.5 µL acetonitrile:water (2:1) was also added and samples were incubated at 4°C for at
176 least 2 hours.

177 Finally, *RapiGest*[™] precipitate and any remaining particulate in the sample was removed
178 by centrifugation (30 min, 13,000 rpm at 4°C). Additionally, two QC pools were prepared
179 containing an equal mix of all WT digests (WT-QC) and another one of all *ssb1Δ* digests
180 (*ssb1Δ*-QC).

181 **LC-MS data acquisition**

182 The digests were analysed on a nanoACQUITY UPLC[™] system (WATERS) coupled to
183 LTQ Orbitrap Velos (linear trap quadrupole with an Orbitrap mass analyser; Thermo).
184 Peptides equivalent to 306,818 cells, and 397,727 cells for the QC runs were loaded onto
185 a C-18 pre-column (Symmetry C18 Column, 100 Å pore size, 5 µm particle size, 180 µm x
186 20 mm, Waters, Manchester, UK) and trapped for 5 minutes at a flow rate of 5 µL/min in
187 99.9% solution A (water, 0.1% (v/v) formic acid). Peptides were then resolved on an
188 analytical C18 column (nanoACQUITY UPLC[™] HSS T3 C18 75 µm x 150 mm, 1.7 µm
189 particle size, Waters, Manchester, UK) with a linear gradient of solution B (acetonitrile,
190 0.1% (v/v) formic acid) from 3% to 35% over 210 minutes at a flow rate of 300 nL/min,
191 followed by a ramp to 90% solution B, column wash and 15 minute re-equilibration. The
192 total run time was 240 minutes. A 30 min blank injection was also performed between
193 each tryptic sample to wash the columns and minimise carry-over between samples.

194 The LTQ Orbitrap was operated in data-dependent mode, with the twenty most intense
195 precursor ions (with charge state ≥ 2) in the survey scan selected for collision induced
196 dissociation (CID) fragmentation. The survey scan was acquired over the range m/z 350-
197 2000 at a resolution of 30,000 (m/z 400) in the Orbitrap analyser and the fragmentation
198 was performed in the LTQ ion trap with the normalised collision energy of 35.

199 **Label-free protein quantification**

200 Mass spectrometry data was acquired in two blocks, WT and *ssb1Δ* samples separately.
201 Acquisition order within each block was randomised and a QC sample was injected at the
202 beginning, intermediately and at the end of each MS run, a total of 5 QC injections. The
203 raw instrument data was then processed using MaxQuant (MQ) software²⁹. Protein
204 identification was performed with the built-in Andromeda search engine³⁰. MS/MS spectra

205 were searched against the *S. cerevisiae* canonical and isoform protein database (UniProt,
206 6721 entries, accessed April 2015) appended with 124 common laboratory contaminants.
207 The parameters were as follows; digest reagent: trypsin, max missed cleavages: 2, max
208 protein mass: 250 kDa, fixed modifications: cysteine carboxymethylation, variable
209 modifications: protein N-terminal acetylation and methionine oxidation and serine,
210 threonine and tyrosine (STY) phosphorylation. The mass tolerance was set to 6 ppm for
211 MS1 and MS/MS to 0.5 Da. The initial MS1 mass calibration was performed within MQ
212 with a 20 ppm tolerance. The false discovery rate (FDR) for accepted peptide spectrum
213 matches and protein matches was set to 1%.

214 For protein quantification, 're-quantify' and 'match between runs' options were selected;
215 match time window was set to 1 min after manual inspection of average chromatographic
216 peak widths in each dataset. Label free quantification was performed with the MaxLFQ
217 algorithm³¹ within MaxQuant. Minimum ratio count was set to 1 and quantification was
218 based on razor and unique peptides only. Razor peptides are determined based the
219 Occam's razor principle where a minimum set of proteins accounting for all identified
220 peptides is used^{32, 33}. All other MQ parameters were left in default. The raw mass
221 spectrometry data was deposited to the ProteomeXchange Consortium³⁴ with the identifier
222 PXD006262

223 **Proteomics data handling procedure**

224 ***Protein significance testing***

225 In order to determine statistically significantly changing proteins with respect to time point
226 T0 we used the MSstats package³⁵ in combination with empirical fold change cut-offs
227 based on technical noise in each experiment. Proteins with an adjusted *p-value* < 0.05 and
228 outside the fold change cut-offs were considered to change expression with respect to T0
229 in response to heat stress. For a detailed explanation of the procedure, see the
230 **Supplementary methods** description and **Supplementary Figure 3**.

231 The final quantification results from MSstats are available in **Supplementary File 1**. The
232 original, unprocessed MQ output proteinGroups tables are available in **Supplementary**
233 **File 2** (WT) and **3** (*ssb1Δ*). The MQ evidence.txt files are uploaded to PRIDE with the id
234 PXD006262.

C-means fuzzy clustering and GO terms enrichment analysis

To identify groups of proteins with similar temporal patterns, c-means fuzzy clustering was performed in R (<http://www.R-project.org/>) using the Mfuzz package³⁶. The number of clusters was determined empirically, by inspecting the results for 2-12 clusters, and assessing biological validity of the resulting clusters (GO processes enrichment) as well as cluster membership and the number of observations in each cluster. The m parameter setting was determined automatically according to Schwaemmle and Jensen³⁷ as implemented in Mfuzz package, 'mestimate' function.

Gene ontology enrichment analysis was performed with the online application Panther^{38, 39}, directly on the Gene Ontology Consortium webpage (<http://pantherdb.org/>). The background set consisted of all proteins identified in a given MS experiment and the target set was the significantly changing proteins, or, in case of c-means fuzzy clustering, the proteins within each cluster and cluster membership > 0.5 . Only terms with a q-value below 0.05 were considered.

From the list of differentially abundant proteins, all molecular chaperones were linked to their attendant protein clients based on the filtered protein interaction dataset used previously^{40, 41}. Based on this information, a bipartite network was created linking proteins by their chaperone-target interactions and a protein-protein interaction network analysis was created using Cytoscape⁴² with an organic layout. Additionally, all the differentially abundant proteins were linked to their Slim GO biological process terms and a bipartite network was created linking proteins by their shared GO terms from which a PPI network analysis was done as before.

Results

Generation of MS-based temporal protein profiles

Our experimental strategy followed the *S. cerevisiae* protein level response to heat stress induced by shift from 30°C to 37°C, the classic 'heat shock'¹⁰. This temperature results in a robust response but is low enough for cells to sustain growth and is physiologically relevant. As expected based on previous literature⁴³, the two strains under investigation; WT (wild type, BY4742, Mat ALPHA) and chaperone mutant *ssb1Δ* (BY4742, Mat ALPHA), had very similar growth rates in glucose-rich, batch cultures at normal

266 temperature 30°C, with estimated doubling times of 2.8 h and 2.9 h, respectively
267 (**Supplementary Figure 1**). Upon heat stress, at 37°C, the growth rate was slowed in both
268 strains, with estimated doubling times during the first 240 minutes increased to 3.8 h in WT
269 and 3.6 h in *ssb1Δ*. The cultures were sampled in quadruplicate at 10 min (T10), 30 min
270 (T30), 60 min (T60), 120min (T120) and 240 min (T240) after transfer to 37°C. The control
271 (mid-exponential cells, 30°C) was obtained just before the shift, at time T0 (**Figure 1A**).
272 After cell lysis, total protein extracts were digested with trypsin and mass spectrometry
273 data was obtained in 48 single-shot LC-MS whole proteome analyses (**Figure 1A**), using a
274 randomised block design⁴⁴. Relative protein quantification was achieved using a label-free
275 approach using MaxQuant^{29, 31}, achieving excellent quantification repeatability in both the
276 WT and *ssb1Δ* blocks of analysis (**Supplementary Figure 2**). Altogether, we quantified
277 2141 proteins, at 1% PSM and protein-level FDR from all WT and *ssb1Δ* runs,
278 representing around 50% of the expressed yeast proteome⁴⁵. After robust statistical
279 analysis (see Material and methods), 1780 proteins in WT and 1970 in *ssb1Δ* were
280 consistently quantified in each of the time points with 1609 proteins overlapping between
281 the two datasets (**Figure 1B**, Venn diagram). This provides good protein coverage and
282 isoform resolution; ~80% proteins in both strains were identified with 3 or more unique
283 peptides across the whole dataset, with an average of 6 unique peptides per protein
284 (boxplots, **Figure 1B**) This supported the resolution and quantification of potentially
285 interesting protein isoforms/paralogues. For example, all of the SSA subfamily members of
286 the ubiquitously expressed HSP70 heat shock protein family could be quantified in the
287 *ssb1Δ* dataset (3 out of 4; Ssa1p, Ssa2p and Ssa4p, could be quantified in the WT).
288 However, because of high sequence similarity (99%) between Ssb isoforms, the two
289 proteins Ssb1p and Ssb2p can not be reliably distinguished by any untargeted MS-based
290 proteomic technique.

291 Temporal profiles of relative protein abundance changes upon heat stress were calculated
292 from the protein ratios at each time point with respect to time 0, represented by a heatmap
293 for each strain shown side-by-side in **Figure 2**. Each cell represents the model-based
294 estimate of fold change from MSstats at a given time (expressed as $\log_2$ of the ratio) after
295 transfer to 37°C. The protein profiles for the WT and mutant cells were additionally
296 clustered row-wise using Euclidean distance. This overview shows the global proteomic
297 response to heat stress in the WT and mutant cells, with many proteins increasing (red)
298 and decreasing (blue) in relative abundance post-stress; however, most show a change of

less than 1.5 fold (corresponding to $\log_2 = 0.58$). Notably, many proteins display coordinated but significantly different behaviour in the mutant cells compared to wild type. For example, clusters 4 and 5 highlight a number of proteins that show increasing abundance post-stress in the wild type cells, but are depleted in the mutant. These highlight several biological processes that the mutant cells is no longer able to sustain in the absence of one of the RAC Hsp70s, including much of nucleotide and amino acid biosynthesis and metabolism.

Other functional enrichments are associated with the clusters, including the archetypal HSR such as clusters 1 and 3 which include up regulated proteins involved in protein folding and response to heat, and clusters 9 and 10 which are enriched for down regulated proteins involved in ribosomal processing and protein synthesis.

Identification of significantly changing protein profiles

Significant protein fold changes across the time points were determined using the MSstats package, adjusting p-values via the Benjamini & Hochberg false discovery rate method⁴⁶ and also applying an empirical cutoff. Subsequently, 267 (WT) and 313 (*ssb1Δ*) proteins were determined to be significantly differentially abundant (see Methods) in at least one time point during the 240 min time course, corresponding to ~15% of all quantified proteins. Only 78 proteins were found to be changing in both strains, a surprisingly small overlap. However, those common changes were highly correlated between the two strains in later time points. In particular, T0/T60 ratio in WT versus T0/T60 ratio in *ssb1Δ* correlated; Spearman = 0.67, p-value < 2.2×10^{-16} and T0/T120; Spearman = 0.74, p-value < 2.2×10^{-16} . This suggests a confined 'core' response that is essentially unaffected by the deletion, supplementing a much greater strain-specific response likely to be linked to the molecular function of the Ssb1 protein, as discussed later. Next, we focused our analysis on the WT heat shock response.

Hallmark proteins of heat stress response (HSR)

The power of the proteomics approach becomes evident from its ability to pinpoint proteins with well-known roles in heat stress. Among the proteins with most statistically significant increases in relative abundance in the WT were the small heat shock proteins with chaperone activity, Hsp26p (adjusted p-value = 1.0×10^{-16}), the membrane associated Hsp12p (adjusted p-value = 3.6×10^{-8}) (**Figure 3A**) as well as Hsp30p, all previously

331 documented to be induced by heat stress⁴⁷⁻⁴⁹. Interestingly, Hsp30p had no detectable
332 peptides and therefore no reported intensity in the T0 time point, but was quantified with
333 an average of 2 unique peptides in all other samples (**Supplementary Figure 4**). We were
334 therefore unable to calculate a fold change for Hsp30p, but based on the distribution of
335 valid fold changes in the experiment we estimate it increases in abundance by at least 3-
336 fold in response to heat.

337 We also quantified both Hsp90 chaperone paralogs, Hsp82p and Hsc82p. Hsc82p was
338 moderately (>2-fold, adjusted p-value 2.4×10^{-5}) induced at T60, 120 and 240.
339 Comparatively, the Hsp82p isoform increased in abundance by over 3-fold two hours after
340 stress (adjusted p-value 5.5×10^{-9}) (**Figure 3A**). This data is in excellent agreement with
341 the transcriptome literature; HSP82 is strongly transcriptionally induced via the Hsf1p
342 factor, while HSC82 is expressed constitutively and also stimulated by Hsf1p, but
343 increases only marginally upon heat stress^{50, 51}.

344 Amongst the 1773 quantified proteins in the WT cells (**Supplementary File 1**), many that
345 displayed significant change and have not previously been associated with the HSR. Most
346 notably, an uncharacterised protein YBR085C-A was induced up to eight-fold over the time
347 course (adjusted p-value 1.4×10^{-6}) (**Figure 3A**). YBR085C-A has been shown to increase
348 in response to DNA damage in a high-throughput study but has not previously been
349 associated with heat stress response⁵². A PSI-BLAST search⁵³ of YBR085C-A against the
350 NCBI non-redundant database found no hits with annotated function. However, a literature
351 search of known DNA-binding regulators revealed a number of transcription factors
352 associated with the promoter region of this gene and thus likely regulating its expression.
353 In particular, a study by Harbison *et al*⁵⁴ provided direct evidence of two heat shock
354 transcription factors, Hsf1p and Msn2p, occupying the DNA regulatory sites of YBR085C-
355 A. The other transcription factors with experimental evidence of YBR085C-A DNA binding
356 are displayed in **Supplementary Table 1**. More generally, we also note that up-regulated
357 proteins at 240 minutes are significantly enriched for targets of several stress response
358 transcription factors⁵⁵ including Msn2p, Msn4p, Hsf1p (adjusted p-value $< 1 \times 10^{-12}$) in all
359 cases.

360 In contrast to widespread upregulation in the WT strain (211 out of the 267 differentially
361 abundant proteins), only 56 proteins exhibited a significant decrease in relative
362 abundance. For example, the most statistically significantly down-regulated proteins were

363 Mup1p (adjusted p-value = 1.1×10^{-5}), Gas3p (adjusted p-value = 3.5×10^{-10}) and Pno1p
364 (adjusted p-value = 3.1×10^{-6}) (**Figure 3A**). The general trends in protein regulation
365 observed here were also recapitulated in other large-scale proteome studies; from the
366 Mann group²⁶ (30 min incubation at 37°C), and a study by Tyagi and Pedrioli⁵⁶ (minimum 4
367 doublings at 37°C). When considering the proteins reported as significantly changing in at
368 least one study, we observed positive, albeit weak pairwise correlation between the
369 relevant time points (inter-study Spearman correlations 0.44-0.62, see **Supplementary**
370 **Figure 5**). This is typical of inter-lab correlations found in methodologically different
371 quantitative proteomic studies⁵⁷. Full protein lists identified in this study are available in
372 **Supplementary Files 2 and 3** for the WT and the *ssb1Δ* samples.

373 **Slow and sustained proteome remodelling in response to moderate heat stress**

374 A distinguishing feature of our experimental strategy compared to previously reported
375 proteome studies is the temporal resolution of the dataset. The 10 and 30 minute time
376 points represent the early HSR, and 60, 120 and 240 minutes the mid to late response,
377 whereas other studies have considered longer times designed to reflect thermo-tolerance
378 adaptations (e.g. 16 h growth at 40°C by Shui & Xiong *et al.*²⁷). We sought to capitalise on
379 this temporal resolution and identify groups of proteins with different profiles across T0,
380 T10, T30, T60, T120 and T240 time points by performing c-means fuzzy clustering^{36, 58}.

381 Clustering analysis of both strains revealed only two major temporal profiles (**Figure 3B**);
382 proteins that were steadily increasing in abundance over time (cluster 1), and those that
383 were slowly decreasing (cluster 2). No dramatic shifts or transitory regulation in protein
384 expression was evident. This is in contrast to observations involving regulation of
385 phosphorylation in response to heat, where up to 15 clusters showing monotonous and
386 adaptation-like profiles could be identified⁵⁹. A GO-term enrichment analysis revealed
387 relatively few significant terms within WT cluster 1 (Bonferroni-adjusted p-value < 0.05) but
388 included expected terms such as protein refolding (p-value = 2.2×10^{-3} , enrichment = 6.2),
389 oligosaccharide metabolic processes (p-value = 4.4×10^{-2} , enrichment = 5.3) and cellular
390 response to heat (p-value = 2.2×10^{-3} , enrichment = 4.4). In contrast, cluster 2 proteins
391 displaying decreasing abundance (**Figure 3B**), had no significantly enriched GO-process
392 terms, consistent with non-specific degradation throughout the heat stress response. Full
393 GO enrichment data are provided in **Supplementary File 4**.

394 A notable example of coordinated regulation in the heat stress response involved proteins
395 annotated with carbohydrate metabolism GO terms involved in the trehalose-synthesis
396 pathway. Trehalose is a storage disaccharide produced in yeast in a relatively simple
397 metabolic pathway (**Figure 4**). It is a general stress protectant and contributes to the
398 suppression of denatured protein aggregation. Following heat stress, the trehalose-
399 synthesis protein (TPS) complex constituents (TPS1p, TPS2p, TPS3p, and TSL1p) were
400 all significantly up-regulated; roughly 2-fold after 120 minutes of heat, with the exception of
401 TPS3p, a regulatory subunit of TPS, which showed much smaller changes (**Figure 4**).
402 Similarly, the most abundant and highest affinity hexose transporter Hxt7p is up-regulated
403 in preference to the others. These examples highlight the ability of proteomics to track the
404 temporal molecular response in a coordinated fashion at the protein level across this
405 pathway. We next turned our focus to the differences observed in the mutant strain.

406 ***ssb1Δ* HSR is different to the wild type response**

407 In the case of the *ssb1Δ* mutant strain that lacks this key member of the HSP70 family we
408 observed notable differences in the membership of the clusters shown in **Figure 3** and
409 their associated gene ontology enrichments. Whilst clusters 1 and 2 retained the same
410 overall trend in the two strains, a closer look at the dynamics of regulation and GO term
411 analysis of the clusters reveals differences in their HSR response (**Supplementary File 4**).
412 Whilst *ssb1Δ* cluster 1 retains proteins with functional enrichments in GO categories
413 'protein refolding', 'protein folding', and 'response to abiotic stimulus', the corresponding
414 increases in relative protein abundance were generally not observed until later time points
415 (T60 at earliest) and showed relatively small increases, between roughly 1.4 and 2-fold
416 change.

417 GO term enrichment analysis of cluster 2 (down-regulated) showed a very different picture
418 in the mutant; 168 proteins were present in this cluster (compared to 48 in WT) with
419 significant enrichment for cytoplasmic translation (p-value = 1.8×10^{-16} , enrichment = 4.4),
420 peptide biosynthetic process (p-value = 6×10^{-10} , enrichment = 2.8) and ribosome
421 biogenesis/assembly (p-value = $9.4 \times 10^{-8}/1.1 \times 10^{-2}$, enrichment = 2.6/3.7). This shows a
422 more pronounced down-regulation of protein synthesis under stress. A direct comparison
423 of the fold changes between WT and *ssb1Δ* at each time point highlights the differences in
424 the global response as well as in individual proteins in each strain (**Supplementary Figure**
425 **6**). This data confirms the trends captured by the clustering, we observed a general lack of
426 response in the mutant strain in the early time points (T0-T60), and an inability to up-

427 regulate key proteins at the later time points (T120-T240). These molecular details are
428 informative when contrasting with the phenotypic response observed in the absence of
429 heat shock⁶⁰, where deletion leads to only a modest decrease in fitness, consistent with
430 our previous study⁶¹. Here, we can see how loss of a key member of the RAC leads to loss
431 of ribosome and proteins involved in translation in general after stress in comparison to the
432 WT response.

433

434 **Proteome, unlike transcriptome is not affected early in HSR**

435 The vast majority of the proteome did not display statistically significant changes in the
436 early time points (T10 and T30). Even proteins with large differential regulation later in the
437 experiment remained mostly unchanged T10-T30. This is in stark contrast to
438 transcriptome-driven gene expression studies such as the seminal microarray study by
439 Gasch et. al.⁹, that reported large mRNA changes within minutes of introduction of heat
440 stress, mirrored in more recent studies by Strassburg et. al.⁶². There the authors measured
441 mRNA changes in yeast cells exposed to heat stress in a time course experiment almost
442 identical to ours. We decided to use this closely matched dataset to investigate the
443 correlation between mRNA expression and protein abundance changes. To take full
444 advantage of the time-resolved data, we performed global correlation analysis where a
445 correlation coefficient is calculated based on all measurements at each time point. For the
446 first analysis, we selected only the protein-transcript pairs for which a significant protein
447 change was measured (resulting in 248 pairs).

448 Despite a strong correlation within experimental sub-types (transcriptome or proteome)
449 and between time points, with R between 0.7-0.9, we observed a poor global correlation
450 between the transcriptome and proteome response ($R < 0.3$) during the early heat shock
451 (**Figure 5A**). However, correlation increased globally over time, with the highest
452 correlations ($R > 0.45$) being observed between the early transcript (15 to 60 min) and late
453 protein changes (120 to 240 min), (**Figure 5A**). These findings are consistent with the
454 immediate molecular response to elevated temperature, by regulating mRNA transcription
455 via Hsf1p (but also potentially through post-translational modifications⁶³ or modulating
456 enzyme activities⁶⁴). The attendant changes in protein level take longer to materialise due
457 to the time it takes to synthesise the responding target proteins. This lag is further
458 supported by the observation that expression of the majority of transcripts peaks between

30 and 60 minutes, while most protein expression peaks 240 minutes after stress (**Figure 5B**). As visible in **Figure 5B**, the average delay in proteome response is estimated to be between 1 and 3 hours. However, the lack of fine-grained resolution makes it only a rough estimate. Furthermore, although the imperfect correlations between the transcriptome and proteome can most obviously be attributed to post-translational regulation, at least some of the variance is likely caused by measurement noise and technical differences between the two datasets.

Global chaperone responses to the proteome under heat shock

As chaperones are the principal molecular responders to heat shock, we examined their relative protein abundance changes across the time course (**Figure 6**), comparing the response in both the WT and mutant strain. This provides a unique picture of the global response for 60 quantified chaperones, in the context of their assigned classes following Gong et al⁴¹. Here, the added value of the temporal experiment is apparent, as consistent step-changes in relative abundance are obvious over time, rather than a simple “before/after” two time-point experiment that could be more prone to false positives. Clear patterns are apparent, highlighting how selected members of the Small chaperone family (Hsp12p, Hsp26p), Hsp60 and Hsp90s (Hsc82p, Hsp82p) all increase over the 240 minutes post-exposure. In parallel, selected Hsp70s increase in relative abundance to help maintain proteostasis. Notably, only a restricted set of chaperones are significantly increased in response to heat shock and indeed a few, as has been described before, decrease; Erj5p being the most obvious. This is consistent with its function as an endoplasmic reticulum-based transmembrane protein, where decrease or loss of function leads to a constitutive increase in the unfolded protein response⁶⁵. A modest decrease in Ssb2p and also Ssb1p is also observed over 240 mins, genes that are also known to be down-regulated under heat stress¹¹.

The global concerted heat shock response in wild type cells is not matched in the *ssb1Δ* mutant. Here, the global effects of Ssb1p absence are observed, leading to a marked attenuation of the main Hsp12p and Hsp26p increase and a generally weaker response across all the related heat shock factors. It is apparent that the loss of this key RAC associated Hsp70 prevents the cell from eliciting a full HSR, and it has knock-on effects on several areas of the chaperone network. For example, Mdj1 increases by 240 mins; Mdj1 is a mitochondrial Hsp40 that is highly connected in the chaperone network and involved in many key functions including folding of nascent peptides and degradation of misfolded

492 proteins^{66, 67} – which are presumed to accrue at higher rates in the absence of Ssb1p
493 under heat stress. Ssa3p also shows a marked decrease at 240 mins and the Erj5p effect
494 is lost.

495 In parallel to the chaperones, we also examined attendant changes in their target
496 proteomes inferred from cognate interactions mapped by affinity-purification studies⁴¹,
497 shown on the right in **Figure 6**. A key feature here is the high correspondence between
498 chaperone abundance changes and their mapped target proteins, which display correlated
499 changes during the heat stress. For each chaperone, we display the fraction of their target
500 proteins present in the differentially abundant proteins (target rate, represented by circle
501 size in Fig 6), coloured by whether they largely increase (red) or decrease (green) in
502 relative abundance. The target rate reaches as high as 60% in some cases. We would not
503 expect to see 100% for several reasons. Firstly, chaperones are expected to attempt to
504 maintain proteostasis (i.e. maintain protein substrate levels) rather than increase them.
505 Therefore it is important to note we do not see a concerted *decrease* in relative abundance
506 for those proteins that are targets of the chaperones that *increase* in abundance.
507 Interestingly, chaperones also function by targeting misfolded clients for degradation
508 pathways, a route to a nominal decrease in abundance. However, we do not observe this
509 phenomenon directly in the quantitative proteomic data. Finally, shotgun proteomics
510 remains a stochastic technique that under-samples the proteome, resulting in some
511 undetected proteins from each chaperone target set. We also note that the data offers
512 strong support for the validity of the protein interactome data, with a direct demonstration
513 of how the specific targets of given chaperones are indeed protected from down-regulation
514 under a heat shock stress.

515 Figure 6 also shows how the targets of the chaperones in the mutant yeast strain are
516 affected, with a matched attenuation of the response to heat. Indeed, in several instances
517 the average level of some chaperone targets is notably reduced, such as those for Apj1p,
518 Ssb2p and most of the CCT complex. This highlights the widespread effects on the cellular
519 proteome of deleting a key member of the RAC complex. By 240 minutes, targets of all
520 three members of the remaining RAC proteins (Ssb2p, Zuo1p and Ssz1p) are reduced in
521 abundance, highlighting the ‘sick’ nature of the cells.

522 The nature of the global response can also be visualised by overlaying this quantitative
523 data on the chaperone-substrate protein interaction network of differentially abundant

524 proteins, as shown in Figure 7. During the HSR, the chaperones and many of their
525 attendant targets increase in the WT cells. For example, after 10 minutes, an increase in
526 the highest-affinity glucose transporter Hxt7p is noted, followed by “small” group
527 chaperones after 30 minutes; by 60 minutes, a clear grouping of Hsp12p, Hsp26p, Hsp26p
528 and Hsp104p has formed, with attendant interconnected targets along with Hsp60p, both
529 HSP90s and selected cytosolic HSP70s. Targets of these chaperones including trehalose
530 biosynthetic enzymes Tps1p and Tps2p are evident by 120 minutes along with the
531 chaperones Sse2p and a growing interlinked network of targets by 240 minutes. This
532 pattern is essentially replicated by the mutant strain but in a much-attenuated form (see
533 the lower half of Figure 7); an increase in Hsp26p levels is not detected at all and indeed
534 by 240 minutes two key HSP70s (Ssa3p and the other RAC-associate Ssb2p) are down-
535 regulated. This down-regulation of Ssb2p affects many of its protein clients including
536 numerous ribosomal proteins.

537 The quantitative changes observed are also reflected in coherent common functional
538 responses (Figure 8, originally noted in Figure 2). Here, we observe global changes to the
539 entire functional network under heat stress, where the links between protein nodes are
540 coloured by common Gene Ontology (biological process) functional categories. Several
541 features are evident here over time, with expansion of the common protein clusters linked
542 to protein folding, response to heat and carbohydrate metabolic processes. Again, the
543 mutant strain exhibits an altered attenuated response pattern particularly in the earlier time
544 points with fewer proteins up-regulated. In contrast, by 240 min a substantial number of
545 proteins are reduced in abundance (green circles), most notably linked to cytoplasmic
546 translation. This group is constituted by the majority of ribosomal proteins, which have
547 depressed abundance levels compared to the cells at T0.

548

549 **Discussion**

550 Budding yeast has been a model organism for elucidating the molecular mechanisms of
551 the heat shock response, most prominently, with global transcriptome profiling^{8, 9, 25}.
552 However, comprehensive time-resolved proteomics studies are so far not available. Here,
553 we addressed this gap and used single-shot LC-MS label-free quantitative proteomics to
554 elucidate both the initial response, and longer term general adaptation to mild heat stress.
555 In contrast to most previous heat shock studies, the shift from 30°C to 37°C investigated

556 here is a smaller fluctuation in temperature. Accordingly, we observed subtle changes in
557 protein expression, typically less than 1.5 fold, throughout the five time points sampled
558 (T10, T30, T60, T120 and T240). This level of confident protein quantification was
559 achieved through a robust normalisation strategy coupled to empirical cut offs derived from
560 observed variance in WT:WT and *ssb1Δ:ssb1Δ* signal. Nevertheless, some limitations
561 inherent to all “relative quantification” experiments remain. For example, since we do not
562 directly measure absolute protein abundances, it is not possible to disentangle the
563 contribution of individual versus global synthesis rates to explain changes in relative
564 protein abundance. Effectively, we measure the percentage contribution of each protein to
565 the total cellular proteome and how this changes over time. We also note that a small lag
566 is expected before the cells truly start experiencing the elevated temperature in our
567 experiments, due to the time it takes for the media inside the culture flasks to equilibrate to
568 the new temperature. Consequently, it makes the precise determination of the early time
569 point kinetics difficult to assess accurately. Nevertheless, the amplitude of protein fold
570 changes across the time-course of the experiment should not be significantly affected.

571 Our data confirm abundance changes in many hallmark HSR proteins, and for the first
572 time, provides a detailed description of temporal patterns of expression for those proteins.
573 In excellent agreement with the vast literature of heat shock response in *S. cerevisiae*^{10 11},
574 we detected a general down-regulation of proteins related to growth and protein synthesis,
575 most notably ribosome biogenesis, and a widespread increase in abundance of stress-
576 related proteins^{47, 68-70}, including the Ssa family proteins whose modest up-regulation
577 under mild heat stress is consistent with their role in preventing aggregation. However,
578 there is a much greater up-regulation of small heat shock proteins, Hsp26p, Hsp12p,
579 Hsp30p and Hsp42, coupled with a moderate induction of Hsp104p (1.5 fold 60 minutes
580 after stress). This indicates that the cell puts greater emphasis on protein stabilisation by
581 preventing aggregation and maintaining membrane and cytoskeletal organisation prior to
582 kick-starting refolding and reactivation of the denatured proteins. We previously measured
583 the absolute abundance changes in chaperone levels under heat shock using targeted
584 mass spectrometry, with Hsp26p increasing to around ~250,000 copies per cell⁷¹. Hsp26
585 and Hsp42 are cytosolic chaperones that form oligomers and bind unfolded target proteins
586 in order to stop them from aggregating. While Hsp42p is active in both normal and stress
587 conditions, Hsp26p functions only in stress conditions⁷⁰, even though both are regulated
588 by Hsf1p and Msn2/Msn4p transcription factors⁷². Interestingly, proteins displaying

589 significant abundance increases between 60-240 minutes were enriched for known targets
590 of several stress response transcription factors (**Supplementary Figure 7**), including
591 Msn2/4p, Hsf1p, Aft1p, Rpn4p. Rpn4p is the key regulator of proteasomal activity, which
592 as expected, is up-regulated to deal with a higher burden of protein degradation
593 associated with misfolded protein accumulation linked to heat shock. Rpn4p is also
594 coupled to other stress response transcription factors such as Pdr1p and Pdr3p, two zinc-
595 finger TFs that activate Rpn4 expression⁷³. Although high-throughput shotgun proteomics
596 is not always sensitive enough to detect transcription factors, it is reassuring to be able to
597 detect the quantitative effects on their targets in a consistent manner.

598 Since chaperones perform multiple proteostatic roles, including disaggregation and
599 refolding, *de novo* folding of nascent polypeptides, and aiding degradation of misfolded
600 proteins, the increased folding workload under stress inevitably leads to increases in
601 abundance of the chaperone itself. In the wider context of established chaperone biology⁷⁴,
602 ⁷⁵, and the known effects of heat on the proteome (for example, protein denaturation and
603 aggregation) our results can be explained by two main scenarios leading to the overall
604 chaperone-target changes observed here. Scenario one, where proteins prone to
605 unfolding and aggregation in heat accumulate in the cell, but are protected by their
606 cognate chaperones from a general down-regulation, they manifest as relative increases in
607 protein abundance; Scenario two, where proteins (other than chaperones) that are
608 functionally important to counter the increase in temperature, such as trehalose
609 biosynthesis pathway components, are actively upregulated. In both cases, there is a need
610 to increase the amount of chaperone activity to either help refold the unfolded/misfolded
611 proteins or to help with *de novo* folding.

612 Our clustering of quantitative protein profiles in **Figure 3** pointed to only two major
613 expression patterns present during heat stress response and minimal transient behaviour
614 was evident. More specifically, we did not detect many protein groups or biological
615 processes displaying complex temporal patterns above simple up- or down-regulation; we
616 did not observe a large set of proteins displaying any effective “recovery” step from the
617 stress. This general behaviour is different to that observed at the mRNA level. Gasch et
618 al.⁹ showed an almost symmetrical transient response of mRNA up- and down-regulation.
619 Unsurprisingly, the time scale of protein response was also different. Gene expression
620 peaks within minutes of stress and returns to a new steady state within hours. These
621 dynamics were not observed for protein level changes, which required longer times to

622 manifest and largely did not appear to return to basal levels. Although the stress and
623 growth conditions differ slightly between our proteomics and the Gasch mRNA studies⁹,
624 the same expression change trends were also observed in another study which integrated
625 matching yeast proteomics and transcriptomics results when adapting to high osmolarity⁷⁶.
626 The authors there also reported relatively large in amplitude and transient mRNA changes
627 and dampened protein levels for over a 1,000 transcript–protein pairs.

628 In addition to the WT heat shock experiment, we performed a corresponding study with
629 cells lacking the Ssb1 chaperone. Although this protein is in fact down-regulated in heat
630 shock, it is an interesting target due to its key role in nascent polypeptide folding. Indeed,
631 as expected, our analyses show how its deletion exacerbates the effects of stress. The
632 level of protein response exhibited in WT cells was lower and the abundance of most
633 proteins did not change until late in the time course. *ssb1Δ* cells do not appear to be
634 capable of a strong heat stress response because the cells either do not sense the
635 stimulus, are already pre-adapted to stress, or are unable to respond appropriately. The
636 latter appears more likely, given the attenuated and delayed response we observe. For
637 example, HSF1 targets continue to be over-represented in proteins up-regulated at 240
638 mins in the *ssb1Δ* cells (adjusted p-value < 4×10^{-10}) although only 16 are detected
639 compared to 64 in the WT. Similarly, we note that Hsp12p and Hsp26p are themselves
640 targets of Ssb1p (as well as many other chaperones), limiting the cells ability to deploy its
641 full complement of stress response molecular repertoire. In the face of this inability to form
642 an appropriate response, for example by producing more chaperones with *de novo* folding
643 function, the cells have a limited RAC functionality. This strategy is consistent with the less
644 protein production in general, and therefore less misfolded and aggregate-prone proteins
645 in the cell. Curiously, less potential for aggregation would reduce the need for expressing
646 stabilising proteins like Hsp12p, Hsp26p.

647 Previously, we have shown that cells deleted for Ssb1p are able to maintain protein
648 homeostasis and do not undergo major proteome remodelling in normal conditions²⁸. We
649 proposed that this is possible owing to other HSP70 chaperones taking over the folding
650 duties of Ssb1p and spreading the workload across the network. Here, we suggest that
651 under heat stress the system is pushed to breaking point leading to a collapse in the
652 requisite chaperone and protein synthetic systems, epitomised by the systemic down-
653 regulation of ribosomal proteins shown in Figure 8 after 240 minutes.

654

655 More generally, our results demonstrate the power of quantitative proteomics coupled to
656 integrated bioinformatics to inform on the molecular events underpinning a cellular
657 response to stress in a temporal fashion. Additionally, by consideration of a deletant strain
658 in parallel, the global consequences of the loss of that gene on the ability of the cell to
659 respond to stress may be characterised and used to rationalise the molecular deficiencies
660 that lead to phenotypes.

661 Overall, the results presented in this study provide a detailed description of the heat shock
662 response in *S. cerevisiae* at the protein level, integrating this information with gene
663 expression changes. This analysis allows us to examine expression patterns of individual
664 proteins in fine detail, and to evaluate the more holistic global response, uncovering
665 important trends on a system level. Finally, the data obtained here are in excellent
666 agreement with the vast, mainly transcriptome-based literature of heat shock response,
667 but emphasises that novel insights can still be gained from carefully controlled large-scale
668 quantitative proteomics studies that cannot be deduced by examining the transcriptome
669 alone.

670

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676

677 **Conflicts of interest**

678 There are no competing or conflicting interests from any of the authors.

679

680

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802 **Figures**

803 **Figure 1 Overview of proteomics strategy and label free quantification results.** Yeast cultures (WT and
804 *ssb1Δ*) were grown at 30°C to mid exponential phase before transfer to 37°C, and subsequent sampling at 0, 10, 30, 60,
805 120 and 240 minutes; the entire experiment was performed in four biological replicates. Cell extracts for each strain
806 were prepared and protein digests analysed by LC-MS/MS using an LTQ-Orbitrap Velos. Mass spectrometry data was
807 acquired for 24 WT and 24 *ssb1Δ* samples in two separate experimental blocks. B) Left panel: Venn diagram shows the
808 overlap of the number of quantified proteins in WT and *ssb1Δ* strains. Middle panel: two boxplots display the number
809 of unique peptides by which each protein was identified. Right panel: barplots indicate the number of unique protein
810 versus protein group identifications in the two datasets.

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814 **Figure 2 Heatmap and functional enrichment of temporal protein profiles during HSR.** Heat map
 815 visualisation of the entire quantitative proteomic profiles under the heat shock response in WT and *ssb1Δ*. At
 816 each time point (column) $\log_2$ ratio to time 0 is plotted. Each row represents a protein and rows were
 817 clustered using Euclidean distance using all timepoints, so that similarities in gene profiles between the two
 818 strains can be observed. This is evident from the coherent clusters which display common Gene Ontology
 819 functional enrichments (See Supplementary File 4) based on GO Slim. Clusters display both common (e.g.
 820 cluster 1) and opposed (e.g. cluster 5) expression patterns between WT and *ssb1Δ* strains.

823 **Figure 3. Temporal protein profiles after heat shock stress in WT and *ssb1Δ* cells.** A) Example protein
 824 profiles for well-established heat stress responders with most significant up-regulation (Hsp26, Hsp12,
 825 Hsp82), as well as YBR085C-A, the highest induced uncharacterized protein. Gas3 and Pno1p are examples of
 826 the most significantly repressed proteins. Red circles signify statistically significant expression change at any
 827 given time point, and the size of the circle represents significance (bigger is more significant). B)
 828 Unsupervised clustering of temporal protein profiles. Two dominant clusters were obtained using fuzzy c-
 829 means algorithm for both WT and *ssb1Δ* cells, displaying proteins with membership greater than 0.7 (210
 830 and 48 proteins in clusters 1 and 2 in WT, and 140 and 168 in the *ssb1Δ* mutant strain). Notably, more
 831 proteins display the cluster 2 “decreasing” abundance profile in the mutant strain.

833 Figure 4. Heatshock induced proteome changes in the trehalose biosynthetic pathway. A bubble-
834 plot overlaid on the trehalose pathway displaying relative protein abundance changes under heat
835 stress of key enzymes. Red indicates abundance increases, green a decrease, with circle size
836 corresponding to statistical significance of the changes. The increases are typically over 2-fold over
837 unstressed cells after 240 minutes in WT cells, but this is attenuated in the *ssb1*Δ mutant.

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843 **Figure 5. Integration of proteome and transcriptome changes in the heat stress response of the wild type**
 844 **strain.** Correlations between yeast transcriptome data taken from Strassburg et al.²⁵, compared to our
 845 quantitative proteomic profiles, comparing relative expression changes at individual time points. The upper
 846 and right-hand quadrants compare intra-technique expression profiles showing high correlations between
 847 transcriptomes, and between proteomes, and lower correlations when transcriptomes and proteomes are
 848 compared. The highest correlations between transcriptome and proteome are indicated on the plot; a time
 849 lag is also evident between early and late time points. B) Histograms showing the number of differentially
 850 expressed transcripts and proteins whose expression peaks at each given time point with respect to
 851 unstressed cells at time zero. Protein expression is still rising in differentially expressed genes at 240
 852 minutes, highlight the lag in the proteome with respect to the transcriptome.

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857 **Figure 6. Global proteome changes in the yeast chaperome under heat stress.** Left: The bubble-plot shows
 858 protein abundance for all chaperones, in both wild type and mutant cells, across the 240-minute time
 859 course. Red circles indicate increasing protein abundance changes according to the key, with green
 860 indicating decrease. The full set of 60 chaperones detected in the label-free mass spectrometry data are
 861 shown, indicating a localised response from a limited set of chaperones, chiefly in the Small class. Right: The
 862 panel shows the corresponding changes in the annotated client proteins, showing the relative fraction of
 863 chaperone-specific targets detected in the differentially abundant protein set at that time point (adjusted
 864 $p < 0.05$), i.e. the “Target Rate”. Circles are coloured by the average abundance change (red increasing, green
 865 decreasing). Protein substrates follow the trends of their parent chaperones. The changes in both the
 866 chaperones and their targets are attenuated for the mutant strain.

Figure 7. Temporal changes to the yeast chaperone-linked protein interaction network under heat shock. Proteins displaying differential abundance are shown as nodes in the graph, linked to cognate protein substrates via edges. Only chaperones or chaperone targets displaying significant abundance changes at each time point are shown, with WT cells in the upper panel and *ssb1Δ* mutant in the lower panel.

Figure 8. Functional network changes to the proteome during heat shock. Differentially abundant proteins during the heat shock response are shown as nodes in the two graphs (WT on the left, and *ssb1Δ* mutant on the right), with edges linking proteins that share common Gene Ontology biological process terms. The growth of the heat shock response is apparent as concerted blocks of common function expand over time, notably involved in carbohydrate metabolism, protein folding and response to heat. Equally, the ability of the mutant *ssb1Δ* strain to respond is evident, particularly in complete down-regulation of cytoplasmic translation capacity as almost all ribosomal proteins are decreased in abundance by 240 minutes.

Supplementary Figures and Tables

A QUANTITATIVE AND TEMPORAL MAP OF PROTEOSTASIS DURING HEAT SHOCK IN *SACCHAROMYCES CEREVISIAE*

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Methods

Detailed description of protein significance testing procedure

In order to determine statistically significantly changing proteins with respect to time point T0 we used the MSstats package [1]. Protein identities, conditions, biological replicates and intensities from MaxQuant were uploaded, retaining protein ID information from the 'proteinGroups.txt' file, conditions and biological replicates from the 'annotation.csv' file, and intensities from the 'evidence.txt' file. Data normalization was performed using the 'equalizeMedians' option and summarization using the 'Tukey's median polish' option. Following this, a condition comparison was performed using the 'groupComparison' option. Log₂ fold changes and adjusted *p* values were obtained for the WT and *ssb1Δ* strains. .

Additionally, we defined empirical thresholds for the minimum magnitude of change a protein had to display in order to be considered significantly regulated. This was based on the technical variance in each block of LC-MS analysis. To that end, a distribution of associated fold changes was generated by calculating ratios between all permutations of the QC sample pairs in either WT-QC or *ssb1Δ*-QC (e.g. QC1 vs QC2, QC1 vs QC3, ..., QC2 vs QC3, etc.) (**Supplementary Figure 3**). The 2.5th and 97.5th percentile values of the resulting Gaussian distributions were then assigned as down- and up-regulation thresholds respectively. The thresholds were $0.69 < \log_2 \text{FC} < -0.958$ for the WT strain and $0.634 < \log_2 \text{FC} < -0.714$ for the *ssb1Δ* mutant. Only proteins with fold changes outside these thresholds and an associated FDR corrected *p*-value < 0.05 were considered significantly changing.

Results

Supplementary Table 1. Transcription factors with experimental evidence of binding to YBR085C-A

Transcription Factor	Study	Experiment type
Hsf1p	Harbison et al. [2]	ChIP-on-chip
Ino4p	Workman et al. [3]	ChIP-on-chip
Msn2p	Harbison et al. [2]	ChIP-on-chip
Pho2p	Harbison et al. [2]	ChIP-on-chip
Skn7p	Harbison et al. [2] and Ni et al. [4]	ChIP-on-chip
Sok2p	Borneman et al. [5]	ChIP-on-chip
Fhl1p	Kasahara et al. [6]	ChIP-on-chip
Sko1p	Capaldi et al. [7] and Ni et al. [4]	ChIP and ChIP-on-chip
Ste12p	Lefrancois et al. [8]	ChIP-seq
Yap1p	Tan et al. [9]	ChIP-on-chip
Gcn4p	Ernst et al. [10]	ChIP-on-chip
Hmo1p	Kasahara et al. [6]	ChIP-on-chip
Cin5p	Ni et al. [4]	ChIP-on-chip
Yap6p	Ni et al. [4]	ChIP-on-chip
Abf1p	Schlecht et al. [11]	ChIP-on-chip

Figures

Supplementary Figure 1 Evaluation of WT and *ssb1*Δ cell growth before and during heat stress.

Optical density (at 600 nm) of the cultures was measured and results are shown as the mean ± standard deviation of the four biological replicates. Dashed line indicates transfer from 30°C to 37°C.

Supplementary Figure 3 Definition of protein fold change thresholds.

The histograms show the distribution of fold changes between all permutations of the QC sample pairs. The dashed lines show the 2.5 and 97.5 percentile cut-offs that were used for determining the magnitude of a fold change at which a protein was considered to be significantly regulated (in addition to passing the FDR-corrected p-value threshold < 0.05). The cut-offs equated to -0.96 (lower bound) and 0.69 (upper bound) for the WT cells, and -0.71 (lower bound) and 0.63 (upper bound) for the mutant cells.

Supplementary Figure 4 Quantification of Hsp30p in the WT samples.

Hsp30p (YCR021C) did not have any detectable peptides in the T0 samples, but was detected and quantified in all later time-points based on three unique peptide sequences: ASGETAIHEPEPEAEQAVEDTA, SITGEVPGIR and LSLTGGFSHHHATDDVEDAAPETK. The solid line represents the average intensity of these three peptides at each time point. The red dashed line represents, for comparison, the intensity value of the lowest quantifiable peptides in this experiment.

Supplementary Figure 5 Correlation of protein fold changes between this and two other yeast heat shock studies: Nagaraj [12] and Tyagi [13].

Supplementary Figure 6. Comparison of protein abundance fold changes between wild type and mutant yeast strains at matched time points after heat shock.

Pairwise comparisons between protein fold changes in the two yeast strains are shown at matched time points after heat shock. Each plot compares the fold change with respect to T0 for each protein in both conditions. The early time points show relatively little remodelling of the proteome has occurred which increases over time post-stress. We note that the correlations between the two strains are modest and effectively non-existent at early time points but increase with time. The data clearly shows a discordance in response between the two strains, whilst some key heat shock response proteins such as Hsp26p remain relatively unaffected in the mutant strain even after 2 or more hours. Interestingly, the novel heat shock responder YBR085C-A is strongly up-regulated in both strains.

Supplementary Figure 7. Enrichment of transcription factor targets in the upregulated proteome over time.

Enrichment in the up-regulated proteome sets for individual transcription factors was calculated using the GeneCodis [14] website taking the sets of proteins deemed to be up-regulated by MSStats at each time point using an adjusted p-value of < 0.05 cutoff. The enrichment in these datasets was then estimated by GeneCodis, which uses Yeastract [15] data on known transcription factor-target relationships, against a background total of 1740 proteins observed at least once across all our experiments. Too few proteins were detected with significant increased abundance at 10 mins, and enrichment p-values were therefore only estimated for 30, 60, 120 and 240 minutes after the initial stress. They were then converted to positive integers for convenience ($-\log_{10}$ p-value), prior to plotting as a stacked-histogram above, ordered from left to right on increasing significance for the 240 time point. The presence of significant enrichments for targets of many key stress response transcription factors is noted, in particular Hsf1p, Msn2p, Msn4p targets which are well reported heatshock transcription factors.

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